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MicroRNA-146a Regulates B Cell Development, Function, and Autoimmunity

A dissertation submitted in partial satisfaction

of the requirements for the degree Doctor of Philosophy

in Cellular and Molecular Pathology

by

Jennifer Kuen-Ling King

2018

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2018

ABSTRACT OF THE DISSERTATION

MicroRNA-146a Regulates

B Cell Development, Function, and Autoimmunity

by

Jennifer Kuen-Ling King

Doctor of Philosophy in Cellular and Molecular Pathology

University of California, Los Angeles, 2018

Professor Dinesh Subba Rao, Chair

MicroRNAs, the short non-coding RNAs that principally act via post-transcriptional repression of target messenger RNAs, are important regulators of gene expression. To date, dysregulated expression of multiple microRNAs have been identified in human diseases, such as cancer and autoimmunity. However, a lack of well-defined function in pathogenesis has hindered advancements leading to clinical translation. MicroRNA-146a has been characterized as a negative regulator of the NFκB and interferon signaling pathways in myeloid and/or T cells, but its function in B cells is not well defined. Because microRNAs often target different pathways in immune cells, this proposal serves to fill this gap in knowledge in B cells to better understand

its dysregulation in human autoimmune diseases, specifically systemic lupus erythematosus.

In the first part of this thesis, we use miR-146a deficient knockout mice (KO) to examine the effects of miR-146a on B cell development. In sum, we found that KO mice show a T cell-independent defect in marginal zone B (MZB) cell development, although all other immature, transitional B cells, follicular cells (FO), and B1 cells were increased. This MZ developmental defect is likely cell intrinsic, as bone marrow progenitors were not different between KO and WT, and there were no differences of immune cell egress from the spleen. We then used high-throughput sequencing to compare the various spleen B cell subsets stage-specific transcriptomes, and determined the MZ cell differentiation was impaired due to decreases in the Notch2 signaling pathway. These findings show an important role of miR-146a regulating normal peripheral B cell development.

We then turn our attention to examining the role of miR-146a on B cell activation and function. We first utilize *in vitro* isolated B cell assays to better characterize KO B cell characteristics compared to WT when stimulated with various T-independent (TI) or T-dependent (TD) antigens. Our results show that KO cells have higher proliferative capacity and activation markers *in vitro* than WT, in particular to TI antigen stimulation. We then perform immunization experiments with TI and TD antigens. Similar to our *in vitro* studies, KOs show higher primary immunization responses to TI antigen immunization, although not with memory responses. We then turn to molecular genetics to define mechanisms of miR-146a target repression in B cells, utilizing RNA-Seq and genetic complementation studies currently underway. The combination of cellular and molecular studies will allow for better characterization of miR-146a in B cells.

Finally, we end this dissertation with a human application currently underway using a large, longitudinal systemic lupus cohort and discuss the importance of exploring microRNAs in human autoimmune research as promising biomarkers in an era of precision medicine.

The dissertation of Jennifer Kuen-Ling King is approved.

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2018

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The work presented in Chapter II is a version of King et al “Regulation of Marginal Zone B-Cell Differentiation by MicroRNA-146a” from the open access journal Frontiers in Immunology, in which the author retains copyright. I designed studies, acquired data, performed analysis and wrote the manuscript. May Paing and Nolan Ung acquired data, performed analysis, and contributed to writing of the manuscript. Dr. Rao designed studies, performed interpretation and analysis, and contributed to writing of the manuscript. Important experimental contributions were from Jorge Contreras, Michael Alberti, Thilini Fernando, Kelvin Zhang, and Matteo Pellegrini.

The work presented in Chapter III is ongoing work with manuscript in preparation. The research is under the direction of PI Dr. Rao. I have planned, conceptualized, executed, and analyzed the experiments. Significant contributions in execution and data analysis are from May Paing, as well as Tiffany Tran and Amit Kumar, and Michael Alberti. David Casero is also involved in bioinformatics analysis.

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CHAPTER I:

Introduction:

“MicroRNAs in B-Cell Gene Regulation and B-Cell Related Diseases”

Non-coding RNA transcripts, which make up approximately 98% of the human genome, play important roles in gene expression. Previously considered “junk DNA”, recent discoveries over the last three decades have described various non-coding RNAs, including microRNAs (miRNAs), long non-coding RNAs, small nucleolar RNAs, and circular RNAs, all with varying regulatory functions in cellular processes (1). MicroRNas (miRNAs or miRs) are a class of 19-23 nucleotide non-coding RNAs that regulate multiple biologic processes, such as cell development, differentiation, metabolism, and immune responses (2). Since their initial discovery in 1993 (3, 4), studies have highlighted the importance of these small, evolutionarily conserved RNAs in biological processes and subsequent dysregulation leading to disease states (2, 5, 6). This dissertation focuses on miRNAs in the immune response, specifically focusing on miR-146a in B cells and its relationship to inflammatory pathways and autoimmune diseases.

miRNA Biogenesis and Function

MicroRNA expression begins with gene transcription via RNA polymerase II, in which many miRNA genes are found in intronic regions with their own promoters (7). Transcription leads to the production of a long primary transcript, called a pri-miRNA. The RNase enzyme, Drosha, with its cofactor protein DGCR8, bind to the pri-miRNA to cleave the 3’ and 5’ strands of the pri-miRNA to produce the pre-miRNA (8). The pre-miRNA is then moved from the nucleus to the cytosol by exportin 5-RAN-GTP (9, 10). Once in the cytosol, the pre-miRNA terminal loop is cleaved into a miRNA duplex via RNase III Dicer and TAR RNA binding protein (TRBP). Then, the miRNA duplex is loaded into the RNA-induced silencing complex (RISC), which involves the argonaute (AGO) family proteins. After unwinding and strand selection, the mature miRNA can bind to the 3’-UTR of target mRNAs via sequence complementarity, leading to degradation/destabilization or translational repression. However, non-canonical functions may

include upregulation of target gene expression, or non-UTR dependent and seedless binding (2, 7). Over 50% of human protein-coding genes are regulated by miRNAs, which are central to normal biologic and physiologic function (8). It is also known that dysregulated miRNA expression can lead to pathologic phenotypes or disease. miRNAs may be altered in disease states due to genetic mutations or polymorphisms, or biogenesis defects in the enzymes that process microRNA, such as in Drosha or Dicer, hence leading to cancer or autoimmune disease pheontypes (6, 7).

miRNA in Immune Responses

While it is now known that miRNAs regulate nearly every aspect of cellular activity, such as development, differentiation, metabolism, proliferation, apoptosis, infections, tumorigenesis, and immunity, we herein focus on miRNAs in hematopoiesis and immune function (Figure 1). miRNAs are pleiotropic in their regulatory response, often targeting multiple genes within one signaling pathway, or many genes in different independent pathways. Their targets and function also may act differently in different tissue or cell types (5).

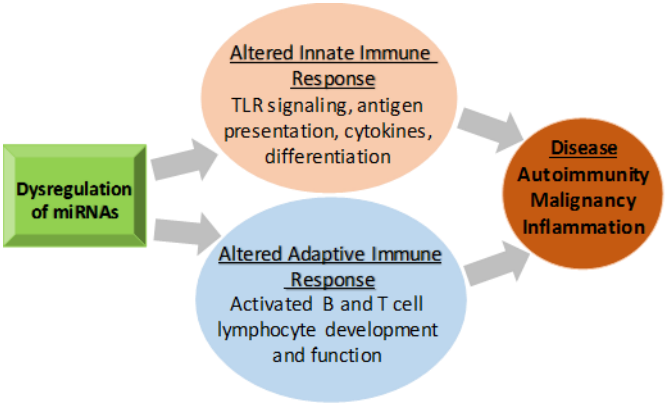

Figure 1. Dysregulation of miRNAs in the immune system.

In the innate immune system, miRNAs are expressed in various cells, such as monocytes, macrophages, dendritic cells, granulocytes, and NK cells, which often act as the first line of defense to outside pathogens. MicroRNAs play a variety of roles, including regulation of pattern recognition receptor signaling (PRRs), toll-like receptor (TLR) expression, and regulation of

myeloid differentiation and maturation (11). In regards to TLRs, various miRNAs regulate TLR4 signaling pathways, including let-7i, let-7e, miR-223, miR-146a, miR-146b, miR-155, and miR-511 (12). For example, miR-155 is a well-studied miRNA that is induced in macrophages after TLR signaling, and transcriptionally regulated by NF-kB (13, 14). It functions as a pro-inflammatory stimulator via repression of certain negative regulators of inflammation, such as *Socs1* (15) and *Ship1* (16*)*, among others. MiR-155 was also shown to be required for dendritic cell activation, functioning as a positive modulator of inflammatory cytokine production, targeting Socs1, p27,and Kpc1 (17). Another important NF-kB regulator is miR-146a, which we will focus on in detail in this dissertation, which was shown in monocytes to repress its targets, *Traf6* and *Irak1*, leading to net downregulation of the NF-kB inflammatory pathway (13, 18, 19). Its expression is also important in dendritic cells, and increases apoptosis of myeloid DCs and decreases production in responses to inflammatory stimuli (20, 21).

In the adaptive immune system, both above examples of miR-155 and miR-146a are also expressed in various immune subsets. MiR-155 is required in CD8+ lymphocytes for optimization of effector activity to infection and tumors (22, 23). In addition, miR-155 promotes Th17 cell differentiation and responses (24, 25). MiR-155 is also necessary for normal B cell differentiation, function, and antibody production (26). Meanwhile, miR-155 overexpression supports pre-B cell proliferation and development of lymphomas (27, 28). Similarly, miR146a is also expressed in adaptive immune cells, such as T cell subsets (29, 30), T regulatory cells (Treg) (31), and B cells (18, 32) . Specifically, miR146a is highly expressed in Tregs and targets *Stat1*, a transcription factor required for Th1 differentiation (31). Thus, miR-146a expression in Tregs is essential for downregulation of the IFN-g mediated pathogenic Th1 response. In synergy with this, miR-146a in traditional T cells (CD4+ and CD8+) were shown to regulate T cell receptor-mediated NF-kB activation via *Traf6* and *Irak1* (29, 30, 33), like monoctyes. Furthermore, in human Th1 cells, mir-146a targets protein kinase Ce (PKCe), and forms a complex with STAT4, which is essential for

Th1 cell differentiation (34). Thus, the same microRNA may have discrete targets in different cells, to provide synergistic and context dependent immune response regulation. A multitude of other miRNAs exhibiting cell specific functions exist in both the innate and adaptive immune system, which often help to “fine tune” immune responses after stimulation.

miRNA in B-cells and B-cell related diseases

The function of B cells is a central process in adaptive immunity. However, dysregulation in the processes of B cell development, activation, proliferation, and differentiation may lead to diseases, such as malignancies or autoantibody-associated autoimmunity. The terminal differentiation of activated B cells is defined by complex changes in gene expression as cells develop into plasma cells or other subsets. Elements that regulate gene expression are of utmost importance. Hence, it is not surprising to see microRNA regulation of gene expression in B cell hematopoietic cell development, immune function, oncogenesis, and autoimmunity.

The events dictating development of B cells from the bone marrow to the periphery are complex and undergo a tightly regulated process of gene expression. Immunoglobulin (Ig) gene rearrangement of the heavy and light chains undergoing V(D)J recombination is regulated by RAG1 and 2 recombinases, and leads to specific B cell receptor affinities on the surface of the B cell. These rearrangements occur during the pro-B cell stage for the heavy chain, and pre-B cell stage for the light chain respectively. Hence, immature B cells will acquire unique antigen specificity through their surface immunoglobulins. Autoreactive B cells will undergo central tolerance processes, while non-autoreactive B cells will differentiate into mature B cells in the periphery (35, 36). There are several miRNA families and individual miRNAs that are important for B cell development (37). Specifically, miR-17-92 cluster includes six mature miRNAs (miR-17, 18a, 19a, 20a, 19b-1, 92a-1) and two paralogs (miR-106a~363 and 106b~25). A series of studies

found that miR-17-92 family is important for pro-B to pre-B stage development, possibly through regulation of the proapoptotic protein, BIM, or other molecular pathways (38, 39). In addition, the pro-to pre-B cell developmental defect in knockout cells could be reversed via lentiviral vector mediated over expression of miR-17 family (38). Separately, miR-34a is also important for the pro-B to pre-B cell development, as miR34a may directly suppress *Foxp1*, a B cell oncogene (40). Distinct miRNAs may show positive and negative regulatory effects on bone marrow B cell development. Specifically, miR-34a, miR-150 (41, 42), miR23a miRNA cluster (43, 44), and miR-212/132 (45) inhibit B cell progenitor survival. However, others such as miR-181 (46) and miR17-92 cluster (38, 39) support differentiation from pro-B cells to pre-B cells. Clearly such differing roles of miRNAs help to keep the body's homeostasis and physiologic function intact for regulating gene expression.

The role of miRNAs in peripheral (i.e. lymphoid organs and blood) development is also important in B cell maturation. Naïve B cells from the bone marrow are released into the periphery and may undergo activation via antigen exposure. Some activated B cells may undergo differentiation into short-lived plasma cells, mainly secreting IgM. Others may enter the follicle and germinal center (GC) to form high-affinity IgG-producing plasma and memory cells. Plasma cell differentiation is regulated by transcription factors BLIMP1 and XBP1 (47). Various miRNAs have been shown to regulate B cell subset development (32), GC reaction (26, 48, 49), Ig isotype switching and somatic hypermutation (50, 51) that demonstrate their importance in peripheral development.

Given the importance of miRNAs in B cell development, it is likely that B cell function is also regulated, and hence, dysregulation may lead to diseases, such as oncogenesis or autoimmunity. Many miRNAs that are involved in B cell development may also be implicated in B cell malignancies, such as B cell lymphomas or leukemias. For example, the miR-17-92 cluster, which is important for bone marrow B cell development and central tolerance (38, 52), also shows

malignant capacity. Increased expression of miR-17-92 cooperates with the oncogene, C-*myc*, to facilitate B cell lymphoma growth (53). In addition, of the aforementioned miRs, miR-155, which was shown to play a role in immune responses, also shows oncogenic potential in B cell lymphoproliferative disorders (54-56). Conversely, deficiencies of miR-146a, which was identified as a negative feedback regulator in the NF-kB and interferon pathways, can lead to lymphoid and myeloid malignancies in older age (57, 58). Both promoters and suppressors of B cell tumorigenesis are present, and may be categorized as: 1) oncogenic miRNAs: miR-17-92, miR-155, miR-21, and miR-217, or 2) tumor suppressor miRNAs: miR-181a, miR-34a, miR-146a, Cluster 15a/16-1, and miR-28 (37). Such miRNAs are of obvious interest, as development of miRNA mimics or inhibitors of miRNAs (i.e. antimiRs), are being explored for clinical therapeutic potentials.

On the opposite side of activation, tolerance in the immune system is a state of low level or minimal responsiveness when encountering antigens. Tolerance ensures that lymphocytes do not attack self-antigens,that may lead to autoimmune conditions. Self-reactive B cells may then lead to autoimmune disease states, such as systemic lupus erythematosus (SLE), Sjogren's syndrome, and rheumatoid arthritis (59). A few studies have identified miRNAs relevant to B cell tolerance and autoimmunity, although more studies are needed to elucidate individual function. Of the above described miR-17-92 cluster, miR-19 regulated B cell tolerance in immature B cells by inhibiting expression of PTEN, a negative regulator for the PI3K-AKT pathway (38). In addition, miR-17-92 overexpressing transgenic mice developed autoimmune disease and higher autoantibody production, leading to increased mortality than controls (52). In addition, an *in vivo* functional screen of a lymphocyte-expressed miRNA library identified miR-148a as an important regulator of B cell tolerance, targeting autoimmune suppressor *Gadd45a*, tumor suppressor *Pten*, and *Bim* (60). Furthermore, increased miR-148a expression led to lethal autoimmune lupus manifestations. Finally, another miR, namely miR-210 was shown in knockout models to develop

autoantibodies, whereas overexpression of miR-210 led to decreased B2 cells and impaired class-switched antibody production (61).

Specifically, we address in this dissertation the role of **miR-146a** in B cell development, function, and autoimmunity. Further identification and description of function of miRNAs that contribute to B cell function and autoimmunity are in need, and may open up potential diagnostic and therapeutic targets for treatment of autoimmune disease conditions.

The exploration of microRNAs with diagnostic or therapeutic functionality is well underway, with a number of pre-clinical or clinical trials ongoing. In this era of precision medicine, microRNA properties, such as ease of detection, relative stability during sample/tissue handling, and relevance in hematopoietic and immunologic biological processes, make them ideal candidates for biomarker and therapeutic development in both malignancies and autoimmune diseases. As proof of clinical application, the aforementioned miR-155 has been shown to be up regulated in several B cell malignancies such as diffuse large B-cell lymphoma (DLBCL) and chronic lymphocytic leukemia (CLL), with overexpression correlating with poor prognosis (62). In addition, studies in various preclinical or clinical stages have applied antimir-155 therapies to cutaneous T cell lymphomas, solid tumors, and lupus lung disease (7, 63). Other select ongoing miRNA clinical trials range from diseases such as diabetes, chronic hepatitis C, solid tumors, and systemic sclerosis (7, 64). However, challenges such as improving delivery systems for miRNA therapeutics to protect against degradation of oligonucleotides by RNases in serum or endocytic cell compartments remain ongoing (7, 64).

miRNA-146a in B-cells and autoimmunity

As described above, miR-146a is an NFκB-induced microRNA that shows expression in immune cells, such as myeloid, T, and B cells (13, 18). Previous studies from our lab and

colleagues using *Mir146a*$^{-/-}$ (KO) mice were found to have hyperactivated T follicular helper cells and enlarged germinal centers (GC) (26), secretion of dsDNA autoAbs, autoimmunity (18), T cell hyperactivation (30), and myeloid and lymphoid tumors (57). This was a consequence of loss of feedback regulation via derepression of miR-146a targets, *Traf6*, *Irak1* and *Stat1* (13, 31). Because microRNAs often target different pathways in different cells, it is important to fully characterize their effects in specific immune cell types and specific biologic contexts. Although the above and other studies have well characterized miR-146a's effects in various immune subsets, the effects on B cells are still not well described, which we describe here.

This author recently found that *Mir146a*$^{-/-}$ (KO) mice show a T cell-independent defect in marginal zone B (MZB) cell development (32), presented in Ch2. To summarize, we performed cellular phenotyping from the hematopoietic stem cells/progenitors and B cell progenitors in the bone marrow, and B cell subsets in the spleen and lymph nodes. We found that in KO mice, marginal zone B cell subsets were decreased, however immature T1, T2, T3 B cells and mature follicular (FO) B cells were increased. Such defects were specific to female mice, revealing a gender bias (as common in autoimmune diseases), and increased with aging up until very old age when the myeloproliferation phenotypes dominate. This marginal zone defect was likely due to a spleen intrinsic defect, with miR-146a targeting the NUMB-NOTCH2 pathway. We then continue to further explore our ongoing work on miR-146a's effect on B cell activation and function (Ch3).

It is known that dysregulation of B cells can lead to a variety of host pathologic conditions, including autoimmune disease such as SLE, rheumatoid arthritis, or Sjogren's syndrome. SLE is a chronic autoimmune disease, often referred to as the quintessential B cell mediated autoimmune disease. It has a complex pathogenesis involving the loss of T and B cell tolerance, autoantibody (autoAb) production, and increased gene expression in the NF-kB and type I

interferon (IFN) pathways (65). Hence, at the cellular and molecular level, studying the role of B cell gene expression dysregulation is important for understanding pathogenesis

Interestingly, in human translational studies, miR146a expression from peripheral blood mononuclear cells (PBMCs) has been shown to inversely correlate with expression of IFN-inducible genes and is lowly expressed in SLE patients with active disease (high disease<low disease) compared to healthy controls (66). In contrast, miR-146a was overexpressed in other autoimmune diseases such as rheumatoid arthritis, Sjogren's, and multiple sclerosis (6, 67), consistent with a reactive process in which miR-146a acts as a negative regulator in inflammatory processes. The low expression of miR-146a in SLE therefore may have a pathogenetic role, leading to dysregulated repression of the SLE inflammatory immune response, including aberrant NFκB and IFN signaling. Hence, we end this dissertation (Ch 3/4) by applying the relevance of miR-146a in B cells to human translational studies, focusing on the autoimmune disease, systemic lupus erythematosus (SLE).

The potential application of microRNAs as diagnostic markers of disease activity in autoimmune diseases such as SLE hold particular importance in the field, as the currently used autoantibody profiles and laboratory tests remain insufficient to consistently and/or accurately capture disease activity in patients. In addition, given the inherent heterogeneity of SLE disease manifestations, it is of utmost importance to accurately identify disease activity markers that will help dictate treatment and avoid unnecessary side effects of chemotherapeutic agents.

CHAPTER II:

"Regulation of Marginal Zone B-Cell Differentiation by MicroRNA-146a"

(reprint)

frontiers in Immunology

Regulation of Marginal Zone B-Cell Differentiation by MicroRNA-146a

*Jennifer K. King[1,2], Nolan M. Ung[3], May H. Paing[3], Jorge R. Contreras[2,3], Michael O. Alberti[3], Thilini R. Fernando[3], Kelvin Zhang[4,5], Matteo Pellegrini[6,7,8] and Dinesh S. Rao[3,6,8]**

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B-cell development in the bone marrow is followed by specification into functional subsets in the spleen, including marginal zone (MZ) B-cells. MZ B-cells are classically characterized by T-independent antigenic responses and require the elaboration of distinct gene expression programs for development. Given their role in gene regulation, it is not surprising that microRNAs are important factors in B-cell development. Recent work demonstrated that deficiency of the NFκB feedback regulator, miR-146a, led to a range of hematopoietic phenotypes, but B-cell phenotypes have not been extensively characterized. Here, we found that miR-146a-deficient mice demonstrate a reduction in MZ B-cells, likely from a developmental block. Utilizing high-throughput sequencing and comparative analysis of developmental stage-specific transcriptomes, we determined that MZ cell differentiation was impaired due to decreases in Notch2 signaling. Our studies reveal miR-146a-dependent B-cell phenotypes and highlight the complex role of miR-146a in the hematopoietic system.

Keywords: B-cell development, marginal zone B-cells, microRNA, notch signaling, gene regulation

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INTRODUCTION

Distinct subsets of immunoglobulin-expressing B-cells have been described in the spleen, including follicular (FO) B-cells, marginal zone (MZ) B-cells, and other, more rare, B-cell subsets. In general, the former subset is important for T-dependent B-cell responses to pathogens, while the latter is thought to play a role predominantly in T-independent B-cell responses and lipid antigen presentation to natural killer T cells (1). Both subsets are thought to derive from bone marrow-derived naïve B-cells that arrive in the spleen, where they can be recognized as immature transitional B-cells (T1, T2, and T3 B-cells). The development of MZ B-cells from these transitional subsets is thought to be regulated by several mechanisms, including (1) strength of B-cell receptor (BCR) signaling, (2) B-cell activating factor (BAFF)/receptor for BAFF (BAFF-R), (3) Notch2 signaling, (4) integrins and chemokines required for MZ retention in the spleen, and (5) NFκB signaling (2). BCR engagement combined with BAFF/BAFF-R signaling promotes B-cell survival and separately MZ B development (3). While both FO and MZ B-cells depend to some degree of BAFF/BAFF-R and NFκB signaling, the cell surface receptor Notch2 is critical specifically for MZ B-cell development. Activation by the ligand Delta-like-1 expressed by endothelial cells of red

pulp venules catalyzes proteolytic cleavage of Notch2, releasing its intracellular domain, and resulting in nuclear translocation and downstream transcriptional effects (4).

It is likely that several other factors play roles in regulating the expression and activity of these key regulators of MZ B-cell development. MicroRNAs (miRNAs) are a family of small non-coding RNAs, 19–23 nt in length, which predominantly act *via* posttranscriptional repression of target messenger RNAs (mRNAs) by binding to the complimentary 3′-untranslated region (UTR) of the mRNA. To date, miRNAs have been implicated in a wide range of biologic processes, including hematopoietic cell development, immune function, autoimmunity, and oncogenesis (5). A single miRNA can target multiple mRNA transcripts and target mRNAs may be controlled by multiple miRNAs, thus adding a layer of complexity to cellular gene expression. Recent work has indicated the general importance of miRNAs in modulating the differentiation of splenic B-cell subsets. A B-cell specific knockout of Dicer, an endoribonuclease required for miRNA biosynthesis, resulted in a preferential development of MZ B-cells in mice (6). In addition to a general role for Dicer, specific miRNA loss or deregulation has been associated with various phenotypes within the B-cell compartment (7).

miR-146a is an NFκB-induced miRNA that shows high expression in spleen tissue, in particular splenic myeloid, T, and B-cells (8, 9). Studies using *Mir146a*$^{-/-}$ (KO) mice were found to have hyperactivated T FO helper cells and germinal centers (10), autoimmunity (8), T cell hyperactivation (11), and myeloid and lymphoid tumors (12) as a consequence of loss of feedback regulation *via* derepression of miR-146a targets, *Traf6*, *Irak1*, and *Stat1* (9, 13). Although these studies have well characterized miR-146a's effects in myeloid and T cell subsets, the effects on B-cells are not well understood. In our study, we found that *Mir146a*$^{-/-}$ mice show an age-independent defect in MZ B-cell development. We have carefully characterized this defect, finding that KO mice show an increase in the preceding transitional B-cell stages and intact splenic retention, indicating a block in development. Using a combination of high-throughput sequencing, molecular biological and cellular-based approaches, we identified that this developmental block results from deregulation of the Notch2 pathway.

MATERIALS AND METHODS

Mice

miR-146a-deficient (*Mir146a*$^{-/-}$) mice were generated as described previously (8). Wild-type (WT) mice (C57B/6) and TCRαβ-deficient (*TCRβ*$^{-/-}$) mice (002118) were purchased from Jackson laboratories and kept under pathogen-free conditions at the University of California, Los Angeles. All mouse studies were approved by the UCLA Office of Animal Research Oversight.

Flow Cytometry

Bone marrow, spleen, and blood were obtained from euthanized mice and red blood cell lysis buffer was used to lyse the single cell suspensions. For intracellular staining, cells were fixed in 2% paraformaldehyde at 37°C for 10 min and then blocked with Fc block for 20 min. Then, they were stained in 1× PBS with 10% FBS and 0.1% Triton X-100 for 20 min in the dark, and subsequently washed with 1× PBS with 4% FBS. Fluorochrome-conjugated antibodies and subset definitions are listed in Tables S1 and S2 in Supplementary Material. Initial spleen gating strategy is shown in Figure S1A in Supplementary Material. Flow cytometry was performed on an LSRII and data were analyzed using FlowJo software.

Sorting of Splenic B-Cell Subsets

Spleens pooled from three to five mice were stained according to FACS surface markers as noted in Table S2 in Supplementary Material. Cells were sorted into T1, T2, T3, FO B-cells, and MZ B-cells *via* FACS Aria.

RNA Sequencing (RNA-Seq) and Analysis

Total RNA was extracted from WT and KO B-cell subsets using Qiazol using the Qiagen miRNEasy mini kit with additional on column DNAse I digestion. Following isolation of RNA, cDNA libraries were built using the Illumina TruSeq RNA Sample Preparation kit V2 (RS-122-2001). An Agilent Bioanalyzer was used to determine RNA quality (RIN >8) prior to sequencing. RNA-Seq libraries were sequenced at the Broad Stem Cell Research Center sequencing core (UCLA). Libraries were sequenced on an Illumina HiSeq 2000 (single-end 100bp). Raw sequence files were obtained using Illumina's proprietary software and are available at NCBI's Gene Expression Omnibus (Accession GSE93252). We first filtered out reads with low quality and reads containing sequencing adapters and then mapped raw reads to the mouse reference genome (UCSC mm10) with the gapped aligner Tophat allowing up to two mismatches. We supplied the UCSC mm10 gene model to Tophat as the reference genome annotation. Only reads uniquely aligned were collected. In total for all libraries sequenced, 365,022,996 reads were uniquely mapped (corresponding to an overall mappability of 91.7%) and used for further analysis. Transcript expression levels were quantified using RPKM units (Reads Per Kilobase of exon per Million reads mapped) using customized scripts written in Perl. Differential expression analysis was performed using both DESeq and edgeR in R (http://www.R-project.org). Raw read counts were used and modeled based on a negative binomial distribution. The multiple testing errors were corrected by the false discovery rate (FDR). We considered genes as differentially expressed if (1) the FDR was less than 0.05, (2) the expression ratio between two time points was >2×, (3) the maximal RPKM value for at least one group in the comparison was >1, and (4) there was agreement between DESeq and edgeR. These differentially expressed genes were then examined from the T2 to MZ and T2 to FO stages in both WT and KO cells. We then focused on genes only found in the T2 to MZ transition in WT and compared them to those in the T2 to MZ transition in KO B-cells. The *Z*-scores for each of these unique gene subsets were calculated, scaled, centered, and displayed as a heat map.

RT-qPCR

RNA was collected from corresponding B-cell samples and reverse transcribed using the qScript reagent (Quanta Biosciences). RT-qPCR was performed with the StepOne Plus Real-Time PCR System (Applied Biosystems) using PerfeCTa SYBR Green FastMix reagent (Quanta Biosciences) or TaqMan MicroRNA Assay (Life Technologies). Primer sequences used are listed in Table S2 in Supplementary Material.

Western Blot

$B220^+$ cells were isolated from WT and KO mice using MACS, treated with LPS, and cultured for 72 h. B-cells were then lysed with RIPA buffer (Boston BioProducts) and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Equal amounts of protein lysate [quantification by bicinchoninic acid protein assay, BCA (Thermo Scientific)] were separated using electrophoresis on a 10% SDS–PAGE and blotted on a nitrocellulose membrane. Numb Rabbit monoclonal (C29G11)(#2756) and β Tubulin Rabbit polyclonal (#2146) antibodies were used (Cell Signaling Technologies). HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology.

Luciferase Reporter Assay

The entire *Numb* 3′-UTR (1,978–3,382 nt; GenBank ID: NM_001136075) containing the miR-146a site was cloned into the pmiRGlo dual luciferase vector (Promega). The miR-146a seed sequence AGTTCTCA (2,596–2,603 nt) was mutated to CTCATAGT and also cloned into pmiRGlo. A similar strategy was used for cloning a 2 kb segment of the *Notch2* 3′-UTR (7,584–9,592 nt; GenBank ID: NM_010928) immediately downstream of the stop codon. The putative miR-146a seed sequence GTTCTCA (8,815–8,821 nt) was mutated to CAGTCTT and also cloned into pmiRGlo. Standard PCR and cloning methods were employed. TargetScan was used to predict miR-146a seed sequences. The *Traf6* 3′-UTR was cloned as previously described (14). HEK-293T cells were co-transfected with luciferase reporter vectors, with or without miR-146a expression vector using BioT transfection reagent (Bioland Scientific) as per the manufacturer's instructions. Cells were lysed after 48 h, substrate was added, and luminescence was measured on a Glomax-Multi Jr (Promega).

Statistical Analyses

Figures are graphed as mean with the SD of the mean for continuous numerical data. Dichotomized or ordinal-type histopathologic data are presented using bar graphs. Data were analyzed with two-tailed Student's *t*-test, conducted using GraphPad Prism software, applied to each experiment as described in the figure legends. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

RESULTS

Deficiency of miR-146a Results in Decreased MZ B-Cells in the Spleen

Previous studies have shown that *Mir146a*$^{-/-}$ mice show age-dependent changes in splenic cellularity and activated T cell phenotypes, starting from 2 to 4 months (10, 11). This is followed by autoimmune inflammatory disease, splenomegaly, lymphadenopathy, and myeloproliferation, and at very advanced ages by splenic myeloid sarcomas, lymphomas, and bone marrow failure (12). Given the high expression of miR-146a in B-cells and features of autoimmune disease in KO mice, we examined splenic B-cell subsets to better characterize miR-146a's effect on B-cell maturation. Beginning with young mice, we confirmed that increases in total splenic cellularity (**Figure 1A**) and B220+ B-cells (**Figure 1B**) were apparent as early as 8–12 weeks old. We characterized immature transitional B-cells (T1, T2, and T3), FO cells, MZ B-cells, and B1 cells and compared them between WT and KO mice (**Figure 1C**) (15). Increased percentages and numbers in precursor T1, T2 (**Figure 1D**; Figure S2 in Supplementary Material), T3, and B1 cells (Figures S1C,D and S2 in Supplementary Material) were seen starting from 8 to 12 weeks in KO compared to WT. In addition, the overall number of FO B-cells started to trend higher in KO mice at 8 weeks of age and statistically significant differences were observed at 12 weeks of age in KO mice (Figure S2 in Supplementary Material). In contrast, MZ B-cells showed a significant decrease as early as 8 weeks of age (**Figure 1D**). This defect persisted until at least 18 weeks of age in the KO mice (Figure S3 in Supplementary Material), after which expansion of the myeloid compartment began to take over in KO spleens (12). In addition, an alternative gating analysis (Figure S1B in Supplementary Material) (1) showed that this defect was also present in MZ precursor (MZP) cells, again confirming a deficiency in the MZ development pathway (Figure S1E in Supplementary Material). Together, these results indicate a specific defect in the MZ subset of splenic B-cells, with an increase in T1 and T2 transitional zone B-cells, suggestive of defective differentiation into MZ B-cells in KO mice.

Decreased MZ B-Cells in miR-146a-deficient Mice Are Due to Spleen-Intrinsic Defect and Not Bone Marrow Development Failure or Lack of Splenic Retention

To fully characterize the origin of differences in splenic B-cell subsets between WT and KO mice, we looked upstream into the bone marrow to assess deficiencies in B-cell development. At the early ages of 8 and 12 weeks, there were no statistically significant differences in total live cells (**Figure 2A**) or B220+ B-cells (**Figure 2B**). We subsequently characterized bone marrow B progenitor cells (15) in WT and KO mice (**Figure 2C**). Fractions A–E in young mice remained similar, although at 12 weeks fraction A showed a decrease in KO mice (**Figure 2D**), which may represent the beginning of myeloproliferative disease or faster flux through this particular stage of development (12, 16). Hardy Fraction F cells, which represent mature bone marrow B-cells as well as recirculating cells, showed a decrease in KO mice, likely representing a decreased recirculating fraction secondary to increased retention of overall B-cells in the spleen. To ensure that this decrease in pre–pro B-cells was not due to decreases in hematopoietic stem cell/progenitor development,

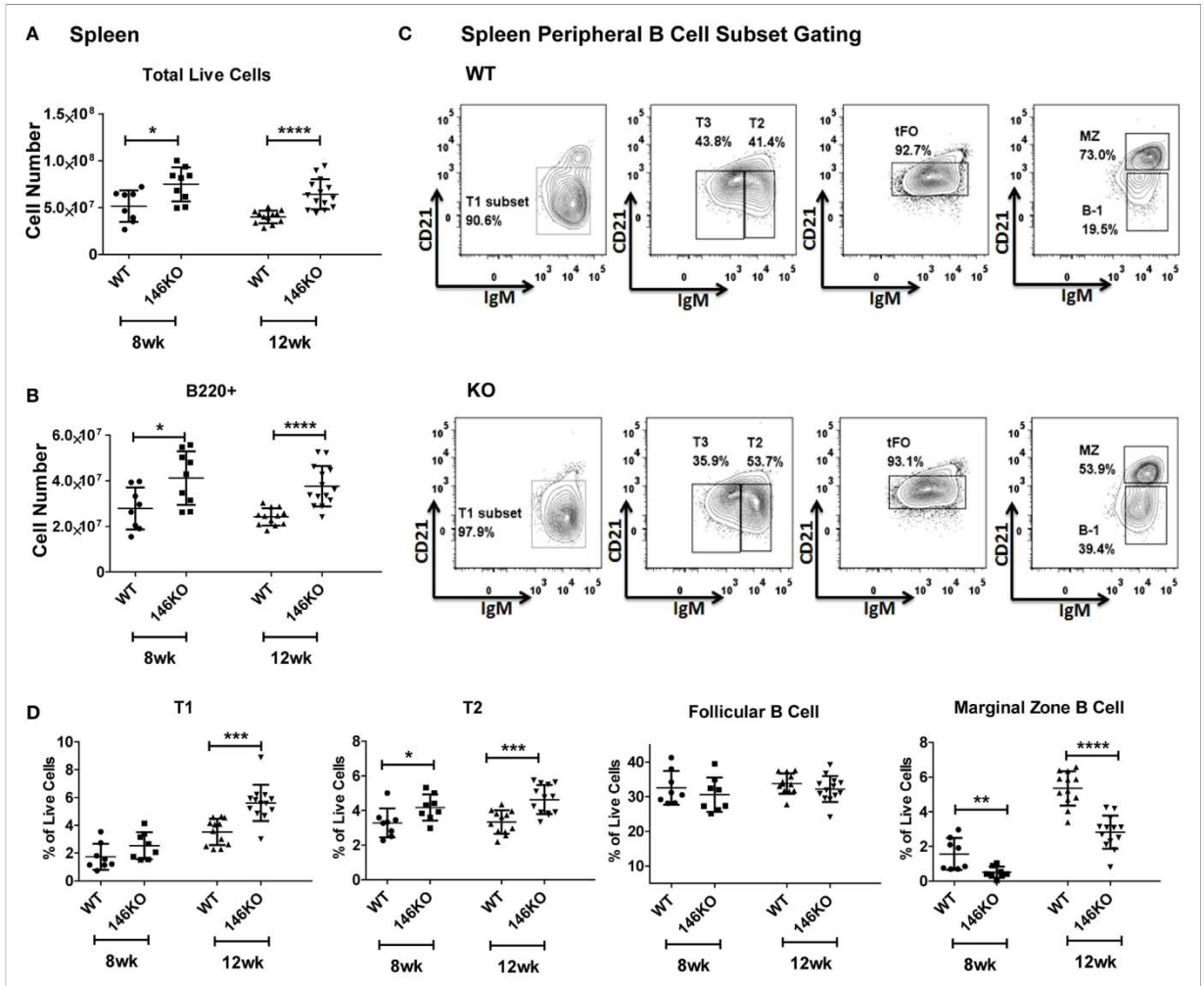

FIGURE 1 | *Mir146a*$^{-/-}$ mice exhibit an increase in total splenic B-cells and immature splenic subsets, except exhibit a defect in marginal zone (MZ) B-cells. Cell numbers of **(A)** total live cells (*$p = 0.015$, ****$p < 0.0001$) and **(B)** B220+ B-cells (*$p = 0.021$, ****$p < 0.0001$) are shown in young mice, ages 8 weeks ($n = 8$–9 mice/group) and 12 weeks ($n = 12$–14 mice/group). Values are mean ± SD. **(C)** Representative FACS plots show maturing B-cell subsets in spleens of wild-type (WT) mice and *Mir146a*$^{-/-}$ knockout (KO) mice at 12 weeks. **(D)** Analysis of spleen subsets from WT mice and KO mice are shown at early ages ($n = 12$–14/group). Increases in the transitional T1 (***$p = 0.0001$) and T2 (*$p = 0.043$, ***$p = 0.0003$) B cells are shown. MZ B-cells are decreased in KO at ages 8 weeks (**$p = 0.008$), 12 weeks (****$p < 0.0001$). Values are mean ± SD.

we examined hematopoietic stem cells (HSC), multipotent progenitors (MPP), lymphoid-primed multipotent progenitors (LMPP), early lymphoid (ELP), and common lymphoid (CLP) progenitors, and found no difference in young mice (Figures S4A,B in Supplementary Material), similar to prior work (16).

In addition, we explored the possibility that a defect in MZ B-cells in KO mice may have been due to a lack of retention of these cells in the splenic niche. However, examination of the peripheral blood (17) showed no difference in MZ B-cells between WT and KO mice (**Figure 3A**). Also, there were no differences in MZ B-cells in lymph nodes (**Figure 3B**). However, overall B220+ cells and FO cells were increased in KO vs. WT, as consistent with spleen findings. Furthermore, because it is known that MZ cells may differentiate into plasmablasts after interacting with antigens presented from macrophages, dendritic cells, or neutrophils, we examined this possibility. It is known that myeloid lineage cells in the spleen of KO mice are significantly increased in older KO mice (8, 12, 16). Hence, we performed FACS analysis for macrophages, dendritic cells, and neutrophils in young 12-week-old mice and found that they were similar in WT and KO at this early age (**Figure 3C**). Furthermore, plasmablasts were then assessed in WT and KO spleens, and also following stimulation with LPS, CpG, or CD40 + IL4, and were not statistically different (**Figure 3D**). Together with a lack of significant changes in

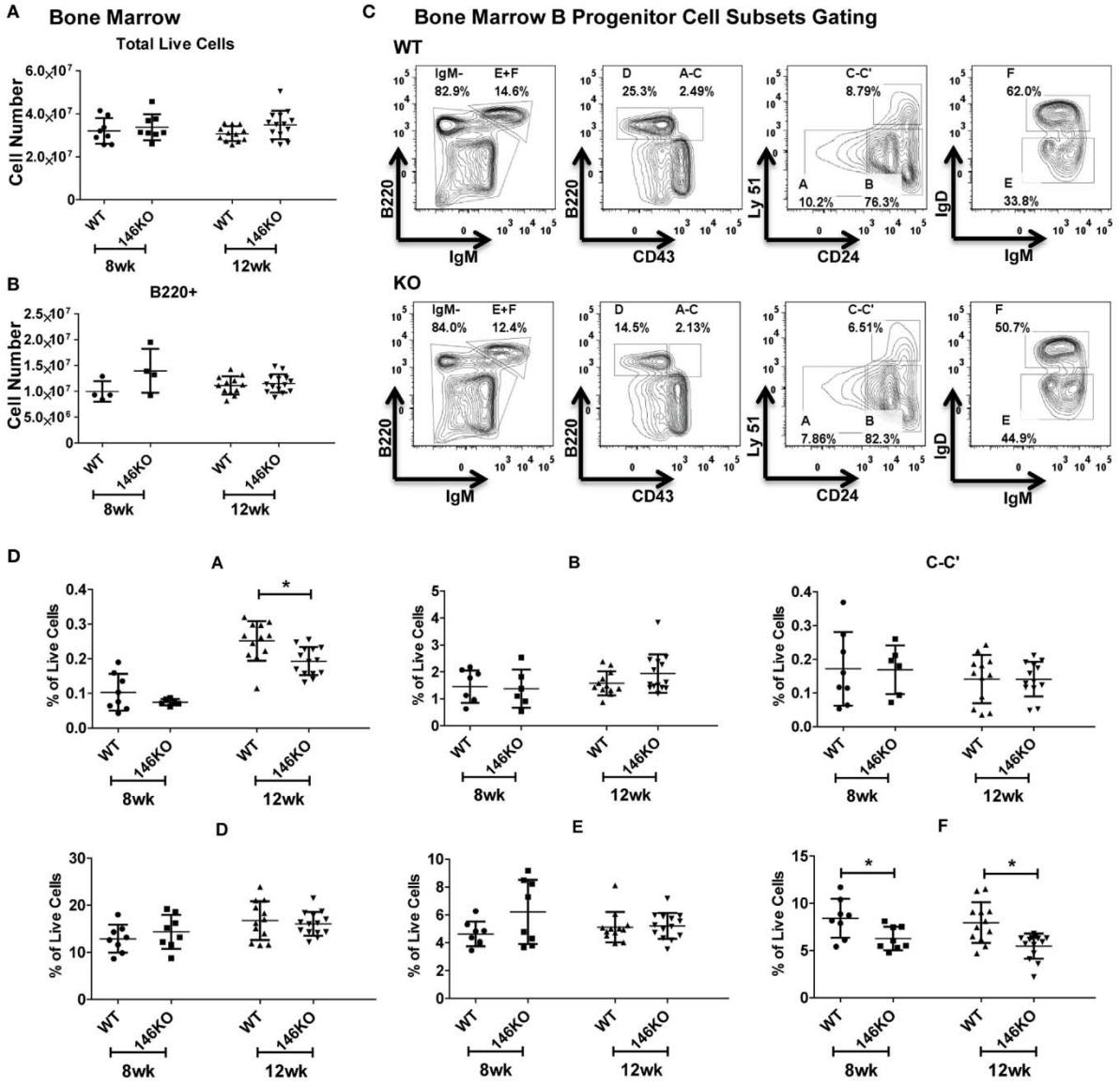

FIGURE 2 | *Mir146a*$^{-/-}$ mice show intact bone marrow B-cell development. Cell numbers of **(A)** total live cells (p = nss) and **(B)** B220+ B-cells (p = nss) are shown in young mice, ages 8 weeks (n = 8 mice/group) and 12 weeks (n = 12–14 mice/group), respectively. Values are mean ± SD. **(C)** Representative FACS plots show bone marrow B progenitors cells (Hardy Fractions) in bone marrow of WT (top panel) and KO mice (bottom panel) at 12 weeks. **(D)** Analysis of B progenitor cell subsets from WT and KO mice at early ages (8 and 12 weeks). Fraction A (*p = 0.006), fraction F at 8 weeks (*p = 0.025) and 12 weeks (*p = 0.001). Values are mean ± SD.

bone marrow development, these findings suggest a local spleen intrinsic defect leading to decreased MZ B-cell development.

Next, we explored whether the decrease in MZ B-cells was related to a developmental defect or due to an altered proliferation and/or apoptosis of MZ B-cells. Using intracellular Ki67 staining in conjunction with staining for B-cell developmental subsets, we determined that the proportion of Ki67 positive cells was statistically unchanged between WT and KO MZP and MZ B-cells (**Figure 3E**). Similarly, Annexin V staining revealed no statistically significant differences in apoptosis of these B-cell subsets (**Figure 3F**). In summary, these data strongly support the idea that the defect in MZ B-cells in *Mir146a*$^{-/-}$ mice is due to a developmental block in maturation of T2 cells into MZ B-cells.

miR-146a-Deficient B-Cells Exhibit Altered Gene Expression during Maturation from Transitional to MZ Stages

To dissect the mechanistic basis of the observed MZ B-cell block in development, we undertook gene expression analyses of the splenic B-cell subsets. First, we examined whether miR-146a was differentially expressed in different splenic B-cells. Indeed, RT-qPCR revealed that MZ B-cells had the highest levels of miR-146a expression, with expression noted in all subsets (**Figure 4A**). Next, we performed RNA-Seq on WT and KO transitional (T1 and T2), MZ and FO B-cell subsets with

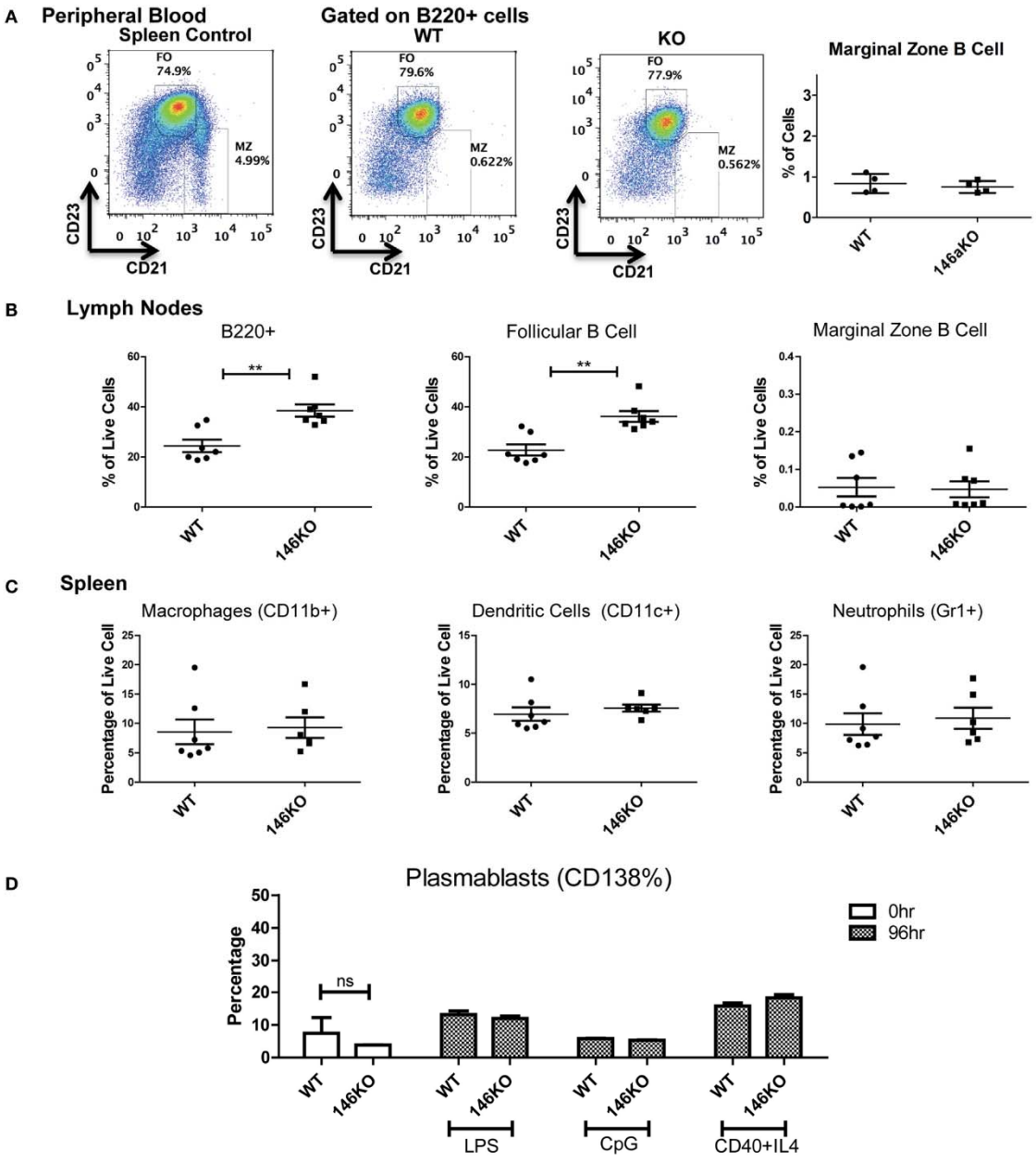

FIGURE 3 | Continued

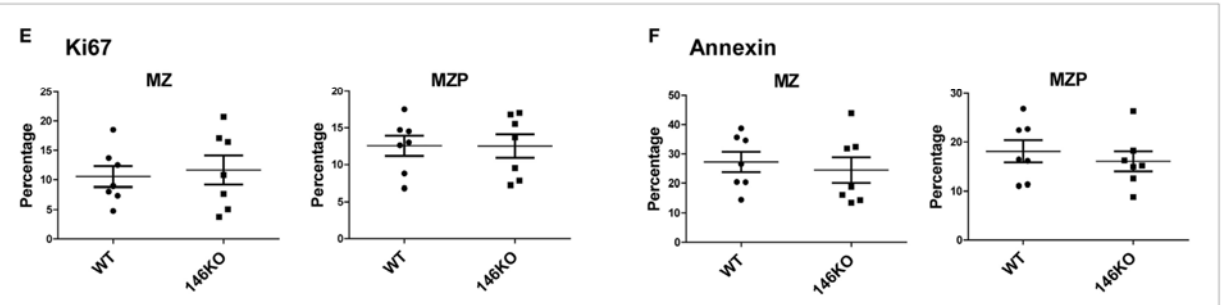

FIGURE 3 | Lower *Mir146a*$^{-/-}$ marginal zone (MZ) B-cells are likely due to a spleen intrinsic developmental block. **(A)** Peripheral blood B220+ cells show no statistically significant difference in circulating MZ or follicular cells ($n = 4$ mice/group). **(B)** Mature B-cell subsets are shown in peripheral lymph nodes (B220+ $^{**}p = 0.002$; FO $^{**}p = 0.001$) ($n = 7$ mice/group). **(C)** Splenic macrophages, dendritic cells, and neutrophils show no differences in WT vs. KO ($n = 7$ mice/group). **(D)** CD138+ plasmablasts are shown in unstimulated spleens (0 h) and 96 h poststimulation with CD40 + IL4, with no significant differences (repeated in duplicate). **(E)** Intracellular Ki67 and **(F)** Annexin V staining are shown for MZ and MZ precursor (MZP) cells ($n = 7$ mice/group).

biological replicates. Surprisingly, when we directly compared the corresponding B-cell stages between the WT and KO mice, only three to six genes showed differential expression. Of this limited group of differentially expressed genes, none were predicted miR-146a targets. The limited numbers of differentially expressed genes using direct comparison of subsets led us to consider alternate approaches. Given that there are very significant changes occurring in gene expression as a T2 cell commits to a MZ fate, we hypothesized that miR-146a alters the set of genes that are changing during this cell fate commitment. To this end, we utilized an alternative analysis comparing transitions between developmental stages of immune cells, similar to an approach that has been previously described (18). Interestingly, the transition from T2 to FO B-cells showed only 57 or 25 differentially expressed genes in the WT and KO, respectively (**Figure 4B**). In contrast, the T2 to MZ transition showed 1,270 or 1,183 differentially expressed genes in WT and KO, respectively (**Figure 4B**), indicating a large change in gene expression needed for commitment to the MZ over the FO fate. Focusing on the T2 to MZ transition, we compared the differentially expressed genes in WT and KO transcriptomes (**Figure 4C**). WT and KO mice showed 1,004 differentially expressed genes in common during the T2 to MZ transition; in addition, there were 266 differentially expressed genes unique to WT mice and 179 unique to KO mice. These unique gene subsets formed two clusters that either increased or decreased in expression during the T2 to MZ transition (**Figure 4D**). Interestingly, the subset unique to the KO mice showed the majority of genes increasing, although these genes as a class were not enriched for predicted miR-146a targets (data not shown).

Notch2 Activity Is Diminished in miR-146a-deficient MZ B-Cells

To further examine what genes may be changing, we analyzed transcriptional targets of pathways known to play a role in MZ B-cell differentiation, namely, *Notch2*, *BAFF*, and *NFκB* (**Figure 4E**). Several known transcriptional targets of the *Notch2* pathway showed lesser fold change of reads per kilobase of transcript per million mapped reads (RPKMs) between T2 and MZ cells in KO compared to WT mice (**Figure 3E**, red). On the other hand, *BAFF*-associated genes (gray) and *NFκB*-associated genes (blue) did not show a consistent change toward reduced or increased fold change at the T2–MZ transition between WT and KO. Because the RNA-Seq data demonstrated decreased expression of *Notch2* targets in KO MZ B-cells, we further validated these findings by examining *Notch2* transcriptional targets by RT-qPCR. First, we used bacterial lipopolysaccharide (LPS) to strongly induce miR-146a expression (9, 16) and screened for *Notch2* transcription targets using RT-qPCR at 72 h poststimulation. RT-qPCR revealed that KO B-cells showed significantly decreased expression of *Notch2* transcriptional targets *Hes1*, *Hes5*, and *Dtx4* (**Figure 4F**) (19, 20). We then examined expression of these candidate genes in primary splenic B-cell subsets, revealing reductions of *Hes1* and *Hes5* and a trend toward reduced *Dtx4*, specifically in MZ B-cells lacking miR-146a (**Figure 4G**). Together, these data suggest that KO mice have an altered gene expression profile during the T2–MZ transition, particularly downstream of *Notch2*, corresponding with the increased expression of miR-146a in the WT MZ population.

Interestingly, *Notch2* itself showed a mild but significant reduction at the mRNA level in KO MZ B-cells (**Figure 5A**). To characterize the effect of miR-146a deficiency on Notch2 protein, we utilized flow cytometry analysis. Notch2 is initially expressed as a surface receptor, and is internalized, cleaved, and endocytosed upon activation. Cell-surface expression of the transmembrane Notch2 receptor was not altered in the absence of miR-146a (Figure S5 in Supplementary Material). Subsequently, we examined intracellular expression of Notch2 in WT mice, noting that the highest expression of intracellular Notch2 was found in MZ and MZ precursor cells (**Figure 5B**). In line with a downstream effect on Notch2-mediated transcription, intracellular staining for Notch2 showed a significant decrease in KO MZ and MZP B-cells (**Figures 5C,D**). We then confirmed these findings *via* Western Blot. To obtain enough protein for analysis, we used LPS to stimulate the WT and KO bulk B-cells and found Notch2 protein was decreased in KO compared to WT cells at 72 h poststimulation (**Figure 5G**).

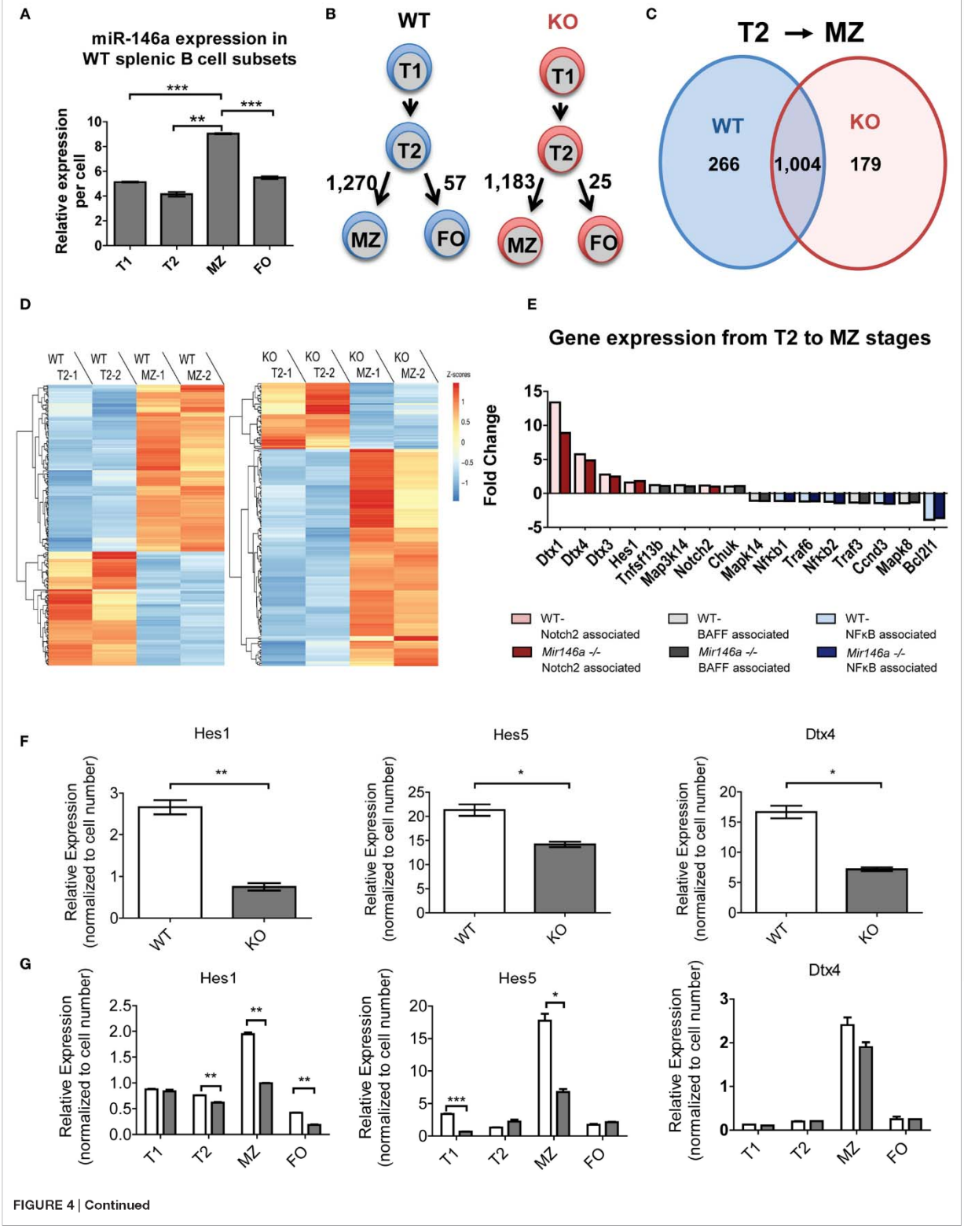

FIGURE 4 | Continued

FIGURE 4 | Continued

RNA sequencing (RNA-Seq) reveals differentially expressed miR-146a-dependent genes at the T2 to MZ transition. (A) Representative RT-qPCR showing relative expression levels of miR-146a in WT T1, T2, MZ, and FO splenic B-cell subsets per cell. (T1-MZ ***$p = 0.0002$; T2-MZ **$p = 0.0015$; MZ-FO ***$p = 0.0009$; repeated in duplicate). Values are mean ± SEM. **(B)** Schematic diagram depicting the number of differentially expressed genes between the T2 to MZ and T2 to FO transitions in both WT and KO mice. **(C)** Venn diagram comparing and contrasting the number of genes unique to and shared between the T2 to MZ transition in WT and KO mice. **(D)** Heat maps from the RNA-Seq data showing differentially expressed genes unique to the T2 to MZ transition in WT vs. genes unique to the T2 to MZ transition in KO (−1 and −2 denote replicates). **(E)** A waterfall plot of the RNA-Seq data displaying notable genes in three signaling pathways influencing MZ B-cell development. **(F)** Representative RT-qPCR analysis of Notch2-associated genes in bulk splenic B-cells 72 h after LPS stimulation repeated in duplicate (*Hes1* **$p = 0.0099$; *Hes5* *$p = 0.032$; *Dtx4* *$p = 0.013$). **(G)** Quantified relative expression of Notch2-associated genes in splenic B-cell subsets using RT-qPCR (Hes1 T2 **$p = 0.0082$, MZ **$p = 0.0014$, FO **$p = 0.0019$; Hes5 T1 ***$p = 0.001$, MZ *$p = 0.011$; repeated in duplicate).

A similar analysis for NFκB transcriptional targets (Figures S6A,B in Supplementary Material) and for two known miR-146a targets, *Traf6* and *Irak1* (Figures S6C–F in Supplementary Material) (8, 9) failed to show statistically significant differences. In addition, a gene set enrichment analysis (GSEA) of the T2 to MZ differentially expressed genes in KO did not show enrichment in NFκB target genes (data not shown). Together, these data demonstrate that the decrease in MZ B-cells is most likely due to the reduction in *Notch2* activity and that miR-146a regulates the levels of Notch2.

Numb, a Regulator of the Notch2 Pathway, Is a Direct Target of miR-146a

Previous studies have indicated that the adaptor protein Numb, a negative regulator of Notch, contains a predicted miR-146a-binding site within its 3′-UTR, but direct targeting has been observed by some groups, but not others (21, 22). One of the canonical functions of Numb is to inhibit Notch signaling by recruiting ubiquitin ligases to degrade cell surface-associated and intracellular Notch proteins (23). Numb may also directly bind to the notch intracellular domain (NICD) in the cytoplasm, thus preventing translocation into the nucleus and subsequent downstream transcription of *Hes1*, *Hes5*, and *Hey1* (19, 24). Here, we hypothesized that if Numb is derepressed in KO MZ B-cells, it may lead to degradation of Notch2 and subsequent inhibition of the gene expression program that is required for MZ B-cell development. Using intracellular FACS staining, we found that Numb was more highly expressed in KO transitional T2 cells (**Figures 5E,F**). Hence, this increase in Numb expression in the T2 subset developmentally precedes the decrease in *Notch2* expression in MZP and MZ B-cells, but the reciprocal relationship was not observed in the same cell types (**Figures 5C,D**). We then confirmed that Numb protein was increased by Western Blot in KO LPS-stimulated B-cells, consistent with a lack of repression by miR-146a (**Figure 5G**).

As mentioned above, the *Numb* 3′-UTR contains predicted miR-146a binding sites (**Figure 5H**). To assess direct repression of *Numb* by miR-146a, we cloned a ~1,300-bp fragment of the *Numb* 3′-UTR into the pmiRGlo luciferase reporter. Repression of the luciferase reporter was observed with co-transfection of miR-146a, and this repression was abolished upon mutation of the miR-146a seed sequence (see Materials and Methods). Of note, although *Notch2* also contained a predicted miR-146a-binding site within its 3′-UTR, luciferase activity was not repressed (**Figures 5H,I**). Together, our findings indicate that *Numb* is a direct target of miR-146a. Downstream, there is inhibition of Notch2 and the gene expression program required for MZ B-cell development. The relationship between miR-146a-mediated inhibition of Numb and Notch2 regulation by Numb requires further study.

miR-146a Regulation of MZ B-Cell Development Is T-Cell Independent

Given that T cells show profound abnormalities in the *Mir146a*$^{-/-}$ mice, another possibility is that there is a T-dependent pathway that leads to increased numbers of FO cells and decreased numbers of MZ B-cells. To test this, we bred mice deficient in αβ T cell receptor (*TCR*β$^{-/-}$) onto *Mir146a*$^{-/-}$ mice to make double knockouts *Mir146a*$^{-/-}$, *TCR*β$^{-/-}$ (DKO), and examined splenic subsets. As expected, *TCR*β$^{-/-}$ and DKO showed higher number of B220+ cells (**Figure 6A**), given the deficiency of T cells skewing the immune populations. Hence, to compare between genotypes, we examined the percentage of B220+ cells for all given splenic subsets (i.e., T2, MZ shown). The T2 subset was similar in KO and DKO mice (**Figure 6B**), and we noted no differences in MZ subsets between *Mir146a*$^{-/-}$ and DKO, indicating that this defect was not T cell dependent (**Figures 6C,D**). Furthermore, when comparing DKO with *TCR*β$^{-/-}$ mice, DKO displayed statistically significant decreases in MZ cells, suggesting that the phenotype is indeed an effect of miR-146a in B-cells (**Figures 6C,D**). Taken together, our results indicate MZ deficit seen in miR-146a-deficient mice is likely B-cell intrinsic and is not dependent on the presence of hyperactivated T cells.

DISCUSSION

Here, we found that miR-146a deficiency has an effect on the differentiation of B-cells in the spleen. miR-146a is expressed more highly in MZ B-cells than in any other splenic B-cell subset, suggesting an important role in these cells. Indeed, the MZ B-cell subset in *Mir146a*$^{-/-}$ mice is markedly reduced. The T1 and T2 subsets that developmentally preceded MZ B-cells are higher in the spleen. Peripheral blood and lymphoid MZ B-cells are similar, suggesting that MZ splenic retention is intact. Furthermore, differentiation into plasmablasts is similar between KO and WT, and alterations in proliferation and apoptosis do not account for the MZ B-cell defect. Hence, we felt that this MZ B-cell abnormality arises from defective development of this subset within the spleen. Exploring the molecular mechanism of this block, we found a reduction in intracellular *Notch2* and its downstream

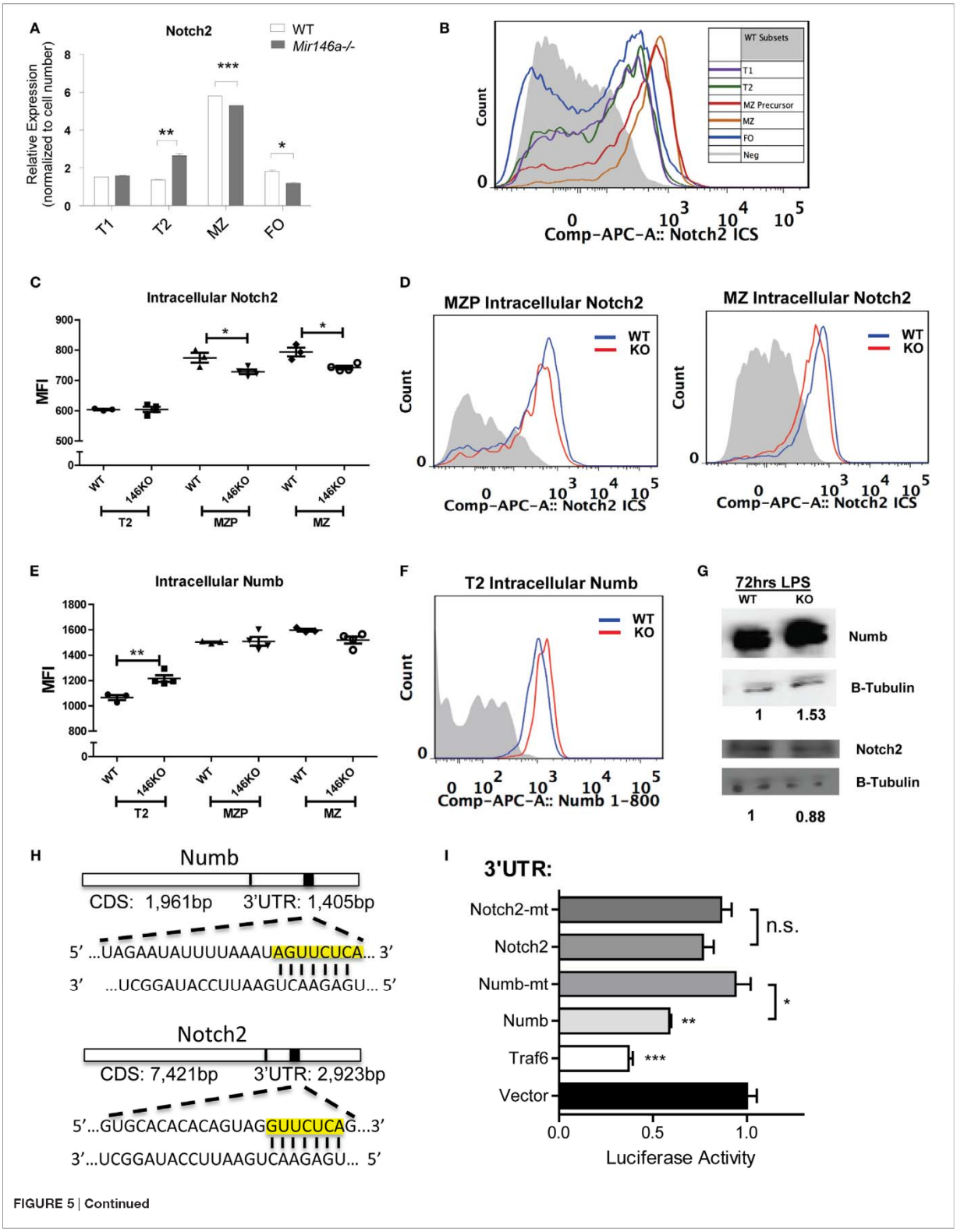

FIGURE 5 | Continued

FIGURE 5 | Continued

***Mir146a*$^{-/-}$ B-cells show decreased Notch2 and increased Numb, a direct target of miR-146a.** **(A)** Representative RT-qPCR quantification of Notch2 expression in splenic B-cell subsets in wild-type (WT) vs. KO (T2 $^{**}p = 0.0057$, marginal zone (MZ) $^{***}p = 0.0002$, FO $^{*}p = 0.016$; repeated in duplicate). **(B)** Representative FACS overlay of intracellular Notch2 expression in WT B-cell subsets. **(C)** Quantitation of median fluorescence intensity (MFI) of intracellular staining of Notch2 in T2, MZ precursors (MZP), and MZ B-cells (MZP $^{*}p = 0.035$; MZ $^{*}p = 0.014$; $n = 3$–4 mice/group, repeated in duplicate). **(D)** Representative overlapping histograms of Notch2 MZ subsets in WT vs. KO mice. **(E)** Quantitation of MFI of intracellular staining of Numb in T2, MZP, and MZ B-cells (T2 $^{**}p = 0.0083$; $n = 4$–5 mice/group). **(F)** Representative overlapping histogram of T2 Numb in WT vs. KO. **(G)** Western blot shows increased Numb protein and decreased Notch2 protein in LPS-stimulated splenic B-cells from WT and KO mice. β-Tubulin was used as a loading control and fold change was calculated using ImageJ (repeated in duplicate). **(H)** *Numb* 3′-untranslated region (UTR) and *Notch2* 3′-UTR containing the putative seed sequences of miR-146a (yellow box) are shown. **(I)** Luciferase assays quantitating repression with MGP/miR-146a relative to MGP alone for each of the UTRs depicted. Each measurement is representative of firefly luciferase normalized to renilla luciferase and was performed in triplicate, with the experiment repeated at least three times (*Traf6* vs. Vector, $^{***}p = 0.0004$; *Numb* vs. Vector, $^{**}p = 0.0016$; *Numb* vs. mutated *Numb*, $^{*}p = 0.012$; *Notch2* vs. mutated *Notch2*). All values are mean ± SEM.

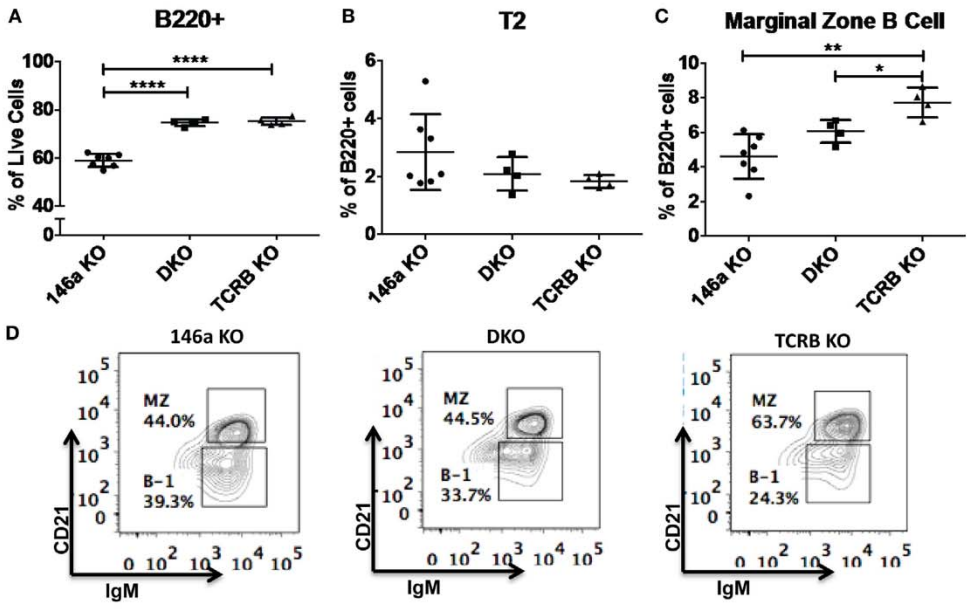

FIGURE 6 | Deficiency of marginal zone (MZ) B-cells in *Mir146a*$^{-/-}$ mice is not T cell dependent. Comparison of percentages of **(A)** total B220+ B-cells ($^{****}p < 0.0001$), **(B)** T2 subset, and **(C)** MZ B-cells ($^{**}p = 0.002$, $^{*}p = 0.022$) in *Mir146a*$^{-/-}$ (146a KO), *Mir146a*$^{-/-}$ *TCR*β$^{-/-}$ (DKO), and *TCR*β$^{-/-}$ (TCRβ KO) mice. All non-annotated comparisons are not statistically significant ($n = 4$–7 mice/group, repeated in duplicate). **(D)** Representative FACS plots showing the MZ fraction in spleen in three mice groups.

transcriptional targets, implicating miR-146a-mediated regulation of a pathway required for the development of MZ B-cells. In identifying this pathway, we utilized a novel method of analysis, where we compared developmental transition-related transcriptomic changes between WT and KO mice. This was productive, as it highlighted many more genes that were potentially important than a simple cross-comparison between WT and *Mir146a*$^{-/-}$ cells at a particular developmental change.

Because miR-146a is an important regulator of NFκB signaling, we wanted to understand whether the MZ B-cell phenotype that we observed is a consequence of hyperactivated NFκB. In myeloid and classic CD4+ and CD8+ T cells, miR-146a is induced by NFκB, and in turn targets the upstream adapter proteins Traf6 and Irak1, to cause negative feedback regulation of the NFκB response (8, 11). Thus, an overall increase in proinflammatory NFκB, as a consequence of loss of negative feedback, is responsible for many of the phenotypes seen in *Mir146a*$^{-/-}$ mice. Knocking out the classical NFkB p50 in these mice resulted in a rescue of the myeloproliferative phenotype, but did not completely rescue the autoimmune pathology (12) and only partially corrected hyperactivation of T cells (11). We did not find telltale increases in key NFκB transcriptional targets at the RNA level and did not observe changes in Irak1 or Traf6 protein. However, Notch2 was clearly affected. NFκB and Notch2 appear to cooperate in MZ B-cell development as demonstrated by heterozygous knockout Notch2$^{+/-}$ p50$^{+/-}$ mice which show loss of MZ B-cells without affecting the FO B-cell population (25). Interestingly, non-canonical NFκB pathway *via* Relb was also shown to be

important for development of MZ B-cells (26, 27). In our data set, NFκB response genes generally are downregulated at the T2-MZ transition, which would be consistent with miR-146a upregulation at that transition. However, we did not see direct evidence of these response genes or of known miR-146a targets (*Traf6 and Irak1*) being dysregulated in *Mir146a*$^{-/-}$ mice. This would indicate that the main thrust of miR-146a activity is not focused on this pathway in MZ B-cells. miR-146a likely targets different pathways in different immune cells/tissues. Indeed, it was shown that in T regulatory cells (Tregs), miR-146a is highly expressed and likely targets *Stat1* in the interferon gamma pathway (IFNγ) (13). Our own recent work demonstrated *Egr1* to be a novel target of miR-146a in Myc-driven B-cell tumors (14). Hence, a multiplicity of targets for miR-146a exists in different cell types and under different contexts.

Our data demonstrate that Numb is a possible novel target of miR-146a in B-cells. Numb is increased in miR-146a-deficient T2 cells, which immediately developmentally precede MZ cells. Although Numb is a known regulatory of Notch, the reciprocal relationship between Numb and Notch2 is not seen in the same cells. Hence, the connection between miR-146a, Numb, and Notch2 cannot be definitively made. Given our findings, we speculate that a combination of the cellular kinetics of differentiation, balance between production and degradation of intracellular Notch2, and asymmetric cell division mediated by Numb (24) may underlie the relationships that we have observed. Indeed, the rates of production and degradation of intracellular Notch2 may be dramatically different once a cell has committed to the MZ B-cell fate. However, teasing out the nature of these relationships will take additional work and effort.

We also found that the MZ phenotype is T cell independent, supporting a B-cell intrinsic mechanism for the MZ defect in *Mir146a*$^{-/-}$ mice. Other investigators have also endorsed the idea that MZ B-cell development is largely T-independent (2). In addition, the conditional knockout of Dicer in B-cells specifically led to an increase in MZ B-cells and decreased FO B-cells, suggesting that miRNAs can drive B-cell intrinsic phenotypes independent of T cells (6).

Our report demonstrates a specific B-cell subset abnormality in *Mir146a*$^{-/-}$ mice, and we have identified a likely novel target in the MZ B-cell lineage. Our future studies will focus on how *Mir146a*$^{-/-}$ B-cells contribute to the autoimmune phenotype, manifested by end-organ inflammation and production of anti-double-stranded DNA antibodies (8) characteristic of murine and human systemic lupus erythematosus (SLE). As the function of MZ B-cells in responses to specific antigen types has been postulated, it will be important to determine how this reduction in MZ B-cells, and the corresponding increase in FO cells, have functional consequences in tilting the balance toward clinical autoimmunity. Interestingly, miR-146a is expressed at low levels in peripheral blood mononuclear cells (PBMC) from patients with SLE (28) and treatment with chemically modified miR-146a agonists improved pulmonary hemorrhage in a pristane-induced lupus mouse model (29). Hence, it will be highly interesting to determine how miR-146a deficiency in B-cells contributes to autoimmune pathogenesis in murine models and ultimately in human disease.

ETHICS STATEMENT

All mouse studies were approved by the UCLA Chancellor's Animal Research Committee (ARC), the local IACUC body for UCLA.

AUTHOR CONTRIBUTIONS

JK designed studies, acquired data, performed analysis, and wrote the manuscript. NU and MP acquired data, performed analysis, and contributed to writing of manuscript. JC, MA, and TF performed data acquisition and analysis. KZ and MP performed data analysis. DR designed studies, performed interpretation and analysis, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00670/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

Regulation of marginal zone B-cell differentiation by microRNA-146a

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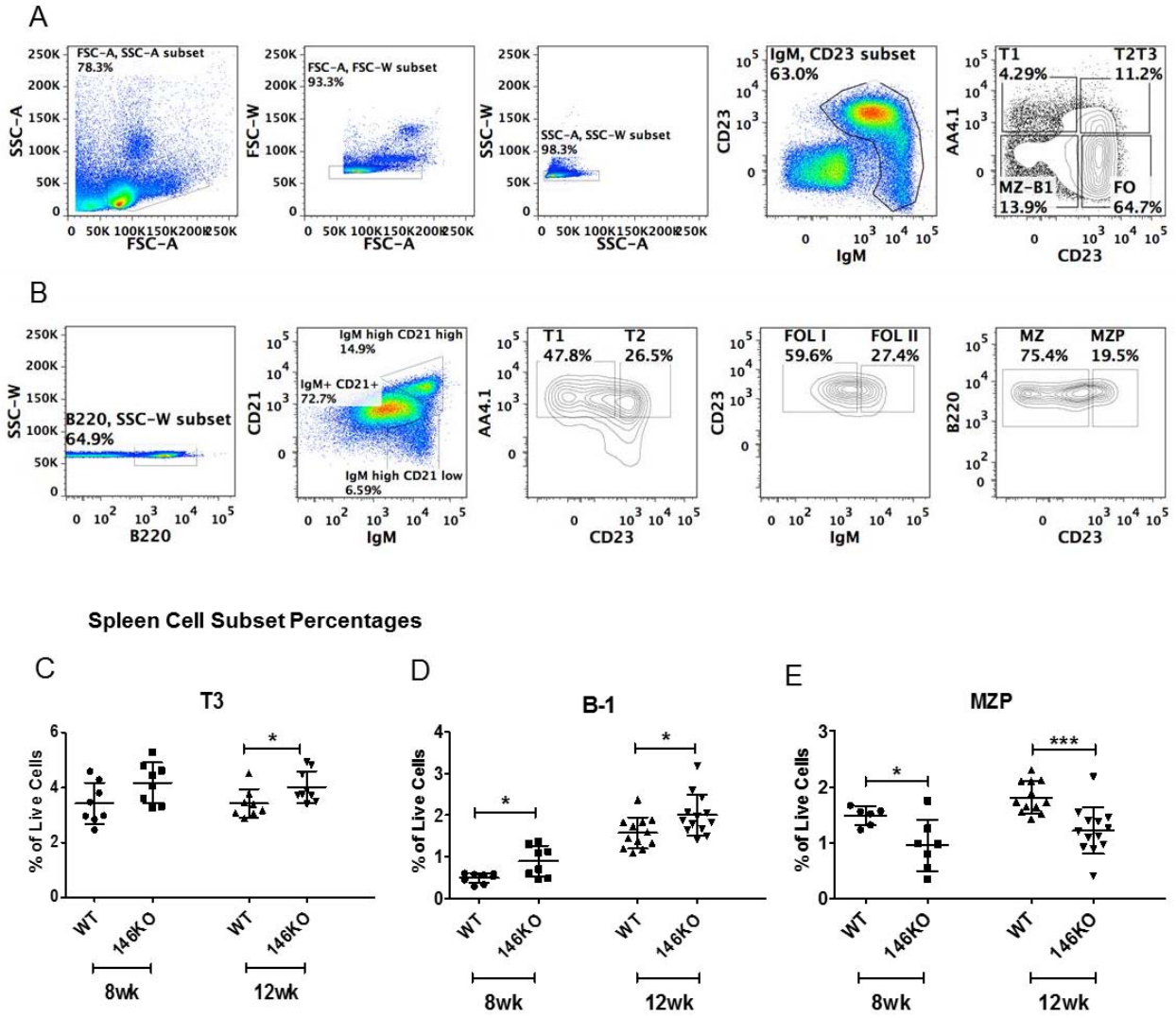
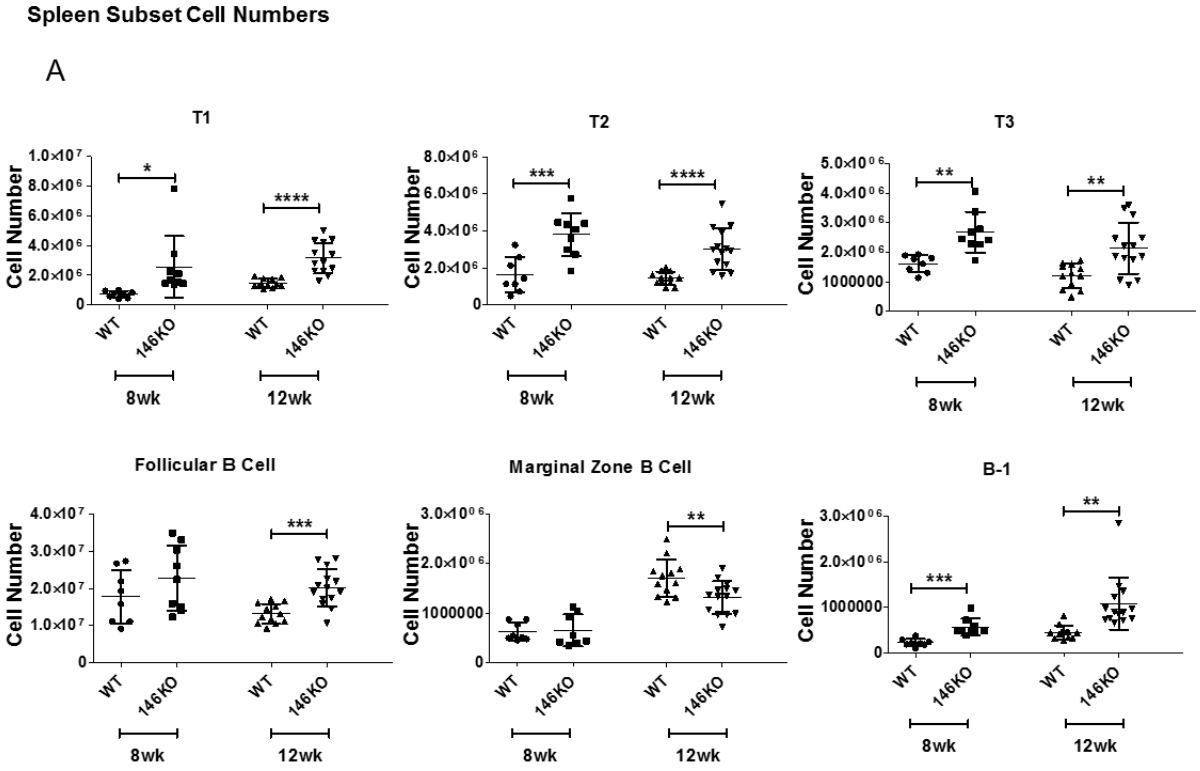
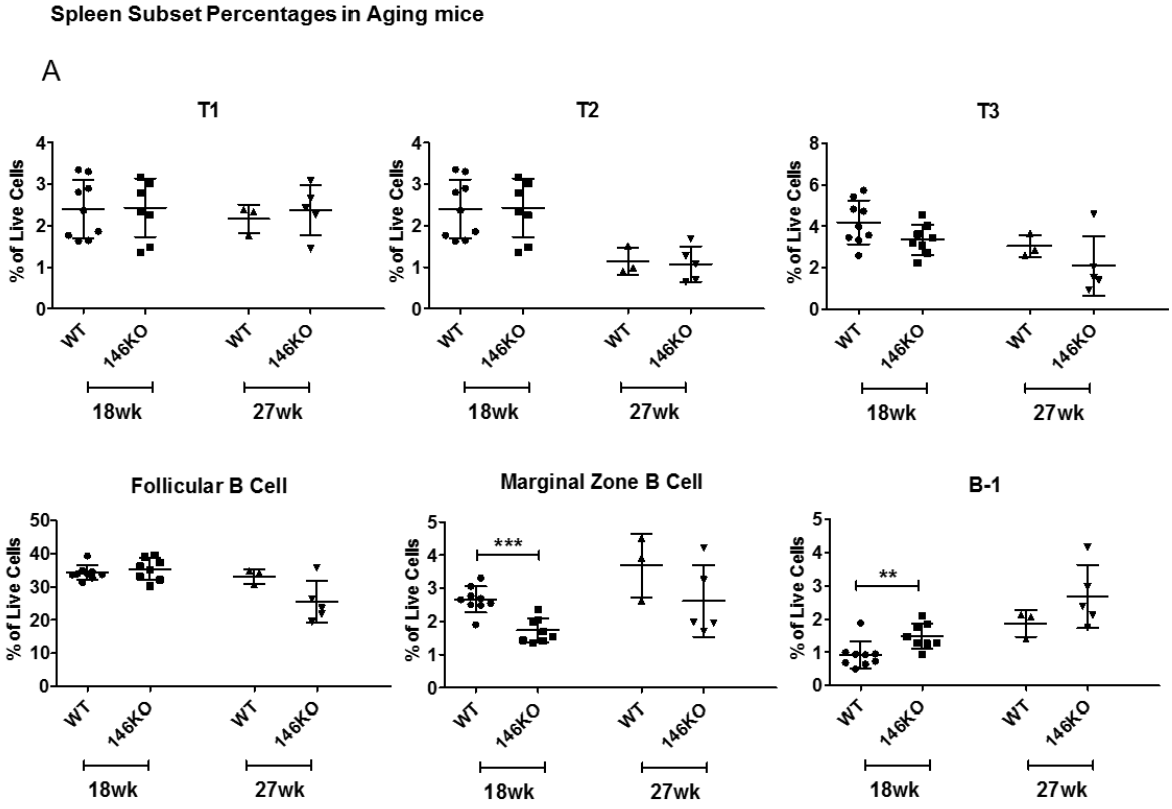


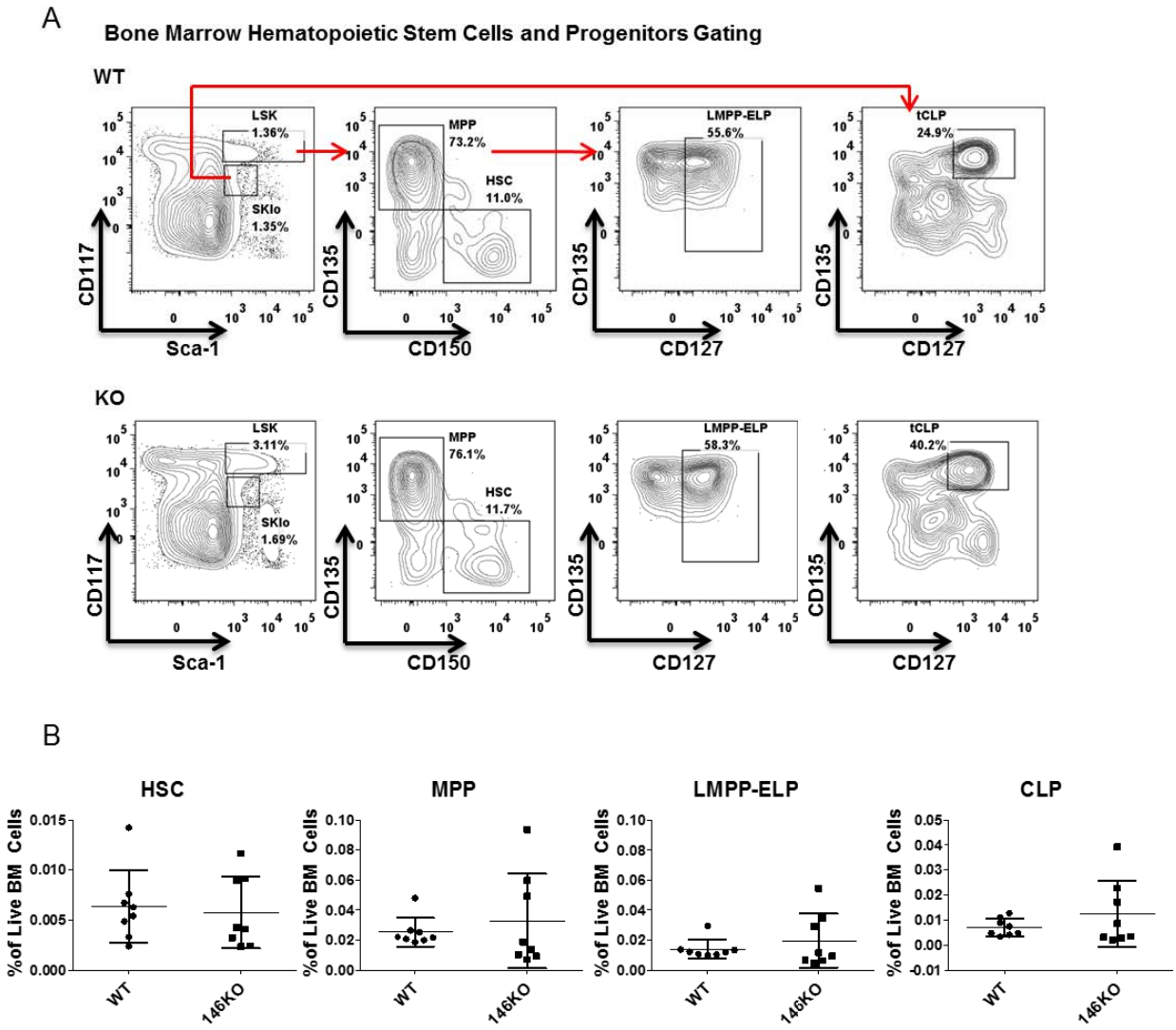
Supplement 1: *Mir146a*$^{-/-}$ mice exhibit increasing level of transitional precursors and B1 cells at a young age, but decreased marginal zone precursor cells. (A) Initial pre-gating FACS plots for spleen are shown. (B) From the SSC-A, SSC-W subset, an alternative gating strategy is shown to evaluate marginal zone precursor (MZP) cells. (C) Spleen transitional cell subset 3 (T3) show trend in 8 week *Mir146a*$^{-/-}$ knockout (KO) mice, and increased percentage of T3 cells at 12 weeks (*p=0.039; n=8-9 mice/group). (D) Splenic B-1 cells are increased in KO mice at 8 weeks (*p=0.012) and 12 weeks (*p=0.022). n= 8-13 mice/group. (E) Splenic MZP cells are decreased in KO mice at 8 weeks (*p=0.0234) and 12 weeks (***p=0.0004; n= 6-13 mice/group). All values are mean + SD.



Supplement 2: *Mir146a*$^{-/-}$ mice have increased cell numbers in all splenic subsets, except marginal zone B cells at a young age. (A) Cell numbers of splenic subsets in 8 week (n=8 mice/group) and 12 week mice (n=12-13 mice/group) respectively are increasing in T1 (*p=0.0253, ****p<0.0001), T2 (***p=0.0007, ****p<0.0001), T3 (**p=0.001, **p=0.002), follicular (***p=0.0003), B1 (***p=0.0003, **p=0.001) cells. In contrast, marginal zone B cells are similar in 8 week-old mice or decreased in 12 week old mice (**p=0.0087) in spite of overall B cell increase in spleen. All values are mean + SD.

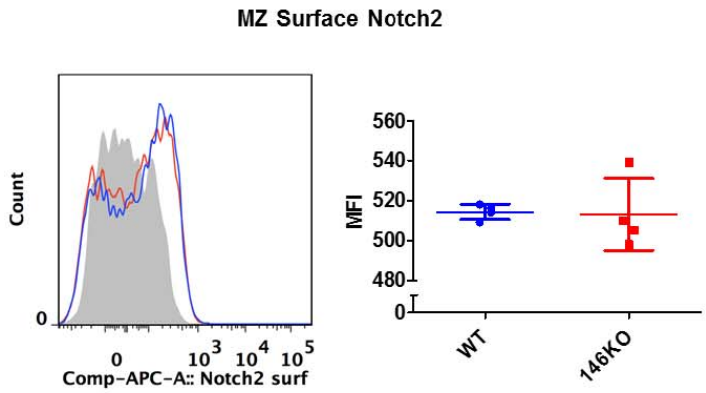


Supplement 3: Age-related *Mir146a*$^{-/-}$ mice inflammatory phenotype leads to decline in splenic B cells compartment. (A) Spleen subsets are shown in mice 18 weeks (n= 8-9 mice/group) and 27 weeks (n=3-5 mice/group) old showing normalization of percentages in T1, T2, T3 and follicular cells. By 27 weeks, the B-1 compartment is also similar between KO and WT. Marginal zone B cells continue to be decreased in KO mice compared to WT up through 18 weeks (***p=0.0002). However by 27 weeks, all B cell subsets show similar percentages in the spleen. All values are mean + SD.

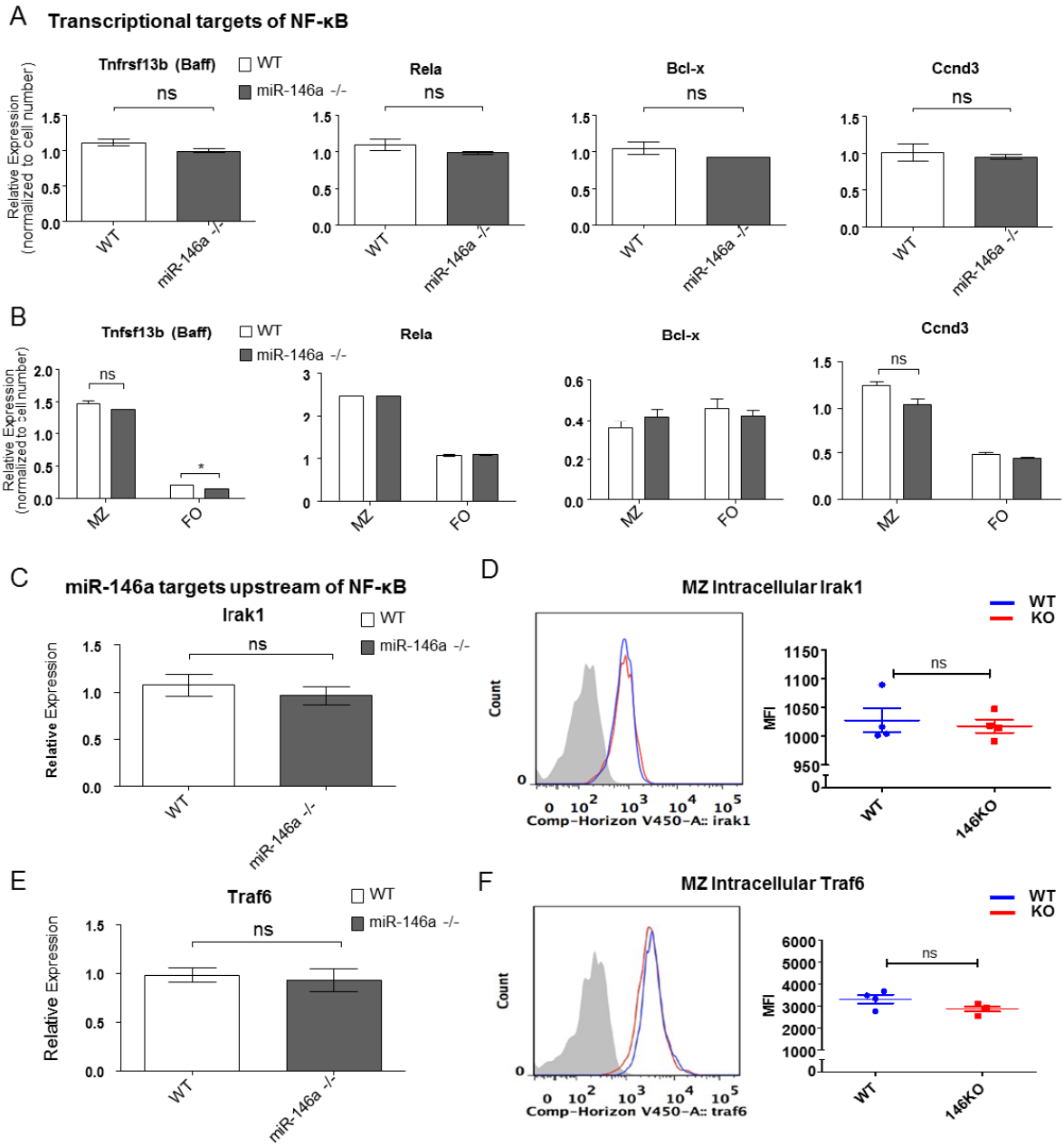


Supplement 4: *Mir146a*$^{-/-}$ mice show no differences in hematopoietic stem cell and stem cell progenitor cells at a young age. (A) Representative FACS plots of hematopoietic stem cells (HSC), multipotent progenitors (MPP), lymphoid-primed multipotent progenitors (LMPP), early lymphocyte progenitors (ELP), and common lymphoid progenitor (CLP). (B) Percentages of live cells of HSC and progenitor cells shown are not different between WT and KO mice at 8 weeks of age. All values are mean + SD.

A



Supplement 5: *Mir146a*$^{-/-}$ and WT mice have similar levels of surface Notch2 expression. (A) Representative FACS overlay of KO vs. WT surface Notch2 expression (left panel). Quantification of MFI surface Notch2 (right panel) show no difference (n=4mice/group).



Supplement 6: Marginal zone B cell defect is not due to a lack of NF-κB. (A) Representative RT-qPCR analysis of NF-kB associated genes in bulk splenic B cells 72hrs after LPS stimulation. Repeated in duplicate. (ns = not significant) (B) Quantified relative expression of NF-κB associated genes in splenic B cell subsets using RT-qPCR (FO *p=0.02; repeated in duplicate). (C) RT-qPCR analysis of Irak1 in bulk splenic B cells 72hrs after LPS stimulation from WT and KO mice (repeated in duplicate). (D) FACS of MZ B cells as an overlapping histogram (left panel) and quantitation of median fluorescence intensity (MFI) (right panel) of intracellular staining of Irak1 (n=4 mice/group). (E) RT-qPCR analysis of Traf6 expression in bulk splenic B cells 72hrs after LPS stimulation from WT and KO mice (repeated in duplicate) (F) FACS of MZ B cells as an overlapping histogram (left panel) and quantitation of median fluorescence intensity (MFI) (right panel) of intracellular staining of Traf6 (n=4 mice per group). All values are mean + SEM.

Supplementary Table 1: A list of antibody stains for FACS analysis.

Bone Marrow B Progenitor Cell Subsets		
Antigen	**Fluorochrome**	**Company**
B220 (RA3-6B2)	PerCP-Cy5.5	BioLegend
IgM	PE	SouthernBiotech
CD43 (S7)	APC	BD Biosciences
CD24 (M1/69)	PECy7	eBioscience
IgD (11-26c.2a)	APC-Cy7	BioLegend
Ly51 (6C3)	Biotin	BioLegend
Streptavidin	APC-eFluor 780	eBioscience
Spleen Subsets		
Antigen	**Fluorochrome**	**Company**
B220 (RA3-6B2)	PerCP-Cy5.5	BioLegend
IgM	PE	SouthernBiotech
CD21 (7E9)	FITC	BioLegend
CD23 (B3B4)	PECy7	BioLegend
CD93 (AA4.1)	APC	eBioscience
Bone Marrow Hematopoietic Stem Cell and Progenitor Cells		
Antigen	**Fluorochrome**	**Company**
CD150 (TC15-12F12.2)	PE	BioLegend
Sca1 (D7)	PerCP-Cy5.5	BioLegend
CD127 (A7R34)	PE-Cy7	BioLegend
CD135 (A2F10)	APC	eBioscience
CD117 (2 B8)	APC-Cy7	BioLegend
CD8a (53-6.7), B220 (RA3-6B2), TCR β (H57-597) , TCR γ/δ (GL3), NK1.1 (PK136), Gr1 (RB6-8C5), Ter119 (TER-119)	Biotin	BioLegend
CD4 (GK1.5), IgM (II/41)	Biotin	eBioscience
Streptavidin	eFluor 450	eBioscience
Intracellular Staining		
Antigen	**Fluorochrome**	**Company**
Notch2 (HMN2-35)	APC	BioLegend
Numb (polyclonal)	APC	Novus
IRAK1 (D51F7)		Cell Signaling
TRAF6 (EP591Y)		Abcam
IgG (Poly4064)	Brilliant Violet	BioLegend

Supplementary Table 2: Hematopoietic Cell Subset Definitions

Bone Marrow B Progenitor Cell Subsets	**Surface Marker Identification**
A	$B220^{+}$ $CD43^{+}$ $CD24^{-}$ $Ly51^{-}$
B	$B220^{+}$ $CD43^{+}$ $CD24^{+}$ $Ly51^{-}$
C	$B220^{+}$ $CD43^{+}$ $CD24^{+}$ $Ly51^{+}$
D	$B220^{+}$ $CD43^{-}$ IgM^{-}
E	$B220^{+}$ $CD43^{-}$ IgM^{+}
F	$B220^{++}$ $CD43^{-}$ IgM^{+} IgD^{+}
Spleen subsets	**Surface Marker Identification**
T1	$CD23^{-}$ $AA4^{+}$ IgM^{high} $CD21^{-/low}$
T2	$CD23^{+}$ $AA4^{+}$ IgM^{high} $CD21^{low}$
T3	$CD23^{+}$ $AA4^{+}$ IgM^{low} $CD21^{low}$
Follicular B cell	$CD23^{+}$ $AA4^{-}$ IgM^{low} $CD21^{int.}$
Marginal Zone B cell	$CD23^{-}$ $AA4^{-}$ IgM^{high} $CD21^{high}$
Marginal Zone B cell Precursor	$CD23^{+}$ $AA4^{-/low}$ IgM^{high} $CD21^{high}$
Bone Marrow Hematopoietic Stem Cell and Progenitor Cells	**Surface Marker Identification**
Hematopoietic Stem Cell	Lin^{-} $CD117^{high}$ $Sca1^{high}$ $CD150^{+}$
Multipotent Progenitor	Lin^{-} $CD117^{+}$ $Sca1^{+}$ $CD135^{+}$
Lymphoid-Primed Multipotent Progenitor	Lin^{-} $CD117^{high}$ $Sca1^{high}$ $CD135^{+}$ $CD127^{-}$
Common Lymphoid Progenitor	Lin^{-} $CD117^{low}$ $Sca1^{low}$ $CD135^{+}$ $CD127^{+}$

Supplementary Table 3: A list of the primers used in the RT-qPCR analysis.

q-m-Ccnd3-F	CGAGCCTCCTACTTCCAGTG
q-m-Ccnd3-R	GGACAGGTAGCGATCCAGGT
q-m-bclx-F	GACAAGGAGATGCAGGTATTGG
q-m-bclx-R	TCCCGTAGAGATCCACAAAAGT
q-m-Tnfrsf13b-F	ATGGCATTCTGCCCCAAAGAT
q-m-Tnfrsf13b-R	ATGGTCGTAGTACCTGCCTTG
q-m-NFkB-F	ATGGCAGACGATGATCCCTAC
q-m-NFkB-R	TGTTGACAGTGGTATTTCTGGTG
q-m-RelA-F	AGGCTTCTGGGCCTTATGTG
q-m-RelA-R	TGCTTCTCTCGCCAGGAATAC
q-m-Irak1-F	ACTCCAGAGAAGTCCCAACCA
q-m-Irak1-R	CAGGAATGCAGGGTAGCAGAG
q-m-Hes5-F	AGTCCCAAGGAGAAAAACCGA
q-m-Hes5-R	GCTGTGTTTCAGGTAGCTGAC
q-m-Irf2-F	AATTCCAATACGATACCAGGGCT
q-m-Dtx3-F	CCCAGCTACGAGAAGTATGGC
q-m-Dtx3-R	TCAAATGCCTTTCGGAACAGG
q-m-Dtx1-F	TACATGCAGAAGGTGAAAAACCC
q-m-Dtx1-R	CGCCCTCATAGCCAGATGC
q-m-Dtx4-F	CGCCCAGTTCGACGAAACTA
q-m-Dtx4-R	TGATGCCGACTTCCATGTCATA
q-m-Tnfrsf13b-F	ATGGCATTCTGCCCCAAAGAT
q-m-Tnfrsf13b-R	ATGGTCGTAGTACCTGCCTTG
q-m-Notch2-F	GACTGCCAATACTCCACCTCT
q-m-Notch2-R	CCATTTTCGCAGGGATGAGAT
q-m-Hes5-F	ATGGCCCCAAGTACCGTGGCG
q-m-Hes5-R	GGTCCCGACGCATCTTCTCC

CHAPTER III:

"MicroRNA-146a regulates B-Cell Function and Autoimmunity"

Abstract

Previous studies have demonstrated the importance of microRNA-146a in negatively regulating the NF-kB inflammatory pathway response. The phenotypes in *Mir146a*-/- (KO) mice include activated T cell phenotypes (30), increased germinal center reactions (26), autoantibody secretion (i.e. dsDNA autoantibodies) (18), myeloproliferation, and malignancies (57), as a consequence of loss of feedback regulation via depression of miR-146a targets involved in the NfKB or IFN inflammatory pathways. Previous studies have described miR-146a's regulatory role in myeloid, T, and other immune cells, but a comprehensive characterization of its role in B cell function remains to be elucidated. **Our central hypothesis is that miR-146a acts as a negative regulator of B cell activation and function via NFkB and IFN signaling pathways.** Using *Mir146a*-/- (KO) mice, we will first characterize miR-146a dependent regulation of B cell activation and function *in vitro* and then *in vivo*. Then, to define mechanisms of gene expression regulation in miR-146a-dependent B cell activation, we use a hypothesis-based approach and discovery based approach to characterize miR-146a gene targets. Furthermore, to functionally characterize the role of these targets, genetic complementation studies using CRISPR/Cas9 gene editing system will be used to delete proposed targets in miR-146a deficient activated B cells. Taken together, the above ***ongoing*** studies will allow for both a cellular/organism level and molecular level understanding of miR-146a's role in B cell immune responses. The *Mir146a*-/- (KO) mice phenotypically resemble aspects of human autoimmune systemic lupus erythematosus (SLE), with its T cell hyperactivity, dysregulated T regulatory capacity, and characteristic production of autoantibodies against dsDNA. Hence, we translate our animal studies into functional human relevance by proposing to evaluate the expression of miR-146a in human SLE patients in B cells (vs. PBMCs) in relationship to disease activity using an established longitudinal cohort of human SLE patients. In synergy with our cellular and molecular studies, we hope our work will lead to a better understanding of SLE pathogenesis and disease activity.

Introduction

MicroRNAs (miRs), the short non-coding RNAs that principally act via post-transcriptional repression of target messenger RNAs, are important regulators of gene expression (2). To date, dysregulated expression of multiple microRNAs in autoimmune and malignant diseases have been identified, although the lack of defined function in pathogenesis has hindered advancements leading to direct clinical translation. Our laboratory and colleagues collective involvement in a description of miR-146a's functions are included in Table 1. However, the role of miR-146a in B cells remains to be better characterized. One study using constitutively expressed miR-146a transgenic mice found a significant reduction in *Fas* expression in germinal center (GC) B cells (68), but gain-of-function approaches are not always physiological. Given its high expression in CD19+ B cells (18), and in line with studies in other immune populations, we hypothesize that miR-146a regulates stimulated B cell activation and function. Hence, we use *Mir146a-/-* mice to characterize miR- 146a's effects on B cell cellular immunology, and subsequently utilize molecular genetics to describe function of miR-146a targets in B cell immune responses.

Immune Cell	Target	Biological Relevance
Monocyte/ Macrophage	*Traf6, Irak1; Irak2*	NF-κB pathway, RIG-1 pathway
T cell	*Traf6, Irak1; Icos; Fadd*	NF-κB pathway; T follicular helper differentiation; Act Induced Cell Death
Treg	*Stat1*	Interferon pathway
B cell	*Fas; Numb*	GC apoptosis; *Notch2 pathway
NK cell	*Stat1*	Interferon pathway
Human PBMC	*IRF5, STAT1*	Interferon pathway

Table 1. miR-146 cell-specific validated targets. GC = germinal center; * = self authorship

Materials and Methods

Mice. MiR-146a deficient (*Mir146a*$^{-/-}$) mice were generated as described previously (18). Wild type mice (C57B/6) were purchased from Jackson laboratories. For CRISPR/Cas9 experiments,

Rosa26-Cas9 knock-in mice were purchased from Jackson laboratories (stock 026179), which constitutively express *cas9*-EGFP (69). These c*as9-eGFP* mice were bred onto miR-146a deficient mice to create *Mir146a*$^{-/-}$ *cas9-eGFP* (M146Cas) mice. All mouse studies were approved by the UCLA Office of Animal Research Oversight.

Flow cytometry. Bone marrow, spleen, and blood were obtained from euthanized mice and red blood cell lysis buffer was used to lyse the single cell suspensions. For intracellular staining, cells were fixed in 2% paraformaldehyde at 37 degrees Celsius for 10 minutes, and then blocked with Fc block for 20 minutes. Then, they were stained in 1x PBS with 10% FBS and 0.1% Triton X-100 for 20 minutes in the dark, and subsequently washed with 1x PBS with 4% FBS. Flow cytometry was performed on a LSRII and data were analyzed using FlowJo software.

***In Vitro* B Cell Activation Assays.** *Mir146a-/-* and WT B-splenocytes were isolated via B220+ magnetic bead separation and culture them in standard media supplemented with toll-like receptor (TLR) ligands or cytokines (i.e. LPS 10ug/ml, CpG 1,000ng/ml, anti-CD40 5ug/ml + ILD-4 10ng/ml, and IgM 5ug/ml + IL-4). Cells were collected at 0, 12, 24, 36, 72, and 96 hours post-stimulation and analyzed. Standardized cell counts were obtained using BD Calibrite beads and calculated via FACS [(# of cells counted/# beads counted) x # of beads added to culture], which demonstrated consistency with manual hemocytometer counting. Supernatants were collected.

***In Vivo* Immunization and Memory B cell Assays.** KO and WT mice were immunized at 6-8 weeks of age with TNP-Ficoll (T cell independent antigen, 100ug) or TNP-KLH (T cell dependent antigen, 100ug) in complete Freund's adjuvant. Serum was collected at days 3, 7, 14, and 28 post-immunization and assessed for TNP-specific antibodies via ELISA. To test immunological memory, KO and WT mice were re-challenged Day 28 with TNP-Ficoll or TNP-KLH, and then examined for specific antibody secretion at day 1, 2, 7 post-re-challenge.

RNA sequencing (RNA-Seq) and analysis. Wild type and miR-146a$^{-/-}$ B-cells were stimulated with LPS or CpG, and collected at day 0, 1, 3, and 4. Total RNA was extracted from using Qiazol using the Qiagen miRNEasy Mini kit with additional on column DNAse I digestion. Following isolation of RNA, cDNA libraries were built using the Illumina TruSeq RNA Sample Preparation kit V2 (RS-122-2001). An Agilent Bioanalyzer was used to determine RNA quality (RIN >8) prior to sequencing. RNA-Seq libraries were sequenced at the Broad Stem Cell Research Center sequencing core (UCLA). Libraries were sequenced on an Illumina HiSeq 2000 (single-end 100bp). Raw sequence files were obtained using Illumina's proprietary software and will be available at NCBI's Gene Expression Omnibus. We first filtered out reads with low quality and reads containing sequencing adapters and then mapped raw reads to the mouse reference genome (UCSC mm10) with the gapped aligner Tophat allowing up to two mismatches. We supplied the UCSC mm10 gene model to Tophat as the reference genome annotation. Only reads uniquely aligned were collected. In total for all libraries sequenced, 365,022,996 reads were uniquely mapped (corresponding to an overall mappability of 91.7%) and used for further analysis. Transcript expression levels were quantified using RPKM units (Reads Per Kilobase of exon per Million reads mapped) using customized scripts written in Perl. Differential expression analysis was performed using both DESeq and edgeR in R (http://www.R-project.org). Raw read counts were used and modeled based on a negative binomial distribution. The multiple testing errors were corrected by the false discovery rate (FDR). We considered genes as differentially expressed if: 1) the FDR was less than 0.05; 2) the expression ratio between two time points was >2X; 3) the maximal RPKM value for at least one group in the comparison was >1; and 4) there was agreement between DESeq and edgeR.

RT-qPCR. RNA was collected from corresponding samples and reverse transcribed using the qScript reagent (Quanta Biosciences). RT-qPCR was performed with the StepOne Plus Real-

Time PCR System (Applied Biosystems) using PerfeCTa SYBR Green FastMix reagent (Quanta Biosciences) or TaqMan MicroRNA Assay (Life Technologies).

Cloning. Single guide RNAs (sgRNAs) were designed for potential miR-146a gene targets and cloned into an MSCV-based retroviral vector. This construct contains the RNA polymerase III U6 promoter (U6) that drives expression of the sgRNAs and scaffold sequence. Transduction will be marked by a mCherry reporter cassette driven under a separate RNA polymerase II promoter. The Cas9 will be supplied via *cas9-eGFP* mice (69), bred onto miR-146a deficient mice to create *Mir146a*$^{-/-}$ *cas9-eGFP* (M146Cas) mice as described.

Western blot. Samples from transduced cell lines were then lysed with RIPA buffer (Boston BioProducts) and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Equal amounts of protein lysate (quantification by bicinchoninic acid protein assay, BCA (Thermo Scientific) were separated using electrophoresis on a 10% SDS–PAGE and blotted on a nitrocellulose membrane.

Results

Mir-146a KO have higher cellular proliferation and activated phenotypes *in vitro* than WT with T-independent antigen stimulation

We utilize an *in vitro* B cell culture system to first examine the *in vitro* cellular phenotypes of B cells (B220+ cells) in *Mir146a-/-* vs. WT B-splenocytes (Figure 1A). We isolated bulk B cells via B220+ magnetic bead separation and cultured them in standard media supplemented with toll-like receptor (TLR) ligands or cytokines, which have been shown to induce miR-146a expression in human PMBCs or have relevance to NFκB and IFN inflammatory pathways. Our data show that miR-146a in B cells are highly induced during activation after 72 hours with

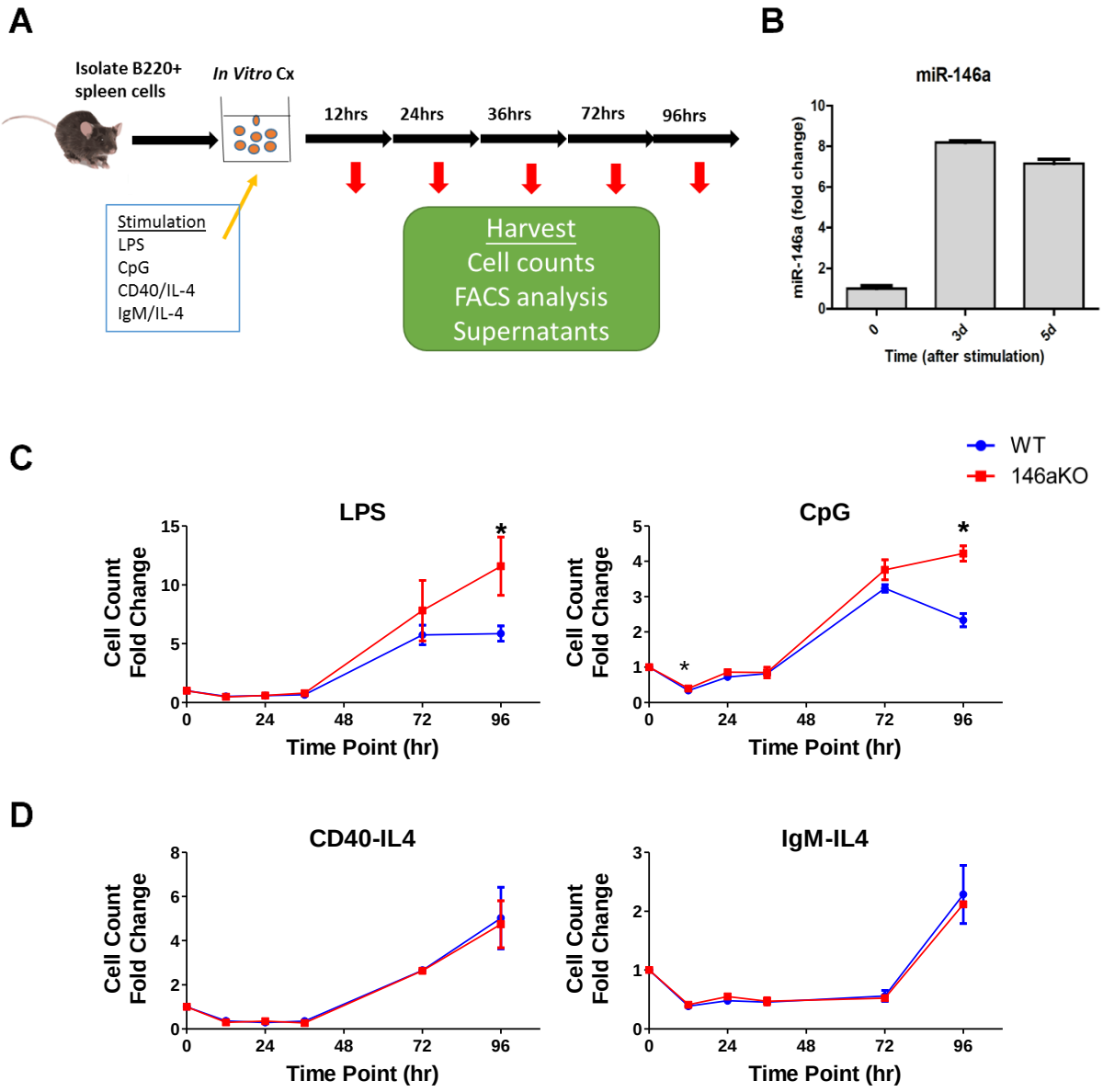


Figure 1. Mir-146a KO have higher cellular proliferation *in vitro* than WT with T-independent antigen stimulation. A) Schema of *in vitro* B cell activation assay. B) RT-qPCR of miR-146a expression in WT cells stimulated with LPS (10ug/ml). C) Cell counts after *in vitro* stimulation with T-independent antigens, LPS and CpG (1,000ng/ml). D) Cell counts after in vitro stimulation with T-dependent (CD40 5ug/ml + IL-4 10ng/ml) and B cell receptor crosslinking IgM (5ug/ml) + IL-4 stimulation.

lipopolysaccharide (LPS) (Figure 1B) in WT mice, consistent with a role as a negative regulator of the inflammatory pathway. T-independent antigens include TLR-4 ligand LPS (10ug/ml) and TLR-9 ligand CpG (1,000ng/ml) were used, which have well-characterized WT B cell *in vitro* responses. (70). We also assessed T-dependent activation via anti-CD40 (5ug/ml) +IL-4 (10ng/ml), as well as B cell receptor crosslinking, IgM (5ug/ml) + IL-4. The experimental design will use pooled B cells from 4 littermate mice (WT or KO), stimulated as above, run in duplicate, and repeated 3x for validation. A mixed effects linear regression model will be conducted for each outcome. These models will include fixed effects for group, stimulation, time and the two-way interactions (i.e. group by time) and three-way interaction (group by time by stimulation). Analyses conducted in Stata Version 13.1, StataCorp LP.

We show that T-independent antigen stimulation of B cells with LPS and CpG in KO show greater cell proliferation than WT, especially at the 96-hour time point (Figure 1C). This time frame of post-stimulatory activity is consistent with B lymphocyte physiology published in the literature in WT mice (70). T-dependent antigen stimulation with CD40 + IL-4 and B cell receptor cross-linking IgM + IL-4 showed the expected physiologic cell proliferation at expected time points, however KO vs. WT mice did not show significant differences (Figure 1D).

We then looked at FACS analysis of bulk B cell expression of surface activation markers: 1) CD69 – a type II C-type lectin with functions in lymphocyte migration and cytokine secretion, and expressed as an early marker of leukocyte activation (71), 2) and 3) CD80 (B7-1) and CD86 (B7-2) – co-stimulatory markers expressed on antigen presenting cells (APCs) that interacts with the CD28 receptor on T cell surfaces, and 4) CD44 – a family of membrane glycoproteins expressed on lymphoid and non-lymphoid cells and shown to be induced in activated B cells (72). KO LPS- stimulated bulk B cells showed higher median fluorescence intensity (MFI) than WT in CD69, CD80, CD86, and CD44 expression (Figure 2A). In addition, KO CpG-stimulated B cells showed higher MFI in CD80 and CD44 than WT (Figure 2B), although not other activation

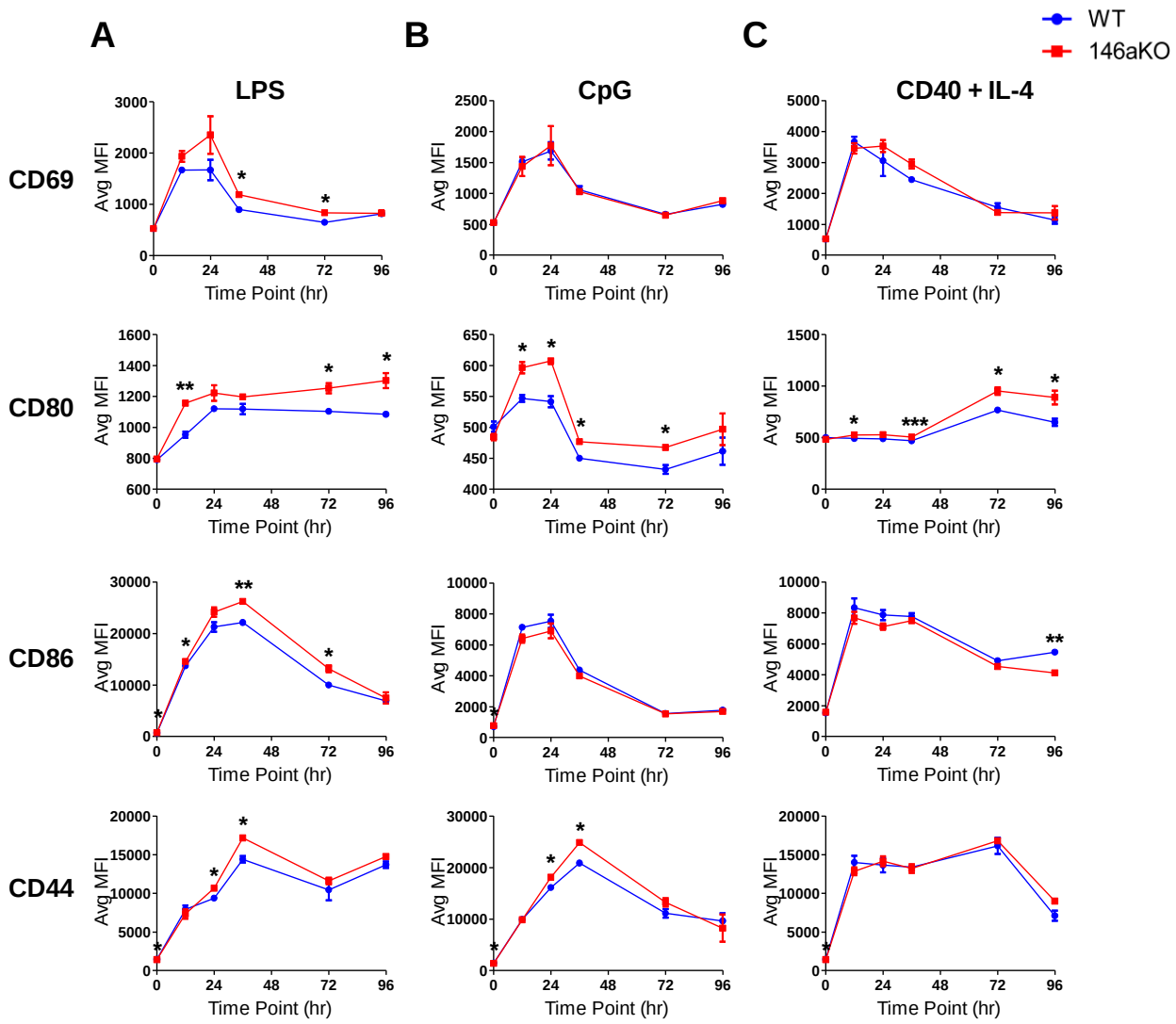


Figure 2. Mir-146a KO have higher activated phenotypes *in vitro* than WT with T-independent antigen stimulation. FACS analysis of B cell surface activation markers from *in vitro* stimulations A) LPS, B) CpG, C) CD40 + IL-4 at various time points.

markers. Of the T-dependent antigen, KOs stimulated with CD40 + IL-4 showed higher CD80 and CD86 MFI than WT (Figure 2C). There was no significant *in vitro* differences in IgM + IL-4 stimulated B cells (Supplement 1). There were no differences in between groups of all stimulations for class switch recombination (surface IgM, IgG, IgA) (data not shown) or plasma cell (CD138+) differentiation at 96 hours (32) (as author previously published).

Together, our *in vitro* studies show that KO mice have higher activated cellular phenotypes than WT, in particular with T-independent antigen stimulation such as LPS.

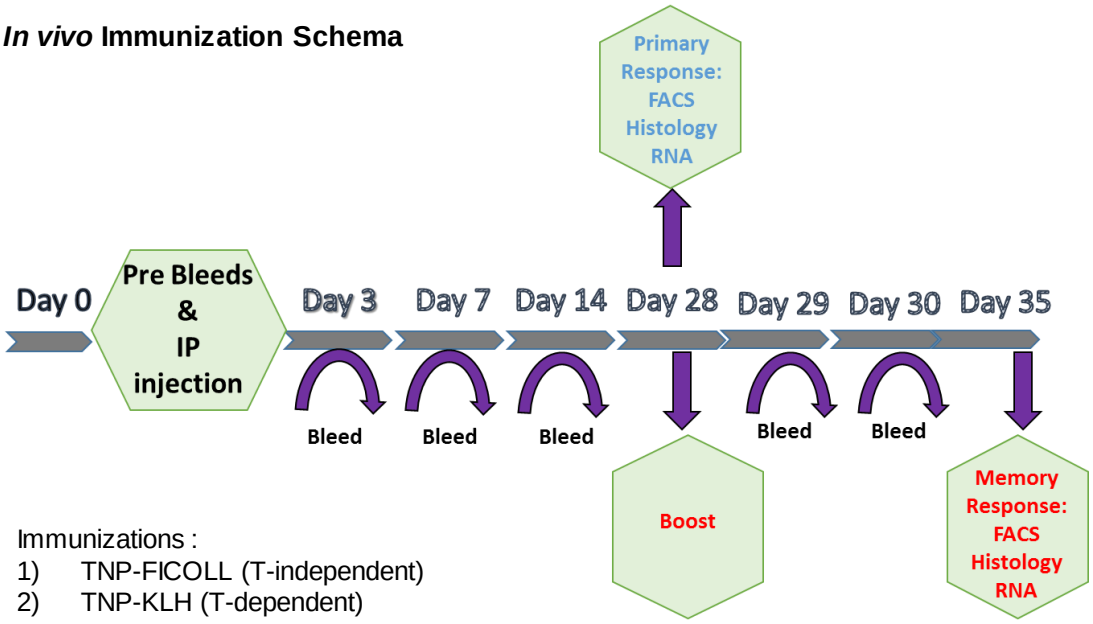
Mir-146a KO has higher *in vivo* immunization responses than WT with T-independent antigen stimulation

Next we examined *in vivo* B cellular phenotypes to ascertain organismal relevance. Young KO and WT mice (4-8/group, to be repeated 3x) were immunized with TNP-Ficoll (T cell independent, TI) or TNP-KLH (T cell dependent, TD) in complete Freund's adjuvant (1:1 volume). Cells and serum were collected post-immunization and assessed for TNP-specific antibodies via ELISA (Figure 3A). Splenic B cell subsets and mature plasma cells in spleens were assessed. Femurs were collected for histological analysis and submitted for processing.

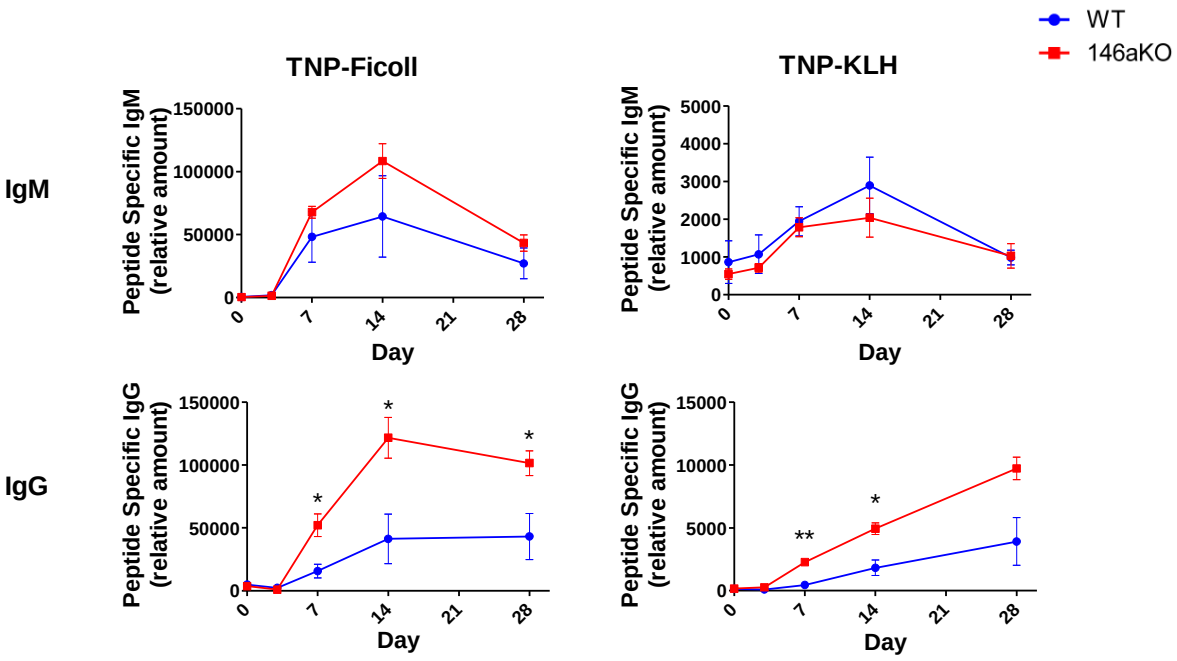
Our results show that for TNP-Ficoll (TI immunization), KOs have a trend of higher peptide specific IgM responses than WT at all post-injection time points, while also having statistically significant IgG response than WT (Figure 3B). For TI-antigen immunizations, we expect that with larger sample sizes that IgM responses will be significant, as would be expected from a TI B cell response. In addition, TNP-Ficoll (a TI-type 2 antigen), has been shown to induce both IgM, as well as significant IgG responses (73), as would be consistent with our results. For TNP-KLH (TD

Figure 3.

A


In vivo Immunization Schema
Primary Response: FACS Histology RNA
Day 0
Pre Bleeds & IP injection
Day 3
Day 7
Day 14
Day 28
Day 29
Day 30
Day 35
Bleed
Bleed
Bleed
Bleed
Bleed
Boost
Memory Response: FACS Histology RNA
Immunizations :
1) TNP-FICOLL (T-independent)
2) TNP-KLH (T-dependent)

B


WT
146aKO
TNP-Ficoll
TNP-KLH
IgM
IgG
Peptide Specific IgM (relative amount)
Peptide Specific IgG (relative amount)
Day
150000
100000
50000
0
5000
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15000
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14
21
28
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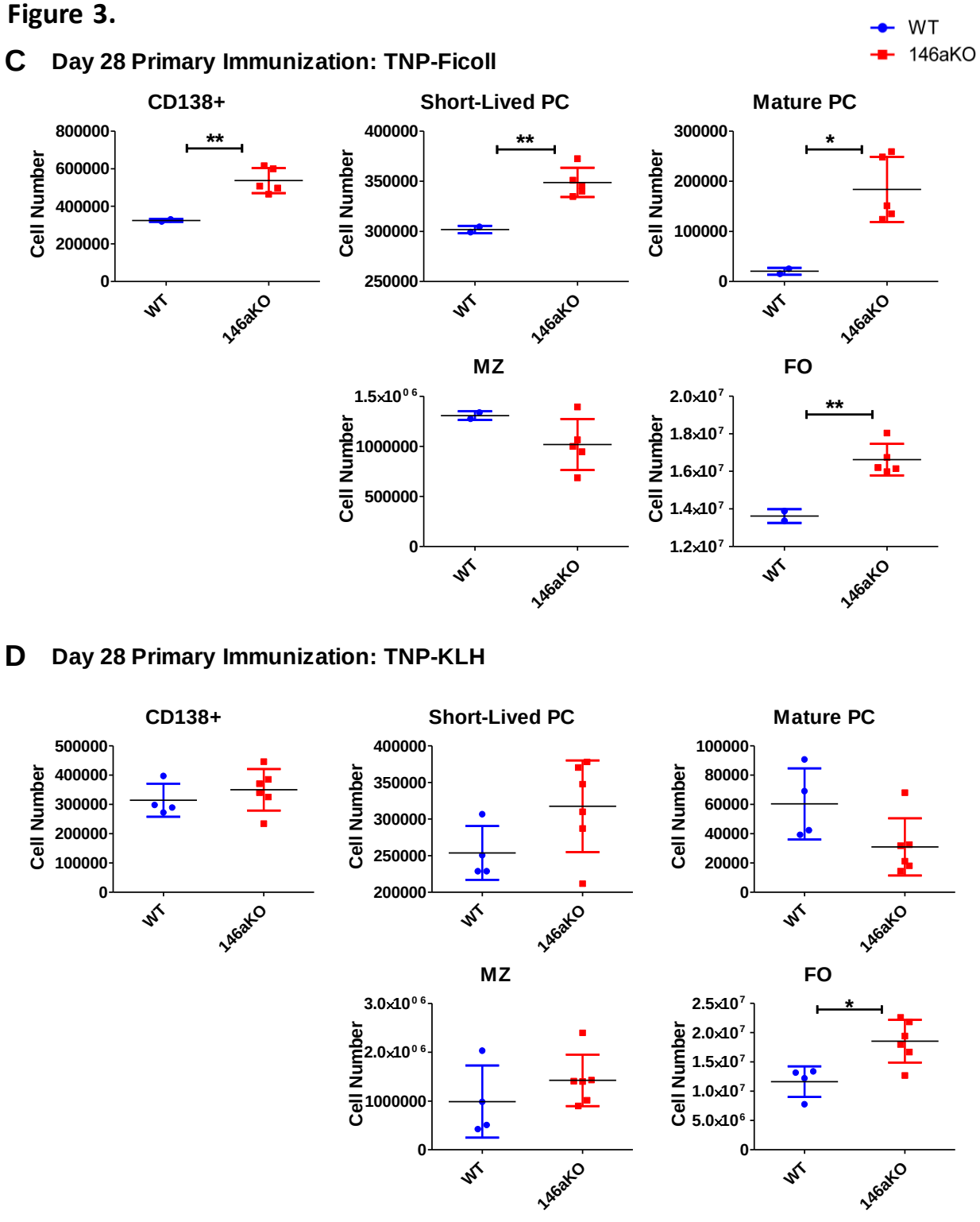
Figure 3.


C
Day 28 Primary Immunization: TNP-Ficoll
WT
146aKO
CD138+
Short-Lived PC
Mature PC
MZ
FO
Cell Number
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D
Day 28 Primary Immunization: TNP-KLH

Figure 3.

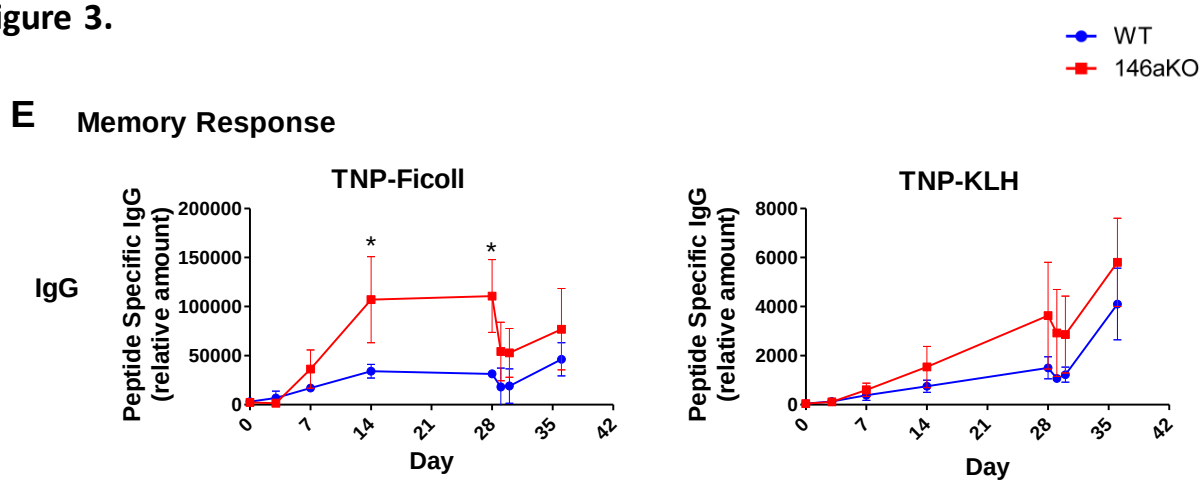


Figure 3. Mir-146a KO have higher *in vivo* immunization responses than WT, especially with T-independent antigen. A) *In vivo* immunization schema of primary and memory B cell responses. B) Primary immunization peptide specific IgM and IgG responses were measured via indirect ELISA at various time points until day 28. C) FACS analysis at day 28 of splenic plasma cells (CD138+) and B cell subsets in TI immunization. D) FACS analysis at day 28 of splenic plasma cells (CD138+) and B cell subsets in TD immunization. E) Memory peptide specific IgG response were measured via indirect ELISA until day 35. *PC = Plasma cell; MZ = Marginal Zone B cell; FO = Follicular B cell.*

immunization), KO cells expectedly showed higher statistically significant peptide specific IgG responses, although not IgM (Figure 3B).

We then examined splenic plasma cell and B cell subsets that might account for the increased peptide specific responses via FACS analysis, as defined in Table 2 (GC stain being optimized). We found TNP-Ficoll immunized KO mice had higher CD138+ plasma cells, both of the short lived and long lived types (Figure 3C), which could account for the higher peptide specific responses seen. Interestingly, as expected, splenic follicular cell numbers were increased (as also seen in native, unimmunized KO mice), although there was no longer a marginal zone number defect (Figure 3C) as previously published (32). This may indicate that in TNP-Ficoll immunization, MZ cells are the contributors to higher peptide specific responses, although further examination of the germinal center and bone marrow cells are warranted to better characterize this. TNP-KLH immunized KO mice did not show statistically significant differences in splenic CD138+ plasma cells from WT (Figure 3D), although again, it would be important to further look in the bone marrow for such $CD138^{hi}B220^{-}IgD^{-}$ cells. It is important to note that miR-146a deficient knockout mice *in vivo* have miR-146a deficiency in other immune cells, such as T cells. This is a significant limitation of performing TD-antigen immunizations *in vivo*, which is further addressed in the discussion.

Table 2. B-cell Subset	FACS Expression Profile
Mature Plasma Cell	$CD138^{high}$, $B220^{-}$
Short-Lived Plasma Cell/Plasmablast	$CD138^{+}$, $B220^{+}$
Follicular B Cell	$CD23^{+}$, $AA4.1^{-}$, IgM^{low}, $CD21^{int.}$, $B220^{+}$
Marginal Zone B Cell	$CD23^{-}$, $AA4.1^{-}$, IgM^{high}, $CD21^{high}$, $B220^{+}$
Activated Germinal Center B Cell	B220+, $GL7^{+}$, CD95 (Fas)+

Furthermore, to test immunological memory, KO and WT mice were re-challenged Day 28 with TNP-Ficoll or TNP-KLH, and then examined for specific antibody secretion at day 1, 2, 7 post-re-challenge. KO memory responses confirm statistical significant in primary responses with TNP-Ficoll in assaying peptide specific IgG, although this was not statistically significant for

memory response (Figure 3E). Memory responses in TNP-KLH show similar higher KO vs. WT trends, although further experiments are underway to assess statistical significance. A simplified power calculation shows that with total 12 mice/group we will have 80% power to detect effect sizes of at least 1.2 with a two-sample t-test and a 0.05 level of significance.

Together, our preliminary *in vivo* studies show that KO mice have higher primary immunization responses than WT, in particular with T-independent antigen immunization.

Traf6 **and** ***Irak1*** **are likely functional gene targets of miR-146a in B cells**

We next proceed from describing the cellular phenotypes of miR146a deficient B cells into defining the mechanisms of gene expression regulation in miR-146a-dependent B cell activation. *We hypothesize that in activated B cells, miR-146a targets genes involved in signaling by NFκB and/or IFN pathways, as described in other cell types.* This ongoing work will use both i) a hypothesis-based approach, focusing on validated targets of miR-146a (i.e.*Traf6, Irak1, Irak2, Stat1, and Irf5),* and ii) a discovery-based approach. Furthermore, to functionally characterize the role of these targets, genetic complementation using the CRISPR/Cas9 gene editing system will be used to delete proposed targets in miR-146a deficient activated B cells. These studies will allow for a molecular level understanding of how miR-146a protects against abnormal responses.

Cultured cells from *in vitro* activating conditions as described above were sent for RNA-Sequencing with biologic replicates. Briefly, using the commercially available Illumina kits, isolated mRNA were routinely processed for RNA-sequencing using polyA selection, as the applicant has done previously (32). To first address our hypothesis based approach, we first curated a list of previously defined miR-146a gene targets, mostly verified in other immune cells. We then selected hypothesis-based B cell miR-146a targets based on the following three criteria: 1) Presence of expression in B cells, 2) Known relevance in B cell activation and selected inflammatory pathways, and 3) Binding of miR-146a to the target gene's mRNA 3' untranslated

Figure 4.

A

Validated miR-146a Targets	Activating Condition	Relevance in SLE
Traf6	TLRs 3, 4, 7, 9	NFκB, IFN signaling
Irak1	TLRs 4, 7, 9	NFκB, IFN, signaling
Irak2	TLR ,4, 7,9	NFκB, IFN, signaling
Stat1	IFN	IFN signaling
Irf5	TLR 7, 9	IFN signaling
Numb	--------	MZ development
Icos	IcosL	Tfh/GC reaction
Fas	FasL	Apoptosis
Fadd	Fas-FasL	Apoptosis

B

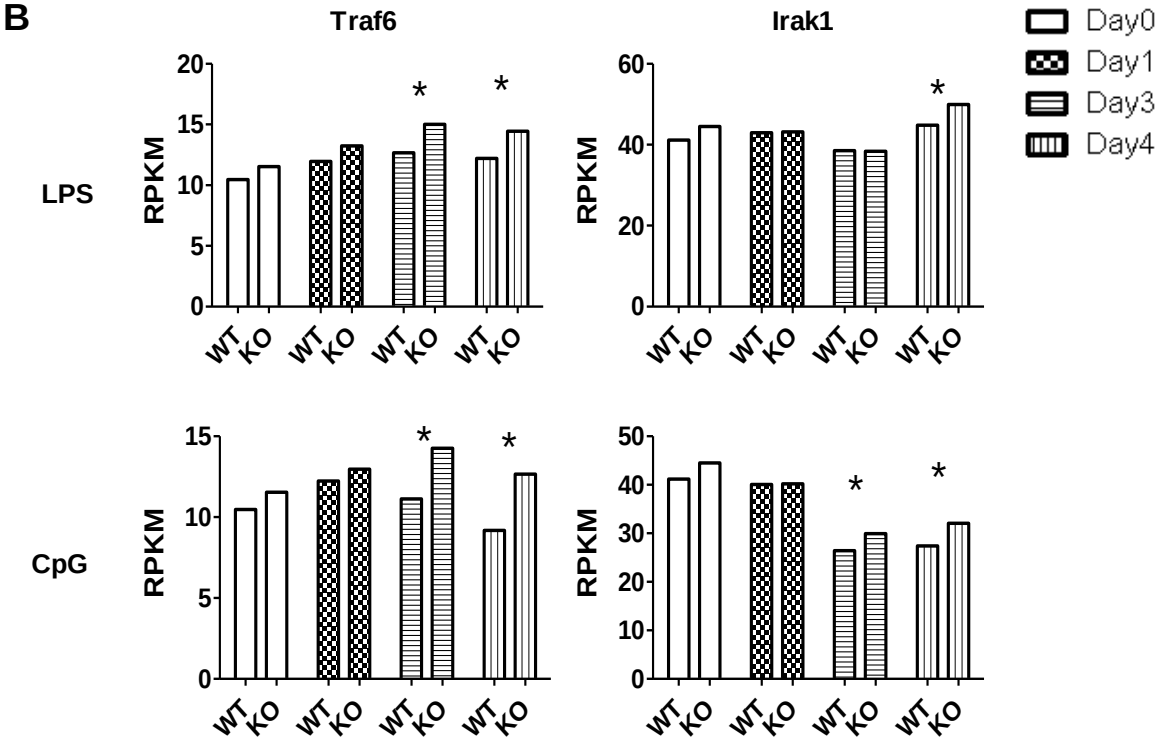
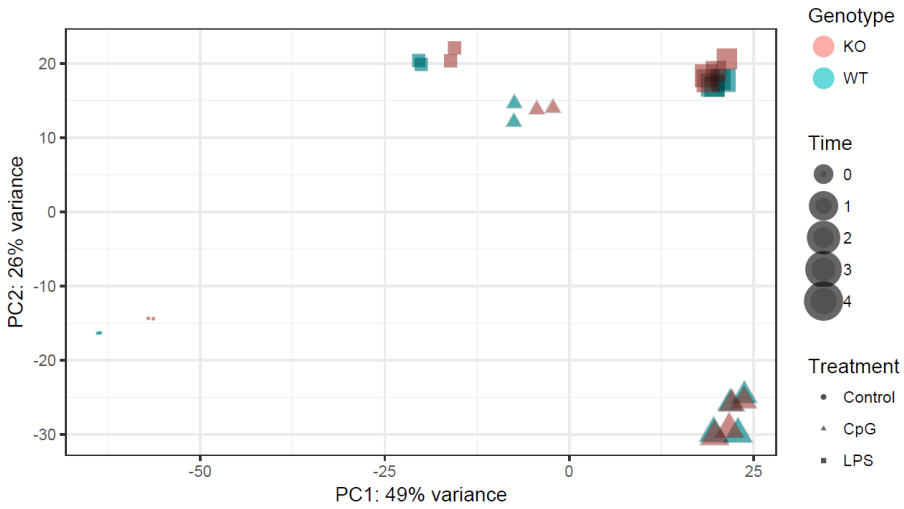


Figure 4.

C



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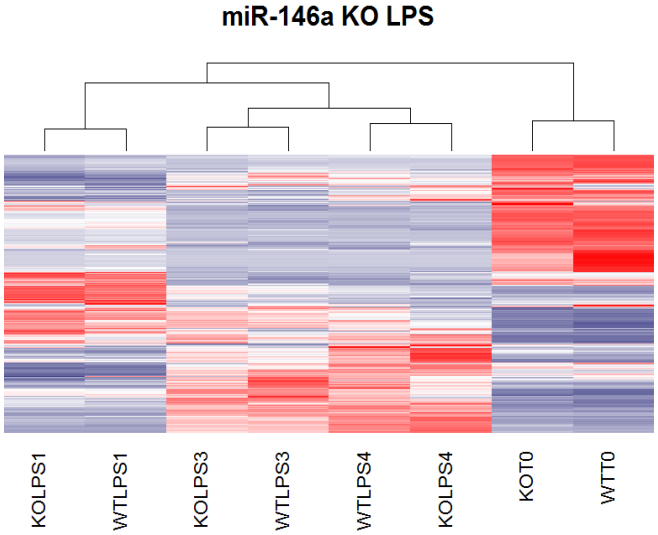


Figure 4. Defining mechanisms of gene expression regulation in miR-146a-dependent B cell activation. A) A list of validated miR-146a targets in immune cells, B) RNA-Seq expression levels of hypothesis-based gene targets from *in vitro* activated B cells at various time points, C) Principle component analysis (PCA) of LPS and CPG stimulated cells, D) Heatmap of LPS stimulated B cells.

region (UTR) (Figure 4A). Of the nine validated miR-146a targets listed in Figure 4A, only *Traf6* and *Irak1* had statistically significant increased levels (indicative of de-repression in miR146a KO) at 72-96 hours with LPS or CpG stimulation (Figure 4B). Assessment of protein levels by Western Blot is in progress.

However, because a singular microRNA often has a multiplicity of targets in various physiologic pathways depending on activating conditions (2), we aim to identify new molecular targets and pathways relevant to miR-146a B cell activation using discovery based approaches. Analysis of RNA-Seq data is in ongoing progress and analysis is described briefly here. Following initial processing of data in association with the UCLA Bioinformatics Collaboratory, the read count matrix file is being analyzed as follows: Pairwise comparisons between both pre- and post-activation samples for each of the stimulation conditions and between different simulation conditions will be carried out using DESeq (74). We are using the Benjamini-Hochberg method to compute the false discovery rate for these analyses. Differentially expressed gene sets will be analyzed for enrichment of specific pathways using the following bioinformatics tools: GSEA (75), IPA Ingenuity®, and GO/KEGG pathway analyses (76). Moreover, differentially expressed genes will be analyzed for new predicted targets of miR-146a by using TargetScan (77) or MiRWalk programs. An initial principle component analysis (PCA) of LPS and CpG KO vs. WT stimulations is shown (Figure 4C). Here we see that the effect of the overall stimulation (i.e. LPS and CpG) is robust in showing differentially expressed genes at various time points, which overpowers the effect of the KO vs. WT phenotype. However, further heatmap visualization of LPS stimulation amongst KO vs. WT do show differentially expressed genes within time points between phenotypes (Figure 3C). Analysis of inflammatory pathways and annotated gene sets are currently underway. As with our hypothesis-based targets, protein levels will be confirmed via Western Blot for miR146a target gene candidates. In sum, both hypothesis and discovery based

approaches of identifying miR-146a B cell targets of repression in the NFkB or other pathways will yield novel insights into mechanism of action.

While the previous section identified miR-146a target genes, we now demonstrate functional relevance of miR-146a targets. By using the CRISPR/Cas9 gene editing system in eukaryotic cells (78), we will demonstrate if target knockdown will lead to cellular phenotype correction *(*of *in vitro* experiments above) in KO mice. Cas9 will be supplied via *Mir146a*$^{-/-}$ *cas9-eGFP* (M146Cas) mice (Figure 5A), as described in Methods. We will design single guide RNAs (sgRNA) using http://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design to target one gene at a time (of candidate miR-146a targets), and will clone them into an MSCV-based retroviral vector, and transduce them into B cell lines for viral collection (Figure 5B). Transduction will be marked by an mCherry reporter cassette. Initially, starting with *Traf6* and control non-targeting (NT) guides, we demonstrate knockdown of TRAF6 compared to NT in the mouse pre-B lymphoblast cell line (70Z/3) previously transduced with Cas9 (70Z3-Cas9) (Figure 5C). We have then isolated 5-fluorouracil-enriched M146Cas primary bone marrow cells and transduced them with the sgRNA vectors targeting gene candidates (i.e. *Traf6, NT*), and injected them retro-orbitally into lethally irradiated syngeneic recipient mice for bone marrow reconstitution (Figure 5D). Our preliminary studies show approximately 30-40% transduction of live mCherry+ sgRNA after spin infection into M146Cas bone marrow cells, as is consistent with previous experiment proportions in our laboratory. However, we do not know yet the percentage of engraftment will be. We opted NOT to sort mCherry+ cells prior to injection into recipient, given the potential for bone marrow infection and trauma shearing exposure to cells after sorting. We will retro-orbitally bleed transplanted mice at Wk 6, 8, and 12 to assess for percentage of GFP$^+$ cells (i.e. marker of engraftment) and mCherry$^+$ cells (i.e. marker of transduction). Following 3 months of reconstitution, we will then sort mCherry$^+$GFP$^+$ cells and isolate B220$^+$ cells to perform *in vitro* experiments as described above to assess for reversal of cellular phenotype. We expect that

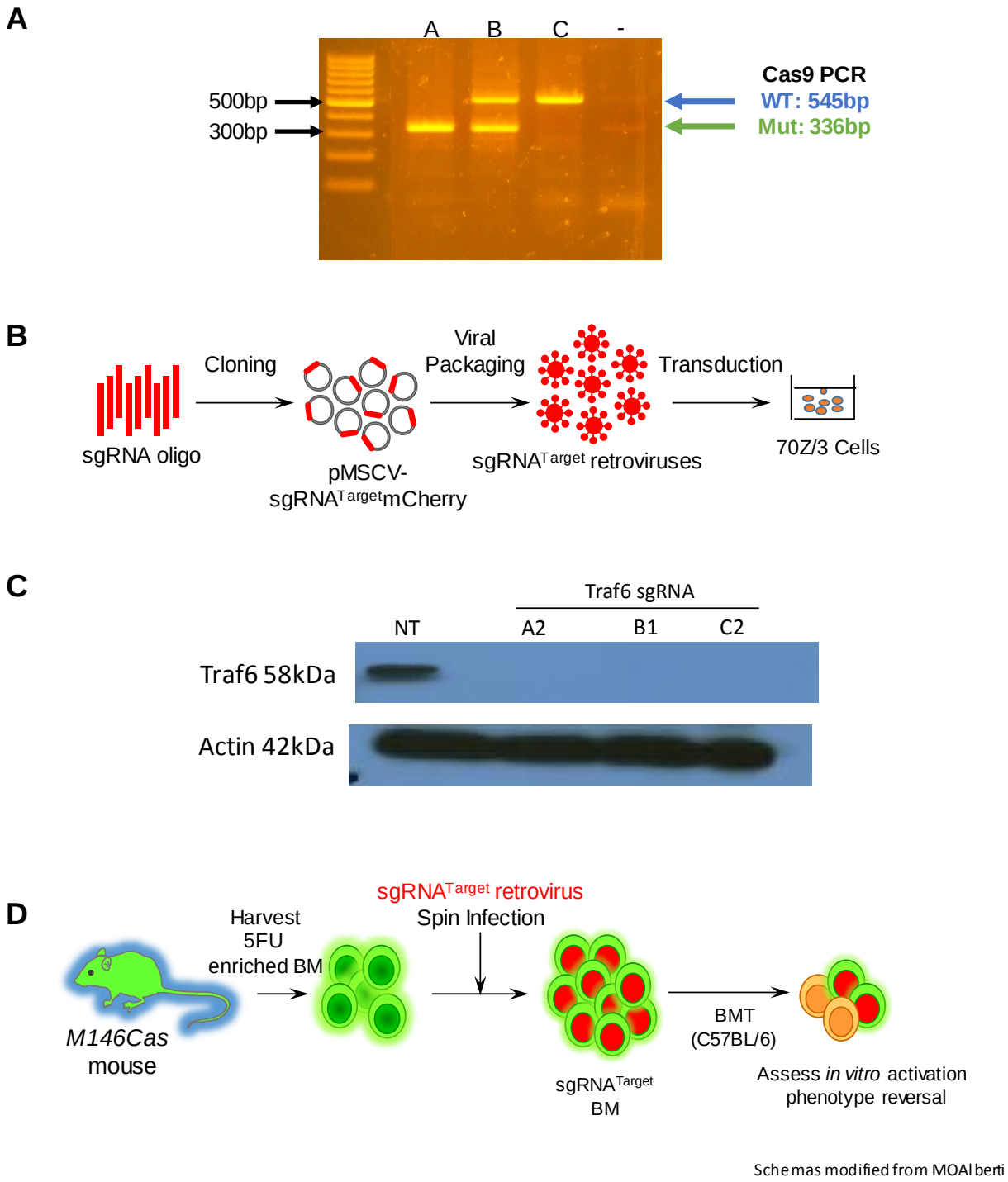


Figure 5. Characterize the functional role of predicted miR-146a targets in B cell activation. A) PCR genotyping for cas9-eGFP mice. B) Schema of sgRNA cloning into 70Z/3 pre-B cell line. C) Western blot of 70Z/3-Cas9 cell lines transduced with specified sgRNAs (NT = non-targeting control). D) Schema of *in vivo* sgRNA guided gene deletion and genetic complementation assay.

using M146Cas cells, knockdown of relevant miR-146a targets will reverse cellular phenotypes back to WT. Bone marrow reconstitution groups will have at least 8 mice, repeated 3x for validation, allowing us to detect effect sizes of at least 0.83 assuming a two sample t-test and two sided significance level of 0.05.

Taken together, our molecular studies will elucidate miR-146a targets in B cells, while validating functional relevance.

Characterizing miR-146a expression in human lupus B cells and relationship to disease activity

Interestingly, human translational studies have identified dysregulated miR-146a expression in systemic lupus erythematosus (SLE) PBMCs (66). However, other studies failed to reproduce these results (79-83). Factors such as diversity of SLE disease severity, ethnicity, and cell detection platforms have been blamed for irreproducibility in other studies (84). Our work in progress will address these concerns by using a large SLE cohort with greater SLE disease activity discrimination based on stricter SELENA-SLEDAI criteria (85, 86) (a commonly used measure of disease activity), including appropriate demographics, and by using confirmatory individual microRNA detection (as opposed to microarray, etc). In addition, we hypothesize that another confounding factor is that the use of PBMCs may mask individual immune subset expression, which may ultimately better reflect disease activity. Notably, miR-146a intrinsic expression varies, being highest in B cells (and polymorphonuclear leukocytes) (18). We thus examine miR-146a in SLE B cells in relationship to disease activity to better address the utilization of miR-146a as a biomarker for SLE disease.

Human PBMCs are comprised of B cells (~15%), T cells (~60%), monocytes (~9%), dendritic cells (~1%), and natural killer cells (~15%) (87), although populations vary across

A

Baseline SLE Characteristics	SLE (Mean ± SD/%)
Age (years)	41.8 ± 13.1
Ethnicity % (n)	
Caucasian	48.0%
Asian or Pacific	13.6%
African American	13.6%
Hispanic	18.9%
Mixed or Other	5.9%
DNA antibody positive	34%
C3	103.9 ± 30.0
History of Renal Disease (past)	15.2%
Any Prednisone Use	47%
Mean Prednisone Dose	5.06 ± 8.4
Hydroxychloroquine	66.5%
Mycophenolate Mofetil	24%
Azathioprine	11.6%
Methotrexate	5.6%
Arava	2%
Mean SELENA-SLEDAI	3.7 ± 3.5

B

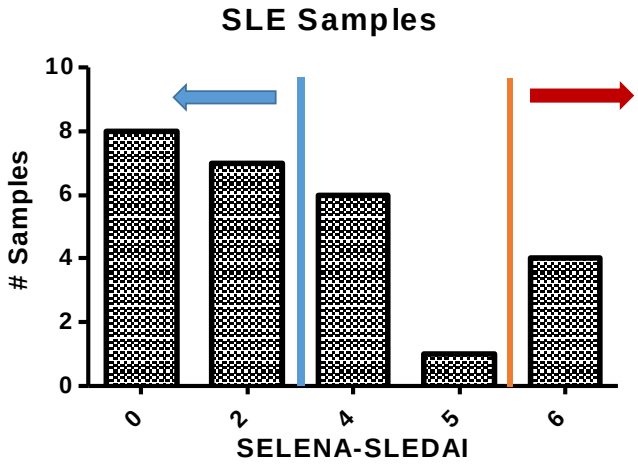


Figure 6. Characterizing miR-146a expression in human lupus B cells and relationship to disease activity. A) Baseline SLE characteristics of patient cohort, b) Human samples collected based on SELENA-SLEDAI score (<=3 is low disease, blue; >=6 is high disease, red). SLE N = 36 samples; Ctrls N = 5 samples.

individuals. Thus, using human PBMCs can present challenges due to varying expression levels of genes in individual cell subsets that may not be accurately reflected in bulk PBMCs. Since microRNAs often target different pathways in immune cells in response to various biologic contexts, it is critical to examine individual cellular microRNA expression and their contributions to activated states. Because 1) B cell- mediated autoantibodies are among the downstream end products contributing to organ damage, and 2) miR-146a is intrinsically highly expressed in B cells (18), we further characterize B cell miR-146a expression in relation to SLE disease activity. In addition, this is to our knowledge, among the first SLE studies examining longitudinal cohorts, and we postulate that microRNA regulation may better reflect disease flare than current standards.

Working with my colleague, Dr. Maureen McMahon, we will be using her well-established, longitudinal SLE cohort, “Biomarkers of Atherosclerosis in Lupus”. This cohort’s initial recruitment consisted of 308 SLE subjects; demographics and baseline characteristics are shown (Figure 4A). Cell specimens are collected at 0, 12, and 18 months, and stored in TRIzol™ as bulk PBMCs, sorted $CD20^+$ B cells, $CD14^+$ monocytes, and $CD3^+$ T cells (Miltenyi Macs columns). RT-qPCR for miR-146a expression will be performed on high quality RNA (RIN>=8) time 0 B cell or PBMC samples, normalizing to human RNU66 (small nuclear RNA) (66), cell number, and/or total RNA. Initial analyses will separately compare baseline B cell and PBMC miR-146a levels between high and low disease activity patients using the Wilcoxon rank sum test. Next, we will construct ROC curves separately for the B cell and PBMC miR-146 values for predicting high vs. low disease activity group. Next, we will compute the area under these ROC curves (AUCs) and compare the AUCs using a paired z-test. This analysis will allow us to evaluate whether B cell miR-146 levels provide a better discriminatory signal than PBMC miR-146 levels for separating high vs. low disease activity subjects. Additionally, we will compute 95% confidence intervals for the AUCs. With the sample size of 50 high disease activity and 50 low disease activity subjects we will have

81% power to detect differences in the AUCs from the B cell miR-146 vs. the PBMC miR-146 of at least 0.135. This assumes a 0.05 level of significance, a correlation of 0.5 between B cell miR-146 and PBMC miR-146, an AUC of 0.7 for PBMC miR-146, and is based on a paired z-test for comparing AUCs. We thus far have collected 36 SLE samples and 5 controls (Figure 4B).

Because our study uses a larger sample size and stricter SLEDAI criteria to better differentiate high and low disease patients than the previous study (which used > 4 vs. ≤ 4 SLEDAI) (66), we anticipate that both B cell and PBMC expression of miR-146a will better discriminate disease activity than the previous study, and that B cell miR146a expression will better discriminate than PBMCs. This is among the first studies to our knowledge to use a longitudinal cohort of patients assessing miRNA expression in specific immune subsets over time, and thus comparing B cell expression to PBMCs.

Discussion

We have thus presented collective animal and human experiments to methodically examine and define the role of miR-146a in B cells in activation, function, and human autoimmune disease. We now use this discussion to discuss pitfalls/alternative strategies and future directions.

For our cellular phenotyping *in vitro* assays, because various TLR ligands and stimulations are known to effect B cell subsets differently (88, 89), based on bulk B cell responses, we will further analyze specific mature splenic subsets of follicular (FO) and marginal zone (MZ) B cells, given their different immune functions. Both FO and MZ cells have been sorted (via Aria) using our extensive gating strategy and data analysis algorithms as described in our recent work (32). Notably, in WT mice, miR-146a is most highly expressed in MZ cells compared to other cell types, but is nevertheless expressed in all transitional and mature subsets. Consistent with this, in the context of B cell development we found that miR-146a deficiency resulted in a relative reduction

of MZ cell development compared to WT mice (32). However, despite their decreased cell numbers, we suspect that MZ KO cells will show heightened responses in the context of activation. Because the innate-like MZ B cell subset is in general more sensitive to TLR signaling (although with TLR4 ligation in particular, both FO and MZ shown vigorous responses) (89) than FO/GC B cells subsets, we expect the MZ B cell KO subset to show greater activated phenotypes than their FO counterparts when using these stimuli. However, in T dependent CD40+ IL4 activation, we expect FO KO phenotypes to be stronger than MZs. Of the TLRs, it will be of particular interest in our KO cells to examine TLR stimulation with RNA or DNA recognition motifs, such as TLR 3,7, and 9, which are elevated in CD19+ B cells in human systemic lupus erythematosus peripheral blood mononuclear cells (PBMCs) (90). Interestingly, SLE B cell increased expression of TLR9 has been shown to correlate with organ damage (91). Studies using TLR-7 agonist imiquimod (R837) (5ug/ml) and TLR-3 agonist poly (I:C) (10ug/ml) are pending. We will also examine *in vitro* cellular proliferation using CFSE staining. Also, annexin V staining would be important to rule out differences in cellular apoptosis. If cultured supernatants do not yield fruitful cytokine data, intracellular cytokine analysis of B cell cultures can be performed.

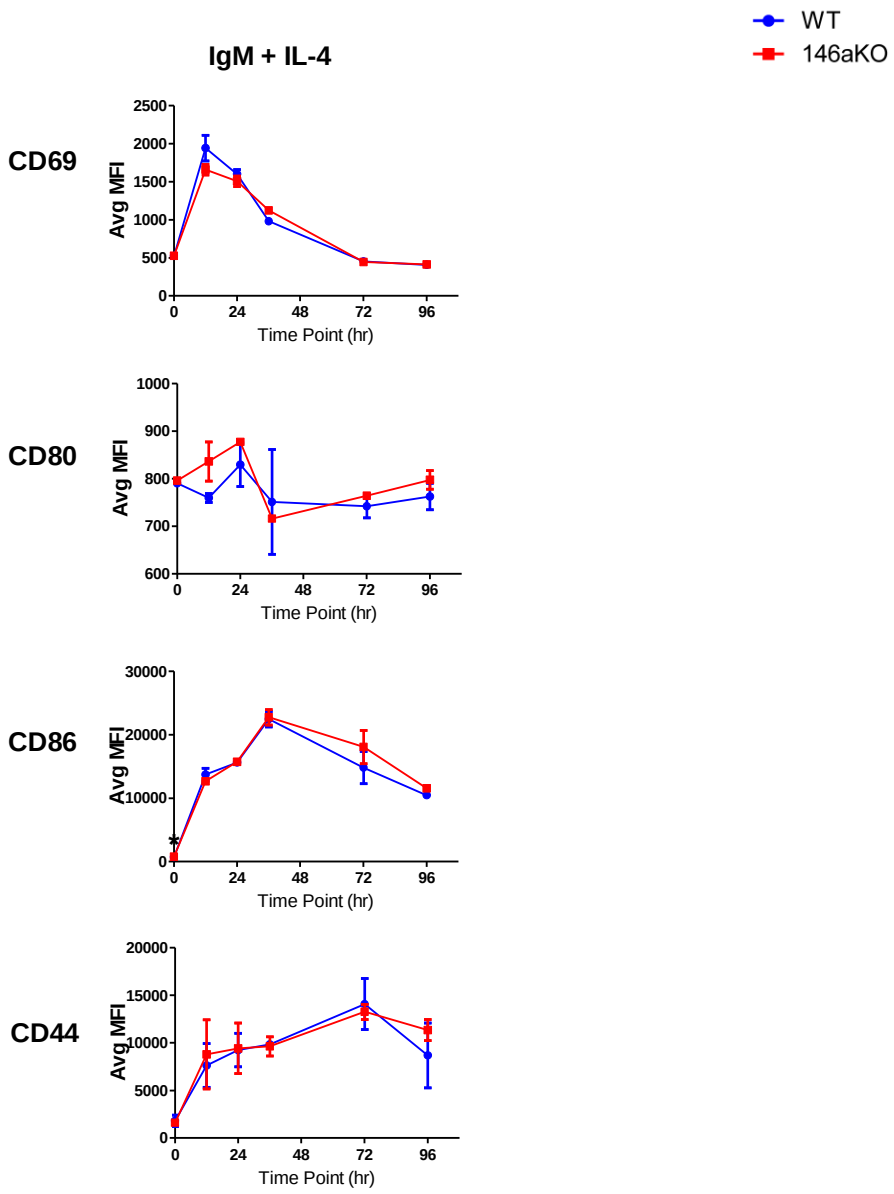
For our *in vivo* immunization assays, it is important to note that using *Mir146a*$^{-/-}$ mice will reflect miR-146a deficiency within other immune subsets as well, as noted in above results. Thus, T dependent responses of KO B cells will reflect B cell extrinsic influences in our immunization studies. However, since miR-146a is also expressed in myeloid and T cells, our results may more accurately reflect true biologic reality. Nevertheless, if only T dependent responses are found, we can utilize a conditional knockout model of floxed miR-146a (92), in which deletion is limited to B cells.

In addition, in our molecular studies, it is possible that we will not find consistent changes in the list of targets that we derived from hypothesis-driven approaches due to an artifact of an *in*

vitro stimulation system. However, the constant addition of algorithms and network-based analyses will assist. Additionally, if sgRNA-mediated knockout is an issue, we may still utilize our lentiviral vectors for siRNA-mediated knockdown (93). Alternatively, *Stat1*$^{-/-}$ mice and *Irf5* floxed mice are commercially available. However, we anticipate a number of miR146-a B cell gene targets of interest, in which case it would be expensive and unfeasible to do multitudes of individual gene target deletions *in vivo*. In this case, we will use our above sgRNA *in vivo* deletions as proof of principle for our deletion system to create a pooled library screen of gene targets important for miR-146a activation, in which identified functional gene targets will be individually validated. Lastly, as with any genetic complementation assay, a single gene may be insufficient to complement the phenotype. If so, we can generate multiple gene knockdowns with sequential transduction of sgRNA/siRNA against multiple targets.

For our human translational studies, the heterogeneity of human SLE disease makes translational studies difficult. Our cohort may not "catch" the specific subset of SLE patients shown previously to have miR-146a under-expression in PBMCs. However, this is unlikely as our sample size is large and encompasses a sizeable Asian population (as in the published study). Nevertheless, because miR-146a is also normally expressed in myeloid and T cell subsets (18), our future studies may include examination of these as a better reflection of overall *in vivo* biologic relevance. It is also possible that miR-146a expression in SLE B cells (or PBMCs) is chronically under expressed, in which case cellular miR-146a expression may correlate less with disease activity, but more with underlying dysregulated feedback mechanisms in SLE pathogenesis.

In this ongoing work, we demonstrate in animal studies that miR-146a KO B cells have enhanced *in vitro* and *in vivo* immune responses than WT, in particular to T-independent antigen stimulation, presumably due to a lack of de-repression of miR-146a targets that regulate inflammatory pathways. These results will be further explored in human SLE B cells (and other immune cells), and correlated with disease activity to assess translational relevance.



Supplementary Figure S1. FACS analysis of B cell surface activation markers from *in vitro* activation with IgM + IL-4.

CHAPTER IV:

Conclusions “Exploring MicroRNAs for Precision Medicine in Rheumatology”

We have outlined a comprehensive examination of miR-146a's impact in B cells on development and function, as well as detail our translational approach to studying relevance to the human autoimmune disease, systemic lupus erythematosus (SLE). Because miR-146a was shown to be underexpressed in human SLE PBMCs, as opposed to other autoimmune diseases which showed overexpression of the negative regulator, it remains an open ended question if under expression of miR-146a has a pathogenetic role, leading to well-described dysregulated NFkB and IFN signaling in human SLE disease.

SLE has a highly complex pathogenesis, including defective apoptosis and clearance, nucleic acid/immune complex stimulation of myeloid and plasmacytoid dendritic cells, loss B/T cell tolerance, dysregulated NFκB and IFN pathways, and autoantibody production. Deposition of anti-dsDNA immune complexes in active SLE renal disease has suggested a primary role of these autoantibodies in SLE pathogenesis (94). A variety of autoantibodies are used as diagnostic markers for disease. However, for predictive disease activity markers**,** aside from general cell, metabolic panels, and acute phase reactants, **our limited arsenal of biomarkers include dsDNA autoantibodies (anti-dsDNA) and complement levels, although their uses are imperfect.** Titers of anti-dsDNA may fluctuate with SLE disease activity, and there is a well-described association between the presence of high titer anti-dsDNA IgG and SLE glomerulonephritis, particularly with concurrent low C3 and C4 levels (94, 95). However, some SLE patients have high titers of anti-dsDNA antibodies and do not flare (96), while other SLE patients have clinical flares without changes in antibodies or complement levels (97). A variety of multifactorial reasons have been proposed for these inconsistencies, including anti-dsDNA antigen avidity, isotype, and isoelectric point. In addition, although it is known that the source of autoantibodies stem from germinal center (GC)-dependent memory B and long-lived plasma cells (98), a recent study suggested that additionally, newly activated naïve cells without the need for affinity maturation contribute as well (99). However, current assays measuring dsDNA

IgG autoantibodies cannot distinguish IgG levels coming from GC or non-GC B cell origins. Because of a lack of reliable disease activity biomarkers, clinicians then rely on complex disease activity measurements, such as the two most commonly used criteria SELENA-SLEDAI (85, 86) and/or British Isles Lupus Activity Group (BILAG) (100). These disease measurement tools rely on a combination of clinical, laboratory, and immune characteristics that have scores in each category. Such criteria are time-consuming, intricate, and impractical as part of a busy clinician's every day practice.

A New England Journal of Medicine article defined precision medicine as treatments targeted to individual patients: *"on the basis of genetic, biomarker, phenotypic, or psychosocial characteristics that distinguish a given patient from other patients with similar clinical presentations. Inherent in this definition is the goal of improving clinical outcomes for individual patients and minimizing unnecessary side effects for those less likely to have a response to a particular treatment." (101)* The field of precision medicine is advancing rapidly, most prominently in the oncologic field. However, as their immunologic counterparts, thinking about developing biomarkers and treatments to allow for precision medicine in autoimmunity remains an underdeveloped necessity. Our application of biomarkers specific to autoimmune diseases is difficult, as unlike cancers, there are often no discrete lesions to sample expression of potential candidates. In contrast, identifying specific biomarkers that reflect the dynamic and dysregulated immune system of an autoimmune patient is the challenge. It is also well-known that clinical syndromes such as SLE represent a wide, heterogeneous constellations of disease manifestations (102) and varying degrees of clinical activity, which may confound large clinical trials testing efficacy of new drug treatment.

Until the recent approval in 2011 of Benlysta (Belimumab), a human monoclonal antibody against B-lymphocyte stimulator (BLyS), no new medications had been approved by the US Food and Drug Administration (FDA) since 1966 (103) However, initial trials (which of

note, excluded active lupus nephritis or CNS involvement) leading up to Phase III trials did not meet their primary endpoints. It was only after serologically active (i.e. dsDNA, ANA) SLE patients were targeted that showed some response that eventually led to FDA approval (103). While in this case, serum autoantibodies (i.e. dsDNA, ANA) are helpful in determining clinical use in a certain subset of SLE patients, the overall effects of Benlysta are still mild in terms of clinical improvement (104, 105). Hence, further defining biomarkers that would allow better prediction of for treatment induction, maintenance, and cessation are warranted.

A recent review (106) expounded on the role of serum autoantibodies in autoimmune diseases as the cornerstone of precision medicine in rheumatology. However, while certain autoantibodies do indeed predict worse prognosis, there are very fewer such autoantibodies in the field that correlate with disease activity, as discussed previously. Hence, identifying more individualized, precise biomarkers to predict disease activity (i.e. to base duration of treatment such as steroids) would be ideal on the rheumatologic field. Because microRNAs are highly responsive to stimuli, biologically dynamic, and sensitive to activated immune states, they may more precisely reflect the body's dynamic physiologic state (84, 107) compared to downstream autoantibody secretion from the various B cells subtypes (i.e. long-lived plasma cells, memory, activated naive). The application of microRNAs to prediction of disease activity in an individual patient in a well-studied, longitudinal manner has not yet to our knowledge, been confirmed. Hence, proof of principle of its application would be a major advancement in the field. Our miR-146a may also serve as a model for which to examine other microRNAs in our human SLE cohort to find particular applications to clinical disease, or potentially to use in conjunction with several microRNAs and/or immune markers. This proposal details a roadmap for potential bench to bedside application, which we look forward to further exploring in the months to come.

The critical regulatory roles of microRNAs in hematopoiesis and immune function have also made them targets of interest for therapeutic potential. MicroRNAs may regulate many gene targets within a signaling pathway to ensure a biologic outcome, or they also may target genes across different pathways. Hence, ensuring miRNA therapeutic targets within the right physiologic or biologic contexts is of utmost importance. Broadly, miRNA-based therapies can be divided into miRNA mimics or miRNA inhibitors (antimiRs) (7). Mimics are often synthetic double-stranded RNA molecules similar to the desired miRNA sequence of interest and are given with the aim to replace dysregulated or dysfunctional miRNAs. In contrast, antimiRs are single stranded antisense oligonucleotides (ASOs) or are modified with locked nucleic acids (LNAs). AntimiRs with a certain 2' O-methoxyethyl modification are named antagomiRs. They have complementary sequences to the miRNA of interest and bind to their targets to block function. However, a major challenge for the utilization of miRNA therapeutics, both using single or double-stranded oligonucleotides) is avoiding degradation of oligonucleotides by serum RNAs or in the endocytic compartment of cells (7, 64). Several strategies for addressing both these issues are currently underway, as a number of pre-clinical miRNA therapies and diseases (i.e. especially in solid tumors, but also hepatitis C, atherosclerosis, cardiac disease, diabetes-related complications, and myocardial infarction) are underway. In regards to the autoimmune disease systemic lupus erythematosus, there are several preclinical examples of miRNA therapies that have been shown to reduce various manifestations of disease. For example, recombinant nanoparticle delivery of miR-146a in lupus mice showed a significant reduction of autoantibodies, proinflammatory cytokines (such as interferon alpha, IL-6), and improvement in progression of disease (108). In addition, *in vivo* administration of miR-130b agomir reduced interferon pathway accelerated progression of lupus nephritis, leading to decreased urine proteinuria, lower levels of immune complex deposition, and improved kidney glomerular lesions (109). Furthermore, *in vivo* administration of miRNA-155 antagomir in mice that developed

pristine-induced diffuse alveolar hemorrhage improved disease progression and pro-inflammatory cytokines production (63). These pre-clinical trials are encouraging, although a number of issues as cited above with delivery and tissue targeting remain. In the autoimmune field though, a single center phase I trial is underway involving a miR-29 mimic in systemic sclerosis (7).

On a broader view, it is important to remember that immune cell development and function are controlled by both transcriptional factors, but also other post-transcriptional regulatory elements, such as microRNAs, other non-coding RNAs (i.e. long noncoding RNA, lncRNA; and circular RNA, circRNA) and RNA-binding proteins (RBPs) (110). Biologically, various post-transcriptional mechanisms exist and often interact to carefully control gene expression or repression. LncRNAs are transcribed by RNA polymerase II and are usually transcripts greater than 200nt, found in sense or antisense orientation to protein coding genes (111). Among their many recently described functions, lncRNAs can act as decoy to sponge away miRNAs from their intended mRNA targets (64). In addition, miRNAs may function closely with RBPs to regulate mRNA stability. RBPs can diminish miRNA-mediated decay by binding directly to the miRNA, or indirectly, by competing for binding motifs in the 3' untranslated region of the mRNA target (110). For example, IGF2BP1 (insulin like growth factor 2 binding protein 1) binds to *BTRC* and *MITF* mRNAs, which prevents miR-183 and miR-340 binding, leading to de-repression of these genes (112-114). It would be interesting to study the interaction of miR-146a and/or lncRNAs and/or RBPs, to better understand functional relevance *in vivo* on inflammatory pathways and immune responses.

A number of lncRNAs have been described in various autoimmune diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis, type 1 diabetes mellitus, and polymyositis/dermatomyositis (115), In particular to SLE, genome-wide associations studies (GWAS) have identified an area on chromosome 1q25 associated with SLE, in which lncRNA

GAS5 remains a prime suspect for lupus susceptibility (116, 117). In addition, the RBP ROQUIN, an E3 ubiquitin ligase, likely plays an interesting role in miRNAs and systemic lupus erythematosus. *Roquin-1 (Rc3h1)* was initially discovered during a mutational screen for SLE symptoms in mice, in which homozygous mutations in mice led to double-stranded DNA, IgG immune complexes, anemia, kidney glomerulonephritis, autoimmune thrombocytopenia, and other abnormalities characteristic of SLE due to ICOS overexpression in T cells (118). *Roquin-1* -deficient mice were shown to have increased miR-146a (~25x) and miR-21 (~14x) expression in T cells, and found to bind to both miR-146a and the RISC subunit Ago2, suggesting a likely mechanism for other miRNA regulation as well (119). Hence further studies examining the collective function of various post-transcriptional regulators that interact with microRNAs are likely to yield novel insights into immune function and disease.

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