MicroRNA control of B cell tolerance, autoimmunity and cancer
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Since the discovery of the first microRNA (miRNA) in 1993, thousands of miRNAs have been identified in humans and mice and many of them have been shown to control a large variety of cellular processes in different cell types including those composing the immune system. MicroRNAs regulate virtually all aspects of immune cell development, differentiation and function. Studies have shown that these molecules are involved in the maintenance of lymphocyte tolerance and, when dysregulated, promote the development of autoimmune diseases. In this review, we focus on the current knowledge about the roles of miRNAs in B cell tolerance and their contribution to autoimmunity, highlighting additional roles for some of these miRNAs in T cell tolerance. Finally, we will comment on miRNAs that promote both autoimmunity and lymphoma.

Introduction
Autoimmune diseases are a group of more than 80 diseases that collectively affect 5% of the world population (1). These diseases are of complex etiology and are believed to arise from a combination of genetic, epigenetic and environmental factors. Autoimmune diseases, including lupus erythematosus (SLE), rheumatoid arthritis, type 1 diabetes and multiple sclerosis are characterized by a breach of immune tolerance, the series of mechanisms that ensure that the immune system reacts against a large variety of invading pathogens without attacking self-tissues. Several immune cell populations are critical for the maintenance of immune tolerance including innate immune cells, B cells and T cells (2, 3). Autoreactive B cells play important roles in the development of autoimmune diseases by producing autoantibodies, presenting autoantigens to T cells and producing proinflammatory cytokines (4). However, the mechanisms underlying the development of self-reactive B cells are poorly understood. Specifically, the roles of microRNAs in B cell tolerance are just starting to be elucidated.

MicroRNAs are small endogenously encoded RNAs about 19-23 nucleotides in length that regulate protein expression by binding with imperfect complementarity to the messenger RNA (mRNA) of their target genes and promoting their degradation and/or translational repression (5, 6). MiRNAs are highly conserved molecules across species. It has been estimated that more than 30% of the protein-coding genome in humans is regulated by miRNAs (7). They are encoded as single miRNAs or miRNAs clusters, with the latter accounting for around one third of all miRNAs. Their primary transcripts are sequentially processed by DROSHA/DGCR8 and Dicer to produce mature miRNAs, which are loaded into the miRNA-induced silencing complex (miRISC) to exert their regulatory function. The first miRNA, lin4, was discovered in Caenorhabditis elegans in 1993, but it was not until 2000 when the scientific community gained interest in this new type of regulatory molecule and launched multiple miRNA expression profiling efforts to identify all the miRNAs expressed in different organisms and cell types. In 2005 Xiao et al. performed a miRNA expression profiling study of murine immune cell subsets and showed that expression of miRNAs in the hematopoietic system changes depending on the differentiation status (8). This and other studies suggested that miRNAs regulate commitment to particular cellular lineages and have a role in cell differentiation and maintenance of cell identity (8-10). Since then, many miRNAs have been shown to play critical roles in the development and function of various immune cell subsets using genetic approaches. MiRNA regulation of each target gene is generally modest and often results in small changes in expression, but the simultaneous regulation of many target genes can exert dramatic effects in cells. The large array of miRNAs that regulate the development and functions of different immune cell subsets have been comprehensively reviewed
before (11-13). In this review we specifically discuss seminal work on miRNA regulation of B cell tolerance and its contribution to autoimmunity.

B cell tolerance

B cell development initiates in the bone marrow. Hematopoietic stem cells (HSC) first commit into a common lymphocyte precursor and then to a pro-B and pre-B cell, the earliest stages of B cell development in which genetic rearrangements occur to produce the heavy and light chain of their immunoglobulin respectively. These rearrangements are known as V(D)J recombination and result in the expression of the first immunoglobulin, an IgM, on the cell surface of an immature B cell. IgM forms a complex with CD79A and CD79B (also known as Igα and Igβ), which are important for signal transduction. This complex constitutes the B cell receptor (BCR). The combinatorial and stochastic nature of V(D)J recombination generates IgM immunoglobulins with a large variety of antigen specificities including self-reactive ones. The first immune tolerance mechanism, known as central tolerance, occurs during this immature B cell stage to eliminate self-reactive B cells and thus prevent them from exiting to the periphery where they can potentially cause damage to our organs. If an immature B cell recognizes self-antigens, it continues to rearrange the light chain to generate a non-autoreactive IgM through receptor editing. If receptor editing is unsuccessful, the autoreactive B cell dies by apoptosis in the process known as clonal deletion. If the newly generated immature B cell is non-autoreactive or loses its self reactivity by receptor editing, it exits the bone marrow and undergoes further maturation in the periphery (14, 15) (Fig. 1). In the spleen, these immature B cells (also called transitional B cells) pass through a second immune tolerance checkpoint, known as peripheral tolerance, which selects against B cells bearing BCRs reactive to self-antigens. The maturation and selection steps of transitional B cells are poorly defined. B cell activating factor (BAFF) acts as a survival factor for peripheral B cells and may play an important role in this process (16).

A third checkpoint is critical for regulating autoreactive B cells during the germinal center reaction, the process by which B cells undergo class switch recombination and somatic hypermutation (SHM) to generate immunoglobulins with improved affinities for pathogens. These processes are both initiated by Activation-Induced Cytidine Deaminase (AID). The largely random nature of somatic hypermutation leads to the generation of more autoreactive B cells in the germinal center that have the potential to differentiate into autoantibody-producing plasma cells. The fact that most pathogenic autoantibodies show the hallmark of SHM and selection strongly suggests that failure to enforce this immune tolerance checkpoint contributes to many autoimmune diseases. T follicular helper (Tfh) cells, a CD4 T cell subset localized in germinal centers, provide critical help to B cells through CD40L and cytokines (i.e. IL-4 and IL-21). There is accumulating evidence that Tfh cells are a limiting factor in germinal centers and aberrant positive selection of autoreactive B cells by dysregulated Tfh cells contribute to autoimmunity (17). Furthermore, Tfh expansion in peripheral blood has been observed in a subset of patients with autoimmune diseases including systemic lupus erythematosus, rheumatoid arthritis, and Sjogren's syndrome. The accumulation of circulating Tfh cells strongly correlates with the severity of disease activity in patients with systemic lupus erythematosus. Tfh cell expansion was also found in several mouse models of lupus and was shown to play a causative role in disease pathogenesis in some models (18-20).

A fraction of germinal center B (GCB) cells differentiate into long-lived plasma cells that reside in the bone marrow, spleen, and inflamed tissues (21). Under normal conditions, the plasma cell population is maintained at a level that supports humoral immunity while preventing the production of excessive amounts of antibody. Plasma cell hyperplasia occurs in autoantibody-mediated autoimmune diseases such as systemic lupus erythematosus (22-24). Therefore, controlling plasma cell differentiation and survival is another important immune tolerance checkpoint. Despite intensive study, our understanding of these checkpoints remains largely incomplete. We and others have been focusing on identifying miRNAs and their target genes that regulate these immune tolerance checkpoints and elucidating their cellular and molecular mechanisms of action.
miRNA regulation of B cell tolerance and autoimmunity

In early studies, Koralov et al. conditionally deleted Dicer (Dcr1), one of the critical enzymes for miRNA biogenesis, in B cells using an Mb1Cre allele which turns on the expression of Cre recombinase from the earliest stage of B cell development. They found an almost complete block of B cell development at the pro- to pre-B cell transition. In addition, although Dicer deficiency did not alter the basic mechanism of V(D)J recombination, it altered the BCR repertoire, suggesting a role for miRNAs in regulating the survival of potentially autoreactive B cells (25).

The first evidence demonstrating a causal role of miRNAs in the development of autoimmunity came in 2008 from the generation of mouse models with increased expression of the miRNA cluster miR-17-92 in lymphocytes (hCD2iCre;miR-17-92 Tg) (26). This cluster is composed of 6 mature miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a, which are processed from the same primary transcript. They are grouped in 4 subfamilies, miR-17, miR-18, miR-19 and miR-92 subfamily, based on their seed sequences. These mice developed lymphoproliferative disease and autoimmunity, and died prematurely. Both T and B cells from these mice exhibited enhanced proliferation and survival upon activation in the periphery. Higher autoantibody levels were also observed in mice with increased lymphocyte expression of miR-17-92. Mechanistically, miR-17-92 suppressed the expression of the tumor suppressor PTEN and the proapoptotic protein Bim. Conversely, miR-17-92 deletion in developing B cells resulted in elevated Bim and PTEN protein levels, cell apoptosis, and B cell deficiency (27).

In 2010, Belver et al. generated B cell conditional knockout mice of Dicer using the CD19Cre allele, which turns on Cre expression at later stages of B cell development. They showed that absence of Dicer in CD19Cre;Dicer1<sup>fl/fl</sup> mice exhibited altered B cell subsets in periphery. Dicer-deficient B cells had skewed BCR repertoire with hallmarks of autoreactivity. In addition, female mice showed autoimmune features with high titers of autoreactive antibodies in serum suggesting a role for microRNAs in late B cell development and in the establishment of B cell tolerance (28). It is interesting to note that CD19Cre;Dicer1<sup>fl/fl</sup> mice showed gender specificity in the development of autoimmunity, while hCD2iCre;miR-17-92 Tg mice did not.

The generation of the IgM<sup>b</sup>-macroself mouse model of B cell tolerance provided an excellent opportunity to study the function of miRNAs and protein coding genes in the regulation of this process. IgM<sup>b</sup>-macroself mice ubiquitously express an engineered superantigen reactive to the constant region of the IgM heavy chain. These mice show normal early B cell development. Once B cells reach the immature stage, they all recognize the engineered superantigen through their membrane-bound IgM. This mimics self-recognition and all B cells are deleted from the repertoire by clonal deletion resulting in mice that have no B cells in their blood, spleen and lymph nodes (29). The C57BL/6 genetic background of these mice allow to use them in bone marrow reconstitution experiments, in which their immune system comes from donor hematopoietic stem and precursor cells (HSPCs) with increased, decreased or absent expression of candidate genes. In this experimental setting, the appearance of B cells in the spleen of IgM<sup>b</sup>-macroself recipient mice after reconstitution would indicate a break of B cell tolerance caused by altered expression of candidate genes.

We performed a functional screen of a library of lymphocyte expressed miRNAs in the IgM<sup>b</sup>-macroself mice. HSPCs from C57BL/6 donor mice were transduced with the library and transferred into irradiated IgM<sup>b</sup>-macroself recipient mice. Splenic B cells in these mice were analyzed 8 weeks post-transfer. This study identified miR-148a as a critical regulator of B cell tolerance. MiR-148a impaired BCR-engagement induced apoptosis (clonal deletion) of autoreactive B cells by regulating its target genes Gadd45a, PTEN and Bim. In addition, increased expression of miR-148a in immune cells accelerated the onset and progression of lethal autoimmunity in MRL-lpr mice, a classical murine model of lupus (30). These results are consistent with previous microRNA profiling studies of samples from lupus patients, which consistently found miR-148a upregulation in their circulating lymphocytes (31-34). The expression of miR-148a was also upregulated in B and T cells of MLR-lpr mice before disease onset (32). Furthermore, miR-148a was the only miRNA upregulated in splenic lymphocytes of NZB/W mice, another model of lupus, at an age before the onset of disease (35).

The IgM<sup>b</sup>-macroself mice also allowed us to demonstrate a role for miR-17-92 in central B cell tolerance. Reconstitution of IgM<sup>b</sup>-macroself recipient mice with HSPCs from donor mice with B cell-specific
transgenic expression of miR-17-92 led to a robust break of B cell tolerance. Functional dissection of the miRNA subfamilies of this cluster showed that the miR-19 subfamily dominated in exerting this effect. Finally, target identification and validation in the IgM\(^b\)-macroself model revealed PTEN as a major mediator of miR-17-92 regulation of B cell tolerance (36). In an independent study, Benhamou et al. used a genetic approach of deletion and complementation to show that the c-Myc/miR-17-92/Pten axis controls the sensitivity of immature B cells to clonal deletion by regulating PI3K activity. Specifically, they overexpressed miR-17-92 in CD19-deficient mice, which have impaired B cell development. Increased expression of miR-17-92 reconstituted the impaired PI3K activity in CD19-deficient cells and restored B cell development in this mouse model (37). Coffre et al. used mice with conditional deletion of Dicer or DGCR8 from the earliest stage of B cell development and found an essential role for miRNAs in the regulation of the PI3K/AKT/FOXO pathway. In addition, they reported a role for miRNAs in receptor editing during B cell maturation (38). These results are in line with the development of autoimmunity in hCD2iCre;miR-17-92 Tg mice and indicate that a break of both B and T cell tolerance underlies disease onset and progression. Concurrently, a correlation between autoimmunity and miR-19 overexpression was reported in lupus-prone MRL-lpr mice, and in airway T cells of asthma patients. The former study measured the expression levels of miRNA in mouse models of lupus and showed increased expression of miR-19a in splenic B and T cells of MRL-lpr mice (35), while the latter study found that miR-19a was preferentially upregulated in asthma airway T cells and promoted pathogenic inflammation in this disease (39).

Interestingly, the studies employing the IgM\(^b\)-macroself mice revealed that miR-148a and miR-17-92 share a common target for the regulation of B cell tolerance but differ in other targets (30, 36). PTEN was found to be the common target, consistent with the important role of PTEN downregulation in B cell tolerance and autoimmunity. Previous studies had shown that PTEN heterozygous knockout mice developed autoimmunity, and that PTEN-deficient B cells were protected from BCR-engagement induced apoptosis (40, 41). MiR-148a targeted Bim, but its expression level was not reduced by miR-17-92 in immature B cells. Bim deficiency also renders immature B cells resistant to BCR-engagement induced apoptosis and Bim heterozygous knockout mice develop autoimmunity (42, 43). Gadd45a was identified as a target for miR-148a but not for miR-17-92. Gadd45a deficient mice develop a systemic autoimmune-like disease with elevated titers of autoreactive antibodies and renal pathology (44). It was previously known that Gadd45a prevents autoimmunity by functioning as a negative regulator of T cell receptor (TCR) signaling through the MEKK4-JNK/p38 pathway in T lymphocytes (45). This novel role of Gadd45a in B cell tolerance should also contribute to the development of autoimmunity in Gadd45a-deficient mice.

Abnormal germinal center responses could also contribute to the production of pathogenic autoantibodies and the development of autoimmunity. MiR-146a has been shown to control germinal center responses through targeting multiple CD40L signaling components in B cells (46). An independent study reported that miR-146a deficient mice accumulated GCB and Tfh cells and exhibited increased ICOSL expression on GCB cells (47). This is consistent with the development of autoimmunity observed in miR-146a deficient mice (48). In addition, polymorphisms of the miR-146a gene have been described and are linked with lupus development in humans (49-51). Germinal center B cell numbers are reduced in miR-155 knockout mice. Concurrently, increased miR-155 expression resulted in GCB cell expansion and enhanced antibody response in mice (52). miR-155 and miR-181b negatively regulate the expression of AID, a critical enzyme required for class switch recombination and somatic hypermutation in GCB cells (53, 54). In addition, miR-217, which is specifically upregulated in GCB cells, is a positive regulator of the GC response and increases the generation of class-switched antibodies and the frequency of somatic hypermutation. miR-217 stabilizes Bcl-6 expression in GCB cells by downregulating the expression of a DNA damage response and repair gene network (55). MiR-17-92 regulates Tfh differentiation through downregulation of PTEN and Phlp2 (56). An independent study reported a role for miR-17-92 in Tfh cells through the regulation of its target Rora (57). MiR-155 promoted Tfh cell accumulation during chronic, low grade inflammation (58). Another study implicated miR-155 in regulating the generation and function of Tfh cells through a miR-155-Peli1-c-Rel pathway (59). Germline miR-146a deficiency in mice resulted in the accumulation of Tfh cells (47), whereas deficiency in both miR-146a and miR-146b was required to observe increased Tfh cell numbers in mice with conditional deletion of these miRNAs in T cells (46).
Furthermore, miR-155 also plays a role in the production of high-affinity class-switched antibodies. B cells lacking miR-155 showed impaired germinal center responses and secretion of high-affinity IgG1 antibodies in mice. The transcription factor Pu.1 was a target for miR-155 in this context (60). The generation of miR-155-deficient MRL-lpr mice revealed that deletion of miR-155 reduced serum IgG but not IgM autoantibody levels and kidney damage in this model of lupus by de-repressing the expression of SHIP-1 (61). Another study established an inhibitory role for miR-210 in the regulation of B cells and autoantibody production through unknown mechanisms. Mice with germline deletion of miR-210 developed autoantibodies. Overexpression of miR-210 impaired class switched antibody responses and caused defects in cellular proliferation and cell cycle entry in splenic B cells (62). MiR-148a has been suggested to promote plasma cell generation by targeting the transcription factors MITF and Bach2 as well as the pro-apoptotic factors Bim and PTEN (63). Finally, increased expression of miR-326 in MRL-lpr mice promoted plasmablast development and antibody production via downregulation of the transcription factor Ets-1 (64). A summary of the miRNAs that regulate B cell tolerance can be found in Table 1 and Fig.2.

Interestingly, some of the miRNAs that regulate B cell tolerance and autoantibody production have been shown to play additional roles in other immune cell subsets that contribute to autoimmune pathogenesis. miR-17-92 also promoted CD4 T cell expansion and Th1 function, and inhibited TGFβ-induced iTreg generation (65). miR-155 is required for Th1 and Th17 responses and regulates CD8 T cell responses (66-70). MiR-146a limits CD4 and CD8 T cell activation and numbers, controls T reg cell regulation of Th1 responses, and Th17 cell differentiation (71-73). Mir-148a regulates survival of repeatedly activated Th1 cells in vitro, and its expression is increased in T cells from rheumatoid arthritis patients (74). MiR-326 regulates Th17 differentiation and is associated with the pathogenesis of multiple sclerosis (75). As new studies investigating the roles of miRNAs in specific immune cell subsets emerge, we will advance towards a more comprehensive understanding of how dysregulation of these molecules contributes to autoimmune diseases. This knowledge might provide new therapeutic targets for the design of better strategies to treat these debilitating diseases.

Dysregulation of common miRNAs in autoimmunity and B cell lymphomas

A potential link between autoimmune diseases and lymphomas has been suggested. There is evidence that several genes and pathways regulating immune tolerance checkpoints are also involved in lymphomagenesis (76). Antigen engagement of BCRs switches on powerful cell growth and survival pathways, mediated by activation or induction of NFκB, PI3K, Myc, Ras/Erk, and anti-apoptotic proteins, while uncontrolled activity of these growth and survival pathways promotes lymphoma development. It has been shown that relentless stimulation of BCRs and lymphocyte growth leads to lymphoma development. Self-antigens pose arguably the greatest potential source for such lymphocyte stimulation. Multiple immune tolerance checkpoints are in place to prevent this from happening. It is reasonable to speculate that compromising these control mechanisms would lead to the outgrowth of autoreactive clones and to lymphomas.

The role of miR-17-92 in lymphomagenesis and autoimmunity is of great interest in this context. We previously showed that B cell-specific miR-17-92 transgenic mice develop lymphomas with a penetrance of approximately 80%. The remaining 20% died of autoimmune diseases (77). MiR-146a functions as a tumor suppressor gene and miR-146a deficient mice developed both autoimmunity and tumors in lymphoid organs (48). Downregulation of miR-146a expression by c-Myc was observed in human and mouse B cell lymphomas (78). The relatively long latency before tumor development in miR-146a deficient mice suggests the need for additional factors to induce malignancy in these animals. The excessive inflammation and autoimmunity seen in miR-146a deficient mice might potentially collaborate to induce cancer in these mice. This is in line with the idea that inflammation and cancer are intimately linked (79-81). Clinical isolates of several types of B cell lymphomas, including diffuse large B cell lymphoma, have 10- to 30-fold higher copy numbers of the miR-155 gene than normal circulating B cells (82), and miR-155 has been implicated in cell proliferation and disease progression in lymphomas and leukemias (83-85). Finally, miR-217 is overexpressed in aggressive human B-cell lymphomas and its increased expression in B cells promoted lymphoma development in mice, providing a potential link between the normal GC response and B cell transformation (55).
Large population studies showed that patients with autoimmune diseases including systemic lupus, rheumatoid arthritis and Sjogren’s syndrome are at increased risk of developing lymphoma (86-88). The drugs used to treat autoimmune diseases were excluded as a cause (89). The miRNAs mentioned above and their corresponding regulatory networks might serve as molecular links between autoimmunity and B cell malignancies. Alternatively, autoimmune diseases could also generate an altered microenvironment that support the development of cancer. It will be interesting to perform in-depth studies focusing on this potential connection of autoimmunity and cancer through the dysregulation of specific miRNAs.

Conclusions and future prospects

We are just beginning to understand the regulation of B cell tolerance at the molecular level. The identification of miRNAs that regulate B cell tolerance is expanding our knowledge on these important mechanisms and uncovering whole regulatory pathways that include protein-coding genes with previously unknown function in this process. Autoreactive B cells play critical roles in autoimmunity. However, autoimmune diseases are complex and multiple immune tolerance defects in various cell types have been shown in disease pathogenesis. The intriguing observation that there are miRNAs that regulate immune tolerance in both B cells and T cell subsets provides new potential targets for the treatment of these elusive diseases. Can systemically administered miRNA-based drugs dampen inflammation during autoimmune diseases and restore immune homeostasis? Although miRNA-based therapeutics are still under development, a series of studies have shown that administration of miRNA inhibitors can exert effects on immune cells and ameliorate some diseases in mouse models and humans (90-94). However, to choose optimal targets we need to understand in depth the role of each specific miRNA in all the immune cell subsets and their complete regulatory networks. A potential miRNA-based drug would then need to be tested for toxicity and undesirable secondary effects in other organs. An alternative would be to deliver the drug to chosen cells. In addition, analysis of the protein-coding genes regulated by these miRNAs could reveal new targets with ideal properties for therapeutic intervention.

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Figure 1. Central tolerance and the IgM<sub>b</sub>-macroself mouse model. A) A schematic describing B cell central tolerance. B) Design of the engineered superantigen ubiquitously expressed by IgM<sub>b</sub>-macroself mice. Single-chain Fv generated from the IgM<sub>b</sub>-reactive hybridoma AF6-78 is linked to the hinge and membrane-proximal domains of rat IgG1, followed by transmembrane and cytoplasmic tail regions (Tm/Cy) of H-2Kb. C) Splenic B cells of irradiated IgM<sub>b</sub>-macroself mice 8 weeks after reconstitution with control hematopoietic stem and precursor cells (HSPCs) or HSPCs transduced with retroviruses encoding the miR-17-92 cluster.
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**Table 1.** Summary of miRNAs involved in B cell tolerance and functions. Abbreviations: B cell receptor (BCR), T follicular helper (Tfh), Activation-Induced Cytidine Deaminase (AID).
Figure 2. MiRNAs that regulate different B cell tolerance mechanisms: central tolerance, germinal center response and plasma cell differentiation. Abbreviations: T follicular helper (Tfh), germinal center B (GCB).
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