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MicroRNA regulation of the germinal center response

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Abstract

The generation of germinal centers (GCs) is a hallmark feature of the adaptive immune response, resulting in the production of high-affinity antibodies that neutralize pathogens and confer protection upon reinfection. The GC response requires interactions between different immune cell types, and the coordination of complex and dynamic gene expression networks within these cells. Here we provide deeper insights into how microRNAs, small endogenously expressed RNAs, regulate the cellular processes involved in the differentiation and function of T follicular helper cells and germinal center B cells, the two main players of the T cell-dependent humoral immune response.

Introduction

Germinal centers (GCs) are specialized structures that form within secondary lymphoid organs during T cell-dependent antibody responses [1]. They are the anatomical site of antigen-specific B cell proliferation and selection events that engender robust high-affinity antibody responses and B cell memory. Naïve $CD4^+$ T cells are primed by dendritic cells in the T cell zone of secondary lymphoid organs and can differentiate into various effector T helper cells (Th1, Th2, Th17, etc.) or T follicular helper (Tfh) cells, which interact with B cells and support GC responses (Figure 1). The transcription factor Bcl6 is necessary and sufficient to induce the Tfh phenotype in activated $CD4^+$ T cells [2–4]. Importantly, Bcl6 induction occurs independently of cognate interactions with B cells at these early stages of the immune response [5]. Induced upregulation of the chemokine receptor CXCR5 and downregulation of CCR7 by these early Tfh cells promotes their migration to the boundary of B cell follicles [6]. Naïve B cells encounter their antigen in the follicle and subsequently localize to these same boundary regions and interact with Tfh cells (Figure 1). This encounter initiates the extrafollicular antibody response in which the activated B cells differentiate into plasma blasts that produce the first wave of antibodies, generally of low affinity [7]. Only very few of the activated B cells, together with Tfh cells, reenter the follicle to establish germinal centers (Figure 1). GC B cells are the predominant antigen-presenting cell type in GCs, and their formation and maintenance requires CD40L provided

by Tfh cells. Thus, Tfh and GC B cells are maintained through reciprocal interactions within GCs [8, 9]. In these multicellular structures somatic hypermutation and affinity maturation lead to the generation of memory B cells and long-lived plasma cells that produce high-affinity antibodies [1]. Most vaccines aim at inducing this second wave of potent antibodies, which provides protection upon re-infection with the same pathogen that elicited the primary response.

Dysregulation of the GC response is a key feature of several autoimmune diseases [10, 11] and GC B cells are the cell of origin for several common types of B cell lymphoma [12]. Harnessing the immunological power of these volatile structures involves orchestrated interactions among several different cell types, and it is not surprising that gene expression within these cells is tightly regulated as well. MicroRNAs are small endogenously expressed RNAs that have emerged as important constituents of gene regulatory networks in the immune system [13, 14]. Here, we discuss our current understanding of how miRNAs contribute to gene regulation and decision-making in the GC response, especially with regard to the two main players, Tfh cells and B cells.

MicroRNA-mediated regulation of T follicular helper cells

Tfh cells are the primary T cell subset that provides help to B cells [15]. They have a distinct miRNA expression profile [4, 16] and requirements for miRNA function that differ from those of other effector Th cell subsets [13]. Global miRNA expression by $CD4^+$ T cells is absolutely required for Tfh cell development, as adoptively transferred *DiGeorge syndrome critical region gene 8* (*Dgcr8*)-deficient T cells, which lack mature miRNAs, failed to generate $CXCR5^+Bcl6^+$ Tfh cells upon immunization with a protein antigen [17]. In addition, activated miRNA-deficient $CD4^+$ T cells failed to downregulate CCR7 and were unable to localize to the T-B zone border and interfollicular regions where wildtype T cells interact with activated B cells [17]. Importantly, this defect was not only attributed to the reduced proliferative capacity of miRNA-deficient $CD4^+$ T cells [13], but also to an intrinsic defect to generate Tfh cells [17]. The observed complete block in Tfh cell differentiation by $CD4^+$ T cells that lack all miRNAs is very striking, since miRNA-deficient $CD4^+$ T cells can still differentiate very efficiently into other Th cell subsets [13].

So far, studies of the particular miRNAs that regulate Tfh cell differentiation and function have been focused mostly on the miR-17~92 cluster, which consists of six miRNAs that comprise four different miRNA families. Overexpression of miR-17~92 in T helper cells causes hyperproliferation and the production of autoantibodies [18]. On the molecular level, miR-17~92 was shown to repress the expression of the anti-apoptotic protein Bim and the tumor suppressor PTEN [18]. Early studies indicated that miR-17~92 may inhibit Tfh cell responses by directly targeting CXCR5 expression, and that miR-17~92 may itself be regulated by Bcl6 [4]. However, more recent data clarified that miR-17~92 in fact promotes Tfh cell differentiation through repression of several target genes, including *Pten* [17, 19]. miR-17~92 also regulates Tfh cell development in part by targeting *Phlpp2*an AKT phosphatase involved in ICOS signaling, which is important for the co-localization of Tfh and B cells [19]. Members of the miR-17~92 family and *Phlpp2* are rapidly induced upon T cell stimulation and follow similar expression kinetics [19]. Inhibition of *Phlpp2* by

miR-17~92 miRNAs might thus be important for adjusting the appropriate strength of ICOS-mediated signaling required for Tfh cell differentiation [19]. Combined deletion of miR-17~92 and its two related miRNA clusters, miR-106a~363 and miR-106b~25, further amplified the defects in Tfh cell differentiation, although miR-17~92 alone was shown to be the main contributor to the observed phenotype [19]. Follicular regulatory T (Tfr) cells share characteristics of thymus-derived Treg cells and Tfh cells and are believed to regulate the germinal center response, although the precise mechanisms remain elusive [20]. Tfr cells seem to be particularly dependent on miR-17~92, as Tfr numbers in mice that either lacked or overexpressed miR-17~92 specifically in T cells correlated with miR-17~92 dose [17].

Tfh cell differentiation is critically supported by multiple inhibitory pathways, including the transcriptional repressor Bcl6 and miRNAs (Figure 1). This indicates that repression of alternative differentiation pathways may be very important for the establishment and maintenance of Tfh cell identity. This idea is further supported by recent data obtained from experiments with conditional miR-17~92-deficient mice in a viral infection model [17]. In wildtype mice, lymphocytic choriomeningitis virus (LCMV) infection primarily generates Th1 and Tfh cells. However, miR-17~92-deficient Tfh cells from LCMV-infected mice upregulated a whole array of genes that are normally associated with Th17 and Th22 cells, including *Ccr6, Il22*and *Rora* [17]. All six miRNAs of the miR-17~92 cluster directly target the *Rora* 3' UTR, and restoring *Rora* expression to its normal lower level in miR-17~92-deficient Tfh cells significantly rescued the inappropriate expression of *Ccr6* and *Il22*. Similarly, miR-10a inhibits the conversion of $Foxp3^+$ Treg cells into Tfh cells under lymphopenic conditions, in part by targeting *Bcl6* and its co-repressor *Ncor2* [21]. Together, these data demonstrate that miRNAs can enforce T helper cell identities by restraining alternative transcriptional programs (Figure 1).

MicroRNA-mediated regulation of the germinal center B cell response

MicroRNAs play essential roles in several aspects of B cell development and immune function, including the GC response [22]. This was revealed through several studies that used Cre recombinase transgenes to conditionally inactivate the miRNA biogenesis enzyme Dicer at different stages of B cell development in mice. Deletion very early in the B cell lineage with Mb1-Cre blocks B cell development at the pro- to pre-B cell transition [23]. Deletion at later stages of B cell development (using CD19-Cre) showed that miRNAs also specifically promote the generation of follicular B cells, while transitional and marginal zone B cell numbers were increased [24]. Interestingly, Dicer deletion at this stage also resulted in a skewed B cell receptor (BCR) repertoire and high titers of autoreactive antibodies [24]. More recent studies using mice that express Cre recombinase only in activated B cells under the control of the activation-induced cytidine deaminase (AID) promoter provided further insights into miRNA regulation of B cell immune function. Global miRNA-deficiency limited to AID-expressing cells strongly impaired GC responses and memory B cell and plasma cell generation [25].

Various human and murine B cell subsets have been profiled by microarrays and sequencing, resulting in a comprehensive assessment of miRNA expression in these cells [16, 26–28]. The specific functional roles of some of these miRNAs have been elucidated.

miR-155 has been the focus of perhaps the most intensive research on miRNA function in any mammalian system. It is highly expressed in GC B cells, and miR-155-deficient and miR-155-overexpressing mice revealed important roles for this miRNA in several aspects of the GC response (Figure 1), including GC size and number, antibody production, class-switch$_{DB2}$affinity maturation, and cytokine production by Th cells [29, 30]. miR-155 negatively regulates the generation of class-switched IgG^+ plasma cells in part through inhibition of the transcription factor Pu.1 [31]. Deletion of miR-155 in lupus-prone *Fas*^{lpr} mice alleviated the systemic autoimmune response by decreasing the levels of autoantibodies in these mice [32]. AID is another important direct target of miR-155 [33, 34]. Since miR-155 promotes the GC B cell response, it is interesting that at the same time it inhibits AID, which is required for somatic hypermutation and also for class-switch recombination. Bcl6 inhibits the expression of several miRNAs, including miR-155, suggesting that coordinated spatiotemporal regulation of Bcl6 and miR-155 expression within the GC may be important for the generation of high-affinity B cells [35].

Several other miRNAs have been implicated to be important regulators of the GC B cell response (Figure 1). For example, miR-185 controls B cell activation through inhibition of Bruton tyrosine kinase, leading to dampened BCR signaling [24]. Like miR-155, miR-181b also impairs class-switch recombination in activated B $_{DB3}$cells by targeting AID [36]. miR-150 is highly expressed in naïve B cells and limits the magnitude of the GC response [37]. In contrast, miR-125b overexpression promotes GC B cell differentiation by inhibiting genes that drive plasma cell differentiation, including Blimp-1 and IRF4 [38]. The catalogue of miRNAs involved in GC responses will undoubtedly grow with further investigation and development of tools for assessing miRNA functions in immunity.

MicroRNAs in the development of GC-derived malignancies

The high frequency of genetic rearrangements in GC B cells makes them a common cell of origin for B cell lymphomas [12]. Dysregulated miRNA expression is a common feature of GC B cell-derived malignancies, and some of these miRNAs appear to be important oncogenes or tumor suppressors [22, 39]. Although miR-155 dampens genomic instability through the repression of AID, overexpression causes B cell leukemogenesis [40]. Indeed, miR-155 is the functional RNA encoded within the avian *bic* proto-oncogene, and it is highly expressed in Hodgin's lymphoma, diffuse large B cell lymphoma (DLBCL), and other hematologic malignancies [41]. In addition to promoting GC B cell differentiation, miR-125b overexpression induces B cell malignancies in transgenic mice [42].

The proto-oncogene miR-17~92 has been shown to drive the development of B cell lymphomas in a c-Myc-induced tumor model system by repressing genes that are important for apoptosis [43]. *Dicer*-deficient B cells exhibit an increase in the expression of miR-17~92 cluster target genes, including the proapoptotic protein Bim [23]. Generation and analysis of conditional miR-17~92-deficient mice contributed greatly to our understanding of the role of this miRNA cluster to normal B cell development *in vivo* [44]. The related miR-106a~363 and miR-106b~25 clusters share some, but not all of miR-17~92's regulatory properties [44]. Subsequent analyses revealed that miR-19 family miRNAs, which are represented once in the miR-106a~363 cluster and twice in miR-17~92,

are particularly important for lymphomagenesis, in part through inhibition of the tumor suppressor gene *Pten* [45, 46]. Transgenic miR-17~92 overexpression in B cells efficiently induces lymphomas [47, 48], and c-Myc-induced tumor formation absolutely requires two functional copies of miR-17~92 [48]. Biochemical analysis of miRNA targeting by photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) showed that miR-17~92 directly targets multiple negative regulators of lymphomagenesis, including genes involved in the PI3K and NF-kB pathways [48]. Thus, miR-17~92 and other miRNAs participate in a complex gene regulatory network, and imbalance in this network can promote tumor development.

Follicular dendritic cells (FDCs) are localized in the light zone of the GC. They capture immune complexes through their abundant expression of complement and Fc receptors and present antigen to GC B cells. This contributes to positive selection of GC B cells and affinity maturation in a T cell-dependent manner. Although data is limited on how miRNAs might regulate FDCs themselves, FDCs have been implicated in regulating the balance between BLIMP-1 and BCL6 expression in B cells in a cell-to-cell contact-dependent manner through miR-9, let-7, and miR-30 [49]. In a different study, adhesion to FDCs was shown to induce miR-181a expression in B cell lymphoma cell lines, which correlated with decreased apoptosis and reduced expression of the pro-apoptotic miR-181a target gene *Bim* [50].

Conclusion

Over the past several years, miRNAs have emerged as important regulators of immune cells. This is reflected in the absolute requirement for miRNAs for the generation of Tfh and GC B cells. Of note, Tfh and GC B cell differentiation pathways are regulated by common transcriptional repressors: While Bcl6 drives the differentiation of both Tfh and GC B cells, Blimp-1 antagonizes Bcl6 function by promoting effector T cell and plasma cell differentiation, respectively [51, 52] (Figure 1). It is tempting to speculate that complex miRNA networks may also cooperate in both Tfh and GC B cells to modulate related gene expression programs that contribute to the cell fate decisions in the GC. For example, miR-155, one of the most heavily studied miRNAs in lymphocytes, has both positive and negative effects on the GC B cell response. This highlights the complexity of miRNAs in regulating cell functions. Interestingly, miR-155 may also regulate Tfh function through c-Maf, a *bona fide* miR-155 target that is important for IL-4 production by Th2 cells [29].

Future studies will further unravel the roles of particular miRNAs and the genetic networks they affect. Given that individual miRNAs can target hundreds of genes, detailed assessment of these global networks will provide unprecedented views on the function of miRNAs in these cells. Continued progress will also yield novel insights into the regulatory pathways that govern immune cell function, and holds promise for the development of therapies that target miRNAs or their target genes in autoimmune diseases and malignancies that derive from dysregulated GC responses.

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•• of outstanding interest:

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Highlights

- Germinal centers (GCs) are hallmark structures of humoral immunity
- MicroRNAs (miRNAs) are important regulators of T follicular helper (Tfh) cell differentiation
- miRNAs regulate several aspects of the GC B cell response
- miRNA dysregulation in T and B cells contributes to the development of autoimmunity and cancer

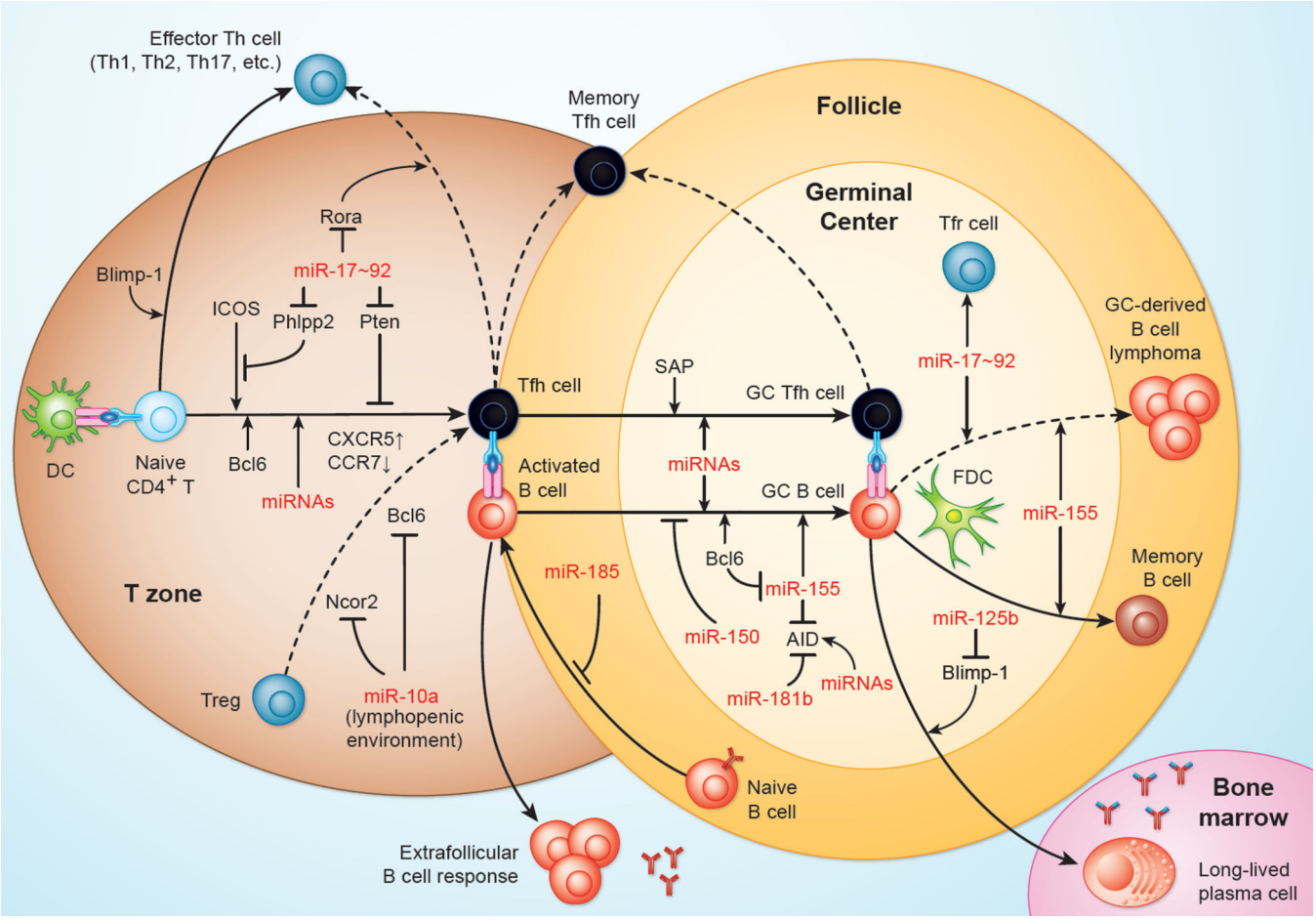

Figure 1. MicroRNA regulation of the germinal center response

MicroRNAs (miRNAs) regulate distinct aspects of the germinal center (GC) response. Upon priming by antigen-presenting dendritic cells (DCs) in the T cell area of secondary lymphoid organs, naïve $CD4^+$ T cells differentiate into effector T helper (Th) cells that migrate into the periphery where they mediate their effector functions. T follicular helper (Tfh) cells are also generated during priming by DCs. These early Tfh cells upregulate the transcriptional repressor Bcl6 and the costimulatory molecule ICOS. Subsequent upregulation of the chemokine receptor CXCR5 and downregulation of CCR7 enables these cells to localize to the T-B zone border and interfollicular regions where they interact with activated B cells in a cognate fashion. The induction of the Tfh cell gene expression program is dependent on miRNA expression by T cells. miR-17~92 promotes Tfh cell differentiation by repressing *Pten* and *Phlpp2*the latter being an important regulator downstream in the ICOS signaling pathway. In addition, miR-17~92 prevents co-expression of effector Th cell genes that are normally not expressed by Tfh cells through repression of *Rora*. The generation of T follicular regulatory (Tfr) cells residing in the GC is particularly sensitive to miR-17~92 dosage. miR-10a prevents the conversion of $Foxp3^+$ Treg cells into Tfh cells under lymphopenic conditions. The Tfh gene expression program may serve as an intermediate state in effector Th cell subset differentiation, and Tfh cells may also persist as memory cells. While most activated B cells enter the extrafollicular pathway, some clones, together

with Tfh cells, reenter the follicle in a process that requires Bcl6 and SLAM-associated protein (SAP) expression by B and T cells, respectively, to establish GCs. In these structures, B cells undergo somatic hypermutation, affinity maturation, and class switch recombination to generate memory B cells and long-lived plasma cells, which produce high-affinity antibodies. miRNA expression by B cells is required for robust GC B cell responses. miR-155 regulates several aspects of the GC reaction, exerting a positive net effect. Nevertheless, miR-155 has also been shown to repress AID, a gene that is required for somatic hypermutation and class-switch recombination. miR-155 itself is regulated by Bcl6. miR-185 inhibits B cell activation, and overexpression of miR-150 or miR-181b inhibits GC B cell responses. Plasma cell differentiation is inhibited by miR-125b through repression of Blimp-1 and other targets. Overexpression of miR-155 or miR-17~92 promotes GC B cell lymphomagenesis. Follicular dendritic cells (FDCs) might regulate miRNA expression in GC B cells through cell adhesion, a process that has also been correlated with lymphomagenesis.