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INSIGHTS

Bhlhe40: Gatekeeper of the GC

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The generation of high-affinity antibodies in the germinal center (GC) requires interplay between GC B cells and T follicular helper cells. Rauschmeier et al. (2021. *J. Exp. Med.* https://doi.org/10.1084/jem.20211406) report that Bhlhe40 restrains GC output through distinct regulatory roles in both arms of the response.

In this issue of *JEM*, Taras Kreslavsky, Meinrad Busslinger, and colleagues reveal that the transcription factor Bhlhe40 is a key player in limiting the size of the germinal center (GC) reaction through playing a separable and cell-intrinsic role in both GC B cells and T cells (Rauschmeier et al., 2021). These findings add to a growing list of transcription factors that act independently in both arms of the humoral immunity, although Bhlhe40 stands out as it acts predominantly as a negative regulator of the early stages of the GC response. Although the downside to this release of restraint in GC was not immediately apparent, aged Bhlhe40 mice developed a GC-like lymphoma at high frequency.

The production of high-affinity antibody is essential for our capacity to ward off microbial infections and underpins the success of most vaccination strategies. This process occurs in a transient micro-anatomical structure called the GC. The GC response requires the interaction between two independently generated lineages of lymphocytes, GC B cells and a specialized subset of $CD4^+$ T cells termed T follicular helper (T_{FH}) cells (Victora and Nussenzweig, 2012). The bulk of the GC is comprised of GC B cells, which are further separated into centrocytes that inhabit the histologically apparent light zone of the GC and centroblasts that are found in the dark zone. The GC reaction is an iterative process where centrocytes interact with T_{FH} cells, receiving selection in the form of "help" that promotes survival and movement to the dark zone where the B cells proliferate and undergo activation-induced cytidine deaminase (AID)–mediated hypermutation of their immunoglobulin genes. Entry and exit from the GC is tightly regulated, as this process has the potential to generate B cells with the capacity to produce high-affinity antibodies against self-antigens leading to autoimmune processes (Young and Brink, 2021), while the GC is also one of the major sources of human lymphomas due to the combination of high proliferation rates and AID-mediated DNA recombination and mutation (Mlynarczyk et al., 2019). Rauschmeier et al. demonstrate in mouse models that Bhlhe40 restrains the size of the GC by limiting the frequency of both GC B and T_{FH} cells (Rauschmeier et al., 2021).

Bhlhe40 has been reported to play multiple roles in immune cell biology, including as a regulator of cytokine production and regulatory T cell frequency in $CD4^+$ T cells and the frequency of tissue-resident memory $CD8^+$ T cells (Cook et al., 2020). As it was reported that Bhlhe40-deficient mice develop a mild autoimmune phenotype with age (Sun et al., 2001), the authors set out to examine the function of this protein in the GC reaction. *Bhlhe40* expression was rapidly induced in activated mouse B cells by both antigen and signals arising from T cell help, supportive of a GC function. Bhlhe40-deficient mice showed a pronounced increase in the frequency of GC B and T_{FH} cells both in the absence of any experimental challenge and after protein immunization. The increase in GC B cells was cell intrinsic and could be recapitulated by the immunization of WT mice that received naive BCR transgenic B cells lacking Bhlhe40. Strikingly, the response to the same antigen delivered in a T cell–independent setting, thus not eliciting a GC response, was equivalent between WT and Bhlhe40-deficiency B cell responses. The competitive advantage of Bhlhe40-deficient GC B cells was detected as early as day 3 after immunization, demonstrating that Bhlhe40 plays a role in the earliest initiation steps of the GC. Crucially, analysis of *Bhlhe40* expression revealed that this factor was largely absent from GC B cells and instead was expressed in antigen-activated B cells that express CCR6. A similar expression in pre-GC B cells can also be observed in human tonsil (King et al., 2021). Analysis of the cell biological consequences of Bhlhe40 loss, including proliferation, apoptosis, and migration, as well as RNA sequencing of the pre-GC compartment, suggested that Bhlhe40 loss did not impact any of these

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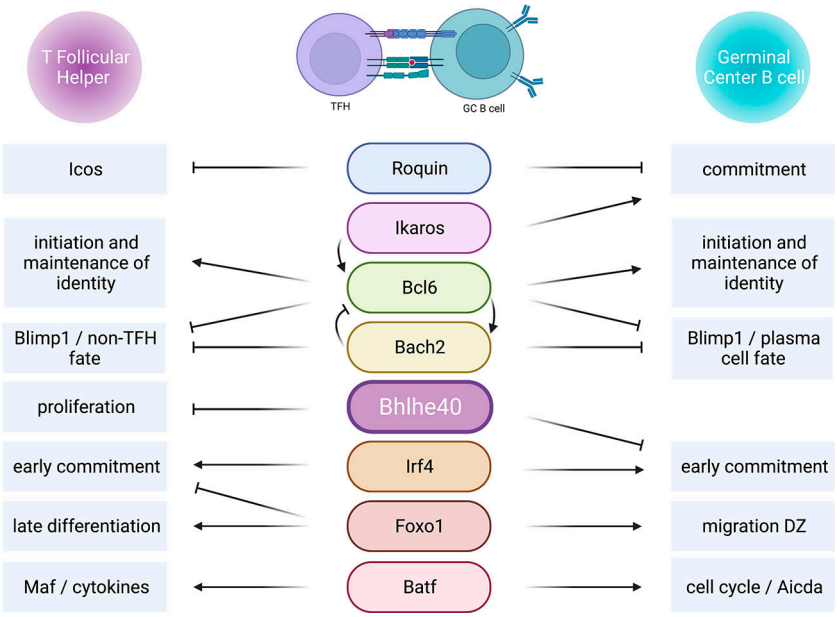

Factors regulating both GC B cell and T_{FH} cell differentiation and function. The transcription factors as well as the RNA-binding protein Roquin that play roles in the regulation of both arms of the GC reaction are listed, along with their major functions. Arrows indicate positive activity; bars indicate a negative regulatory role of the protein on the function indicated. Rauschmeier et al. (2021) identified Bhlhe40 as a repressor of both GC B and T_{FH} cells. DZ, dark zone. Created with BioRender.com.

essential processes and instead pointed to the possibility that Bhlhe40 acts by limiting the differentiation of activated B cells to the GC B cell fate. Although the changes in gene expression were surprisingly mild, analysis of Bhlhe40 binding sites using chromatin immunoprecipitation coupled with deep sequencing supported a broad role for this protein in repressing GC B cell genes in activated B cells.

Although the above data clearly point to a cell-intrinsic function for Bhlhe40, the authors also generated a new floxed allele and were surprised to find the B cell–specific loss of Bhlhe40 resulted in a milder increase in GCs than pan-blood cell inactivation. This suggests a role for a non–B cell population in the phenotype. Indeed, analysis of mice lacking Bhlhe40 in T cells revealed a similar partial recapitulation of the Bhlhe40-null phenotype. In contrast to GC B cells, the role of Bhlhe40 in T_{FH} cells appears to be the inhibition of proliferation, as the deficient T cells showed hyperproliferation and expression of genes associated with cell division.

This elegant series of experiments definitively shows that Bhlhe40 joins a small group of transcription factors, including Bcl6, Batf, Irf4, FoxO1, Bach2, and Ikaros family members, as well as the RNA-binding protein Roquin, that are required for the GC response through the regulation of both the B and T cell arms (see figure; Crotty, 2019; Hart and Laufer, 2021; Song and Matthias, 2018). Bcl6 is the best characterized of these factors, being essential for the formation of both GC B and T_{FH} formation, although its mode of action appears highly specialized for each cell type (Choi and Crotty, 2021). Bcl6 also appears to be limiting for GC formation, with its overexpression resulting in a similar increase in GC formation to that outlined after Bhlhe40 loss (Robinson et al., 2020). GC B and T_{FH} cells are also lost in mice lacking either Irf4 or Batf, factors that can cooperatively bind DNA. Irf4 mirrors Bhlhe40 in that its expression is up-regulated in naive B and T cells by antigen receptor signaling, and then rapidly silenced in GC B cells (Willis et al., 2014). It appears likely that Bhlhe40 and Irf4 are important in the same activated fraction in the earliest stages of the B cell response. It is curious that while Bhlhe40 is a clear negative regulator of the GC differentiation process, the authors were unable to find a direct role for this factor in the expression of any of the above-mentioned major regulators of GC biology (see figure). Thus, the exact role of Bhlhe40, particularly in GC B cells, and its relationship to other established regulators of the GC reaction remains enigmatic.

Positioning Bhlhe40 in the transcriptional network controlling GC B cell biology is further hindered by the limited understanding of its mechanisms of action. Bhlhe40 is expressed in a wide range of cells and has been shown to act as both activator and repressor of transcription, with its activity likely depending on its selective interaction with an ensemble of partner proteins (Cook et al., 2020; Kiss et al., 2020). As a repressor, Bhlhe40 has been proposed to recruit HDAC1 in nonimmune cells, as well as displacing activating transcription factor binding. Interestingly, one of these potentially displayed transcription factors is IRF4 (Cook et al., 2020), which is crucial for the up-regulation of *Bcl6* (Ochiai et al., 2013). Further complicating the interpretation of its function, the repression of proliferation by Bhlhe40 can also be mediated by nontranscriptional mechanisms, as it can bind to, and stabilize, cyclin E, thus preventing cell cycle progression (Kiss et al., 2020).

Although, Bhlhe40-deficient mice were previously reported to display some symptoms of autoimmunity, including the presence of autoantibodies (Sun et al., 2001), the current study did not report any overt pathological consequences of the increased GC formation in knockout mice. However, aged Bhlhe40-deficient mice develop a clonal lymphoma-like pathology with high penetrance. The individual lymphomas displayed a variety of immunoglobulin isotypes and had ongoing *Aicda* (encoding AID) expression that may support tumor progression. Whether *BHLHE40* is mutated in similar human mature B cell lymphomas is unknown and represents a key area for future enquiry. Assuming that the enhanced differentiation of activated B cells to GC B cells seen in young mice and the lymphoma observed in aged mode are mechanistically linked, this suggests that relatively mild perturbations in the dynamics of GC entry can have devastating consequences later in life.

Despite being essential components of a healthy immune system, it is also apparent that GCs are a source of danger to the body, as they have the capacity to produce both pathogenic autoantibodies (Young and Brink, 2021) and the malignant clones that ultimately result in lymphoma (Mlynarczyk et al., 2019). The findings of Rauschmeier et al. reveal just how tight the regulation of this process is, with Bhlhe40 acting at a very discrete time and developmental stage of the GC response to put a break on the capacity of

activated B and T cells to form GC B and T_{FH} cells.

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