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Germinal Center Structure and Function: Lessons form CD19

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Abstract

The germinal center is a critical locus of production of protective immunity, but its function is poorly understood. Studies of mutant forms of CD19 revealed differences in signaling in different compartments *inside* the germinal center. Structural findings indicate a selective role in the interaction with follicular dendritic cells in the GC. Loss of these signals leads to surprising changes in germinal center B cells that challenge previous models of GC function.

Keywords

CD19; germinal center; B lymphocyte; antibodies; follicular dendritic cells

Introduction

The germinal center reaction is central to the adaptive humoral immune response, but the mechanisms involved are poorly understood. It is also a fascinating problem in cell biology, with rapid cell turnover, migration, and selection, accompanied by mutation, breaking and recombination of DNA. We know some of the signals required for successful formation of GC, but less of how these signals control B cell responses inside the GC [1].

Mice deficient in CD19 fail to produce GC or specific antibodies after antigen challenge [2]. However, we identified a mutant form of CD19 that, when expressed in mice lacking native CD19, was sufficient to rescue a sustained GC reaction in immunized mice, but the GC fail to produce B cells capable of producing specific antibodies to the immunizing antigen [3,4]. Thus, it appeared that these mice might have a defect *inside* the GC, and thus might provide information about how signals operate in the GC. This review is to summarize what we have learned about the signals that regulate GC from study of these mice. Readers are referred to other recent reviews for a more general overview of GC function [5,6].

CD19 Transgenes and Function in vivo

We and others had extensively characterized signaling by CD19 induced by stimulating B cells in vitro [7–9]. Intracellular proteins that bind to phosphorylated residues in the CD19 cytoplasmic domain mediate CD19 signaling [10,11]. There are nine such residues. Some of these tyrosines share common motifs in the flanking amino acids, and indeed these can be shown to bind SH2 domains from the same proteins [12]. To test the relevance of the studies

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of signal transduction in vitro to in vivo physiology, we expressed CD19 transgenes that encoded either wild-type CD19 protein or forms of CD19 containing paired mutations of CD19 tyrosines with similar function. Lines of mice were selected that expressed levels of CD19 that were comparable to native CD19. These lines were then tested for the ability to rescue the known physiologic functions of CD19 when crossed onto the CD19^{-/-} background. To make a long story short, the only pair of tyrosines that were essential for CD19 function was Y482/Y513, the tyrosines on CD19 that bind Phosphatidylinositol 3-kinase [3].

Mice that expressed CD19 containing mutations in these two tyrosines ("CD19 Y482/513F") were equivalent to CD19 knockout mice in most respects. Numbers of B1 and marginal zone B cells were reduced to similar levels, and there was no increase in titers of specific antibodies after immunization. The one difference was that, although substantially reduced in comparison to wild-type mice, an increase in the numbers of PNA⁺, Fas⁺ GC B cells was apparent in the spleen after ip immunization of CD19 Y482/513F mice, whereas no response could be measured in mice lacking CD19.

The ability of CD19 Y482/513F mice to form GC but their failure to produce specific antibodies after immunization suggested a problem in the GC reaction in these mice. Indeed, unlike other mutant mice in which GC form but rapidly collapse, the GC in CD19 Y482/513F mice are sustained for at least three weeks. So the question remains, what are the CD19-dependent B cell responses inside the GC?

Initial Induction of Germinal Center B Cells

The earliest step in the differentiation of B cells into the GC is manifest as an increase in expression of Fas and in the ability to bind PNA [13,14]. This first step is likely to involve an antigen-dependent, and hence B Cell Receptor (BCR)-dependent, signal. In transgenic systems, B cells with extremely low affinity can enter GC, although not if competing cells with higher affinity are present [15]. This is followed by a second differentiation step in the GC, in which surface expression of IgD and, in mice, CD38 are reduced [16].

In C57Bl/6 mice, GC are rare (although not entirely absent) in mice lacking CD19 (Myers and Carter, unpublished). Thus, CD19 signaling plays a critical, although not essential, role for the induction of the first step in differentiation of B cells into the GC response, most likely through modulation of the BCR signals generated by initial encounter of antigen, which is predominantly low affinity. CD19 is phosphorylated and binds cytoplasmic signaling molecules after ligation of the BCR alone [17–21], and this may be the mechanism of CD19 signaling in this initial activation step of follicular B cells. Mice lacking CD19 form GC in Peyer's Patches [22]. Peyer's Patches are unique in their anatomical location to the gut and as such, they are continuously exposed to antigen. Thus, continuous, high levels of antigen exposure seems to be sufficient to drive B cells without CD19 into the GC, but these GC were smaller compared to wild type mice, so even here CD9 plays an important enhancing role.

The number of PNA⁺, Fas⁺ GC B cells was reduced in CD19 Y482/513F mice immunized with ip SRBC (a strong antigen), compared to wild-type mice, but were present to a substantially greater extent than in mice lacking CD19. Histologically, GC were present and persisted in the mice with mutant CD19, although the GC were smaller than in wild type mice. Thus, the CD19 Y482/513F mice were readily able to form organized GC, unlike CD19-deficient mice.

To analyze the progression of GC B cells in the CD19 Y482/513F mice through the initial steps in differentiation, GC B cells that were not switched (IgM⁺) were analyzed for expression of IgD and CD38. The number of B cells at the initial stage of GC differentiation (PNA⁺, Fas⁺, IgM⁺, IgD⁺, CD38⁺) was equivalent in wild-type and CD19 Y482/513F mice [3]. Thus,

the surprising observation was that signaling by CD19 Y482/Y513, although required for all other known functions of CD19, is not required for the induction of the initial step in GC differentiation. This may reflect the fact that minimal antigen binding (or, at least minimal *affinity* of binding) is sufficient to induce B cells to enter the GC reaction [15]. The number of B cells at the initial step in the GC reaction is a balance of their production and of the movement of cells out of this compartment [23,24], so a downstream block may also increase the number of cells at the first stage (see below). Therefore, CD19 Y482/Y513 may still play a modulating, although not essential, role in the induction of the first step in GC differentiation.

The fact that B cells are activated to enter the GC reaction in the CD19 Y482/513F mice but not detectably in CD19^{-/-} mice implies a signaling function for the CD19 tyrosines *other* than Y482 and Y513 [3]. To date, this is the only observation that implies any function of the CD19 tyrosines that bind proteins other than PI3K. Our preliminary data with a form of CD19 in which all tyrosines *except* Y482 and Y513 are mutated implies that these two tyrosines are *sufficient* to mediate the role of CD19 in induction of GC (Crowder and Carter, manuscript in preparation), but the data in CD19 Y482/513F mice imply that other CD19 tyrosines are functional and sufficient to enable the first step in GC differentiation when association of PI3K with CD19 is abrogated by mutation of CD19 Y482 and Y513.

Although B cells in CD19 Y482/513F mice can enter the GC reaction, further differentiation of GC B cells in these mice was partially blocked. This was manifest as normal numbers of B cells with an early GC phenotype (PNA⁺, Fas⁺, IgM⁺, IgD⁺, CD38⁺), but substantially reduced numbers of PNA⁺, Fas⁺, IgM⁺, CD38⁻, IgD⁻ B cells. Histologically, in wild-type mice, the border between the IgD⁺ follicular B cells and the IgD⁻ GC B cells was sharp, implying rapid down-regulation of IgD once B cells entered the GC. In contrast, IgD⁺ cells were readily apparent inside splenic GC of CD19 Y482/513F mice [4]. The IgD⁺ cells were only present in the region of the GC that includes Follicular Dendritic Cells (FDC) (GC light zone). This was surprising in light of previous models [1,25], which suggest that B cells first undergo proliferation in the dark zone of the GC, and then migrate into the light zone, where they undergo selection. In contrast, in the presence of a block of differentiation *within* the GC in CD19 Y482/513F mice, B cells with an immature GC phenotype are observed in the FDC (light zone) (Fig. 1). These cells are present in the well-developed GC, and are unlikely to have undergone proliferation (see below), so it is more likely that these cells have entered the GC after the GC was established. Such cells are difficult to visualize in wild-type mice because they normally differentiate rapidly into IgD⁻ cells [16]. These cells have undergone initial GC differentiation, so they may not correspond to non-specific B cells that have been demonstrated in GC in intra-vital imaging studies. Possibly the extensions of FDC that reach up into the follicular mantle are able to recruit such new cells into the GC. These observations also raise the question of whether the mechanism of recruitment of cells into an established GC differs from what happens during the initial formation of the GC. However, in the early GC, at day 2 after immunization, the clusters of PNA⁺ cells were in contact with FDC in carefully done confocal imaging [3]. Only after that did distinct collections of PNA⁺ cells outside the FDC zone appear. It remains possible that the mutant form of CD19 has somehow resulted in the abnormal localization of B cells with an early GC phenotype, so that they appear in the FDC zone. However, we favor the hypothesis that interaction with FDC drives GC B cells to the second step of differentiation, manifest as the decrease in surface IgD and CD38.

The presence of cells in the early stages of the GC reaction is supported by previous work from the Rajewsky lab [26]. Ig gene sequences derived from cells in the FDC zone of the GC encoded IgM heavy chains with few mutations. More recent intra-vital imaging studies have demonstrated that B cells, other than adoptively transferred B cells specific for the immunizing antigen, are present in the FDC zone [27]. We do not yet know the relationship of these cells to the B cells we observe with an early GC phenotype (PNA⁺, Fas⁺, IgM⁺, IgD⁺, CD38⁺),

which normally appears to be a short-lived transitional stage, but these observations do support the concept that additional cells are recruited into the FDC zone in an ongoing GC response. However, the motility characteristics of these cells differ from those of transferred, antigen specific GC cells in these studies, and the number of such B cells newly entering an established GC that can out-compete the resident antigen-specific cells is likely to be small.

Our observations that the earliest detectable aggregates of PNA+ B cells (day 2 after SRBC immunization in B6 mice) occur in close association with FDC and that cells with an early GC phenotype appear in the FDC zone of the GC at later stages (albeit more apparent in the presence of a partial block in further differentiation in mice with mutant CD19) differ from the previous model that GC B cells first undergo proliferation in the dark (non-FDC) zone before entering the light (FDC) zone [25]. Models of GC using B cells expressing a BCR transgene also support the model of B cells moving to the T-B border before migrating back deeper into the follicle to form the GC (24, 25). Several caveats to these results need to be considered. In the studies that led to the initial model, the mice were pre-immunized to a carrier before challenge with the hapten-carrier conjugate. Perhaps the pre-existing immunity in the T cell compartment altered the nature of T-B interactions after secondary challenge. With the transgenic models, the BCR start with a relatively high affinity receptor. In several such models, close analysis of the transgenic B cells reveals some level of activation above that of truly resting follicular B cells. One possibility is that the higher baseline affinity and some level of pre-existing activation leads the transgenic B cells to respond in a manner analogous to marginal zone B cells, which migrate to the T cell zone in response to immunization with appropriate antigens. Our current work and that of others suggests that some follicular cells express a partial marginal zone phenotype (“marginal zone wannabe”). It may be difficult to dissociate a marginal zone-like response, with migration to the T-B border, from a truly naïve B cell GC response, when using BCR transgenics, particularly after adoptive transfer. Indeed, Manser has reported GC formation in the absence of T cells [28].

Differential Regulation of Proliferation of Light-zone and Dark-zone Germinal Center B Cells

Rapid turnover is a hallmark of a GC B cells [29,30]. Indeed, aggregates of proliferating cells, detected by binding of Ki67 or by BrdU incorporation, can be used to identify GC. Previous models indicated that proliferation occurred in the dark (non-FDC) zone, and that cells exited the cell cycle before moving to the light (FDC) zone to undergo selection [25,31]. Previous work by Berek initially suggested that, at least in the mouse, proliferating B cells were in both zones [32,33]. Our observations were similar, whether using Ki67 binding or BrdU incorporation [4]. One could argue that such cells had actually undergone cell division in the non-FDC zone, but had not yet lost the hallmarks of proliferating cells when they reached the FDC zone. However, our time course studies (Wang and Carter, unpublished) indicate that BrdU incorporation is apparent in both zones at the earliest time points at which labeled cells can be detected. Labeling may be more prominent in the non-FDC zone, but not any earlier than in the FDC zone. Secondly, in our high-resolution immunofluorescence studies, IgM-expressing cells were only apparent in the FDC zone (acknowledging that such studies are complicated by the presence of IgM captured on FDC and that Waldschmidt interpreted images from his group differently [16]), and IgM+ GC B cells are in cell cycle. Finally, we could detect mitotic cells in the outer regions of a GC on conventional H&E stains [supplemental data in 4], although differentiation of light and dark zones is difficult on sections of mouse spleen.

Although BrdU+ or Ki67+ B cells were readily detectable in the FDC zone in GC of wild-type B6 mice, the numbers of such cells were reduced in GC of CD19 Y482/513F mice [4]. The specificity was apparent in that these indicators stain the nuclei, in contrast to the halo appearance of PNA staining. Thus, together they form a “donut and hole” appearance. Most

of the holes in the PNA⁺ donuts were filled in the FDC zone of wild-type mice, while the holes were empty in the donuts in the FDC zone of CD19 Y482/513F mice (Fig. 2). In contrast, the percentages of B cells in cell cycle were equivalent in the non-FDC zone of wild-type and CD19 Y482/513F mice. Thus, CD19 appears dispensable for proliferation in the non-FDC zone, but critical for proliferation in the FDC zone of splenic GC in mice. The histological analysis was supported by flow cytometry, which demonstrated reduced numbers of IgM⁺ cells in cell cycle in mice with mutant CD19, while the percentages of switched, IgG⁺ cells in cell cycle was equivalent in mice with mutant or wild-type CD19, coupled with our finding that IgM⁺ cells were largely restricted to the FDC zone.

The broader significance of these observations is that proliferation in the FDC and non-FDC zones of the GC are governed by different signals. We interpret our results to indicate that relatively limited BCR signaling, which does not require amplification by CD19, is sufficient to drive proliferation in the non-FDC zone. Perhaps non-FDC zone proliferation is driven largely by other signals, such as CD40 because when CD19-deficient B cells are stimulated with anti-CD40 *in vitro*, there is no significant difference in proliferation compared to wt B cells (16). Teleologically, this also makes sense if the non-FDC zone is viewed as a reservoir of B cells with some level of antigen specificity that can undergo mutation linked with expansion, which then serves as a pool from which higher affinity B cells are selected. Consideration of such a model might be important in interpreting results of studies that test the role of antigen in the formation of GC [34,35]. Our results suggest that limited BCR signals might be sufficient to drive the formation of a normal non-FDC zone.

Regulation of Class Switching, Mutation and Selection in the Germinal Center

Switched GC B cells were readily apparent in CD19 Y482/513F mice, despite the partial block in differentiation and proliferation of IgM⁺ cells in the GC of these mice. CD19 may play some role in signals for switching, as the absolute numbers of switched GC B cells were reduced in spleens of mice with mutant CD19, but the percentage of GC B cells that had undergone switching was higher than in wild-type mice. Most likely switching is more dependent on other signals, such as TNF-family receptors and cytokines [6]. Unlike some previous reports, we could not find any deficit in CD40 function *in vitro* in B cells from mice with mutant CD19 (Wang and Carter, unpublished).

Despite the fact that CD19 appears to play a limited role in switching *per se*, it is important in determining subclasses. CD19-deficient mice are able to switch to IgA in Peyer's Patch GC [22] and to subclasses important for T-independent type 2 responses when immunized with high-density TNP-ficoll conjugates [36]. However, the normal predominance of IgG1 cells after immunization with SRBC or NP-CGG is much reduced in CD19 Y482/513F mice. This appeared not to result in a change in the preferred subclass, but a re-distribution across all subclasses of IgG. Whether this is the result of altered signals prior to switching, such as induction of sterile transcripts, or selection after switching remains to be determined.

Like class switching, mutation of Ig sequences occurred at near normal frequencies in CD19 Y482/513F mice. However, selection was clearly disrupted. Using the phOx-CSA system (35–37), canonical changes that are known to increase affinity were observed in 62% of sequences from mice with wild-type CD19 but in none from CD19 Y482/513F mice.

Hypothesis: FDC Zone Signaling

We interpret the deficiencies observed in the GC of CD19 Y482/513F mice to represent disrupted signaling in B cells in the FDC zone. Although other signaling pathways mediated by CD19 play a role in the initial induction of B cells to enter the GC differentiation pathway, signaling by CD19 Y482 and Y513 is not essential. However, once B cells have entered this

pathway, the next steps, which include down-regulation of expression of CD38 and IgD and entry into cell cycle (prior to switching), are heavily dependent on CD19 Y482/Y513 signaling. Given the restriction of the IgM-expressing GC B cells to the FDC zone (at least in our system) and the fact that the decrease in cell cycling appears limited to the FDC zone histologically, the deficits we observe in IgM+ GC B cells are likely the result of the failure of signaling during the encounter of the B cell with the FDC. Again, this implies an important encounter of the B cells with the FDC prior to proliferation and switching. Despite these deficits, the percentage of switched cells is increased, implying that the ability to switch per se is not dependent on the induction of proliferation within the IgM compartment (the failure of proliferation within the IgM compartment also contributes to the relative increase in the percentage of switched cells).

Once cells switch, proliferation and mutation proceed unimpeded in the GC of CDY482/513F mice. These functions are probably linked and are preserved in the relatively normal non-FDC zone of these mice. The net result of the GC reaction in these mice is no increase in specific antibody or in B cell memory, implying that the non-FDC events are not sufficient to produce a specific antibody response, most likely due to the failure of selection. We presume that the inability to select high affinity mutations is also a result of failure in the interaction of the B cells with FDC, although we have no direct evidence of this. However, when CD19 signaling is engaged, B cells adhere to stromal cells at much higher frequency compared to resting B cells [37], further supporting the idea that CD19 signaling impacts B cell-FDC interactions. If the deficiencies seen in GC B cells from CD19 mutant mice are as a result of defective interactions of B cells with FDC, the interesting question is whether this selection event occurs at the same interaction that is responsible for inducing the differentiation and proliferation at the IgM+ stage, or a second interaction with FDC after switching. Understanding the answers to such question will require integrating results from intra-vital imaging (although spleen and lymph node GC may differ), single cell sequence analyses, mathematical modeling, and studies of altered signaling, such as through CD19.

abbreviations

GC	germinal center
FDC	follicular dendritic cell
ip	intraperitoneal
SRBC	sheep red blood cells

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Fig. 1. IgD⁺ B cells in the GC of mice with mutant CD19. Spleen sections from wild type (top) or CD19 Y842/513F mice that had been immunized with SRBC were stained for PNA (green), IgD (red) or CD35 (blue). In the CD19^{+/+} mice, the border between IgD follicular cells and PNA⁺ GC cells is clear (arrow), and very few IgD⁺ GC B cells are apparent. In contrast, in mice with mutant CD19, PNA⁺, IgD⁺ cells are present in the GC, in the region of the GC that contains FDC (yellow arrows), but not in the region of the GC that does not contain FDC (blue arrows). *modified from [3], used with permission.*

Fig. 2.

Proliferation in the FDC and non-FDC regions of the GC. Spleen sections from wild type (top) or CD19 Y482/513F mice that had been immunized with SRBC were stained for PNA (green), BrdU (red) or CD35 (blue). In the CD19^{+/+} mice, BrdU⁺ cells are seen in both the FDC (red arrow) and the non-FDC (blue arrow) regions of the GC. *modified from [3], used with permission.* In the mice with mutant CD19, while BrdU⁺ cells are abundant in the non-FDC zone (blue arrow), few GC B cells in the FDC zone are BrdU⁺ (red arrows).