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Directing traffic in the germinal center roundabout

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B-cells undergo iterative rounds of somatic hypermutation and selection for high-affinity antigen binding in the germinal center (GC) microenvironment. Two new studies provide insights into the temporal and spatial control mechanisms that act within B-cells and follicular dendritic cells (FDCs) to jointly govern B-cell differentiation and cell traffic within the GC.

In T-cell-dependent immune responses, antigen-activated B-cells undergo repeated cycles of IgV gene somatic hypermutation and selection for high-affinity antibodies before differentiating into memory B-cells and plasma cells. The making of highly specific B-cells is a rapid, dynamic process that occurs in a confined histological environment in the lymph nodes called GC, which consists of two zones – a dark zone (DZ) and a light zone (LZ) – that contain B-cells with different biological functions called centroblasts, or DZ B-cells, and centrocytes, or LZ B-cells. Recent studies have provided a good understanding about the dynamic movements of B-cells between DZ and LZ and on the molecular control of the major stages of GC B-cell differentiation[1]. However, GC

B-cells show a marked phenotypic heterogeneity, and the transitions between different developmental cell stages remain incompletely defined. In addition, it is largely unclear how B cell-interacting reticular cells (BRCs) within the GC microenvironment function to direct these movements. Two works in this issue of *Nature Immunology* aim at solving this puzzle. Pikor et al. used a chemokine reporter system combined with single-cell transcriptomics to molecularly characterize DZ FDCs and define their function in the GC reaction[2]. Kennedy et al. identified two functionally distinct, spatially separated B-cell populations in the DZ and uncovered their relationship to LZ B-cells, thus providing new insights into how GC B-cells segregate, and transition between, diverse biological programs[3].

Stromal cells within lymphoid tissues provide an architectural framework that facilitates the spatial organization required for effective immune responses[4]. Recent work using single-cell transcriptomics has highlighted the heterogeneity within lymphoid stromal cell populations, most notably in T-cell areas of the lymph node[5]. However, little is known about the heterogeneity and molecular identity of BRCs due to difficulties in the isolation and enrichment of these cell types for molecular analysis. BRCs are thought to be primarily composed of FDCs occupying the center of the B-cell follicle and marginal reticular cells (MRCs) that reside in the outer part of the follicle beneath the subcapsular sinus.

Within the GC microenvironment, DZ-LZ polarization is thought to be accomplished by differential distribution of BRCs expressing the chemokines CXCL13, which signals through CXCR5, in the LZ and CXCL12, which signals through CXCR4, in the DZ[6,7]. FDCs are one type of BRCs present in the primary follicle where they mediate critical organizational functions in part due to their high expression of CXCL13. Upon immunization, FDCs increase in number and occupy the GC LZ. FDCs can harbor antigen for long periods of time, a function that is critical for the selection of high-affinity

B-cells in the LZ. A second population of BRCs occupying the DZ that express CXCL12 has more recently been described[7,8]. Much less is known about the cellular origin, molecular identity and function of this cell type.

Pikor and colleagues made use of an elegant CXCL13-reporter mouse line to more fully characterize the breadth of stromal cell types present in B-cell areas of the lymph node[2]. Employing this sensitive reporter system, they detected CXCL13-expressing BRCs throughout all B-cell areas in the lymph node. Single-cell transcriptomic analysis on CXCL13-expressing cells unexpectedly revealed that the known BRC subsets, which mainly consist of MRCs and FDCs, comprised only a minority of cells in both naïve and immunized experimental settings. The remainders were composed of previously uncharacterized populations of cells that occupied distinct cellular niches at the T-B border and interfollicular areas.

Most interestingly, Pikor et al. have now more fully characterized the population of CXCL12-expressing BRCs present in the GC DZ[2]. They demonstrate that these cells, which they termed DZ FDCs, share a common cellular origin with their well-described FDC counterpart in the GC LZ (LZ FDCs) [**Fig.1** left]. DZ FDCs were also present in primary follicles and the number of both cell types increased following immunization. Further work is needed to determine whether this expansion is due to the proliferation of pre-committed precursors, as proposed by the authors, or from precursor populations of MRCs or pericytes, as previously suggested[9,10]. Finally, through a series of well-executed experiments, Pikor et al. show that the polarization of the GC into DZ and LZ requires crosstalk between $CXCR4^{hi}$ DZ B-cells and CXCL12-expressing DZ FDCs. In the absence of CXCL12 in DZ FDCs, polarized GCs did not develop [**Fig.1** right], resulting in poor quality T-dependent humoral immune responses, similar to findings made in a previous study that focused on CXCR4-deficient GC B-cells[7].

Now, turning to the inhabitants of this newly described FDC meshwork, the B-cells moving back and forth within this scaffold while drastically changing their phenotype. A landmark study demonstrated that DZ and LZ B-cells can be separated based on differential CXCR4 and CD83 cell-surface expression, which allowed the molecular analysis of flow-cytometrically isolated $CXCR4^{+}CD83^{lo}$ DZ and $CXCR4^{lo}CD83^{+}$ LZ B-cells, providing deep insights into GC biology[11]. Since then, genetic studies in mice have revealed a functional heterogeneity of GC B-cells extending well beyond the DZ-LZ categorization, reflecting the developmentally distinct GC stages associated with proliferation, hypermutation, selection, post-GC differentiation and the continual differentiation of B-cells recirculating in the LZ-DZ roundabout.

Kennedy et al. reasoned that since GC B-cells visualized by CXCR4 and CD83 staining in effect appear like a (somewhat banana-shaped) continuum rather than comprising two demarcated populations, functionally distinct, smaller subpopulations may have been concealed in the transcriptional analysis[3]. Hence, the authors sorted GC B-cells into three fractions: $CXCR4^{+}CD83^{-}$, $CXCR4^{-}CD83^{+}$, and $CXCR4^{+}CD83^{+}$, which they referred to as DZ, LZ, and a newly defined gray zone (GZ) fraction, and performed transcriptional profiling, genome accessibility analysis, and mass spectrometry (proteome, phosphoproteome). In all experimental approaches, DZ, LZ and GZ populations clustered into distinct groups, with the pattern of the transcriptomic, epigenomic and proteomic differences indicating that the respective subsets exert unique functions during GC B-cell development. Most notably, the GZ fraction comprised a unique cluster of genes that was enriched for cell cycle-associated genes, including Cyclin B1. This finding was verified by mass-spectrometry analysis that revealed strong Cyclin B1 upregulation and enrichment of phosphopepides of cell cycle-related pathways in the GZ fraction.

Where in the GC are these Cyclin B1$^+$ B-cells localized? Using multicolor-confocal microscopy, Kennedy and colleagues could locate discrete clusters of Cyclin B1$^+$ B-cells within the histologically defined DZ in mice immunized with two different T-dependent antigens[3]. In a parallel experiment employing flow-cytometry, the authors effectively sliced up the CXCR4/CD83-stained GC population into about a dozen small pieces based on differential expression of these markers and determined Cyclin B1 protein expression in the gated cell populations. The results showed that Cyclin B1 expression was highest in the population that stained most strongly for both CXCR4 and CD83, and which comprised the highest percentage of cells in S-phase among the fractions. Overall, the findings point towards the existence of two functionally distinct GC B-cell populations located at different areas in the DZ, which Kennedy et al. refer to as DZp, for "DZ proliferation", and DZd, for "DZ differentiation" [**Fig.1** middle]. Of note, AID and AID expression-inducing transcription factors were upregulated in DZd B-cells, suggesting that somatic hypermutation is confined to these cells. This begs the question as to what is the temporal relationship of DZp B-cells to the DZd and LZ B-cells? A combination of single-cell transcriptomic and pseudotime analysis could position the DZp cells at the transition from LZ to DZd B-cells.

Previously, single-cell RNA-seq and genetic studies provided initial evidence for the occurrence of proliferating GC B-cells in the CXCR4$^+$CD83$^+$ fraction that, following selection, transition from the LZ to the DZ[12,13], possibly expressing the transcription factor AP4[14]. By employing an integrated approach including transcriptome, proteome and epigenomic analyses, Kennedy et al. have now identified and comprehensively characterized a distinct proliferating GC B-cell population localized in the DZ that is developmentally situated in-between antigen-selected LZ B-cells and "hypermutating" DZ B-cells[3] [**Fig.1** middle]. Whether further heterogeneity within the DZ FDC population exists to support these distinct DZ B-cell states merits additional investigation. Overall, a

picture emerges from this and other molecular studies suggesting that a biological program aimed at fuelling an antigen-selected LZ B-cell with metabolic nutrients prior to cell division is set up in the LZ B-cell through the joined activity of several signaling pathways (including MYC, NF-κB, mTORC1)[3,15](and reviewed in ref.[1,16]). The well-fed LZ B-cell then transits to the DZ for cell division[3], requiring AP4 activity[14]. This DZp B-cell may employ fatty acid oxidation (FAO) to meet its bioenergetic demands, as suggested by recent findings showing that proliferating GC B-cells use FAO rather than aerobic glycolysis as major energy source[17].

Finally, GC B-cell lymphomas, which form a phenotypically, genetically and clinically heterogeneous group, are commonly thought to originate from the malignant transformation of GC B-cells at various DZ or LZ developmental stages[16]. Can the identification of heterogeneity in GC B-cells and the stromal networks that support them further increase the sharpness of the current cell-of-origin picture? Perhaps it does, as suggested by recent work that provides evidence for an altered GC differentiation program in the presence of a MYC signature in a clinically distinct genetic subtype of GC B-cell lymphoma[18].

In summary, the studies of Pikor et al. and Kennedy et al. provide a framework for understanding the microenvironmental cues that support the molecular changes in B-cells at different stages of the GC reaction. These studies advance our knowledge of the signals that are necessary to orchestrate the development of high-affinity antibodies required for effective host defense and may also provide clues to identifying microenvironmental or stage-specific signals that promote or suppress GC-derived B-cell malignancy.

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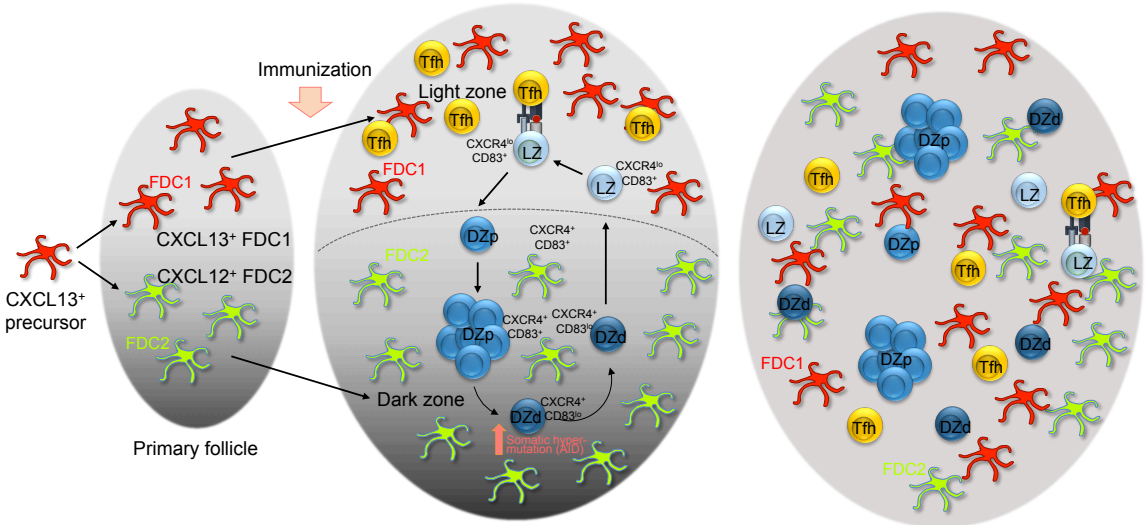
Figure Legend

Figure 1. Temporal and spatial control mechanisms governing B-cell differentiation and cell traffic within the germinal center (GC). (Left) The primary follicle in the lymph nodes comprises $CXCL13^+$ follicular dendritic cells (FDC), referred to by Pikor et al. as FDC1, and $CXCL12^+$ FDCs, named FDC2, that originate from a common $CXCL13^+$ precursor cell[2]. The distinct FDCs localize in areas of the primary follicle that upon immunization become the light zone (LZ) and dark zone (DZ), respectively, in the established GC (see middle panel)[2]. (Middle) LZ-DZ recirculation in the GC as suggested by the work of Kennedy and colleagues[3]. During selection for high-affinity antigen binding, a T-follicular helper (Tfh) cell provides signals to a LZ B-cell that induce a metabolic cell-growth program and migration of this cell to the DZ. The cell differentiates into a DZp B-cell, for "DZ proliferation", and undergoes cell division in certain areas of the DZ. The descendants differentiate into DZd B-cells, for "DZ differentiation", that activate the somatic hypermutation mechanism, before migrating back to the LZ and differentiating into a LZ B-cell. The CXCR4/CD83 cell-surface marker profiles of the corresponding DZ and LZ B-cell subsets are indicated. (Right) The genetic ablation of CXCL12 in FDCs impedes the correct positioning of LZ and DZ FDCs (FDC1 and FDC2) in the corresponding areas within the GC[2]. This precludes the development of a polarized GC and thus the establishment of an ordered LZ-DZ traffic, as a result markedly thwarting the T-dependent humoral immune response.



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