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The microanatomy of B cell activation

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The logistic problem of B cell antigen encounter in the lymph node has recently been studied by dynamic imaging using two-photon microscopy. These studies combined with the early studies of antigen transport have yielded a more complete picture of the orchestration of B cell activation *in vivo*. Here we summarize the recent advances and focus on the specialized macrophages that are critical to this process and the role of B cells themselves as antigen transporting cells.

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Introduction

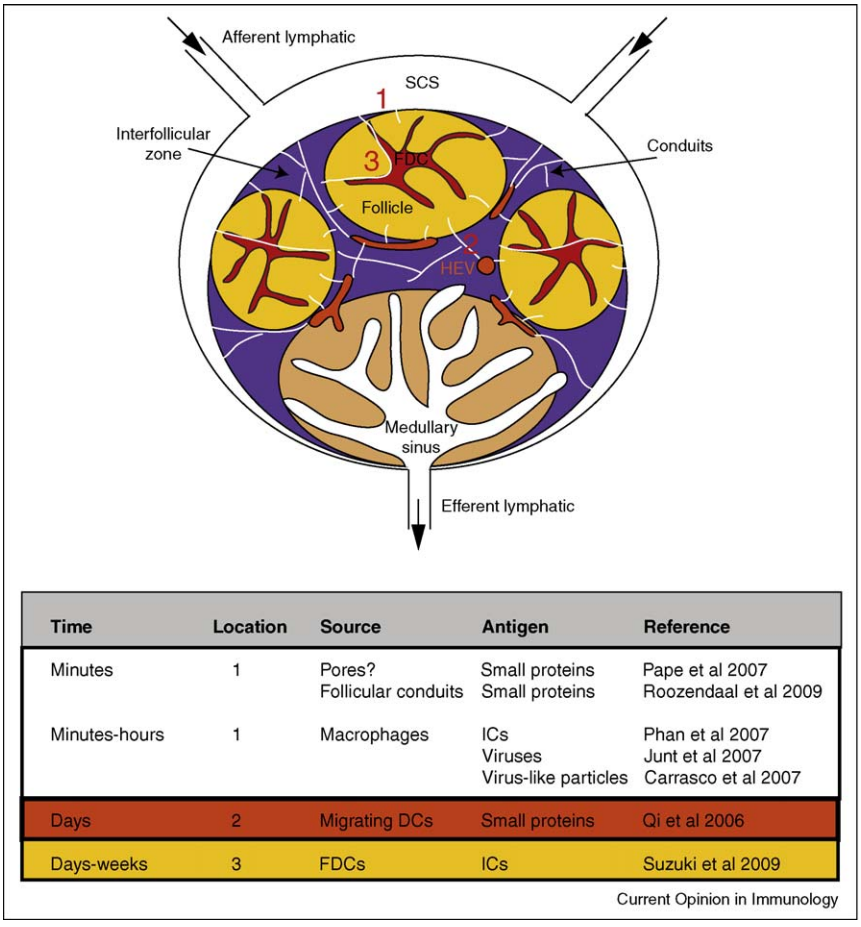
The immune system comprises a complex system of molecular, cellular, and structural components that protects the host against infectious agents and transformed cancer cells by means of exclusion (via mucocutaneous barriers), eradication (via innate and adaptive effector mechanisms), and containment (via granuloma formation). Secondary lymphoid organs (SLOs) such as lymph nodes (LNs) and the spleen are critical for efficient orchestration of the response as they provide a supporting framework within which antigens and rare antigen-specific T and B cells can be efficiently brought together to initiate the adaptive response. As such, understanding the dynamics of cellular migration and antigen trafficking in SLOs and the requirements for antigen encounter in the LN are important steps toward developing a global picture of adaptive immunity. In this review we will discuss recent advances in our understanding of B cell activation with the advent of intravital two-photon microscopy, a technology that has allowed direct visualization of the spatial and temporal organization of the B cell response in intact LNs in real-time. We will examine the logistic problems of bringing antigen into contact with rare antigen-specific B cells and how specialized macrophages that line the subcapsular sinus (SCS) sample the afferent lymph for antigen to present to B cells. We will discuss some biological differences between these SCS macrophages and those that line the medullary sinuses. Finally, we will review the data for B cells as antigen transporting cells and the potential significance of this novel B cell function in FDC antigen deposition and the antibody response.

Barriers to B cell antigen encounter

The primary role of the LN is to filter the lymph and trap any pathogens that have breached the initial mucocutaneous barriers. The pattern of lymph flow has been extensively studied in the past in small and large animals using colloidal particles, radiotracers, and electron-dense particles (reviewed in [1]). Afferent lymphatic vessels drain lymph into the SCS, a sinus that forms an anatomical and functional barrier to the free diffusion of lymph-borne particles into the parenchyma [2–4]. Lymph then slowly percolates through cortical and medullary sinuses that, like the SCS, are lined by LyVE-1-expressing lymphatic vascular endothelial cells [5]. The medullary sinuses comprise a dense labyrinth of branching vessels and are richly populated with macrophages [6•] where the bulk of lymph-borne soluble and particulate antigen is trapped and catabolized [3,7,8]. SCS macrophages and the majority of medullary macrophages express the sialic-acid-binding immunoglobulin-like C-type lectin sialoadhesin (CD169) recognized by the MOMA-1 mAb [9], a property they share with the metallophilic macrophages that line the marginal sinus of the spleen [10]. Despite some confusion in the literature, $CD169^+$ macrophages lining the SCS can clearly be distinguished from those lining the medullary sinuses by the expression of F4/80 [11,12], SIGNR1 [13,14], and MARCO [14,15] by medullary but not SCS macrophages.

The secondary role of the LN is to stage the adaptive immune response and this creates a paradox given that T and B cells are located in distinct anatomical compartments beneath the impermeable barrier formed by the floor of the SCS. For T cells this is partly overcome by the conduit system comprising an interconnected network of collagen fibers ensheathed by fibroblastic reticular cells (FRCs) that express ERTR-7 [16]. Many of these conduits begin in the interfollicular region below the SCS and traverse the T cell zone in the parenchyma to anchor near high endothelial venules (HEVs) [17] (Figure 1). They provide a mechanism for rapid delivery of small molecules <70 kDa with a hydrodynamic radius <5.5 nm to the T zone [18] where resident dendritic cells (DCs)

Figure 1



Time	Location	Source	Antigen	Reference
Minutes	1	Pores? Follicular conduits	Small proteins Small proteins	Pape et al 2007 Roozendaal et al 2009
Minutes-hours	1	Macrophages	ICs Viruses Virus-like particles	Phan et al 2007 Junt et al 2007 Carrasco et al 2007
Days	2	Migrating DCs	Small proteins	Qi et al 2006
Days-weeks	3	FDCs	ICs	Suzuki et al 2009

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Microanatomy of the lymph node. Schematic of a lymph node showing the locations for antigen presentation to B cells. (1) Subcapsular region. (2) Lymphoid follicle. (3) T zone (paracortex) in the vicinity of HEVs. Lymph drains in the afferent lymphatic to the subcapsular sinus (SCS) and flows to the medullary sinuses. The lymphoid follicle (orange) is situated beneath the SCS and is traversed by occasional follicular conduits (white). Follicular dendritic cells (FDCs) are brown. The T zone (blue) contains HEVs (red) through which lymphocytes enter the lymph node. The medulla (light brown) contains numerous macrophages and cords of plasma cells. Three general locations of B cell access to antigen are labeled, and the table summarizes the kinetics and properties of antigens arriving at these sites.

associated with conduits may sample antigen for presentation to T cells [19,20]. In addition, antigen may be transported from the periphery by DCs traveling in the afferent lymph that must also cross the SCS to reach the T zone. In this case, the network of stromal cells with underlying conduits provide a scaffold for T cells during their random migration as they survey the parenchyma for processed antigen presented on the surface of these DCs [21•].

Follicular stromal cells that express CD157 recognized by the BP-3 mAb [22,23] form the stromal cell network and likely substratum for B cell migration in the follicle. Although some studies have emphasized that follicles have few conduits [1] others have noted that the conduits present have similar properties to those in the T zone [24–26,27••]. In addition, some EM studies have detected occasional gaps 0.1–1 μm wide in the floor of the SCS through which small particulate antigen may freely diffuse [28,29], but these may be transient migration pores created by the transendothelial migration of lymphocytes [29,30]. Moreover, there is a large discrepancy between their diameter and the physical limits imposed by the barrier to follicular diffusion. An intriguing recent study reported that a bolus of fluorescently labeled hen egg lysozyme (HEL) injected subcutaneously into the ear was detectable in the follicle by epifluorescence microscopy if the cervical LNs was carefully prepared to avoid washing away the small (14.3 kDa) highly cationic protein [31•]. This study raised the possibility that small antigens may rapidly diffuse across the floor of the SCS into the follicle. In more recent work, Roozendaal *et al.* tracked the distribution of fluorescently labeled turkey egg lysozyme (TEL) in real-time by two-photon microscopy and found that it first traveled into the follicle via conduits before reaching follicular B cells [27••]. Further comparisons and perturbations of these systems

are needed before we can know the exact extent to which small antigens access the follicle via SCS pores versus conduits. The fraction of pathogen-derived antigens that would have the size and physiochemical properties to access follicles via these routes is not clear but seems likely to be small. Moreover, the inability of antigens opsonized by antibody (>150 kDa) or C3b (~180 kDa) to enter conduits suggests that the antigens traveling through these structures will only be transiently available for encountering rare cognate B cell. So how do most antigen types, including viral particles and opsonized antigens, get from outside in? Here a prominent role for the specialized SCS macrophages has emerged.

Locations of B cell encounter with particulate antigen

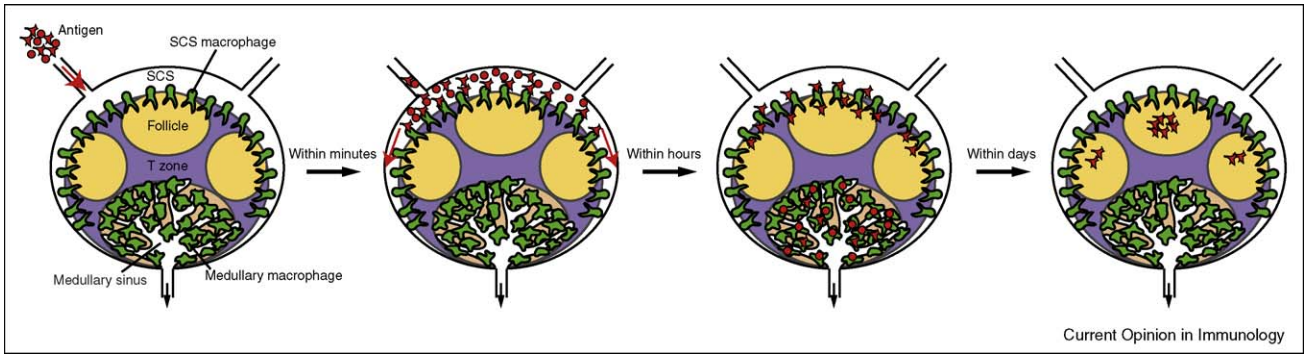
Mature recirculating B cells enter the LN via HEVs situated in the outer T zone and interfollicular regions and migrate to the follicle where they are retained for up to 24 hours before they exit the LN and re-enter the circulation [32,33]. This 'inside out' B cell migration pattern runs counter-current to the 'outside in' trafficking of antigen and provides multiple opportunities for B cell antigen encounter (Figure 1). Indeed, B cell activation in the vicinity of HEVs has been observed in a study which found $CD11c^+$ splenic DCs pulsed with very high concentrations of HEL migrated to the popliteal LN following footpad injection and were able to activate newly arrived B cells [34•]. This study is remarkable for its elegant use of intravital two-photon microscopy to show a recirculating B cell entering the T zone via the HEV fluxing calcium upon immediate contact with an antigen-pulsed DC. Antigen-pulsed DCs have occasionally been shown to induce humoral immune responses after adoptive transfer [35,36], and it would appear that certain DCs do not degrade all the antigens they internalize but recycle some to the cell surface. In the case of immune complexes (ICs), this can occur in an FcγRIIB-dependent manner [37]. Notwithstanding these reports, the chances of antigen encounter are probably highest inside the follicle itself where B cells spend most of their transit time [38]. Consistent with this, early antigen capture studies showed that while most of the antigens are trapped and degraded in the medullary region, small amounts did manage to infiltrate the follicle and can be retained there on FDCs for prolonged periods of time [7,8,39]. This retention is most prominent when the antigen can form ICs [3,40] and may last up to seven weeks [41]. Thus, a common view held that B cells migrate to follicles to 'survey' for antigen displayed on the surface of FDCs.

Early antigen capture studies also noted that antigen–antibody complexes were initially trapped in the floor of the SCS but rarely persisted there for more than a day [7,8,39] (Figure 2). Therefore, B cells may also sample antigens in the subcapsular region of the follicle, but this possibility was largely overlooked for several decades because of the relatively short-lived nature of their subcapsular retention compared to their retention on FDCs. In fact, the focus then shifted to finding the mechanism of FDC antigen deposition and, subsequently, a nonphagocytic antigen transport cell with dendritic morphology was proposed to shuttle antigen away from the SCS to the follicle for deposition on FDCs [8,42]. This oversight has recently been redressed by three dynamic imaging studies using two-photon microscopy which independently identified SCS macrophages as important sites for particulate antigen encounter with B cells within the first few hours of the immune response [43••,44••,45••].

SCS macrophages capture and present intact antigen to follicular B cells

Two-photon microscopy allows optical sectioning of intact LNs to the depths of up to 200 μm [46] and therefore is ideal for high-resolution dynamic imaging

Figure 2



Dynamics of antigen capture in the lymph node. Schematic showing the fate of opsonized (irregular shape) and nonopsonized (circle) antigens draining in the afferent lymphatics. Within minutes SCS macrophages have sampled some of the opsonized antigens while the bulk of antigens continues on to the medullary sinus. Within hours SCS macrophages have translocated some of the opsonized antigens from the lumen into the follicle whereas medullary macrophages have phagocytosed the remaining antigens. Opsonized antigens are then relayed to B cells for deposition on FDCs within a day where they can persist for weeks. In contrast, the remaining antigens are catabolized by medullary macrophages.

of the subcapsular region and superficial follicle. Although the three studies utilized different antigens in different experimental systems, the collective data are consistent with a model whereby SCS macrophages capture and present intact antigen (in the form of ICs [44••], viruses [45••], and virus-like particles [43••]) to activate follicular B cells. Intravital imaging of the SCS in live animals with intact lymphatic flow and blood circulation demonstrated macrophage capture of particulate antigen in the form of ICs [44••] and viruses [45••]. Follicular B cells routinely migrated to the subcapsular region where they interacted closely with SCS macrophages as measured by their slower median velocities and increased turning angles in the macrophage vicinity [44••]. Antigen-specific B cells accumulated in the subcapsular region [43••,45••], acquired antigen from the SCS macrophages and subsequently migrated with antigen capped in their uropod with a reduced median velocity [43••,44••] consistent with antigen engagement of their BCR [47]. B cell activation was also demonstrated by their downmodulation of the BCR [43••,45••], upregulation of the activation marker CD86 [43••,44••], and relocalization to the T–B border [43••,44••,45••]. Indeed, when macrophages were depleted by subcutaneous injection of liposomal clodronate, B cell activation was impaired [45••]. As expected, generalized ablation of macrophages in these experiments also led to a failure to contain viruses within the LN and their systemic dissemination. Taking these real-time imaging studies and the antigen catabolism studies together, there appears to be compartmentalization of LN function with the medullary macrophages being indispensable for the primary function of antigen catabolism and SCS macrophages playing a central role in the secondary function of generating an adaptive humoral response.

Not surprisingly, SCS macrophages may themselves become infected directly following the capture of lymph-borne pathogens such as viruses [48•]. Consequently, SCS macrophages in the interfollicular zone have been suggested to be a source of antigen presented by nearby DCs to activate $CD8^+$ antiviral T cells [48•]. Neutrophils, macrophages and DCs infected with agents such as *Toxoplasma gondii* draining in the afferent lymphatics may also be trapped by SCS macrophages, possibly via adhesion to CD169 [49], and thereby act as Trojan horses to indirectly infect them [50•]. Of note, early electron microscopy studies of horse-radish peroxidase-containing ICs showed they were deposited in the SCS floor where they were sequestered on the cell surface or in plasma membrane infoldings suggesting they were non-phagocytic [42]. Consistent with the poorly phagocytic nature of these cells, subcutaneous administration of liposomal clodronate results in widespread ablation of medullary macrophages within one day but SCS macrophage ablation takes much longer [51]. In this regard, the propensity of SCS macrophages to retain rather than degrade antigens may make them a safe haven for further pathogen replication.

Mechanisms of SCS macrophage antigen capture and presentation

Ultrastructural studies had earlier shown that SCS macrophages often send cytoplasmic protrusions into the sinus to sample the lymph [3,30,42]. Indeed, it was observed by intravital two-photon microscopy that macrophages with 'heads' that protruded more into the lumen of the SCS were well positioned in the lymph flow to capture ICs as they drained from the afferent lymphatic vessel [44••]. The array of macrophages interspersed between lymphatic vascular endothelial cells in the floor of the SCS resembles the network of epidermal dendritic Langerhans cells in the skin [52] and contrasts against the dense filtration mesh created by macrophages in the medulla [6•]. Furthermore, the rate of SCS lymphatic flow is much faster than the sluggish flow in the cortical sinuses and medulla [44••,6•]. Thus, SCS macrophages appear to sample newly arriving lymph and become coated with antigenic complexes as shown by intravital two-photon microscopy [44••] whereas medullary macrophages serve to stringently filter and trap all and sundry particulates (Figure 2). This is consistent with the restricted expression pattern of phagocytic receptors by SCS macrophages and in particular the absence of certain C-type lectins and scavenger receptors.

Despite all these studies, the mechanism of antigen capture is still unknown. A potential mechanism for the capture of opsonized antigen involves the use of complement receptors CR3 (CD11b/CD18 or Mac1) [53] and CR4 (CD11c/CD18) [54]. Like other macrophage types, SCS macrophages are thought to express CR3 [45••] although this may not uniformly be the case [12]. Classical complement activation occurs when pathogens are opsonized with pre-existing 'natural antibodies' [55] or antibodies generated in a primary antibody response. Antigen may also be opsonized by the recognition of carbohydrate moieties on bacterial surfaces by C1q or mannose binding lectin to activate the complement cascade independent of antibody binding [56]. Micro-organisms that lack complement inhibitory molecules can become tagged by spontaneously generated C3b, allowing the activation of the alternative pathway. Macrophages also typically express Fc receptors and it can be anticipated that this class of receptors will be involved in IC capture. The mannose receptor is shed into the circulation and lymph and may act as a circulating innate pathogen recognition receptor [57]. SCS macrophages bind to the cysteine-rich domain of the mannose receptor, possibly via CD169 [58,59], providing another potential mechanism for antigen capture. In fact, CD169 itself has been proposed to be involved in pathogen recognition and capture [60] although the capacity of

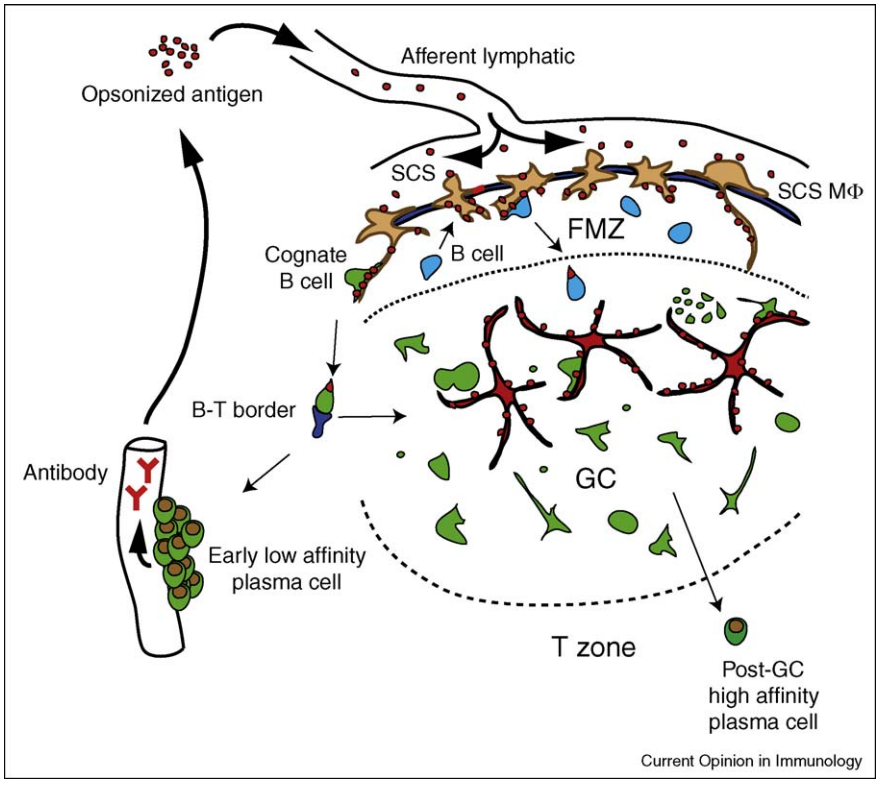
CD169-deficient SCS macrophages [61] to capture and present antigen has yet to be tested. Interestingly, the capture of viruses by SCS macrophages appeared to be independent of complement C3 [45••] raising the possibility that other as yet undefined pathogen recognition receptors may be involved.

Interestingly, similar to FDCs that are also able to present intact antigen to B cells, SCS macrophages express high levels of the adhesion molecules VCAM-1 and ICAM-1 [45••]. It is tempting to speculate that this improves cell–cell interactions and facilitates the formation of an immunological synapse between SCS macrophages and B cells [62]. There is considerable evidence for a role of these adhesion molecules *in vitro* but *in vivo* data are still lacking. Nevertheless, by two-photon microscopy B cells were observed to gather antigen along macrophage processes in a 'contraction-spreading' pattern [43••,44••]. One implication of this macrophage membrane-bound mode of presentation is that the avidity of soluble antigen will be much higher thereby potentiating any BCR signals that may drive plasmablast differentiation [63].

Follicular B cells as antigen transporting cells

Another insight from two-photon microscopy has been the recognition of follicular B cells themselves as antigen transport cells [44••] (Figure 3). In fact, this concept is not new and has been around for several decades [64,65]. As it turns out, the high intrinsic motility of B cells, which was only recognized with the development of two-photon microscopy, and their abundance make them ideal candidates to transport ICs from the SCS to the follicle. Thus, noncognate B cells were observed to capture ICs from SCS macrophages and transport them at their uropod in a CR2-dependent manner [44••]. It was envisioned that B cell capture of ICs was driven by the higher affinity of CR1/CR2 for complement than the receptor(s) used by SCS macrophages to capture ICs (potentially CR3/CR4 or Fc receptors), and the subsequent IC deposition on FDCs was driven by the higher density of CR1/CR2 on FDCs than on B cells. A similar role of follicular shuttling of ICs has been suggested for marginal zone B cells in the spleen [66,67•]. In another variant on the theme of B cells as antigen transport cells, IgE-containing ICs were captured by circulating B cells, most likely via the low affinity IgE

Figure 3



Model for role of B cell antigen transport in the primary antibody response. Cognate B cells (green) acquire antigen from SCS macrophages (light brown) and migrate to the T–B border. Upon receipt of T cell help they differentiate into early plasmablasts and migrate to the medullary cords where they secrete antibodies; alternatively they differentiate into GC B cells. Early antibodies feedback to opsonize incoming antigen and these ICs are relayed by SCS macrophages to noncognate B cells (blue) that transport them into the GC light zone. These antigen-containing ICs are then available for deposition on FDCs (dark brown) and affinity-based selection of GC B cells to become high affinity plasma cells. FMZ, follicular mantle zone.

receptor (CD23), and delivered rapidly to splenic follicles [68]. Basophils have also recently been implicated in the transport and presentation of IgE-containing ICs during a recall B cell response [69].

So why is follicular B cell antigen transport important? SCS macrophages have only been shown to retain and display ICs for a period of hours. Antigen deposition on primary follicle FDCs is likely to ensure that rare cognate B cells arriving in the LN more than one day after antigen exposure can be activated. Indeed, very recent two-photon microscopy work has shown cognate B cells capturing opsonized antigen directly from FDC processes one day after immunization [70••]. Even though the antigen was provided as a single bolus, FDCs displayed sufficient antigen to allow ongoing activation of B cells newly arriving in the LN over a period of more than a week [70••]. Thus the antigen-specific B cell precursor frequency and dynamics of lymphocyte recirculation under steady state and inflammatory conditions — as well as the properties of the antigen — will impact the site of B cell antigen encounter. Intriguingly, naïve follicular B cells have been observed by two-photon microscopy to routinely enter the germinal center (GC) light zone [71••,72••]. It has been argued that this allows for the recruitment of naïve B cells into an ongoing GC response [72••]. However, another exciting possibility is that these noncognate B cells transport antigen-containing ICs into the GC to facilitate affinity maturation of the primary antibody response (Figure 3).

Conclusion

T cell antigen presentation and activation by DCs in the LN has been extensively mapped at the molecular, cellular, and structural level over the past decades. In contrast, the logistics of B cell antigen presentation and activation is only beginning to be re-evaluated in the last few years with two-photon microscopy. These studies have 'rediscovered' the critical role of SCS macrophages in capturing and presenting antigen to B cells in the earliest phase of the immune response. However, relatively little is known about these specialized macrophages and the mechanisms by which they handle antigen for cognate B cell activation. Another major finding is the role of B cells themselves in the relay of antigen from SCS to follicle and FDCs. The importance of this B cell antigen transporting function in the antibody response remains to be fully elucidated. Clearly these recent advances would not have been possible without two-photon microscopy, and it is expected that further experiments using this technique will shed more light on dynamic aspects of the immune response *in vivo*.

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