B cell Acquisition of Antigen in vivo

Santiago F. Gonzalez1, Lisa A. Pitcher1, Thorsten Mempel2, Franziska Schuerpf3, and Michael C. Carroll1,4

1Immune Disease Institute, Program in Cellular and Molecular Medicine, Children's Hospital, Boston, MA 02115
2Department of Medicine, Harvard Medical School, Boston, MA 02115
3DecImmune Therapeutics, INC, Boston, MA 02115
4Departments of Pathology and Pediatrics, Harvard Medical School, Boston, MA 02115

Summary

The fate of B lymphocytes is dictated in large part by cognate antigen and the environment in which it is encountered. Yet we are only now beginning to understand where and how B cells acquire antigen. Recent studies identify multiple pathways by which lymph-borne antigens enter the B cell follicles of LNs. Size is a major factor as particulate antigens and large IC are bound by subcapsular sinus macrophages. By contrast, small antigens (under 70 KDa) are rapidly channeled into follicles via conduits secreted by fibroblastic reticular cells (FRC). Interestingly, the conduits not only deliver antigen to follicular dendritic cells (FDC) but provide a rich source of B cell chemokine, i.e. CXCL-13. Thus, the follicular conduits provide an “antigen highway” for B cells trafficking within the LN. These new findings provide an important discovery in understanding how B cells acquire cognate antigen.

Introduction

While the complex manner in which T cells recognize antigen is relatively well understood, until recently how antigen enters the B cell follicles and encounters B cells remained an enigma. In the last 2 years, a number of reports were published addressing this question. It is concluded that multiple pathways are involved and that the innate immune system lies at the heart of providing antigen to B cells in an efficient manner.

Follicular Dendritic Cells concentrate antigen in lymphoid tissues

Early histology studies were instructive in tracking uptake of antigen on follicular dendritic cells (FDC) within the B cell follicles of peripheral lymphoid tissues [1,2]. FDCs are most prominent in germinal centers (GC) as the stromal network is highly expanded during an ongoing secondary immune response. Through the elegant work of Browning and colleagues, it is clear that FDCs originate from stromal cells and differentiate into their characteristic morphology in response to lymphotoxin [3-7]. Although, FDCs remain somewhat enigmatic due to the inherent difficulty in obtaining sufficient numbers for characterization, they are not only important in retention of antigens but also are a source of B cell chemokine CXCL-13.
Thus, FDC are dependent on B cells as a source of LT-beta and B cells are dependent on FDCs for attraction to the follicles.

Early studies tracking uptake of immune complexes (IC) onto FDC led to the finding that binding was dependent on Fc receptors (FcRIIb) and complement receptors (CR), i.e. CD21/CD35[9-13]. CR are co-expressed on FDC and B cells in the mouse and represent splice products from the Cr2 locus[14,15]. Both receptors bind iC3b and C3d but CD35, which includes an additional 6 short consensus repeats (SCR), also binds C3b and C4b.

A current view is that FDC retention of antigen is essential for clonal selection of B cells within GC[16-18]. Whether antigen retention is also required for maintenance of memory and effector (antibody producing cells) B cells is less clear. Early studies by Gray and colleagues support a role for antigen persistence in maintenance of B cell memory [19,20]. More recent studies using chimeric mice in which antigen retention on FDC is impaired due to an absence of CR expression on FDC demonstrate a defect in maintenance of both long-term memory and antibody response[21,22]. However, other studies using genetic approaches to engineer mice that switch BCR specificity after immunization suggest that antigen retention is not required for a memory response[23].

Whether FDC retention of antigen is required for initiation of primary antibody responses is not clear. The finding that the IgM primary response appears normal in chimeric mice in which FDC are deficient in CR, suggests that other sources of antigen may be sufficient to trigger early B cell activation [13,21]. For example, the presence of excess antigen following a primary immunization, especially in the presence of adjuvants that concentrate antigen within the lymphoid compartment, could circumvent the necessity for deposition on FDC. Studies by Shlomchik and colleagues using an Ig transgenic model in which B cells do not secrete antibody, identify apparently normal secondary responses[24]. However, in their model, CR expression is required for B cell memory [25]. They propose that surface Ig on B cells is sufficient to capture antigen and present it to other B cells for activation. Surprisingly, they found that binding of antigen by cognate B cells leads to activation of C3 on the B cell surface, which could explain a source of C3-ligand for stimulation of the co-receptor[26].

**Lymph-borne antigens bind sub-capsular sinus macrophages**

The development of intravital imaging using multi-photon microscopy (MP-IVM) has provided a valuable new technique to address how lymph-borne antigens gain entry into the B cell follicles within peripheral lymph nodes (LN)[27,28]. Recent studies using this technology have shown that the size and nature of the antigen influences its pathway of entry. Large particulate antigens such as VSV (vesicular stomatitis virus)[29], inert beads coated with antigen [30] and IC composed of large (> 70 kDa) antigens and Ig are trapped by macrophages lining the LN sub-capsular sinus (SCS-macrophages)[31]. This sub-set of macrophages which are CD169+ CD11c lo and bind the cysteine-rich domain of the mannose receptor[32], act as sentinels to bind to incoming pathogens and antigens and, in some cases, shuttle them to underlying B cells. For example, Junt et al [29] and Carrasco et al [30] independently reported that cognate B cells residing in the underlying follicles sample antigen from the SCS macrophages. Thus, this pathway may provide a source of specific antigen for circulating B cells. However, unlike FDC, SCS macrophages do not appear to maintain antigen on their surface over long periods. Therefore, the pathway might be more important for secondary responses to pathogens when an increased number of cognate B cells are present in the draining LN.

Naïve B cells provide an alternative pathway for delivery of IC into the B cell follicles and retention on FDC [31]. Immune complexes are bound by SCS macrophages and in the presence of complement C3 transferred to naïve B cells in the underlying follicles [31]. Subsequently,
naïve B cells transport IC in a CR-dependent process to the FDC. How IC are off-loaded to FDC is not clear, but our previous studies suggest that the process is dependent on the presence of CR on FDC. Given the high concentration of CR on FDC relative to B cells, it is possible that the transfer is a passive event. A similar pathway is involved in transport of C3-coated IC from the marginal sinus of the spleen into B cell area. These findings are consistent with other (?) previous studies showing a requirement for marginal zone (MZ) B cells and complement in the uptake of T-independent antigens and delivery to FDC[33-36].

**Small antigens enter B cell follicles via conduits**

Not all lymph-borne antigens require binding by SCS macrophages to enter the LN follicles. Pape et al found that small protein antigen (HEL) rapidly entered the B cell follicles in draining LNs in a SCS-macrophage-independent manner [37]. They proposed that small antigens bypass SCS macrophages and enter the follicles via small gaps in the floor of the LN sinus where it is bound by cognate B cells.

In our own studies using turkey lysozyme (TEL), we identified a novel pathway in which lymph-borne antigen enters the B cell follicles directly via discrete conduits or channels[38]. Like the study of Pape et al, TEL did not appear to bind to SCS macrophages even in the presence of specific antibody. To compare uptake and transport of IC formed with either large (>70 Kda) or small antigen, mice were passively immunized with a mixture of rabbit antibody specific for phycoerythrin (PE) (approximately 250 Kda) and lysozyme (12 Kda).

Subsequently, mice were injected in the hind flanks with a mixture of the two antigens. The inguinal LNs were harvested at various time points following injection and the B cells analyzed by flow cytometry and FDC uptake imaged by confocal microscopy. As reported by Cyster and colleagues [31], IC composed of large PE antigen were taken-up by naïve B cells in a CR-dependent manner within 2-4 hours and optimal deposition on FDC was observed by 8-24 hrs (Fig 1) [38]. By contrast, TEL complexes were observed deposited on FDC in less than 2 hrs independent of B cell transport. Thus, naïve B cells are not required for transport of small antigens to FDC.

To track entry of small antigen into the draining LN, mice were injected in the footpad with labeled TEL and imaged by 2-photon microscopy in real time. Remarkably, within seconds of filling the sub-capsular sinus, the small antigen entered into the follicles through discrete conduits. It is important to note that antigen was observed first within the conduits and only later did it enter the follicular interstitial space (Fig 2). While this study does not rule out entry of lysozyme via gaps in the SCS floor, it suggests that the majority of lymph-borne antigen enters the follicles through conduits first.

Anderson first described conduits in rat LNs in the 1970’s[39], proposing that the extensive network of fibers were important for distribution of antigen into the LN parenchyma. More recent studies determined that gp 38+ FRC were the source of the collagen-rich fibers, which represent a major stromal component of the LN[40,41]. Although it was reported that FRC fibers were present in both the T and B cell areas, their density was much greater in the cortical region. Therefore, much of the characterization was focused on fibers within the T cell area. Our characterization of follicular (FO) conduits by electron microscopy (EM) (Fig 3) and immuno-staining (Fig 4) demonstrated that they are structurally similar to those in the T zone [38]. Thus, both cortical and FO conduits are composed of a core of collagen fibers surrounded by a basement membrane and enveloped by FRC. The conduits have an overall diameter of 0.5-1.0 um; however, the tight spacing (approximately 5-8 nm) between the individual collagen fibers limits the size of molecules that enter, causing the conduits to act as molecular sieves and only accept structures less than 5-8 nm in diameter. How charge and polarity affect entrance is not known, but they are likely important in the overall physico-chemical properties limiting inclusion.
In our study, the rapid draining of TEL from the SCS into the FO zone suggested that the conduits directly open into the sinus. Transmission EM analysis of LNs identify collagen-rich conduits extending from the open sinus deep into the FO region (Fig 3). This observation is in agreement with published studies using both light microscopy [40,41] and scanning EM [42] to characterize draining of small molecules from the sub-capsular sinus into the paracortical region.

**B cells sample antigen in conduits**

The elegant studies of Gretz and Shaw [40] and Sixt et al [41] support a role for conduits in delivery of T cell antigens to dendritic cells (DC) which are positioned along the FRC fibers. EM images suggest that DC sample the contents of conduits and possibly process them for presentation to T cells which migrate along the fibers in response to presence of CCL19 and CCL 21chemokines[43]. Our characterization of the FO conduits by EM suggests a similar pathway in the B cell zone. However, in contrast to the cortical area, B cells appear to sample antigen directly. Given the gaps between the inner collagen core and the FRC, B cells could gain access to small antigen. As discussed further below, B cells are attracted to the conduits through the release of CXCL-13 secreted by the stromal cells (Fig 4).

To test whether B cells take-up antigen directly from the conduits, a mix of labeled-naïve and -MD4 B cells were adoptively transferred into mice 18 hrs prior to footpad injection with a mixture of large and small antigen, i.e. PE-TEL and TEL. Analysis of the popliteal LN by intravital imaging demonstrated rapid filling of the SCS space and almost immediate draining of the small TEL but not PE-TEL into the FO conduits (Fig 2 and Movie S1). Notably, migration of MD4 B cells slowed within minutes following antigen injection as many cognate cells bound to the labeled TEL. By contrast, naïve B cells did not slow in their movement and did not take-up antigen. Thus, the results demonstrate that cognate B cells take-up antigen directly from the conduit possibly at the gaps between the FRC and the conduit as identified by EM.

**Do FO conduits deliver antigen directly to FDC?**

Our results showing that TEL-IC are taken-up by FDC independent of naïve B cells suggests conduits may deliver antigen directly to FDC. To examine whether FO conduits contact FDC, LN sections were co-stained with antibodies specific for collagen type 1 (conduit marker) and CD35 (FDC marker). Confocal analyses identified extensive overlapping of the dendritic processes and FRC-fibers. The results support a model in which small antigens drain from the conduits onto or at FDC and in the presence of antibody and complement bind to the FDC surface. Therefore, specific antibody produced locally by activated B cells (or pre-existing natural Ig or lectins such as MBL or Ficolin which are found in the lymph) could bind antigen as it emerges from the conduit and activate C3 at the FDC surface. Our earlier studies have shown that cells within the LNs secrete C3 [10,44] and, under certain circumstances, local production is required for an efficient humoral response[45,46]. Alternatively, it is possible that complement is also activated and binds covalently to antigen as IC form either in the tissues or within the SCS lymph. As a result, the antigen+ C3d ligand (approximately 35 Kda) both drain via the conduit to the FDC.

**Conduits as antigen highways for B cells**

Stromal cells –both FDC and FRC-like cells within the B cell follicles are known sources of the B cell chemokine CXCL-13. To test whether FO conduits are carriers of chemokine, LN tissues were stained with a panel of antibodies specific for conduit markers such as ERTR-7, collagen type 1 and fibronectin and anti-CXCL-13. Confocal analyses identified co-localization of the chemokine and conduits, suggesting that they are a source of CXCL-13 (Fig 4) [38]. The resulting general model is that FRC within the B cell area secrete CXCL-13 that percolates through the conduit system providing an attractant to B cells. Thus, FO conduits
may provide not only a highway for B cells navigating through the follicles but also a rich source of antigen.

In future experiments it will be important to understand the structural and functional relationship between the FRC conduits and the FDC. For example, do the conduits open directly into FDC and thus provide a direct pipeline of small antigen?

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

The authors thank Ms. Alexandra Gillmore for assistance in assembling and editing the manuscript and Ms. Maria Ericsson at the Harvard Medical School EM Facility for technical assistance.

**Literature Cited**


_Curr Opin Immunol_. Author manuscript; available in PMC 2010 June 1.


Figure 1. Uptake of Ag on FDCs in peripheral LNs is size dependent
(A) Confocal fluorescence micrographs of histological sections of pLNs show delay of KLH deposition on FDCs (blue, Cy5-anti-CD35) relative to TEL deposition, 6 hr after injection of IC. The scale bar represents 50 μm. Images are representative of at least three independent experiments. (B) The percentages of B220+ cells that have acquired TEL-IC or PE-IC are indicated and are represented in the graph as he mean values ± standard error of the mean (SEM) from at least three LNs. Statistical significance was determined with a one-tailed, paired Student’s t test.
Figure 2. Small Ags are rapidly delivered to LN follicles through FO conduits
A633-TEL (red, containing 50ng TEL) and TEL_PE (green, containing 5μg TEL) were injected into the footpads of mice, and their entry into the draining LN was monitored by MP-IVM. (A) Intravital micrographs depicting the distribution of the small (A633-TEL, red) and large (TEL-PE, green) Ag in a B follicle early (55 s, left panel) or late (16 min, 40 s, right panel) after footpad injection (WT B = white; MD4 B= blue). The scale bar represents 30μm.
Figure 3. Ultra structural characterization of follicular conduits
(A) Schematic drawing of a conduit in the B cell follicle. Red arrows indicate the direction of small antigens from the afferent lymphatics to the B cell follicle. FRC and FDC represent fibroblast reticular cell and follicular dendritic cell, respectively. (B) Transmission electron micrograph and (C) schematic drawing of a transverse section of a FRC located in the subcapsular sinus (ss) area of the lymph node. Upper and lower arrows indicate the conduit lumen and a FRC junction respectively. (D) Electron micrograph of a longitudinal section of a FRC in the ss area in the lymph node. White arrow indicates a FRC junction, the conduit structure inside the FRC is indicated in light blue. (E) Electron micrograph of a B cell in close contact with the lumen of a conduit.
Figure 4. CXCL 13 is associated with FO conduits

(A) Immunofluorescence micrographs of B cell follicles stained with antibodies against fibronectin (green), ER-TR7 (red), and CD35 (blue). T-B cell boundaries are indicated by hatched lines. Scale bars represent 100 μm (top) and 20 μm (bottom). (B) LN cyrosections stained for CXCL 13 (red), fibronectin (green, left and middle columns [scale bars represent 100 and 200 μm]), and gp38 (green, right column [the scale bar represents 20 μm]).