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Article

B Cells Acquire Particulate Antigen in a Macrophage-Rich Area at the Boundary between the Follicle and the Subcapsular Sinus of the Lymph Node

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SUMMARY

Adaptive B cell responses are initiated by B cell receptor–antigen engagement. Despite its importance, the early stages of B cell interaction with antigen in vivo remain elusive. Using fluorescent particulate antigen in combination with multiphoton microscopy and immune staining, we identified the follicle-subcapsular sinus (SCS) boundary of the lymph node as a site for B cell-antigen acquisition. In this macrophage-rich region, antigen accumulated shortly after administration, where specific follicular B cells were retained for long periods of time. These B cells acquired antigen cumulatively and became the main antigen carriers inside the follicle before polarizing to the B cell-T cell border. These observations not only provide evidence of a B cell role as antigen transporters into the follicle, but also highlight the boundary between the follicle and the SCS as a site for initiation of B cell responses.

INTRODUCTION

B cell activation by antigen is a critical step to initiate an adaptive immune response. After antigen stimulation, activated B cells migrate to the border between the follicle and the T cell zone in the lymph node (Garside et al., 1998). Once in this region, antigen-loaded B cells form stable interactions with antigen-specific helper T cells, which in turn provide signals to allow them to proliferate (Okada et al., 2005). Although some of their progeny become extrafollicular antibody-secreting plasma cells, others form germinal centers in which B cells undergo somatic mutation and affinity maturation (MacLennan et al., 2003). These high-affinity B cells can then differentiate into long-lived memory or plasma cells (Möller, 1987).

Very little is known about how and where B cells encounter antigen in vivo. Antigens can be transported passively to the spleen and lymph nodes by the blood or lymph. Once there, soluble antigens of small molecular size filter through the conduit system to gain direct access to lymphoid follicles (Gretz et al., 2000; Sixt et al., 2005). In contrast, larger antigen complexes cannot diffuse passively into the follicles and therefore require active transport by accessory or resident cells. Several cell types have been associated with this activity. For instance, dendritic cells (DCs) that express high amounts of mannose receptors are associated with the active transport of antigen into the B cell follicle (Berney et al., 1999). It was recently shown that marginal-zone B cells, which express high amounts of complement receptor (CR1/2), transport immune complexes (ICs) into the follicle, and transfer them onto follicular dendritic cells (FDCs), suggesting that B cells themselves play a key role in antigen transport (Ferguson et al., 2004).

Antigens are also transported actively from peripheral tissues into the lymph nodes. DCs can capture and internalize antigens in the periphery and retain them unprocessed (Delamarre et al., 2005; Wykes et al., 1998). Several groups have shown that DCs can present antigens to B cells. For instance, bone marrow-derived myeloid DCs pulsed in vitro with *Streptococcus pneumoniae* elicit in vivo immunoglobulin responses (Colino et al., 2002). In addition, blood-borne particulate antigens (i.e., bacteria) are taken up by DCs and transported to the spleen marginal zone, where they associate with antigen-specific B cells (Balazs et al., 2002). Binding to the inhibitory Fcγ receptor IIB (FcγRIIB) on the DC surface has been suggested as a possible mechanism to facilitate antigen presentation to B cells (Bergtold et al., 2005). Newly arriving B cells encounter antigen-loaded DCs located close to the high endothelial venules (HEV), where antigen can be presented (Qi et al., 2006). It is therefore likely that there is no single defined pathway of antigen presentation to B cells; it will depend on several parameters, such as B cell location in lymphoid organs, the route by which antigen reaches these tissues, and the physical characteristics of the antigen itself.

Here we developed an approach that allowed in vivo tracking and quantification of the fate of particulate antigen. Using highly fluorescent particles the size of a virus, we demonstrated that, shortly after administration,

antigen was deposited inside the draining lymph node and was specifically trapped by a macrophage sheet located beneath the subcapsular sinus (SCS). Specific follicular B cells initially encountered the antigen close to this subcapsular region and accumulated it over time, suggesting multiple rounds of antigen acquisition. By multiphoton microscopy, we observed that antigen recognition reduced the high motility of follicular B cells and promoted prolonged residency in a confined area. These B cells were attached to a cluster of particulate antigen, resembling the formation of an immunological synapse.

RESULTS

In Vivo Visualization of Particulate Antigen: Early Deposition on Macrophages Followed by Accumulation on FDCs

To track the fate of particulate antigen in vivo, avidin-coated highly fluorescent particles of small molecular size (0.2 μm, similar to the size of a virus) were injected subcutaneously into the footpad of C57BL/6 (B6) recipient mice. We followed the kinetics of antigen arrival to the draining lymph nodes by fluorescence microscopy of cryosections obtained from excised popliteal and inguinal lymph nodes at different times after antigen injection. To better visualize lymph node architecture and the follicle boundary, we costained tissue sections with the B cell marker B220. As early as 1 hr after injection, fluorescent particles accumulated in large numbers in the subcapsular and medullary sinus areas of popliteal lymph nodes (Figure 1A). Similar results were obtained with fluorescent bioparticles, such as bacteria (Figure 1A), validating this distribution as characteristic of particulate antigen of 1 to 0.2 μm diameter arriving via the lymph.

Even at this early time point, some particulate antigen was also observed inside the follicle (Figure 1B). Staining with markers of different accessory and resident cells showed an initial, long-lasting association of particles, mainly with the macrophages (Mac-3$^+$) located just beneath the SCS (Figure 1C). We found almost no colocalization with fibroblastic reticular cells (ERTR7$^+$), DCs (CD11c$^+$) or primary follicular dendritic cells (FDCs) (FDC-M2$^+$) at early time points. By 24 hr after injection, large numbers of particles were found deeper in the follicle, associated with FDCs (Figures 1B and 1C). In contrast, no particles were observed in the paracortex of the popliteal lymph node (data not shown); similarly, we detected no fluorescence in phosphate buffered saline (PBS)-injected mice or in sections from inguinal lymph nodes (data not shown). Quantitative analysis of the fluorescence image revealed that, despite its bulk, the amount of antigen in the follicles increased over time (Figure 1D). This approach thus provided a highly sensitive method of tracking particulate antigen in vivo, showing that viral-sized particles can gain access to the follicle shortly after injection and accumulate mainly through a macrophage sheet located just beneath the SCS.

High-Affinity Specific B Cells Acquire Particulate Antigen at the Boundary Between the Follicle and the Subcapsular Sinus of the Lymph Node

We used this technique to study the early stages of B cell-antigen acquisition. We transferred SNARF-1-labeled hen egg lysozyme (HEL)-specific transgenic B (tg-B) cells (5×10^6) into syngenic B6 mice. We allowed 20 hr for B cells to equilibrate within lymphoid tissues; then, we injected avidin fluorescent particles, alone (particles) or loaded with HEL (HEL particles), into the mouse footpads. Quantification of the amount of protein loaded into the particles indicated that the total quantity of HEL administered to the mice was less than 1 μg. Tissue cryosections were analyzed by fluorescence microscopy as described above. By 24 hr after antigen injection, the majority of follicular B cells had encountered antigen and most were polarized toward the B cell-T cell boundary area (Figure 2A), as previously reported (Garside et al., 1998; Okada et al., 2005). These B cells showed hallmark signs of activation, as judged by major histocompatibility complex (MHC) class II (MHC class II) expression, CD86 upregulation, as well as immunoglobulin M (IgM) down-modulation (Figure 2B). At early time points after injection, although the majority of HEL-particles still appeared to be associated with the subcapsular macrophages, we detected antigen-loaded tg-B cells near the subcapsular region (Figure 2A). By 24 hr after injection, most of the particles in the follicle were associated with tg-B cells, and not with FDCs, as observed in the absence of antigen-specific B cells. This indicated that antigen was consumed immediately on its arrival in the follicle or, more likely, did not reach the FDCs.

Cumulative Particulate-Antigen Acquisition by Specific B Cells

To further examine the process of antigen acquisition by B cells, we used high-resolution confocal microscopy and three-dimensional reconstruction analysis. Maintenance of consistent high fluorescence intensity by the particles allowed for both the clear visualization and precise quantification of the antigen acquired by B cells. The proportion of tg-B cells carrying detectable amounts of HEL particles increased over time (Figure 3A). Indeed, by 6 hr after injection, 70% of the follicular tg-B cells had acquired antigen; by 24 hr, all tg-B cells showed signs of antigen acquisition. Antigen acquisition was dependent on B cell receptor (BCR) recognition of antigen, because less than 10% of tg-B cells associated with unloaded particles (Figure 3A). In some cases, particles also associated with other cells, although the nature of these cells was unclear.

We then counted the number of HEL particles associated with each of the tg-B cells. This analysis showed that, at early time points, each follicular tg-B cell loaded with antigen carried between one and five particles; however, by 24 hr, most had accumulated more than five particles each (Figures 3B and 3C; Movie S1 in the Supplemental Data available online). This was consistent with the idea that B cells probably acquire antigen in sequential encounters inside the follicle before migrating into the B cell-T cell border. We also found that the amount

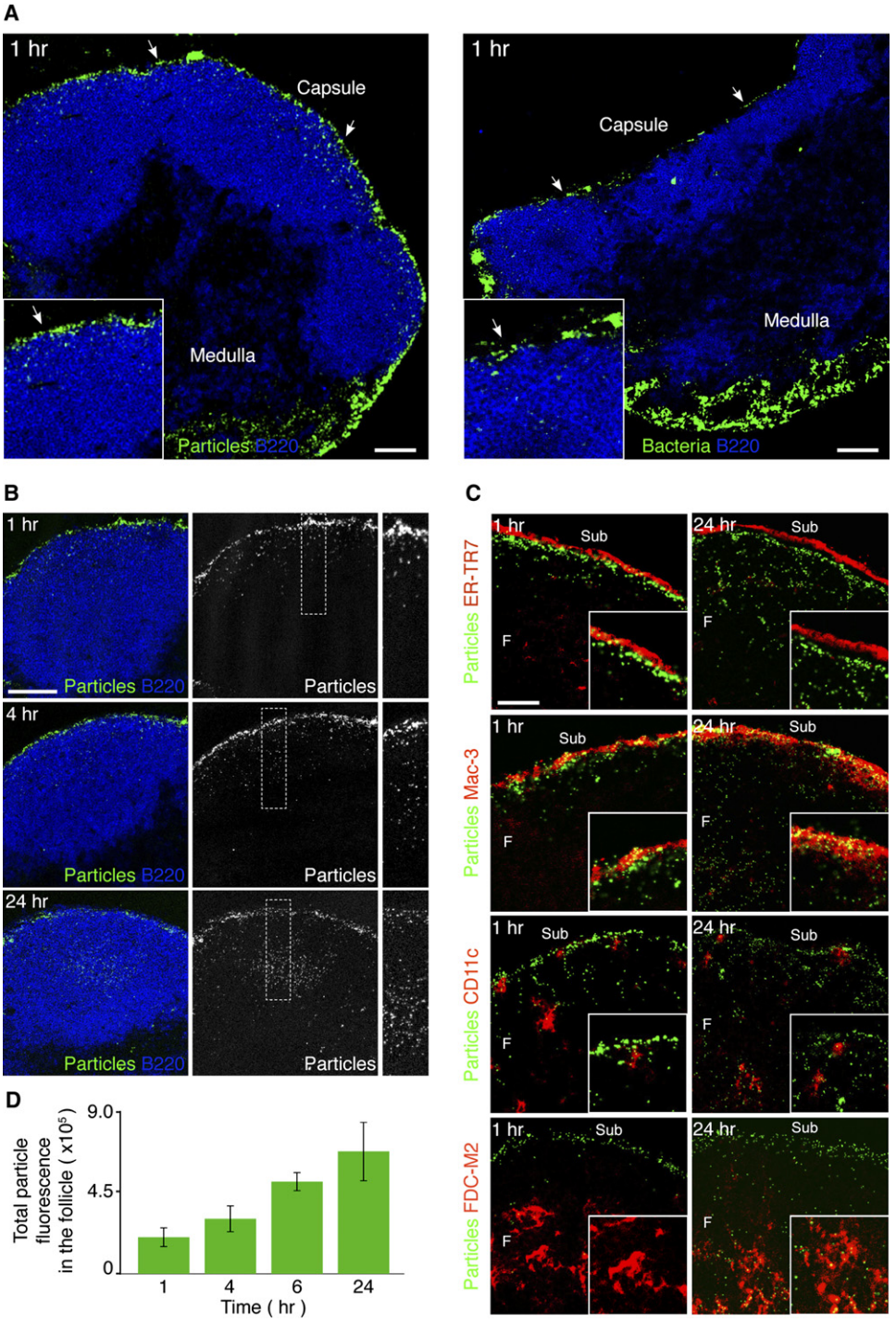

Figure 1. Detection of Particulate Antigen within the Follicle at Early Time Points after Footpad Injection

C57BL/6 recipient mice were injected in the footpad with avidin-coated fluorescent particles or Alexa-488-conjugated bacteria (*E.coli*) prepared as specified in the Experimental Procedures. At different time points after antigen administration, popliteal lymph nodes were isolated, frozen, and prepared for serial cryosection. Finally, lymph node tissue sections were stained as described in the Experimental Procedures.

(A) Representative tissue sections of popliteal lymph nodes from recipient mice, showing the distribution of avidin-coated fluorescent particles (left panel) and fluorescent bacteria (right panel) (green) 1 hr after injection. Tissue sections were stained with B220 (blue) to identify the B cell follicles. Inner panels show a higher magnification of the SCS area, where particulate antigen accumulates (white arrows).

(B) The entrance and accumulation of avidin fluorescent particles inside the follicular area are shown in the tissue sections of representative B cell follicles (identified by B220 staining, blue) of popliteal lymph nodes at different time points after injection. Magnification of a small section of each follicle (pictured in the middle panels) is shown on the right panels.

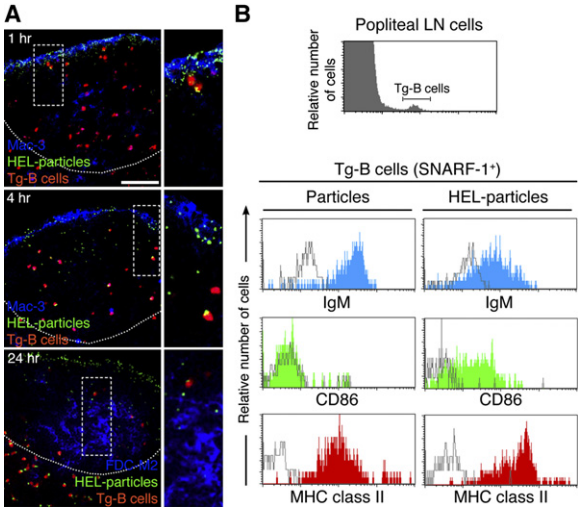

Figure 2. Particulate-Antigen Acquisition by Antigen-Specific Follicular B Cells

SNARF-1 labeled tg-B cells (red) were adoptively transferred into the tail vein of recipient mice. Twenty hours later, these mice were injected with biotinylated-HEL-loaded avidin fluorescent particles (HEL particles; green) into the footpad. Popliteal lymph nodes were then isolated, frozen, and prepared for serial cryosection at different time points after injection. Lymph node tissue sections were prepared for immunostaining as described in the Experimental Procedures; the HEL particles and tg-B cells present in the tissue section were detected by their fluorescence. Twenty-four hours after antigen injection, popliteal lymph nodes were isolated for flow-cytometry analysis and prepared as explained in the Experimental Procedures.

(A) Representative images of B cell follicles in popliteal lymph node tissue sections stained for macrophages (Mac-3$^+$) and FDCs (FDC-M2$^+$) (blue) at the indicated time points after HEL-particle injection. The dotted white line shows the B cell-T cell border identified by B220 staining in sequential tissue sections. Right panels show enlargements of the small sections pictured on the left panels (dashed white line). The scale bar represents 100 μm.

(B) Flow-cytometry analysis of the surface expression of IgM, CD86, and MHC class-II in tg-B cells (gated as SNARF-1$^+$, showed in the top panel) of popliteal lymph nodes 24 hr after injection of avidin fluorescent particles that were loaded or not with biotynilated HEL. Overlaid gray-line histograms correspond to the isotype staining control of the tg-B cells (SNARF-1$^+$) in each case.

Data shown are representative of four experiments.

of particulate antigen acquired by follicular B cells was dependent on the quantity of antigen administered. Reducing the amount of HEL particles injected by 90% resulted in only 50% of antigen-associated tg-B cells at 24 hr, with a maximum of one to two particles per cell (Figures 3D and 3E). Polarization of B cells into the B cell-T cell border was almost undetectable (data not shown).

At early time points, most antigen-bearing tg-B cells appeared to be located around the subcapsular region (Figures 2A and 3B). To quantify this phenomenon, we subdivided the follicles into two regions: one being the subcapsular half (upper follicle) and the other being next to the B cell-T cell border (lower follicle) (Figure S1). We evaluated the distribution of antigen-loaded tg-B cells in both areas and found that at 1 hr, the majority of antigen-bearing tg-B cells were located in the upper-follicle region (Figure 4A). In contrast, antigen-associated tg-B cells were almost undetectable in the lower-follicle area. At later time points (4–6 hr), however, the proportion of antigen-experienced tg-B cells increased notably in this area. Quantification of the distribution of follicular tg-B cells revealed a discrete accumulation of these B cells at 4 hr in the upper part of the follicle, and this accumulation preceded a marked polarization toward the B cell-T cell border by 24 hr (Figure 4B). These results indicate that the lymph node subcapsular region is a site of particulate-antigen acquisition by B cells.

Analysis of the Dynamics of Follicular B cells upon Particulate-Antigen Injection by Multiphoton Microscopy Reveals Two Different B Cell Behaviors

To study the dynamics of the early stages of B cell-antigen acquisition, we performed multiphoton microscopy of explanted lymph nodes (Figure 5A). Equal numbers (5×10^6) of SNARF-1-labeled tg-B cells and control 7-amino-4-chloromethylcoumarin (CMAC)-labeled wild-type B (non-tg-B) cells were adoptively cotransferred into B6 recipients. We analyzed cell behavior at different time points after injection of HEL particles into mouse footpads. Our existing microscope settings allowed for the detection of B cells to depths of approximately 150 μm from the capsule. Although this was too superficial to allow for detailed analysis of distal parts of the follicle, it was sufficient to permit the study of B cell behavior at the subcapsular follicle boundary and within the follicle.

Images of intact popliteal lymph node (LN) obtained from untreated mice showed that tg-B cells move by a random walk, concurring with previous reports (Miller et al., 2002). Their velocity was similar to that of non-tg-B cells throughout the follicles (Figure 5B), however slightly slower than previously reported (Miller et al., 2002; Okada et al., 2005). Similar to the observations in T cells, this was likely due to the region of the lymph node imaged with our microscope settings (Huang et al., 2004). After antigen injection, however, follicular tg-B cells showed a gradual decrease in migration over time, with reduced track length and a steady decline in mean velocity from 3.5 to a minimum of 1 μm/min at 24 hr (Figure 5C). This reduction in velocity was dependent on antigen recognition because non-tg-B cells remained equally motile at all time points analyzed. By 24 hr, those tg-B cells that reached the

(C) Representative images of B cell follicle sections show the different level of colocalization of avidin fluorescent particles with fibroblastic reticular cells (ERTR7$^+$), macrophages (Mac-3$^+$), DCs (CD11c$^+$), and FDCs (FDC-M2$^+$) 1 hr and 24 hr after injection. Inner panels show an enlarged area of the images. "F" indicates follicle; "Sub" indicates subcapsular region.

(D) Quantification of the accumulation of avidin fluorescent particles inside the follicles over time (error bars indicate the standard deviation [SD]). The follicular area analyzed in the tissue sections was identified as positive for B220.

Scale bars represent 100 μm.

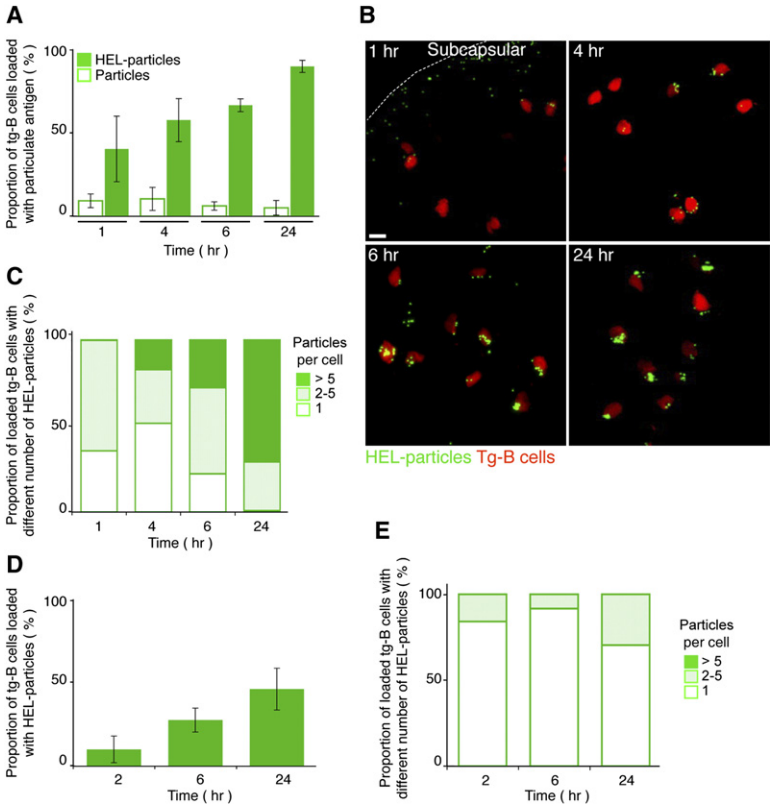

Figure 3. Cumulative Particulate-Antigen Acquisition by Follicular B Cells over the Time

Recipient mice were transferred with SNARF-1 labeled tg-B cells (red) and 20 hr later injected into the footpad with avidin fluorescent particles that were loaded or not with biotinylated HEL (green). Popliteal lymph node tissue sections were removed at the indicated time points and prepared for immunostaining with B220 antibody (see also Experimental Procedures); finally, stained-tissue sections were analyzed with confocal microscopy. Particulate antigen and tg-B cells were detected by their preserved fluorescence; B220-staining pattern was used to identify B cell follicles.

(A) Quantification of the proportion of follicular tg-B cells loaded with particulate antigen (particles or HEL particles) over time in popliteal lymph node tissue sections (error bars indicate the SD).

(B) Representative fields in the follicular area showing the encounter and accumulation of HEL particles on tg-B cells over the time at high resolution. The scale bar represents 5 μm.

(C) Quantification of the proportion of loaded tg-B cells detected in (A) with different numbers of HEL particles.

(D) Quantification of the proportion of follicular tg-B cells loaded with HEL particles over the time in popliteal lymph node tissue sections obtained from mice injected with only 10% of the quantity of HEL particles used in (A) (error bars indicate the SD).

(E) Quantification of the proportion of loaded tg-B cells detected in (D) with different numbers of HEL particles.

Data shown are representative of three different experiments.

B cell-T cell boundary region recovered high motility, probably searching for T cell help, as reported (Okada et al., 2005) (Movie S2). The images also showed two distinct tg-B cell behaviors within the follicle. First, motile tg-B cells had a migratory shape; during migration, the tg-B cells that had acquired antigen appeared to transport it in the uropod (Figure 5D and Movie S3). Second, some tg-B cells were attached to the site of antigen binding, confining these cells to a small area of movement (Figure 5E and Movie S4). In some cases, the time that B cells remained attached to the site of antigen binding was prolonged because it lasted for the duration of the imaging sessions.

Cell-Tracking Analysis Reveals Prolonged Periods of Confinement of Antigen-Specific B Cells at the Boundary between the Follicle and the Subcapsular Sinus of the Lymph Node

To determine whether these distinct behaviors were associated predominantly with antigen engagement and/or differential B cell localization in the follicle, we compared the migratory features of tg- and non-tg-B cells in the subcapsular region or deeper within the follicle (Figure 6 and Movie S5). We tracked 35 non-tg- and tg-B cells for more than 40 min in the subcapsular area. All showed signs of reduced motility compared to that of B cells in deeper follicle areas (Figures 6A and 6B; representative tracking data for seven tg-B and five non-tg-B cells, $p = 0.001$). Furthermore, in the subcapsular region, tg-B cells showed a greater reduction in motility than did non-tg-B cells (Figure 6B; 21 tg-B cells versus 14 non-tg-B cells, $p = 0.0146$), indicating that antigen recognition might have a role in further slowing B cell movement in this region. In these tg-B cells, antigen appears to be clustered at one side of the cell, and this represents one of the hallmark features of the B cell immunological synapse; in many cases, this technique allowed for the visualization of the antigen-gathering process (Figure 6C; Movie S6). When we analyzed the time of B cell residency within a confined area of 20 μm diameter, we found that more than 80% of antigen-specific cells remained for prolonged periods (>10 min), compared to less than 30% of non-tg-B cells (Figure 6D); this suggests that tg-B cells were attached to a still-undefined antigen-presenting surface. This behavior was almost absent in both tg- and non-tg-B cells deeper within the follicle. These results thus show not only that subcapsular B cell localization is associated with decreased B cell motility, but also that antigen recognition is associated with B cell retention in this area for long time periods.

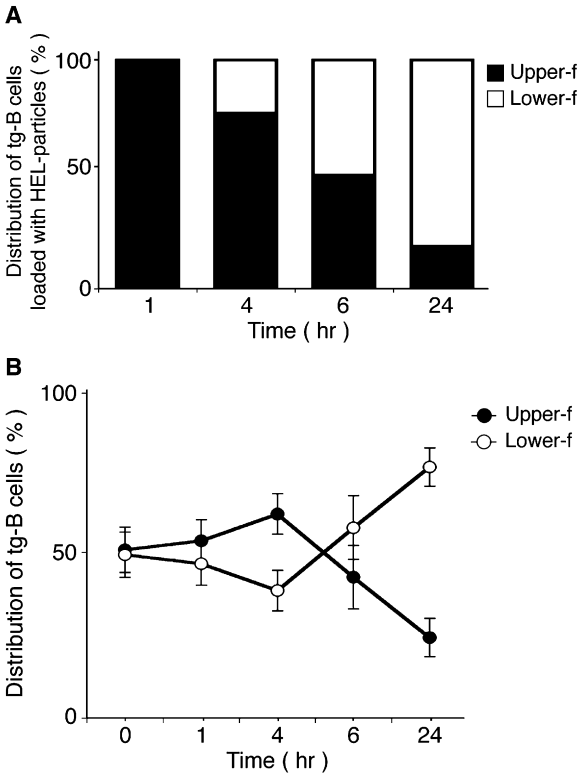

Figure 4. Initial Encounter of Particulate Antigen by Specific B Cells in the Follicular Area Close to the Subcapsular Sinus

(A) Quantification of the distribution of follicular tg-B cells loaded with HEL particles in upper-follicle ("Upper-f") and lower-follicle ("Lower-f") regions in popliteal lymph node tissue sections over time.

(B) Quantification of the ditribution of tg-B cells in the upper-follicle ("Upper-f") and lower-follicle ("Lower-f") regions in popliteal lymph node tissue sections at different time points after HEL-particle injection (error bars indicate the SD).

Data shown are representative of three different experiments.

DISCUSSION

Here we visualized the very early events associated with in vivo B cell recognition of particulate antigen. We established that, shortly after injection, particulate antigens accumulated in a macrophage-rich area at the boundary of lymph node follicles with the SCS. In this area, follicular B cells showed reduced motility compared to that of B cells deep within the follicle, and that cognate B cells acquired antigen. Many of these cognate interactions were long lasting; during this time, B cells were attached and confined to a small area at the site of antigen binding. These in vivo observations resembled the in vitro formation of the immunological synapse. In contrast, B cells from deep within the follicle had a migratory shape and more-rapid velocity. Those elongated B cells that had already acquired antigen transported it in the cell uropod, apparently as a single concentrated cargo. Finally, after 24 hr, antigen-carrying B cells showed characteristic features of activation and migrated toward the T cell region, as reported (Garside et al., 1998; Okada et al., 2005). We thus propose that macrophages collect and concentrate particulate antigen that arrives via the lymph at the SCS, where it is then encountered and acquired by follicular B cells.

Our finding that B cells acquired particulate antigen in a macrophage-rich area at the follicle-SCS boundary sheds light on a way by which antigen arriving via lymph is made available to follicular B cells. This is particularly important, considering the physical barrier to large-molecular-weight antigens imposed by the conduit network (Sixt et al., 2005), and because follicular B cells form the largest available B cell repertoire in vivo. The strategic location of these lymph node macrophages is reminiscent of that reported for spleen marginal-zone macrophages. Marginal-zone macrophages concentrate T-independent-2 and particulate antigens, such as bacteria found in the blood (Gray et al., 1985; Humphrey and Grennan, 1981). Thus, macrophages could play an essential role in filtering antigen arriving either via the lymph or blood from the site of infection and presenting them to follicular B cells.

The finding that macrophages could play a role in presenting intact antigen to B cells is intriguing. Macrophages express pattern-recognition receptors and scavenger receptors, which aid in the clearance of pathogens (Taylor et al., 2005); however, the mechanisms by which they are able to retain and present antigens to B cells is yet to be elucidated. It is tempting to speculate that several receptors could be involved. In this line, the C-type lectin SIGNR1, a murine homolog of human DC-SIGN, which is expressed in macrophages, could be implicated in this process. SIGNR1-deficient mice lack protective responses to infection, as they fail to clear bacteria from blood and lack a rapid antibody response to *S. pneumoniae* (Koppel et al., 2005). The molecular mechanism underlying this response is not yet completely understood, but, like DC-SIGN, SIGNR1 interacts with pathogen polysaccharides such as mannan and dextran (Kang et al., 2003). In DCs, the route seems to involve antigen internalization to nondegradative compartments and then recycling back to the cell surface (Kwon et al., 2002). In addition, other receptors expressed on the surface of macrophages are likely to be involved in this process (Taylor et al., 2005; Taylor et al., 2004). This suggests a general mechanism for antigen presentation to B cells, leading us to predict a role for macrophages as antigen-presenting cells for B cells (Carrasco and Batista, 2006a).

Although our model proposes subcapsular macrophages as the initial antigen-presenting cells for follicular B cells, FDCs might also have an important role. Our observation that particulate antigen was deposited on FDCs in the absence of high frequencies of specific B cells (approximately 3 in 100) suggests that, in normal conditions of clonal abundance (approximately 1 in 100,000), B cells might initially encounter antigen on the FDC surface (Figure 7). Although B cells deep in the follicle are more motile than are those at the boundary with the SCS, FDC expression of certain ligands, such as VCAM-1 and ICAM-1, which mediate B cell adhesion (Carrasco

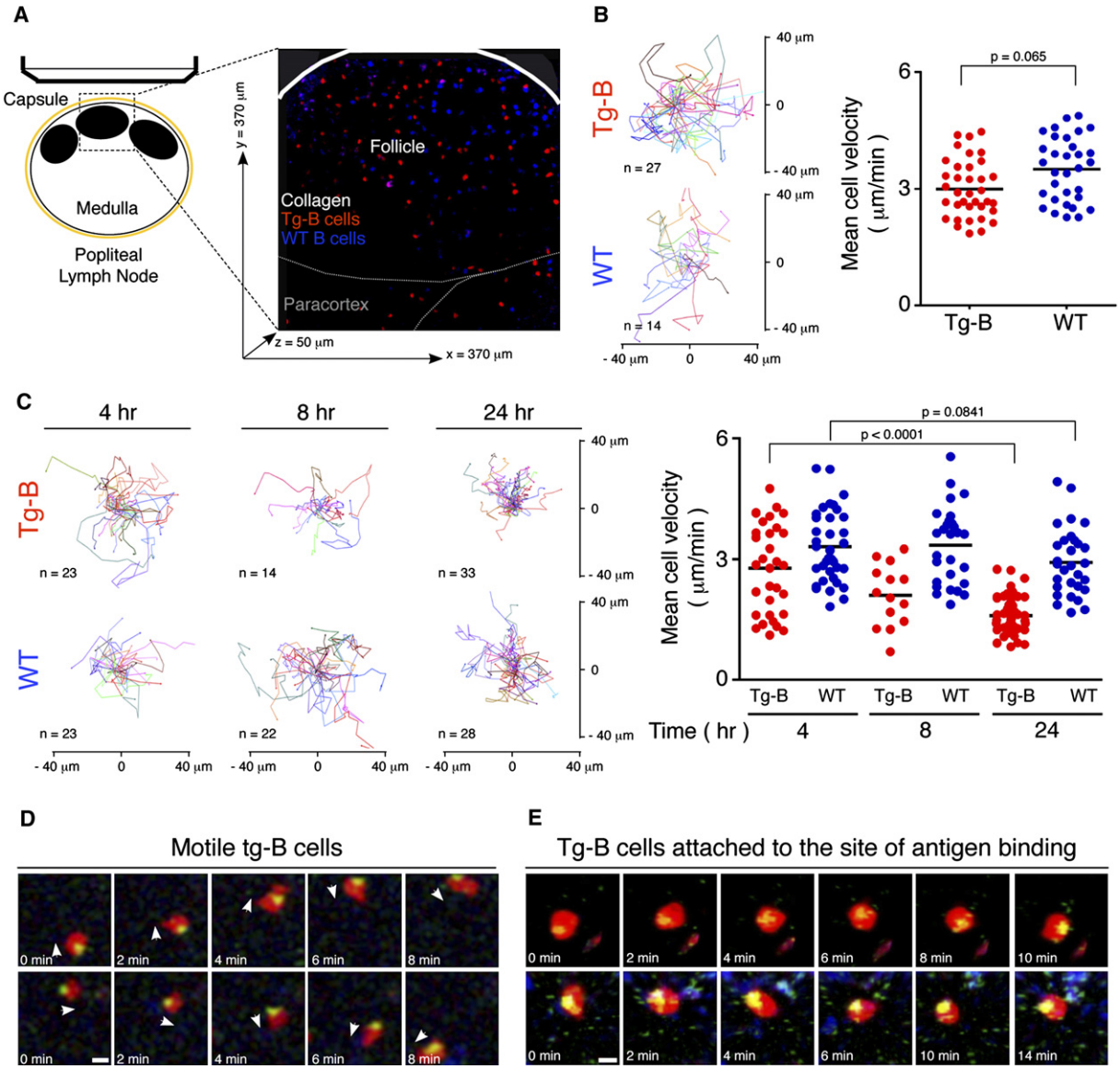

Figure 5. Dynamics of Wild-Type and Tg-B Cells upon HEL-Particle Encounter in the Follicle by Multiphoton Microscopy

SNARF-1 labeled tg-B cells (red) and CMAC-labeled wild-type (WT) B cells (blue) were cotransferred into C57BL/6 recipient mice. Twenty hours later, these mice were injected in the footpad with HEL particles (green). Popliteal lymph nodes were then isolated and prepared for multiphoton microscopy as described in the Experimental Procedures.

(A) Schematic diagram of an example imaged region of the popliteal lymph node by multiphoton microscopy, and its dimensions. The white dashed line represents the approximate location of the B cell-T cell border.

(B) Relative tracks and mean velocity of WT and tg-B cells in popliteal lymph nodes in steady state. WT and tg-B cells move with similar mean cell velocities (Student's t test, p value = 0.065, not significant).

(C) Effects in track length and mean velocity of WT and tg-B cells over the time in the presence of HEL particles. The differences in mean cell velocity of tg-B cells over the time were significant by Student's t test ($p > 0.0001$); no significant changes in mean velocity were detected in WT B cells ($p = 0.0841$).

(D) Representative time-lapse images of two motile tg-B cells (red) with a migratory shape carrying HEL particles (green) in the uropod, located within the follicle. White arrows indicate the migratory direction.

(E) Two examples of tg-B cells (red) attached to the site of antigen binding (HEL particles, green) are shown. Scale bars represent 5 μm.

Data for this figure were pooled from movies of three different experiments.

and Batista, 2006b; Carrasco et al., 2004), could facilitate antigen recognition.

In the absence of high frequencies of specific B cells, we observed particulate antigen trapped on the surface of FDCs. Although this antigen accumulation has long been thought to be important for immune responses, the mechanism by which antigens gain access to the follicle and are deposited on FDCs has not yet been described. A number of groups have addressed this question, proposing various mechanisms that include passive diffusion,

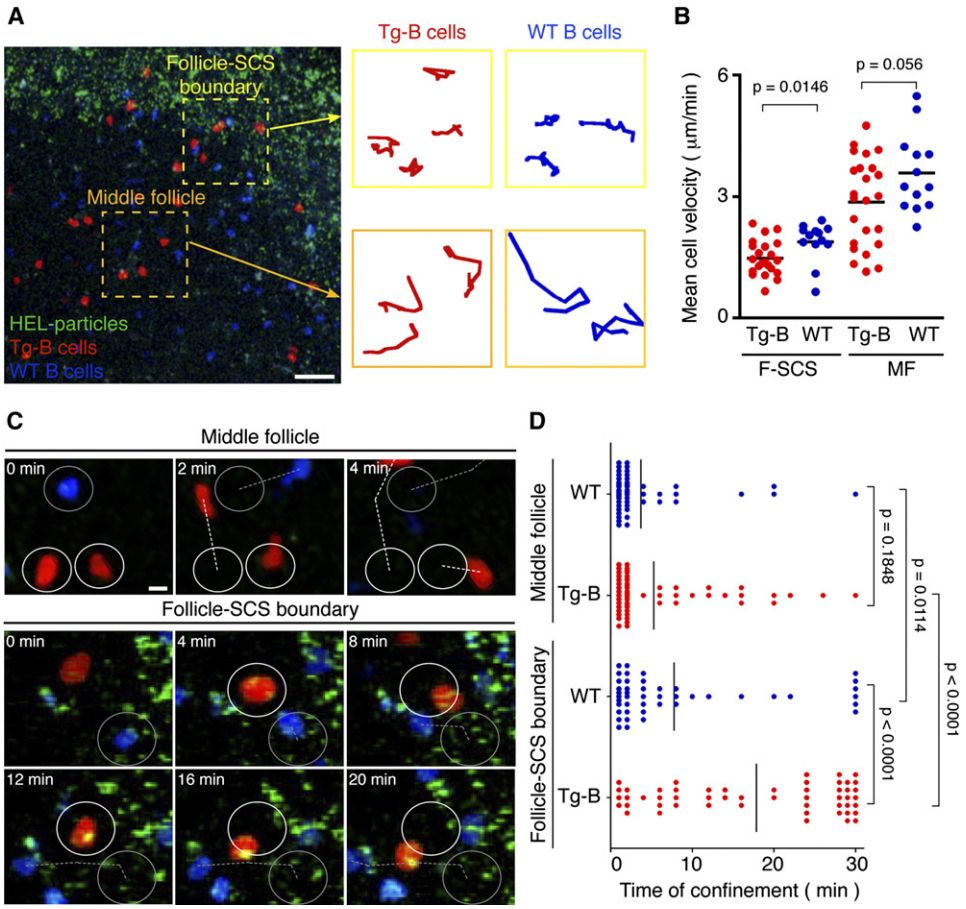

Figure 6. B Cells Showed Reduced Motility in the Follicle–Subcapsular-Sinus Boundary and Prolonged Time of Confinement after Particulate-Antigen Recognition

Recipient mice were adoptively transferred with equal numbers of SNARF-1 labeled tg-B cells (red) and CMAC-labeled wild-type (WT) B cells (blue). Twenty hours later, these mice were injected in the footpad with HEL particles (green). Popliteal lymph nodes were isolated 3–4 hr after injection and prepared for multiphoton microscopy as described in the Experimental Procedures. Dynamics of WT (blue) and tg-B (red) cells were analyzed in the follicle–subcapsular-sinus (SCS) boundary and middle follicle.

(A) Tracking of representative WT and tg-B cells in both areas. Tracked B cells are inside the regions (dashed line) in the projection of the 3D volume shown on the left panel. The scale bar represents 25 μm.

(B) Mean cell velocity of WT and tg-B cells in the follicle-SCS (F-SCS) boundary and middle follicle (MF) regions. The differences in mean cell velocity of WT and tg-B cells in the F-SCS boundary were significant by Student's t test ($p = 0.0146$); no significant differences in mean cell velocity of WT and tg-B cells were detected in the middle follicle ($p = 0.056$).

(C) Time-lapse images of representative WT (blue) and tg-B (red) cells 3–4 hr after HEL-particle injection. Tracking over time of the indicated cells (contained into circles) is shown for tg-B cells (white dashed line) and WT B cells (gray dashed line). The scale bar represents 5 μm.

(D) Quantification of the time of confinement of WT (blue dots) and tg- (red dots) B cells in the follicle-SCS boundary and middle-follicle regions. The time of confinement was estimated as the time that a B cell remains in a confined area of 20 μm diameter. Student's t test analysis was applied to the data; the differences in time of confinement of WT and tg-B cells in the follicle-SCS boundary and of tg-B cells in the follicle-SCS boundary and in the middle-follicle region were significant ($p < 0.0001$ in both cases).

Data for this figure were pooled from movies of three different experiments.

as well as active transport by B lymphocytes (Brown et al., 1970; Gray et al., 1984), macrophages (White et al., 1970), or FDC precursors (Szakal et al., 1983). In the spleen, the involvement of marginal-zone B cells and of complement and natural IgM in antigen transport probably constitutes the most compelling argument in support of a major role for B cells in this process (Ferguson et al., 2004). We also observed noncognate B cells in close juxtaposition with antigen-rich zones in the subcapsular region, where they exhibited decreased motility. Although the cause of this speed reduction is still unknown, it is plausible that close contact with an antigen-rich area could decorate the B cell surface with antigen. Such antigen transfer, which is likely to be complement mediated, might lead noncognate B cells to transport antigen deep into the follicle, where it could be passed to FDCs (Figure 7). We found that 10% of tg-B cells associated with particles, even when they were not loaded with HEL. Extrapolation of this proportion to the total pool of B cells resident in the follicle suggests that the total amount of antigen

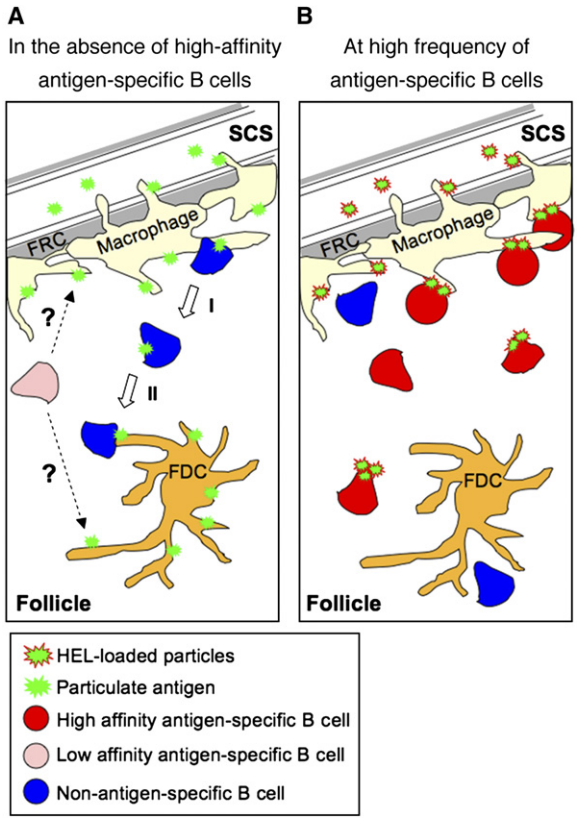

Figure 7. Model for Particulate-Antigen Acquisition by Follicular B Cells

Particulate antigens, such as virus or bacteria, reach the lymph node via lymph from peripheral tissues. Once there, antigens accumulate in a macrophage-rich area beneath the subcapsular sinus (SCS). Macrophages probably filter these antigens into the follicle by mechanisms that are still unknown.

(A) In the absence of high-affinity B cells, particulate antigen can be carried in a B cell receptor (BCR)-independent manner from the SCS and deposited on FDCs. Low-affinity follicular B cells could then recognize antigen in either of these two locations.

(B) When a high frequency of antigen-specific B cells is present in the follicle, they mainly capture antigen in the macrophage-rich region beneath the SCS and become the main carriers of antigen in the follicle.

carried by the B cells, in a BCR-independent manner, is considerably high. Thus, these findings support an important role of B cells in antigen transport from the SCS to the FDCs in the lymph node.

This study complements the very recent demonstration that soluble antigens can gain access to the follicle as a wave diffused from the SCS (Pape et al., 2007). These authors nonetheless rule out subcapsular recognition of soluble antigens on the basis of the detection of antigen-loaded B cells deep in the follicle shortly after antigen administration (3.5 min). Although soluble antigens such as toxins might freely gain access to the follicle, our findings suggest that most antigens that arrive via the afferent lymphatics to the SCS, such as proteins on viral or bacterial particles, will accumulate and be presented to follicular B cells by resident macrophages.

Our results showed that B cells encountered particulate antigen very shortly after its administration. In contrast, no particulate antigen was detected in the T cell zone before the arrival of antigen-loaded B cells from the follicles. A period of 12–24 hr is required for migration of antigen-loaded DCs from peripheral tissue to the draining lymph node (Kamath et al., 2002). Together, these data reveal B cells as initial sensors and effectors of the adaptive immune response. The absence of particulate antigen in the T cell area and the later appearance of antigen-loaded DCs in the lymph node also indicate that the recently described route of antigen encounter by B cells before homing to follicles (Qi et al., 2006) would participate in B cell-response amplification rather than its initiation. This would facilitate the activation of newly recruited B cells to the lymph node.

The immunological synapse has been implicated in the activation of T cells, natural killer (NK) cells and B cells; this structure is characterized in vitro by the molecular segregation of receptors at the zone of membrane contact between lymphocytes and antigen-bearing cells (Carrasco et al., 2004; Davis et al., 1999; Grakoui et al., 1999; Krummel et al., 2000; Monks et al., 1998; Stinchcombe et al., 2001). A hallmark of the B cell synapse is antigen accumulation to form the central supramolecular activation cluster (cSMAC) (Batista et al., 2001; Fleire et al., 2006). Despite its extensive in vitro characterization, however, there is only limited evidence of the in vivo existence of the immunological synapse. Tissue-section studies suggest the formation of prototypic T cell synapses in vivo (Barcia et al., 2006; Reichert et al., 2001). These findings are extended by visualization of CD43 exclusion in live tissue (Stoll et al., 2002). The sensitivity of our approach allowed for the clear visualization of antigen-specific B cells in the subcapsular region, attached to the site of antigen binding. In many cases, we also observed a spatiotemporal sequence of antigen gathering by B cells that much resembles immunological synapse formation in vitro (Batista et al., 2001; Fleire et al., 2006). We subsequently detected the upregulation of CD86 and MHC class II activation markers, as well as IgM downregulation, in these cells. These results strongly suggest that this synapse-like structure has an in vivo role in the B cell-activation process.

Multiphoton microscopy is a powerful technique that allows for the imaging of living cells within intact lymphoid organs, and its application in the B cell field has uncovered important aspects of the humoral immune response. Three recent reports address the dynamism of the germinal center, describing it as an open structure in which antigen-specific B cells shuttle between dark and light zones (Allen et al., 2007; Hauser et al., 2007; Schwickert et al., 2007). The authors propose that this high motility, together with the entry of follicular B cells into the germinal center, enhances competition and ensures selection of high-affinity B cells. A previous study (Okada et al., 2005) focused on B cell migration to the T cell zone after activation and the formation of B cell-T cell conjugates, stages that occur previous to germinal-center formation. We concentrated on the very early events that initiate the humoral

immune response, studying antigen encounter by follicular B cells in vivo. Our system allowed for the in vivo tracking of particulate antigen, from its arrival to the draining lymph node via lymph, recognition by B cells in the follicle, and transport by B cells to the T cell zone. We propose the follicle-SCS boundary as the site for initial B cell-antigen encounter in the lymph nodes.

Our results provide an important piece of the complex puzzle that defines the spatiotemporal behavior of B cells in the early stages of particulate-antigen recognition. Under steady-state conditions, B cells show random motility within the follicle. Possibly attracted by chemokines, they approached the follicle edge, where we observed slow B cell motion and particulate-antigen accumulation. A similar mechanism could underlie antigen transport by marginal-zone B cells, which are also located in a macrophage-rich region of the spleen. If specific antigen engagement occurs, B cells gather the antigen and remain at the site of antigen binding for a prolonged period of time, similar to the formation of an immunological synapse. Before B cells migrate to the B cell-T cell border, they could make repeated trips to acquire antigen at the follicle-SCS boundary. It would be very interesting to follow the fate of such particulate antigens in later stages of the B cell response. A series of recent studies showed germinal-center B cells crawling over the surfaces of FDCs 6 days after immunization (Allen et al., 2007; Schwickert et al., 2007); it remains to be determined whether antigen is present on these FDC membranes. The method we developed here may well constitute a useful approach to answer these types of questions.

EXPERIMENTAL PROCEDURES

Mice and Cells

Wild-type (WT) and HEL-specific B cells from spleens of wild-type and MD4 mice, respectively, were enriched by negative selection to > 98% purity, as previously described (Carrasco et al., 2004). Purified B cells were labeled with 2 μm SNARF-1 or 50 μm CMAC long-term dyes (Molecular Probes) for 15 min at 37°C, washed, and left in culture for 1 hr for dye equilibration. Five to ten millions of SNARF-1-labeled MD4 B cells or 5 millions of SNARF-1-labeled MD4 B cells plus 5 millions of CMAC-labeled WT B cells were then adoptively transferred by tail-vein injection into 4–6 week old C57 BL/6 recipient mice. After 20 hr, recipients were injected in the footpad with avidin-coated fluorescent particles prepared as follows: Alexa-488-conjugated avidin microspheres of 0.2 μm diameter (Molecular Probes) were loaded or not with biotinylated HEL (Sigma) in PBS 1% bovine serum albumin (BSA), washed, and resuspended in 25 μls of PBS for injection into the footpad. Approximately 1 μl of the stock of fluorescent particles (approx. 10^8–10^9 particles), or ten times less, where indicated, were prepared per injection. A similar quantity of Alexa488-conjugated *E.coli* bacterial particles (Molecular Probes) were injected in the mouse footpad at a final volume of 25 μls of PBS, where indicated. Mice were then sacrified by cervical dislocation at different time points, and popliteal and inguinal lymph nodes were removed and prepared for multiphoton imaging, flow cytometry, and tissue immunofluorescence. All experiments were approved by the Cancer Research UK Animal Ethics Committee and the United Kingdom Home Office.

Flow Cytometry

Popliteal lymph nodes were removed 24 hr after antigen administration, treated with collagenase IV + DNase I for 30 min at 37°C, and finally disgregated to obtain a cell suspension. Cells were then stained with fluorescein (FITC)-conjugated rat anti-mouse CD86 or rat anti-mouse MHC class-II ($I\text{-}A^b$) (BD Biosciences) plus Cy5-labeled goat anti-mouse IgM (Jackson Immunoresearch) and analyzed by flow cytometry. MD4 B cells were identified as SNARF-1^+.

Tissue Immunofluorescence

Cryostat sections (10 μm thickness) of popliteal and inguinal lymph nodes were fixed in 4% paraformaldehide for 10 min, blocked with PBS 1% BSA and 10% goat serum for 30 min, and stained with rat anti-mouse CD45R/B220 (BD Biosciences), rat anti-mouse FDC-M2 (ImmunoKontact), rat anti-mouse ER-TR7 (Bachem), hamster anti-mouse CD11c (Caltag), or rat anti-mouse Mac-3 (BD Biosciences) plus Alexa633-conjugated goat anti-rat IgG (Southern Biotechnology). Imaging was carried out on a Zeiss Axiovert LSM 510-META inverted microscope, and the image analysis was performed with LSM 510 and ImageJ software.

Multiphoton Imaging

Popliteal lymph nodes were prepared for multi-photon imaging as previously reported (Miller et al., 2002). In brief, lymph nodes were cemented through the hylum to the base of a glass-bottom 35 mm culture dish by veterinary topical tissue adhesive Nexaband (WPI) and were continuously perfused with warmed (37°C) Roswell Park Memorial Institute (RPMI) 1640 medium without Phenol Red (GIBCO, Invitrogen) bubbled with carbogen (95% O_2 5% CO_2). The system was set up inside the environmental chamber that covers the microscope, keeping the temperature at 37°C; the temperature was monitored throughout imaging session. Image acquisition was performed through the capsule of the lymph node with an upright Zeiss Axioplan microscope fitted with a multi-immersion 25×/0.75 objective and a Meta900 Titanium-Saphire Laser (Coherent) tuned to provide an excitation wavelength of 800 nm. For four-dimensional imaging (x, y, z, and time), stacks of eleven squared xy planes spanning 370 μm by 370 μm with 5 μm z spacing were acquired every 60 s over 45–60 min. Emission wavelengths were detected through 390–420 nm (second harmonic collagen), 430–485 nm (CMAC), 500–550 nm (Alexa488), and 585–615 nm (SNARF-1) band-pass filters. Sequences of image stacks were transformed into volume-rendered four-dimensional movies and analyzed with Volocity software (Improvision).

Supplemental Data

One figure and six movies are available at http://www.immunity.com/cgi/content/full/27/1/160/DC1/.

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