Visualizing B cell capture of cognate antigen from follicular dendritic cells

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The prominent display of opsonized antigen by follicular dendritic cells (FDCs) has long favored the view that they serve as antigen-presenting cells for B cells. Surprisingly, however, although B cell capture of antigen from macrophages and dendritic cells has been visualized, acquisition from FDCs has not been directly observed. Using two-photon microscopy, we visualized B cell capture of cognate antigen from FDCs. B cell CXCR5 expression was required, and encounter with FDC-associated antigen could be detected for >1 wk after immunization. B cell–FDC contact times were often brief but occasionally persisted for >30 min, and B cells sometimes acquired antigen together with FDC surface proteins. These observations establish that FDCs can serve as sites of B cell antigen capture, with their prolonged display time ensuring that even rare B cells have the chance of antigen encounter, and they suggest possible information transfer from antigen-presenting cell to B cell.

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Abbreviations: CFP, cyan fluorescent protein; CR, complement receptor; DEL, duck egg lysozyme; FDC, follicular dendritic cell; GC, germinal center; HEL, hen egg lysozyme; IC, immune complex; SA, streptavidin; SCS, subcapsular sinus macrophage; tg, transgenic.

B cells must encounter intact antigen to mount humoral immune responses. At any one time, most B cells in the body are situated inside lymphoid follicles of spleen, LNs, and mucosal lymphoid tissues, sites which are shielded from direct access to most fluid-borne antigens. Some 40 yr ago, antigen-tracking studies showed that opsonized antigens became distributed inside lymphoid follicles in a reticular fashion, and this led to studies identifying and characterizing follicular dendritic cells (FDCs) as the specialized antigen-trapping cells within follicles (Nossal et al., 1964; Szakal et al., 1989). FDCs expressing complement receptor (CR) 1 and CR2 are present within primary follicles, whereas the FDCs within germinal center (GC) light zones express additional surface markers including FcγRIIb (Szakal et al., 1989; Allen and Cyster, 2008). FDCs also express the integrin ligands ICAM-1, VCAM-1, and MAdCAM-1 (Szakal et al., 1989; Allen and Cyster, 2008). Follicular stromal cells, including FDCs, are a source of the chemokine CXCL13, and migration of B cells into lymphoid follicles depends on expression of the CXCL13 receptor CXCR5 (Allen and Cyster, 2008).

Despite the long period of study, the sites of first encounter between B cells and antigen have only recently been visualized. Three studies identified subcapsular sinus macrophages (SCSs) in LNs as an important site of cognate encounter with particulate antigen in the first hours of the response (Carrasco and Batista, 2007; Junt et al., 2007; Phan et al., 2007). It was also shown that noncognate B cells could capture opsonized antigen via CR1/2, and these cells delivered the antigen to FDCs (Phan et al., 2007). Another study used a model system to demonstrate B cell antigen capture from T zone dendritic cells during entry into LNs (Qi et al., 2006). In two further studies, follicular B cell antigen encounter with a small (~14 kD) soluble protein antigen appeared to occur by free diffusion of the antigen into the follicle (Pape et al., 2007) or after traveling via follicular conduits (Roozendaal et al., 2009). Given these advances, the lack of in vivo data on B cell antigen capture from FDCs is a notable omission. In vitro studies demonstrated that B cell activation by immune complexes (ICs) is enhanced when they are encountered in the presence of FDCs (Wu et al., 2008). Two-photon microscopy of GCs showed B cell migration in close association with GC FDCs, but these studies did not use fluorescently labeled antigens and could not track antigen capture

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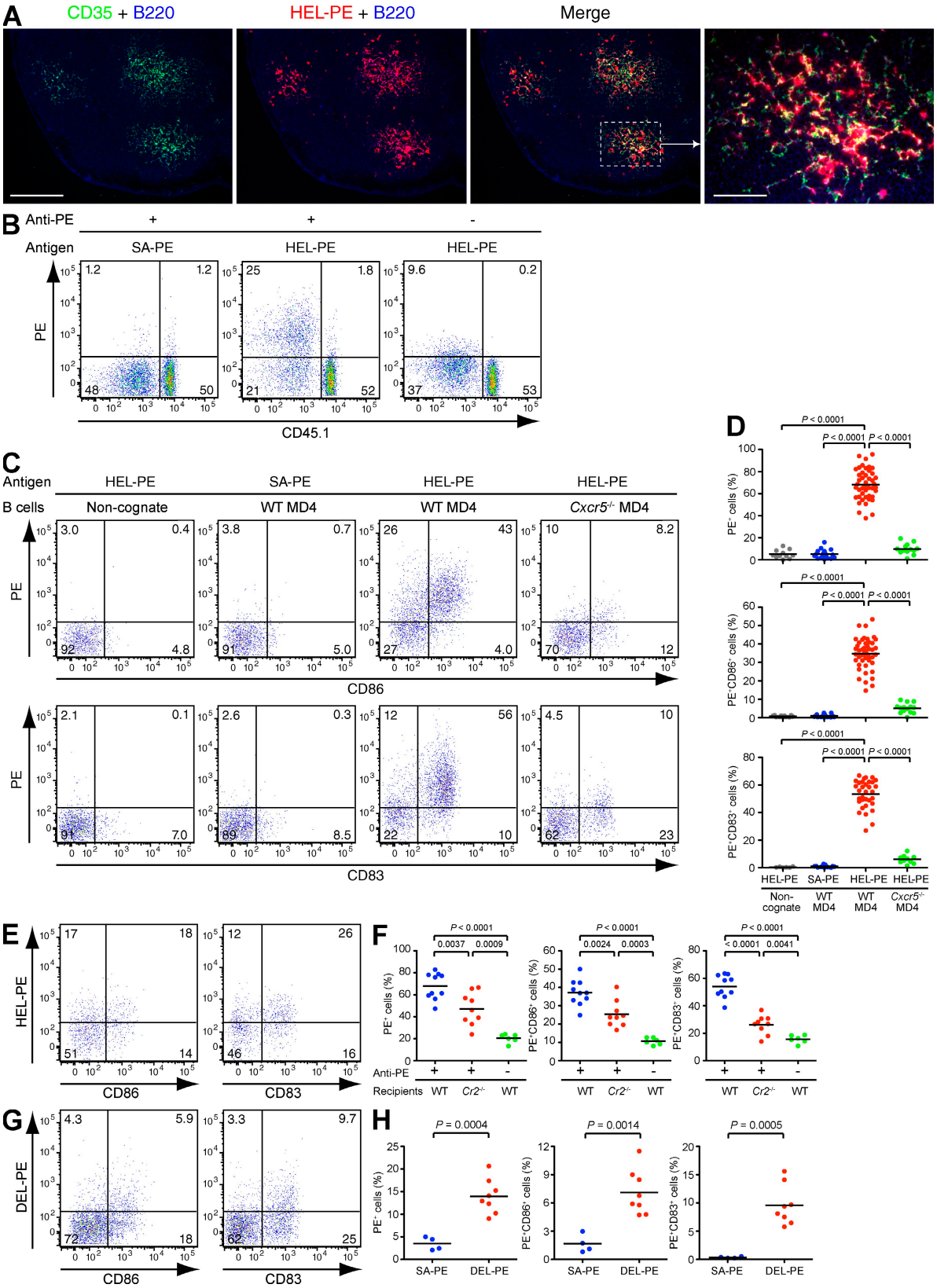

Figure 1. Deposition of HEL-PE ICs on FDCs and CXCR5-dependent encounter by cognate B cells. (A) LN section showing distribution of HEL-PE and staining for CD35 (green) and B220 (blue), 24 h after immunization. Bars: (left) 200 µm; (right) 50 µm. Data are representative of follicles from six inguinal LNs in three experiments. (B and C) Flow cytometric analysis of B cells 12 h after transfer to mice that had received anti-PE antibody and been

(Allen et al., 2007; Hauser et al., 2007; Schwickert et al., 2007). In this paper, we use two-photon microscopy to visualize B cell antigen capture from FDCs in primary follicles. We use mice passively immunized against a surface of the antigen distinct from the region recognized by the labeled B cells, modeling secondary exposure to a mutated or modified form of the priming antigen. We show that FDCs can function for prolonged periods as antigen-presenting cells for naive B cells, providing a possible mechanism to ensure antigen can be encountered by rare antigen-specific B cells traveling from distant sites. Moreover, we show that B cells often acquire FDC surface proteins during cognate antigen capture.

RESULTS AND DISCUSSION

System for studying antigen capture from FDCs

To take advantage of the intense fluorescence of PE and the high-affinity hen egg lysozyme (HEL) binding of MD4 Ig transgenic B cells (Goodnow et al., 1988), we generated multivalent HEL-PE antigen using biotinylated HEL and streptavidin (SA)-PE. Mice were passively immunized with polyclonal anti-PE IgG and, 1 d later, immunized s.c. with 10 μg HEL-PE. Analysis of draining LN sections taken at various time points showed that the majority of the HEL-PE became restricted to FDCs 1 d after antigen injection (Fig. 1 A and Fig. S1). Staining of adjacent sections for GC markers GL7 and FcγRIIb confirmed that these were primary follicle FDC networks (Fig. S2). As an approach to favor the likelihood that cognate B cells encountered antigen for the first time on FDCs, MD4 B cells were intravenously transferred into the recipients at 1 d and analyzed at various times by flow cytometry for acquisition of antigen. Within 12 h of transfer, 70% of MD4 B cells in the draining LN were PE^+, indicating capture of HEL-PE (Fig. 1 B), whereas cotransferred noncognate B cells did not capture the HEL-PE at this time (Fig. 1 B). In control recipients that had been immunized with PE that was not coupled with HEL there was no PE capture (Fig. 1 B), and in mice receiving HEL-PE in the absence of antibody to PE there was minimal antigen capture by cognate B cells (Fig. 1 B).

After antigen capture, cognate B cells up-regulated the costimulatory molecule CD86 (Fig. 1 C) and also CD83 (Fig. 1 C), another early marker of B cell activation (Prazma et al., 2007), whereas the transferred noncognate B cells remained quiescent. In contrast to wild-type MD4 B cells, CXCR5-deficient MD4 B cells that are unable to enter LN follicles (Ansel et al., 2000) showed little antigen capture or evidence of activation at the 12-h time point (Fig. 1, C and D). Defective encounter with FDC-bound antigen may contribute to the reduced antibody responses mounted by CXCR5-deficient B cells (Junt et al., 2005). Indeed, although wild-type MD4 B cells were able to promote proliferation of cotransferred HEL-specific CD4 T cells, CXCR5-deficient MD4 B cells showed minimal ability to promote T cell proliferation (Fig. S3). Moreover, wild-type MD4 B cells were induced to differentiate to GC B cells, whereas very few CXCR5-deficient cells underwent differentiation (Fig. S3). These observations indicate that B cells capturing antigen from FDCs are able to internalize, process, and present the antigen to T cells and to respond to T cell help.

Further supporting the conclusion that antigen encounter at this time point required presentation by FDCs, when CR1/2-deficient mice were immunized very little HEL-PE became deposited on FDCs, as expected (Roozendaal and Carroll, 2007), and antigen capture by wild-type MD4 B cells was significantly reduced (Fig. 1, E and F). The low continued capture that did occur (Fig. 1 F) suggests that some encounter may occur within the CR1/2-deficient follicle, perhaps reflecting residual display by SCS macrophages or low-level FcγRIIb-mediated display within the follicle. Transfer of MD4 B cells to mice that had been preimmunized with duck egg lysozyme (DEL)-PE, an antigen that binds the MD4 B cell receptor with ∼1,000-fold lower affinity ($K_a \sim 10^7\ M^{-1}$) than HEL (Lavoie et al., 1992), provided evidence that low-affinity B cells could capture FDC-associated antigen and become activated (Fig. 1, G and H).

Visualizing cognate antigen capture from FDCs

Using the system described in the previous section, LNs were imaged by two-photon microscopy to detect CFSE-labeled MD4 B cells, cyan fluorescent protein (CFP) transgenic (tg) control B cells, and HEL-PE antigen. Within 12 h of B cell transfer to preimmunized hosts, B cells could be observed migrating through the HEL-PE–laden FDC network, and some cells carried HEL-PE at their uropod (Fig. 2 A and Video 1). In some cases the image quality allowed resolution of a punctate or beaded aspect to the HEL-PE distribution along the FDC processes (Fig. 2 B), reminiscent of the IC–coated bodies or iccosomes observed in fixed-tissue ultrastructural studies (Szakal et al., 1988, 1989). A punctate PE distribution within

immunized with HEL-PE or SA-PE, or mice receiving HEL-PE alone. Plots were pregated for $CD19^+CD45.1^+$ or $CD45.2^+$ B cells. (B) HEL-PE capture by MD4 B cells ($CD45.1^-CD45.2^+$) compared with nontransgenic (noncognate) $CD45.1^+CD45.2^+$ control cells. (C) CD86 and CD83 expression on noncognate wild-type (WT) MD4 or *Cxcr5*$^{-/-}$ MD4 B cells in passively immunized mice that had received HEL-PE or SA-PE as indicated. (D) Summary of flow cytometric data of the type in B and C, pooled from 18 experiments. Each point indicates an individual LN (at least six mice per group). Bars indicate means. (E and F) Flow cytometric analysis of wild-type MD4 B cells transferred into *Cr2*$^{-/-}$ hosts (E) and summary of HEL-PE binding, CD86 and CD83 expression for MD4 B cells transferred to anti-PE–treated wild-type, and *Cr2*$^{-/-}$ recipients and wild-type recipients that did not receive ant-PE (F). Bars indicate means. (G and H) Flow cytometry of wild-type MD4 B cells transferred into mice that had received anti-PE antibody and been immunized with DEL-PE (G) and summary of antigen capture and activation marker expression (H). Plots were pregated for $CD19^+CD45.2^+$ B cells. Data are from two (E and F) or three (G and H) experiments. Each point (F and H) indicates an individual LN (at least four mice per group). Bars indicate means. Numbers in quadrants (B, C, E, and G) indicate percentage of gated cells.

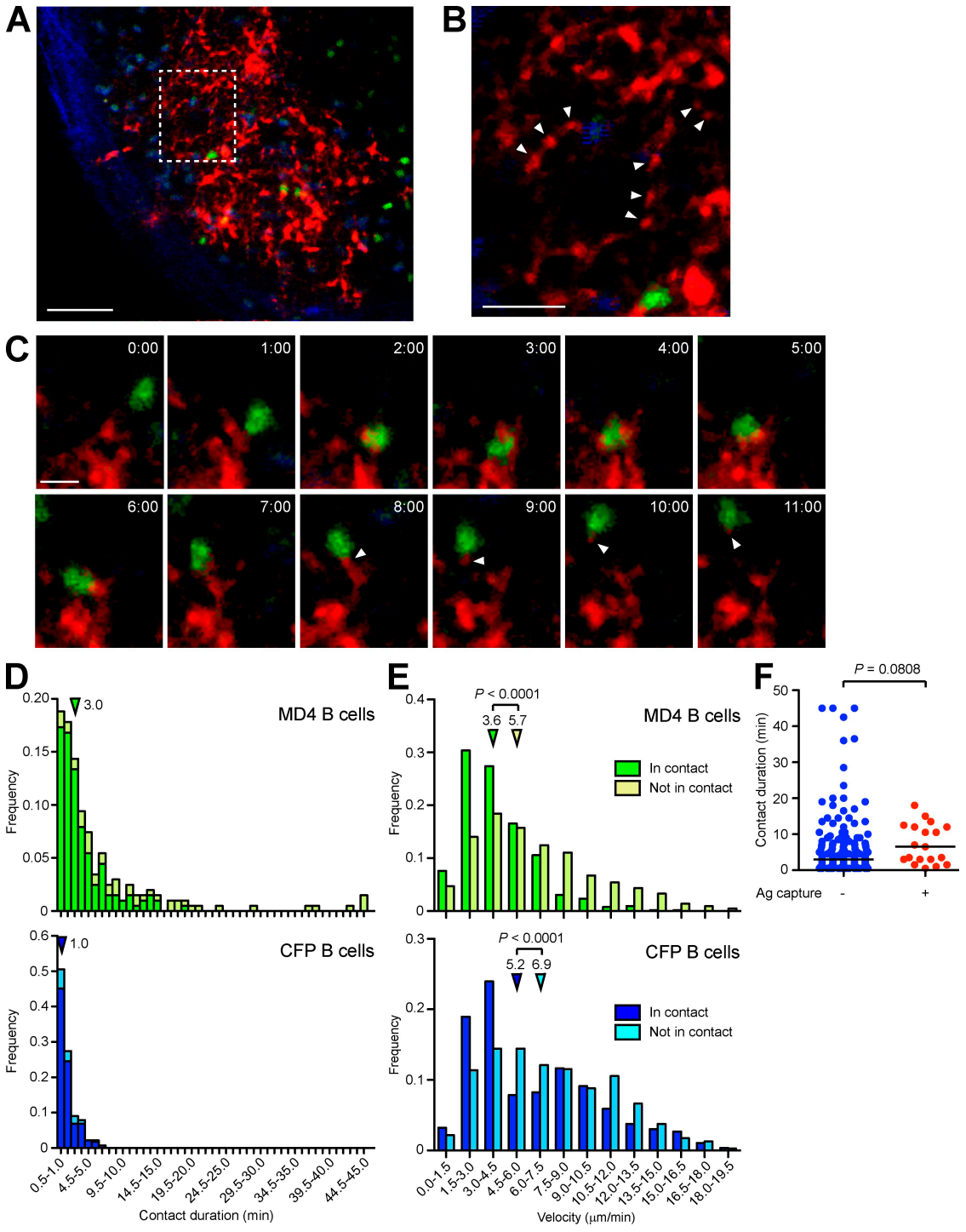

Figure 2. Visualization of cognate antigen capture from FDC by two-photon microscopy. (A) Two-photon microscopy of a follicle within an explanted LN from a mouse that had been HEL-PE (red) immunized 36 h earlier and had received CFSE+ MD4 B cells (green) and CFP tg polyclonal B cells (cyan) 12 h before. The capsule is visible in blue by second harmonic emission from collagen. (B) Enlarged view of boxed region in A highlighting several beaded HEL-PE+ processes (arrowheads). (C) Time-lapse images of a CFSE+ MD4 B cells encountering a HEL-PE+ process within the follicle, capturing and moving away with a large aggregate of HEL-PE (arrowheads), as presented in Video 2. Elapsed time is shown as minutes:seconds. Data in A–C are representative of three experiments with five mice. (D) Frequency of CFSE+ MD4 B cells or CFP tg noncognate B cells contacting HEL-PE+ FDC processes for the indicated amounts of time (202 contacts of 127 MD4 B cells, 277 contacts of 81 CFP B cells). Dark shading indicates that the contact was observed for its entirety during the imaging period; light shading indicates that the contact had formed before imaging began or persisted after the end of imaging. (E) Velocities of CFSE+ MD4 B cells or CFP tg noncognate B cells that were contacting HEL-PE+ FDC processes (in contact, dark shading) or were not contacting such processes (not in contact, light shading). Median velocities are indicated by numbers and arrowheads. (F) Contacts between CFSE+ MD4 B

the FDC network was also observed in immunofluorescence analysis of cryosections (Fig. S1), though particle sizes could not be directly compared because of the different methods of image detection. In the absence of cognate B cells, the majority of PE codistributed with the FDC marker CD35 (CR1; Fig. 1 A and Fig. S1), allowing us to use PE fluorescence as a reasonable surrogate for FDC in the imaging analysis. PE ICs and injected FDC-reactive antibodies have previously been used to highlight the GC light zone FDC network in studies tracking the migration dynamics of GC B cells (Allen et al., 2007; Hauser et al., 2007; Schwickert et al., 2007). By tracking individual MD4 B cells over time, acquisition of antigen from primary follicle FDCs could be observed, often involving capture of large antigen aggregates (Fig. 2 C and Video 2). Measurement of contact time with antigen-laden FDC processes showed that cognate B cells had increased contact times (median of 3 min), with some cells maintaining contact for >30 min (Fig. 2 D and Video 3), compared with noncognate B cells where interactions with antigen-labeled FDC processes rarely lasted for more than 1–2 min (Fig. 2 D). These interaction dynamics share similarities with the behaviors observed previously by two-photon microscopy of B cells and FDCs within the GC, where many transient interactions and occasional long interactions were observed (Allen et al., 2007; Hauser et al., 2007; Schwickert et al., 2007). Both cognate and noncognate cells moved with lower mean velocities during periods of contact with antigen-decorated FDCs, though the reduction in velocity was greatest for cognate cells (Fig. 2 E). Cognate B cells also had a reduced velocity when not in contact with the PE^+ FDCs (Fig. 2 E), which is consistent with the previous finding that B cells recently engaged by antigen moved with reduced velocity (Okada et al., 2005). The cognate B cells also had sharper turning angles than noncognate cells, both during contact and when not in contact with the PE^+ FDCs (Fig. S4). These observations indicate that B cells interact closely with FDCs and that cognate cells are able to capture large amounts of antigen during such encounters. Of 127 cognate cells that contacted HEL-PE–laden FDCs, 19 cells captured detectable amounts of HEL-PE. The median contact time during the antigen capture event was 6.5 min (Fig. 2 F). The increased interaction time may facilitate integrin-mediated B cell spreading and contraction on the FDC in a process that can facilitate collection and capture of membrane-bound antigen (Fleire et al., 2006).

Cognate B cells acquire FDC surface antigens

The observation that cognate B cells often captured large units of antigen together with the beaded aspect of some FDC processes led us to ask whether cognate B cells were capturing fragments of FDCs. Indeed, by flow cytometry about one-third of the PE-bright B cells were also shown to be positive for the follicular stromal marker BP-3 (Fig. 3, A and B; McNagny et al., 1991; Ngo et al., 1999), whereas noncognate B cells showed little staining for this marker (Fig. 3, A and B). To test whether this capture extended to additional FDC markers, $Cr2^{-/-}$ B cells that lack surface CR1 (CD35) expression were transferred to preimmunized wild-type recipients and the capture of CR1 was examined. Similarly to BP-3, the cognate, but not noncognate, B cells captured CR1 (Fig. 3, C and D). B cells capturing BP-3 or CR1 corresponded to the brightest PE^+ cells, suggesting a relationship between the extent of antigen capture and the probability of acquiring FDC surface markers. Alternatively, only by capturing an FDC fragment could cognate B cells acquire the highest amounts of antigen. Immunofluorescence microscopy of B cells purified 12 h after transfer revealed cells with single large clusters of PE at one pole (Fig. 3 E). On PE-bright cells where BP-3 could be detected, it was typically located as a single cluster overlapping with or closely adjacent to the PE signal (Fig. 3, E and F). In the case of cells with lower amounts of PE signal, the BP-3 was sometimes situated in a separate cluster (Fig. 3, E and F). Collectively, these observations suggest that cognate B cells are able to acquire FDC surface markers during antigen capture.

Previous work using the MD4 system established that recombinant HEL-C3d-C3d was a much more potent stimulus of B cell activation than HEL alone (Dempsey et al., 1996). We were therefore surprised to find that $Cr2^{-/-}$ MD4 and wild-type MD4 B cells showed equally efficient activation upon encountering opsonized HEL-PE in the follicle (Fig. S5, A and B), suggesting that activation by multivalent HEL complexes is less dependent on B cell–intrinsic CR2 signaling. Notably, however, when complexes were formed with the low-affinity antigen DEL, CR2 in the cognate B cells made a prominent contribution to the efficiency of both antigen capture and B cell activation (Fig. S5, C and D). This observation adds to the extensive evidence that the CR2 complex acts intrinsically to amplify BCR signaling (Dempsey et al., 1996; Roozendaal and Carroll, 2007) to suggest that it can also augment antigen capture by cognate B cells.

Prolonged period of encounter

During early studies of antigen deposition on FDCs, it was established that the antigen could be retained in an intact form for periods of weeks (Tew and Mandel, 1978). The importance of this sustained presentation has been emphasized in the context of driving GC responses. However, it has not been determined whether this prolonged retention facilitates ongoing encounter by naive B cells that enter the LN late, perhaps after traveling from a distant site. Given the presence of at least 20 secondary lymphoid tissues in the healthy mouse and the evidence that B cells spend about a day in any given lymphoid tissue (Tomura et al., 2008), if the cell were to sample lymphoid

cells and $HEL\text{-}PE^+$ FDC processes that resulted in antigen capture (Ag capture +) or were not accompanied with detectable antigen capture (Ag capture −). Median contact durations (bars) are 6.5 min for contacts with capture (n = 19) and 3.0 min for contacts without capture (n = 183). Bars: (A) 50 µm; (B) 20 µm; (C) 10 µm. Data in D, E, and F are pooled from three experiments.

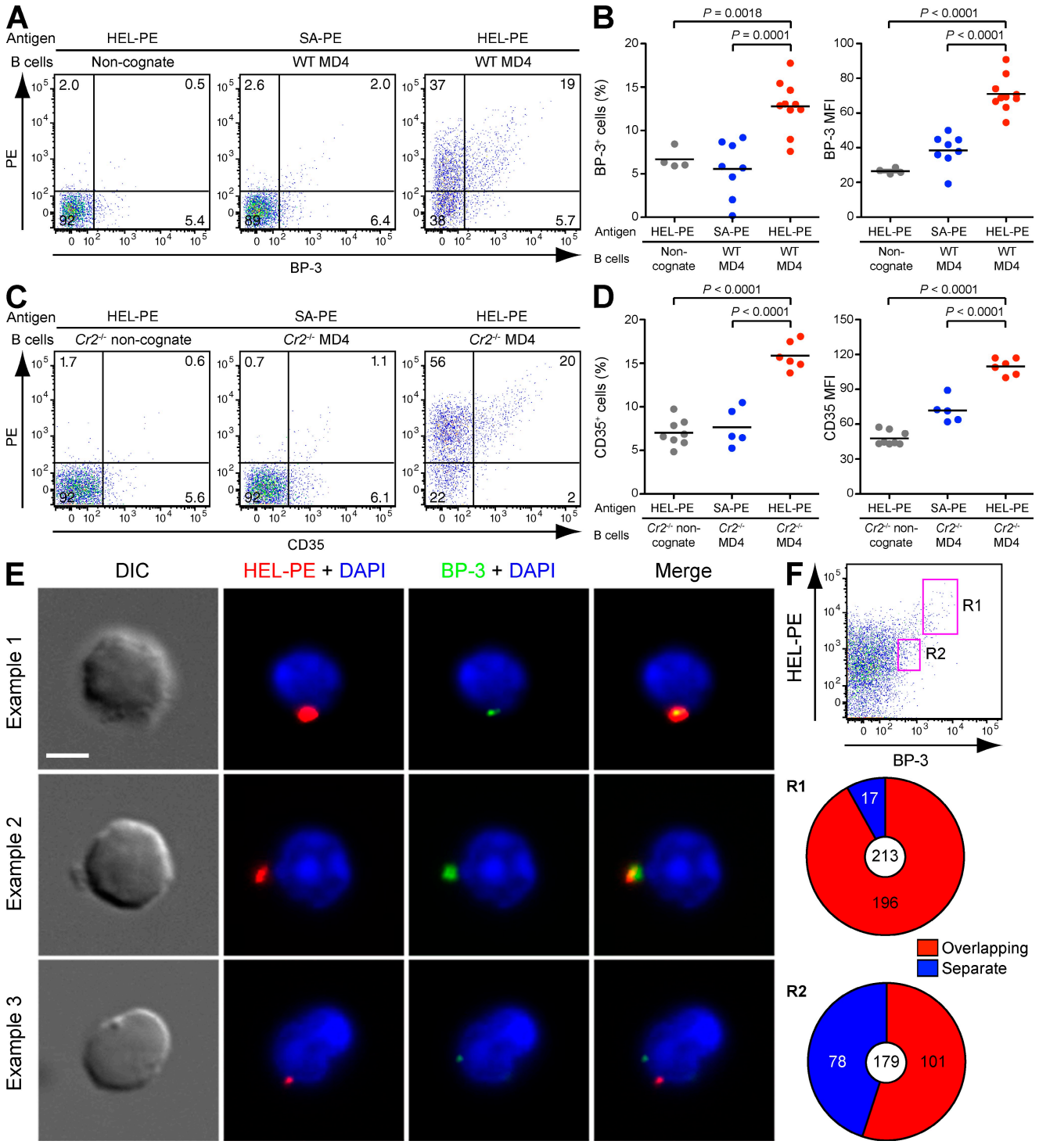

Figure 3. Cognate B cells acquire FDC surface antigens. (A) Flow cytometric analysis of wild-type (WT) noncognate or MD4 B cells, 12 h after transfer to recipients immunized as indicated, stained to detect BP-3. (B) Summary of data obtained as in A. (C) Flow cytometric analysis of *Cr2*$^{-/-}$ noncognate or MD4 B cells, 12 h after transfer to recipients immunized as indicated, stained to detect CR1 (CD35). (D) Summary of data obtained as in C. Each point in B and D indicates a single LN, and two inguinal LNs were analyzed from each mouse (at least three mice per group). Bars indicate means. Data are from four (A and B) or two (C and D) experiments. Numbers in quadrants in A and C indicate the percentage of gated cells. (E) Fluorescence microscopy of transferred MD4 B cells isolated from LNs 12 h after transfer, visualizing bound HEL-PE (red) and stained with antibodies to detect BP-3 (green) and with DAPI to detect nuclei (blue). Bar, 3 µm. Three examples are shown that are representative of cells analyzed in two experiments. (F) Gating scheme for sorting PE^{hi}BP-3^{hi} (R1) or PE^{lo}BP-3^{lo} (R2) transferred MD4 B cells and pie charts showing the number of cells that had overlapping or separate clusters of PE and BP-3 signals in these gates. The total numbers of cells analyzed are indicated in the inner circles. Data are pooled from two experiments.

organs randomly, a single B cell has a 50% chance to localize to a given LN within 2 wk (see Materials and methods). The time is likely to be shortened by inflammatory insults that increase entry and decrease exit rates for a draining lymphoid tissue. In time-course experiments, cognate B cells that newly entered a HEL-PE draining LN could be observed capturing FDC-displayed antigen 9 d after the initial immunization (Fig. 4, A and B). These encounters continued to lead to B cell activation as assessed by CD83 and CD86 induction (Fig. 4 B). Thus, FDCs provide a mechanism to increase the chance of antigen encounter even by rare antigen-specific B cells. Starting from day 6, much of the deposited PE was located within GL7$^+$ GCs (Fig. S2). These are likely to be GCs generated by endogenous B cells in response to the HEL-PE ICs. Recent work has visualized naive B cell entry to the GC light zone (Schwickert et al., 2007), providing a possible mechanism for naive B cell encounter with antigen in this compartment.

Concluding remarks

Through the use of two-photon microscopy and brightly labeled high-affinity antigen, we have visualized B cell capture of opsonized antigen from FDCs in primary follicles. In some cases, B cells appear to capture the antigen as a large unit that also involves acquisition of FDC membrane markers, which is consistent with evidence from fixed tissue analysis that B cells can become associated with IC–coated bodies or iccosomes (Szakal et al., 1988, 1989). The frequently beaded appearance of HEL-PE on the FDC may also fit with this concept. The correlation between acquisition of FDC surface antigens and acquisition of the largest amounts of cognate antigen suggests that by capturing FDC fragments, cognate B cells are able to increase the amount of antigen they acquire. Preformed iccosomes may not, however, be necessary to account for the FDC membrane antigen capture, as in vitro studies demonstrated the ability of antigen-binding B cells to

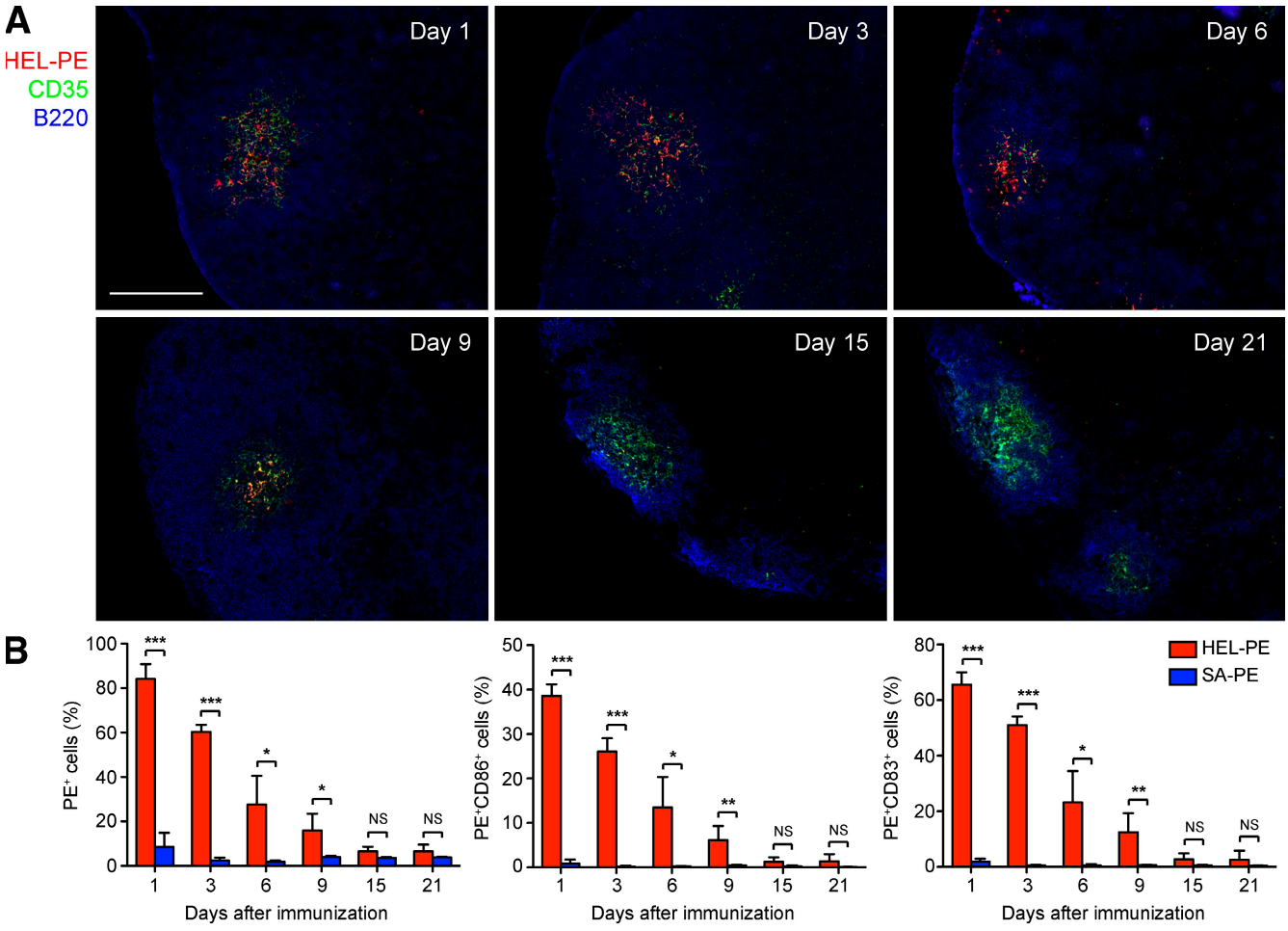

Figure 4. Late entering B cells continue to encounter FDC bound antigen. (A) Immunofluorescence analysis of LN sections taken at the indicate time points after HEL-PE immunization, visualizing PE (red) and stained to detect CD35 (green) and B220 (blue). Bar, 200 µm. Data are representative of follicles from at least four inguinal LNs at each time point in two experiments. (B) Summary of flow cytometric data showing the frequency of transferred MD4 B cells that were PE$^+$, PE$^+$CD86$^+$, or CD83$^+$ when the cells were harvested 12 h after transfer to mice immunized with HEL-PE or SA-PE the indicated number of days before the cell transfer. Error bars indicate means + SD of at least six LNs from four mice in two experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

extract antigens from cell membranes (Batista et al., 2001) and exchange of membrane between closely opposed cells has been observed in a variety of contexts (Williams et al., 2007). The finding on some of the cells with lower amounts of HEL-PE of separate PE^+ and $BP\text{-}3^+$ foci suggests that the antigen and stromal marker can sometimes be captured separately though the possibility that antigen and BP-3 become separated after capture is not excluded. BP-3 is expressed on the entire network of follicular stromal cells and is not limited to the antigen-presenting $CR1^+$ FDC (Ngo et al., 1999). Perhaps the integrin activation induced by B cell receptor engagement (Spaargaren et al., 2003; Carrasco et al., 2004) can promote increased adhesive interactions with the follicular stromal cell network that increase capture even of antigen-negative $BP\text{-}3^+$ membrane fragments. Whether captured as a single unit or separately, these observations suggest that additional signals may be delivered from follicular stromal cells to B cells during cognate antigen recognition.

Many antigens can become opsonized independently of high-affinity antibody, via alternative or lectin pathways of complement activation, and some can become decorated by low-affinity natural antibody, providing pathways by which FDCs can be antigen loaded in the initial phases of a primary response. Collectively, the findings in this paper and the recent work visualizing cognate antigen encounter on SCS macrophages (Carrasco and Batista, 2007; Junt et al., 2007; Phan et al., 2007) can propose that in the first hours after arrival of opsonized antigen in a LN, B cells have the opportunity to encounter antigen displayed by macrophages. At later time points, however, encounter with FDC-bound antigen may predominate, especially in responses involving nonreplicating antigens. In cases where antigen is continuously produced, both antigen display sites may operate in synchrony. Whether the fate of B cells encountering antigen on SCS macrophages and FDCs is the same remains to be investigated. The time course over which naive B cells can encounter antigen on FDCs is likely to vary widely depending on the properties and amounts of antigen, the frequency of specific B cells, and the nature of any prior B cell response. Our finding that encounter could occur 9 d after immunization, a time point when the FDC networks were GC associated, is in accord with the finding that transferred cognate B cells could join a day-9 GC response (Schwickert et al., 2007). Another context where we envision a role for antigen encounter on primary follicle FDCs is after the first antibody production of a primary response. Here, the higher affinity cells may respond first, giving rise to antibody that promotes antigen deposition on FDCs and increasing the chance of productive encounter by low-affinity cells that recognize distinct epitopes. In the case of a secondary response, because we passively immunize with high-affinity IgG specific for PE but not HEL, we suggest our findings may model secondary encounter with a mutated or otherwise modified form of the priming antigen. Finally, although we have visualized the process of naive B cell capture of antigen from FDCs in the primary follicle, the similar migration behaviors of GC B cells with respect to light zone FDCs (Allen et al., 2007; Hauser et al., 2007; Schwickert et al., 2007) suggests that similar antigen capture from FDCs can take place within the GC during antibody affinity maturation.

MATERIALS AND METHODS

Mice. 6–12-wk-old male wild-type and Ly5.2 (CD45.1) congenic C57BL/6 were purchased from either the National Cancer Institute or The Jackson Laboratory. MD4 mice (Goodnow et al., 1988) were from an internal colony. Animals were housed in a germ-free environment in the Laboratory Animal Research Center at the University of California, San Francisco (UCSF) and all experiments conformed to ethical principles and guidelines approved by the UCSF Institutional Animal Care and Use Committee.

In vivo generation of HEL-PE and DEL-PE ICs. HEL- or DEL-PE was produced by incubating HEL- or DEL-biotin in molar excess with SA-PE and removing unbound HEL- or DEL-biotin using the BioSpin 30 column (Bio-Rad Laboratories). ICs were generated as previously described (Phan et al., 2007). In brief, mice were first passively immunized with 2 mg of rabbit anti-PE IgG (Rockland Immunochemicals). Recipient mice were passively immunized in this way unless otherwise indicated. 1 d later, mice received HEL- or DEL-PE s.c. in the flank and tail base to drain the inguinal LN (2.5 μg/site). Unless otherwise stated, mice received transfers of cells 24 h after antigen immunization and, 12 h later, they were analyzed by flow cytometry and sectioning or the LNs were explanted for two-photon microscopy.

Flow cytometry. Draining LNs were teased apart with microforceps into DME containing 2% fetal bovine serum, penicillin/streptomycin, and Hepes buffer. Single cells were washed and blocked with anti-CD16/32 (2.4G2; UCSF Hybridoma Core Facility) and stained for flow cytometric analysis as previously described (Allen et al., 2007; Phan et al., 2007). Data were acquired on a LSRII flow cytometer (BD) and were analyzed with FlowJo software (Tree Star, Inc.).

Immunofluorescent microscopy for BP-3 capture. Transferred MD4 B cells ($CD45.2^+$) were sorted as $CD19^+CD45.1^-PE^{hi}BP\text{-}3^{hi}$ or $PE^{lo}BP\text{-}3^{lo}$ subsets from the LNs of CD45.1 congenic recipients with a FACSAria (BD). Cells were then fixed with 4% paraformaldehyde (Sigma-Aldrich) on 8-well chambered coverslips (Invitrogen) and stained with DAPI (Invitrogen). Images were obtained with an inverted microscope (Axio Observer Z1; Carl Zeiss, Inc.).

Two-photon microscopy. Explanted LNs were prepared for imaging as previously described (Okada et al., 2005; Allen et al., 2007). Images were acquired with Video Savant (IO Industries), and maximum-intensity time-lapse images were generated with MetaMorph (MDS Analytical Technologies). Videos were processed with a median noise filter. Excitation wavelength was 910 nm. Emission filters were 440–480 nm for second harmonic, 470–500 nm for CFP, 525–575 nm for CFSE, and 605–675 nm for PE. Cell tracks were made with Imaris 5.01 ×64 (Bitplane), and automated tracks were verified and corrected manually. Contact durations of B cells with FDCs were measured manually for each track by counting time points with a 30-s interval, in which a B cell–derived fluorescent signal was in contact with PE signals. The velocities and turning angles of cells between each imaging frame were calculated with MATLAB (The MathWorks, Inc.). Annotation and final compilation of videos were performed with After Effects 7.0 software (Adobe). Video files were converted to MPEG format with AVI-MPEG Converter for Windows 1.5 (FlyDragon Software).

Statistical analysis. Prism software (GraphPad Software, Inc.) was used for all statistical analyses. For comparison of two nonparametric datasets, the Mann-Whitney *U* test was used. Means of two groups were compared with the one-tailed Student's *t* test. The number of cell recirculation attempts (N) was calculated according to the equation $P = (1 - qN)$, where P is the probability to localize to a particular lymphoid organ (out of 20 equivalent ones) at least once after N trial attempts and q is the probability to localize to any other lymphoid organ in a single trial (for 20 lymphoid organs $q = 19/20$).

Online supplemental material. Detailed description of methods can be found in the supplemental text. Fig. S1 is a magnified image of a LN section to depict punctate distribution of HEL-PE in the FDC network. Fig. S2 shows LN sections stained to detect GC markers FcγRIIb and GL7, as well as HEL-PE. Fig. S3 shows T cell proliferation and GC B cell differentiation driven by antigen on FDCs. Fig. S4 shows turning angles of B cells calculated based on two-photon imaging. Fig. S5 shows antigen acquisition and activation of *Cr2*$^{-/-}$ MD4 B cells. Video 1 shows real-time imaging of B cell migration in the FDC network. Video 2 shows real-time imaging of antigen capture from FDCs by cognate B cells. Video 3 shows real-time imaging of prolonged interaction of B cells with FDCs. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090209/DC1.

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The authors declare that they have no competing financial interests.

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REFERENCES

Allen, C.D., and J.G. Cyster. 2008. Follicular dendritic cell networks of primary follicles and germinal centers: phenotype and function. *Semin. Immunol.* 20:14–25.

Allen, C.D., T. Okada, H.L. Tang, and J.G. Cyster. 2007. Imaging of germinal center selection events during affinity maturation. *Science.* 315:528–531.

Ansel, K.M., V.N. Ngo, P.L. Hyman, S.A. Luther, R. Förster, J.D. Sedgwick, J.L. Browning, M. Lipp, and J.G. Cyster. 2000. A chemokine driven positive feedback loop organizes lymphoid follicles. *Nature.* 406:309–314.

Batista, F.D., D. Iber, and M.S. Neuberger. 2001. B cells acquire antigen from target cells after synapse formation. *Nature.* 411:489–494.

Carrasco, Y.R., and F.D. Batista. 2007. B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. *Immunity.* 27:160–171.

Carrasco, Y.R., S.J. Fleire, T. Cameron, M.L. Dustin, and F.D. Batista. 2004. LFA-1/ICAM-1 interaction lowers the threshold of B cell activation by facilitating B cell adhesion and synapse formation. *Immunity.* 20:589–599.

Dempsey, P.W., M.E. Allison, S. Akkaraju, C.C. Goodnow, and D.T. Fearon. 1996. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science.* 271:348–350.

Fleire, S.J., J.P. Goldman, Y.R. Carrasco, M. Weber, D. Bray, and F.D. Batista. 2006. B cell ligand discrimination through a spreading and contraction response. *Science.* 312:738–741.

Goodnow, C.C., J. Crosbie, S. Adelstein, T.B. Lavoie, S.J. Smith-Gill, R.A. Brink, H. Pritchard-Briscoe, J.S. Wotherspoon, R.H. Loblay, K. Raphael, et al. 1988. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature.* 334:676–682.

Hauser, A.E., M.J. Shlomchik, and A.M. Haberman. 2007. In vivo imaging studies shed light on germinal-centre development. *Nat. Rev. Immunol.* 7:499–504.

Junt, T., K. Fink, R. Forster, B. Senn, M. Lipp, M. Muramatsu, R.M. Zinkernagel, B. Ludewig, and H. Hengartner. 2005. CXCR5-dependent seeding of follicular niches by B and Th cells augments antiviral B cell responses. *J. Immunol.* 175:7109–7116.

Junt, T., E.A. Moseman, M. Iannacone, S. Massberg, P.A. Lang, M. Boes, K. Fink, S.E. Henrickson, D.M. Shayakhmetov, N.C. Di Paolo, et al. 2007. Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Nature.* 450:110–114.

Lavoie, T.B., W.N. Drohan, and S.J. Smith-Gill. 1992. Experimental analysis by site-directed mutagenesis of somatic mutation effects on affinity and fine specificity in antibodies specific for lysozyme. *J. Immunol.* 148:503–513.

McNagny, K.M., R.P. Bucy, and M.D. Cooper. 1991. Reticular cells in peripheral lymphoid tissues express the phosphatidylinositol-linked BP-3 antigen. *Eur. J. Immunol.* 21:509–515.

Ngo, V.N., H. Korner, M.D. Gunn, K.N. Schmidt, D.S. Riminton, M.D. Cooper, J.L. Browning, J.D. Sedgwick, and J.G. Cyster. 1999. Lymphotoxin α/β and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. *J. Exp. Med.* 189:403–412.

Nossal, G.J., G.L. Ada, and C.M. Austin. 1964. Antigens in immunity. Iv. Cellular Localization of 125-I- and 131-I-labelled flagella in lymph nodes. *Aust. J. Exp. Biol. Med. Sci.* 42:311–330.

Okada, T., M.J. Miller, I. Parker, M.F. Krummel, M. Neighbors, S.B. Hartley, A. O'Garra, M.D. Cahalan, and J.G. Cyster. 2005. Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS Biol.* 3:e150.

Pape, K.A., D.M. Catron, A.A. Itano, and M.K. Jenkins. 2007. The humoral immune response is initiated in lymph nodes by B cells that acquire soluble antigen directly in the follicles. *Immunity.* 26:491–502.

Phan, T.G., I. Grigorova, T. Okada, and J.G. Cyster. 2007. Subcapsular encounter and complement-dependent transport of immune complexes by lymph node B cells. *Nat. Immunol.* 8:992–1000.

Prazma, C.M., N. Yazawa, Y. Fujimoto, M. Fujimoto, and T.F. Tedder. 2007. CD83 expression is a sensitive marker of activation required for B cell and CD4+ T cell longevity in vivo. *J. Immunol.* 179:4550–4562.

Qi,H., J.G. Egen, A.Y. Huang, and R.N. Germain. 2006. Extrafollicular activation of lymph node B cells by antigen-bearing dendritic cells. *Science.* 312:1672–1676.

Roozendaal, R., and M.C. Carroll. 2007. Complement receptors CD21 and CD35 in humoral immunity. *Immunol. Rev.* 219:157–166.

Roozendaal, R., T.R. Mempel, L.A. Pitcher, S.F. Gonzalez, A. Verschoor, R.E. Mebius, U.H. von Andrian, and M.C. Carroll. 2009. Conduits mediate transport of low-molecular-weight antigen to lymph node follicles. *Immunity.* 30:264–276.

Schwickert, T.A., R.L. Lindquist, G. Shakhar, G. Livshits, D. Skokos, M.H. Kosco-Vilbois, M.L. Dustin, and M.C. Nussenzweig. 2007. In vivo imaging of germinal centres reveals a dynamic open structure. *Nature.* 446:83–87.

Spaargaren, M., E.A. Beuling, M.L. Rurup, H.P. Meijer, M.D. Klok, S. Middendorp, R.W. Hendriks, and S.T. Pals. 2003. The B cell antigen receptor controls integrin activity through Btk and PLCγ2. *J. Exp. Med.* 198:1539–1550.

Szakal, A.K., M.H. Kosco, and J.G. Tew. 1988. A novel in vivo follicular dendritic cell-dependent iccosome-mediated mechanism for delivery of antigen to antigen-processing cells. *J. Immunol.* 140:341–353.

Szakal, A.K., M.H. Kosco, and J.G. Tew. 1989. Microanatomy of lymphoid tissue during humoral immune responses: structure function relationships. *Annu. Rev. Immunol.* 7:91–109.

Tew, J.G., and T. Mandel. 1978. The maintenance and regulation of serum antibody levels: evidence indicating a role for antigen retained in lymphoid follicles. *J. Immunol.* 120:1063–1069.

Tomura, M., N. Yoshida, J. Tanaka, S. Karasawa, Y. Miwa, A. Miyawaki, and O. Kanagawa. 2008. Monitoring cellular movement in vivo with photoconvertible fluorescence protein "Kaede" transgenic mice. *Proc. Natl. Acad. Sci. USA.* 105:10871–10876.

Williams, G.S., L.M. Collinson, J. Brzostek, P. Eissmann, C.R. Almeida, F.E. McCann, D. Burshtyn, and D.M. Davis. 2007. Membranous structures transfer cell surface proteins across NK cell immune synapses. *Traffic.* 8:1190–1204.

Wu,Y., S. Sukumar, M.E. El Shikh, A.M. Best, A.K. Szakal, and J.G. Tew. 2008. Immune complex-bearing follicular dendritic cells deliver a late antigenic signal that promotes somatic hypermutation. *J. Immunol.* 180:281–290.