Follicular Dendritic Cells Help Resting B Cells to Become Effective Antigen-presenting Cells: Induction of B7/BB1 and Upregulation of Major Histocompatibility Complex Class II Molecules

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Summary

This study was designed to investigate whether follicular dendritic cells (FDC) can activate B cells to a state in which they can function as effective antigen-presenting cells (APC). High buoyant density (i.e., resting) B cells specific for 2,4-dinitro-fluorobenzene (DNP) were incubated with DNP-ovalbumin (OVA) bearing FDC, after which their capacity to process and present to an OVA-specific T cell clone was assessed. The efficacies of alternative sources of antigen and activation signals in the induction of B cell APC function were compared with those provided by FDC. Only FDC and Sepharose beads coated with anti-immunoglobulin (Ig)κ monoclonal antibody provided the necessary stimulus. FDC carrying inappropriate antigens also induced B cell APC function in the presence of exogenous DNP-OVA. However, in circumstances where soluble DNP-OVA was limiting, FDC bearing complexes containing DNP, which could crosslink B cell Ig receptors, induced the most potent APC function. Analysis by flow cytometry revealed that within 24 h of coculture with FDC, a significant percentage of B cells increased in size and expressed higher levels of major histocompatibility complex class II. By 48 h, an upregulation of the costimulatory molecule, B7/BB1, occurred, but only when exposed to the FDC bearing DNP. Taken together, the results demonstrate that FDC have the capacity to activate resting B cells to a state in which they can function as APC for T cells. The stimuli that FDC provide may include: (*a*) an antigen-dependent signal that influences the upregulation of B7/BB1; and (*b*) possibly a signal independent of crosslinking mIg that results in Ig internalization. The relevance of these findings to the formation of germinal centers and maintenance of the humoral response is discussed.

Germinal centers are sites of antigen-driven B cell proliferation that can develop in two circumstances: an antigenic challenge (1) and depletion of circulating antibody levels (2). They arise within primary follicles, becoming a zone where B cells divide rapidly (3, 4), switch their Ig isotypes (5, 6), and undergo affinity maturation (7–9). The net result is the generation of an antigen-experienced, memory B cell population (10, 11) and the induction of antigen-specific plasma cells (2, 12, 13).

Formation of germinal centers is at least in part dependent upon the presence of immune complexes (10). These molecules are trapped and retained by follicular dendritic cells (FDC)[1] that reside in primary follicles and the light zone of germinal centers (14, 15). After an antigen insult, the set of events giving rise to a germinal center involves a deposition of antigen in the form of immune complexes on FDC, followed by contact, internalization, processing, and presentation of the immunogen by specific B cells to T helper cells in the area (16–18). At this point, the B cells begin to divide and go on to form the germinal center.

In addition to an antigenic challenge, germinal centers are also generated after a decrease in circulating Ab titers. Taking at least a third of a mouse's volume of blood induces germinal center formation in association with antigen-specific plasma cells (2). During this reaction, the B cell proliferation observed may aid in the maintenance of circulating memory B cell clones. Periodic contact with the antigen retained by FDC seems necessary for maintaining memory since transferring cells from an immune animal to a naive one in the

[1] *Abbreviations used in this paper:* DNP, 2,4-dinitro-fluorobenzene; FDC, follicular dendritic cells.

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absence of antigen leads to a loss of the memory population within 12 wk (19). However, providing antigen that becomes localized on FDC will maintain the memory response over much longer periods. Under physiological conditions when antibody levels fall, the exposure of antigenic epitopes on FDC and the subsequent B cell proliferation observed may represent the mechanism by which memory B cell clones are cyclically maintained (13, 20, 21).

In several experimental systems, resting B cells are poor at processing and presenting antigen to T cells (22–26). As this study and those of others have previously shown, a monovalent antigenic signal appears to be insufficient for B cell activation (27–30). Crosslinking membrane (m)Ig is required for such an effect (31). How then does such crosslinking occur in vivo; do specialized cells exist that "present" antigen to resting B cells, be they naive or memory B cells? FDC are obvious candidates for this function since their cardinal feature is to trap and retain unprocessed antigen for months on the extracellular surface of their plasma membrane (14, 20). While we have shown that antigen-specific B cells can obtain antigen from FDC and subsequently process and present the antigen (17, 18), it is still not known if this process can induce resting B cells to become competent antigen presenters. In this report, we demonstrate that FDC have the capacity to induce a variety of phenotypic changes in B cells that lead to this activation state. While the induction of some changes in B cell physiology appears to be antigen independent, the induction of optimal B cell APC function is generated after an antigen-specific interaction with FDC.

Materials and Methods

Animals. The Sp6 transgenic mouse line was generated by introducing a μ,κ construct from an anti-TNP hybridoma of BALB/c origin into the germline of an outbred mouse (32). The line was maintained on a BALB/c background by Dr. L. Forni at the Basel Institute for Immunology. Transgenic progeny were identified by their high titers of circulating anti-TNP antibodies as assessed by standard ELISA techniques. Mice were screened after 4 wk of age and used up to 6 mo old.

Female C57BL/6 mice were obtained from Iffa-Credo (L' Arbrasle, France) and used between the ages of 1 and 6 mo. C57BL/6 mice rendered IL-4 deficient by gene targeting were kindly provided by Drs. M. Kopf and G. LeGros (Max Planck Institute, Freiburg, Germany) (33).

Immunizations. For targeting of antigen to FDC in vivo, circulating specific antibody was initially generated by giving a primary immunization behind the neck with antigen precipated in aluminium sulfate containing 5×10^8 heat-killed *Bordetella pertussis* (20 μg/0.1 ml per mouse). 2–6 wk later, the mice received a second injection of the same antigen subcutaneously in each limb (10 μg/50 μl per limb), which localized on the FDC within 24 h in the form of immune complexes.

The antigens used included OVA (fraction V, A-5503; Sigma Chemical Co., St. Louis, MO), KLH (374817; Calbiochem-Behring Corp., La Jolla, CA), and 2,4-dinitro-fluorobenzene (DNP, D-1529; Sigma Chemical Co.). OVA or KLH were coupled to DNP as previously described (34, 35).

Preparing Isolated FDC. The low density, nonadherent cell fraction was obtained form the lymph nodes of immune C57BL/6 mice 2 d after whole body irradiation (600 rad, cesium source). The radiation treatment is an essential step in order to isolate FDC with far fewer contaminating lymphocytes. C57BL/6 mice were used as the source of FDC in order to avoid antigen presentation by cells in this population (which are H-2b) to the H-2d-restricted T cell clones. The association of B cells intertwined within the FDC's dendritic processes makes standard depletion techniques unsuccessful (18). Once excised from the mice, the collagen of the tissue was digested using an enzyme cocktail according to the method developed by Schnizlein et al. (36) with modifications reported elsewhere (35). Enrichment steps were done as specified by Schnizlein using continuous Percoll gradients and an adherence step. To deplete T cells, the preparations were then treated with anti-Thy-1 (T24)–, anti-CD4 (GK1.5)–, and anti-CD8 (53.6.7)–coated magnetic Dynabeads (Dynal A.S., Oslo, Norway) according to the manufacturer's specifications. These steps lead to a cell suspension containing between 25 and 40% FDC as quantitated using the rat anti–mouse FDC-specific mAb antibody, FDC-M1 (35), <1% CD4- or CD8-positive cells, and 50–60% Ig-positive cells (which would include the 25–40% immune complex retaining FDC and with the 20–25% remaining being B cells). The percentage of undefined cells upon culturing for several days have the morphological characteristics of fibroblasts. A small percentage of these are also macrophages, some of which contain tingible bodies (i.e., apoptotic lymphocytes). For the T cell proliferation assays, the isolated FDC preparations were irradiated with 1,000 rad before culturing. In all procedures and experiments, the complete medium used was IMDM containing 5% FCS and supplemented with 5×10^{-5} M mercaptoethanol plus penicillin/streptomycin.

Preparing the Non-FDC Population, Macrophages, and Dendritic Cells. The cells defined as non-FDC were generated using a protocol in which the tissue is mechanically disrupted instead of enzymatically digested. Due to their extensive network of dendritic processes, FDC do not survive this harsh break-up of the tissue (37). The resulting non-FDC-containing single-cell suspension was then treated in parallel with the cells obtained using the enzymes (i.e., percoll gradient sedimentation, 1-h adherence, T cell depletion, and irradiation).

The macrophage and dendritic cell populations were obtained from the cells left behind after washing of the tissue culture plate during the adherence step of the FDC isolation procedure. Gentle removal using a single sweeping stroke of a rubber policeman yielded >90% viable cells.

Preparing Resting and Activated B Cells. SP6 mice transgenic for μ,κ Ig isotype specific for TNP or DNP were used as the source of antigen-specific B cells (32). Single-cell suspensions were generated from their spleens by passing the tissue through a nylon mesh. After lysis of red blood cells, cells were fractionated into a resting or an activated population by buoyancy using discontinuous Percoll density gradients. The resting, high buoyant density B cells were isolated at the 1.079–1.085-g/cc interface and the activated, low buoyant density cells between 1.06 and 1.07 g/cc (38). The high density fraction was then depleted of T cells using a cocktail of Thy-1 (T24)–, CD4 (GK1.5)–, and CD8 (53.6.75)–coated magnetic Dynabeads. The resulting population as determined using a FACS® (Becton Dickinson & Co., Mountain View, CA) was <1% positive for CD4 or CD8 cells (mAbs L3T4 and Ly2, respectively; Caltag Laboratories, San Francisco, CA), and 90–95% positive for sIg ($F[ab']_2$ fragments of rat anti–mouse IgG, H and L chain specific; Jackson Immunoresearch Labs., Inc., West Grove, PA) and IE (mAb 14.4.4; American Type Culture Collection, Rockville, MD).

The activated, low density population was incubated for 1 h

at 37°C to remove adherent cells such as macrophages and dendritic cells. By this procedure, the cells were 85–90% positive for sIg and B220 (mAb 14.8; American Type Culture Collection) and 90–95% positive for IE. Although the low density fraction was highly enriched for activated B cells, a mixture of plasmablasts, plasma cells, and fibroblasts contributed to the remaining Ia-negative percentage of cells. The remaining Ia-positive, sIg/B220–negative fraction reflected a macrophage contribution as assessed by morphology and possibly dendritic cells. Since this population served only as a comparative positive control for APC function, care was not taken to remove every non-B cell. For the T cell proliferation assays (see below), the B cell populations were added to cell culture wells with the appropriate combinations of the other cell types or antigens, and then irradiated with 1,000 rad before adding the OVA-specific T cell (T-ova) clones.

Preparation of Immune Complex or Anti-κ-coated Beads. DNP immune mouse serum was incubated with sheep anti-mouse Ig-coupled magnetic Dynabeads, washed, and then incubated in either DNP-OVA or DNP-KLH (100 μg of antigen/ml of beads). This order of sequential formation of immune complexes on the beads was specifically conducted in order to: (*a*) avoid aggregate formation; and (*b*) render inaccessible the Fc portion of the antibody. The beads were added to the cultures at a ratio of four beads per cell.

The rat anti-mouse κ light chain mAb, 187.1, was covalently linked to Sepharose beads. They were then added to the cultures at a concentration of ~20 beads/3–4 × 10^5 cells per well.

A polyclonal mouse anti-OVA antiserum was mixed with DNP-OVA at a concentration that caused formation of precipitated immune complexes. A final concentration of 10 μg/ml of DNP-OVA was added to the cultures.

Antigen Presentation Assay Measured as an Index of T Cell Clone Proliferation. To assess the antigen presentation capacity of resting B cells, 3 × 10^5 high density B cells were cultured with 3 × 10^4 H-2^d-restricted, OVA-specific T cell clones (a gift from Dr. B. Stockinger, National Institute for Medical Research, London) in the presence or absence of a source of antigen and/or activation signals. Activated, low density cells (3 × 10^5/well) were used for comparison and positive controls. FDC, the adherent cell population, or the non-FDC containing population were added at a concentration of 5 × 10^4 cells/well. DNP-OVA, DNP-KLH, OVA, or KLH were added at a final concentration of 10 μg/ml (except in Fig. 1). As described above, all non-T cell groups were plated in round-bottomed 96-well plates (Costar, Cambridge, MA) in IMDM supplemented with 5% FCS, 5 × 10^{-5} M mercaptoethanol, and penicillin-streptomycin. Antigen, immune complexes, or coated beads were then added and the plates irradiated with 1,000 rad. The T-ova clones were then added and the plates placed at 37°C, 5% CO_2. After 48 h, 1 μCi of [^{3}H]thymidine was added to each well and incorporation by the T cell clones was assessed 18 h later in a liquid scintillation counter.

Analysis by FACS®. To compare forward and side scatter profiles and levels of expression for MHC class II molecules and the B cell activation antigen, B7/BB1 (39, 40), 3 × 10^5 resting B cells were incubated for 24 h with and without 5 × 10^4 FDC, 20–30 anti-κ beads, or 10 μg/ml DNP-OVA in U-bottomed wells. Class II was detected using the IE-specific mouse mAb, 14.4.4, which was biotinylated and then visualized using streptavidin-FITC (Southern Biotechnology Associates, Birmingham, AL). Surface IgM was detected using a goat anti-mouse IgM conjugated to PE (Southern Biotechnology Associates). T cell contamination was assessed using a FITC-conjugated anti-CD4 (L3T4; Caltag Labs., San Francisco, CA) and a PE-conjugated anti-CD8 (Ly2; Caltag Labs.). B7/BB1 expression was evaluated using the fusion protein made from the extracellular portion of the mouse CTLA-4 gene and the human IgG1 constant region. This molecule was generated in house by Dr. P. Lane and colleagues (Basel Institute for Immunology) and has been reported to act as a ligand for B7 (41). The binding of this protein to the cells was visualized using an $F(ab')_2$ FITC-conjugated mouse anti-human IgG (Jackson ImmunoResearch Labs., Inc.).

Results

Follicular Dendritic Cells Induce Resting B Cells to Become Efficient APC. The ability of resting B cells to process and present antigen to T-ova clones was compared with that of activated B cell–enriched populations. To increase the frequency of DNP-specific B cells, both high (resting) and low buoyant density (activated) B cells were isolated from spleens of BALB/c mice transgenic for a rearranged μ,κ encoding a specificity for DNP. As previously described (42), up to 50% of the B cells in these mice bind DNP-conjugated proteins. As illustrated in Table 1, DNP-specific resting B cells were not able to present soluble DNP-OVA to T-ova clones, while the activated B cells could.

To determine whether FDC bearing antigen–antibody complexes can induce APC function in resting B cells, FDC were isolated from mice that had been hyper-immunized with DNP-OVA. To eliminate the possibility of direct antigen presentation to the H-2^d-restricted T-ova clone, FDC were isolated from C57BL/6 mice (H-2^b). As shown in Table 1, DNP-OVA provided on FDC was acquired, processed, and presented by both the resting and activated DNP-specific B cells. No T cell activation was observed in the absence of B cells (T-ova clone and FDC control wells).

The possibility that other cell types in the FDC preparations contributed to this function was tested using cell suspensions devoid of FDC. First, macrophages and dendritic cells contained in the adherent population were collected or, alternatively, immune lymph nodes were mechanically disrupted using nylon mesh. This latter procedure destroys FDC due to the extensive association of their dendritic processes with the collagen matrix of the organ. As shown in Table 1, T cell proliferation induced by either of these non-FDC containing groups was no higher than background. In addition, adding soluble DNP-OVA to these cultures still did not rescue the APC function of the resting B cells, while the activated population efficiently used the exogenous antigen (Table 1). Taken together, these results confirm the interpretation that it is the FDC component of the population that provides the observed induction of APC function by the resting B cells.

To assess whether soluble mediators produced by cells in the FDC preparations were supporting B cell antigen presentation in this system, FDC isolated from C57BL/6 mice hyper-immunized with KLH were used. Even in the presence of exogenous KLH, the FDC_{KLH} population supported only 10% of the level of thymidine incorporation by T-ova clones as compared with that obtained in the presence of FDC from DNP-OVA immune mice (Table 1). Thus, the

Table 1. *Antigen Presentation Capacity of Resting and Activated B cells Stimulated by FDC vs. Non-FDC-containing Populations*

	Thymidine incorporation*			
	Resting B cells (high density)		Activated B cells (low density)‡	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
	cpm			
Medium alone	433§	152	467	161
+ DNP-OVA‖	365	122	645	171
+ T cells	453	212	1,036	909
+ T cells + DNP-OVA	544	889	30,827	48,192
+ T cells + $FDC_{D\text{-}O}$¶	36,049	28,905	24,130	32,948
+ T cells + $M\emptyset/DC_{D\text{-}O}$¶	522	1,605	4,059	3,729
+ T cells + $M\emptyset/DC_{D\text{-}O}$ + DNP-OVA	–	6,157	–	52,711
+ T cells + non-$FDC_{D\text{-}O}$**	397	–	591	–
+ T cells + non-$FDC_{D\text{-}O}$ + DNP-OVA	1,335	–	16,410	–
+ T cells + FDC_{KLH}‡‡	–	3,271	–	5,480
+ T cells + FDC_{KLH} + KLH	–	3,622	–	5,723
+ $FDC_{D\text{-}O}$	758	1,521	729	1,995
+ FDC_{KLH}	587	143	300	167
Additional controls:				
T cells + $FDC_{D\text{-}O}$	1,066	1,348		
T cells + MØ/DC		152		

* B cells, FDCs, MØ/DC, and non-FDC populations were irradiated with 1,000 rad; B cells were added at 3×10^5/well, T cells at 3×10^4/well, and all others at 5×10^4/well; [^{3}H]thymidine was added for the last 24 h of a 76-h culture period.

‡ The activated B cell preparation contained up to 90% $B220^+$ cells and <5% MØ and DC.

§ All values represent the average of triplicate wells; all standard deviations for these values fall within 10% of the mean.

‖ DNP-OVA: 10 μg/ml of exogenous antigen was added per well.

¶ FDC and MØ/DC were obtained from mice primed and boosted with DNP-OVA; the B cells, T cells, and MØ/DC were then incubated with or without exogenous DNP-OVA (10 μg/ml).

** Non-FDCs were from preparations in which the FDCs were removed. For this, spleens from immune C57BL/6 mice were mechanically disrupted instead of being enzymatically digested. The resulting non-FDC-containing single-cell suspension was then treated in parallel with the cells obtained using the enzymes (i.e., Percoll gradient sedimentation, 1-h adherence, T cell depletion, and irradiation with 1,000 rad).

‡‡ FDC were obtained from mice primed and boosted with KLH; the B cells, T cells, and FDCs were then incubated with or without exogenous KLH (10 μg/ml).

T cell proliferation observed in these cultures cannot be attributed to nonspecific soluble mediators derived from cells in the FDC preparations.

Mimicking the Effects of FDC Retained Antigen. The next series of experiments were designed to characterize the FDC-derived signal(s) that induce B cell APC function. FDC retain antigen on the extracellular face of their plasma membrane in the form of immune complexes in a spatially ordered arrangement. Thus, by presenting a solid matrix of antigen, one of the essential signals provided by FDC may be the efficient crosslinking of the B cell's antigen receptors. Whether or not such crosslinking was sufficient to induce B cell APC function was tested by adding beads coated noncovalently with immune complexes to cultures containing resting B cells and T-ova clones. As shown in Table 2, the immune complexes coated to beads did not support the induction of resting B cell APC function, even in the presence of exogenous DNP-OVA. However, the activated DNP-specific B cells were able to acquire, process, and present DNP-OVA provided on the beads (Table 2). In addition, attempts to induce APC function in resting B cells using DNP conjugated to irrelevant antigen (KLH), in the presence of exogenous DNP-OVA, were without success (Table 2). Precoating culture wells with DNP-OVA was similarly unsuccessful, and incubating the immune complex–coated Dynabeads with the FDC and resting B cells did not inhibit their activation (not shown). Crosslinking of mIg using anti-κ-coated beads efficiently induced the APC function of resting B cells in the presence of exogenous DNP-OVA (Table 2). In this respect, anti-κ-coated beads were as efficient as FDC obtained from

Table 2. *Antigen Presentation Capacity of Resting and Activated B cells Using Various Sources of Antigen*

	Thymidine incorporation*			
	Resting B cells (high density)		Activated B cells‡ (low density)	
	Soluble DNA-OVA§		Soluble DNP-OVA‡	
	−	+	−	+
	cpm			
Antigen on beads				
+ DNP-OVA beads¶	765‖	693	31,633	51,376
+ DNP-KLH-beads¶	589	478	2,609	58,975
+ anti-Ig-beads**	501	16,477	5,847	61,535
Soluble antigens				
+ DNP-OVA§	–	1,393	–	78,666
+ D-O-anti-D-O complexes‡‡	–	3,177	–	51,824
FDC-associated antigen				
+ $FDC_{D\text{-}O}$§§	16,164	–	18,427	–
Controls:				
B cells alone	211	472	269	341
+ T cells	743	798	1,891	78,041
+ $FDC_{D\text{-}O}$	920	–	889	–
Additional control:				
T cells + $FDC_{D\text{-}O}$	1,217			

* B cells and FDCs were irradiated with 1,000 rad before culture; [^{3}H]thymidine was added for the last 24 h of a 76-h culture period.
‡ The activated B cell preparation contained up to 90% B220$^+$ cells and <5% MØ and DC.
§ D-O, DNP-OVA: 10 μg/ml of exogenous antigen were added per well.
‖ All values represent the average of triplicate wells; all standard deviations for these values fall within 10% of the mean.
¶ DNP immune mouse serum was incubated with sheep anti-mouse Ig coupled magnetic Dynabeads, washed, and then incubated in either DNP-OVA or DNP-KLH (100 μg of antigen/ml of beads). The beads were added to the cultures at a ratio of four beads per cell; the presence of DNP-KLH on the beads was confirmed by using them in antigen proliferation assay using the KLH-specific hybrid, 10H/A.
** The rat anti-mouse κ light chain mAb, 187.1, was covalently linked to Sepharose beads, then added to the cultures at a concentration of ~20 beads/3 × 10^5 cells.
‡‡ A polyclonal mouse anti-OVA was mixed with DNP-OVA in order to form immune complexes. A final concentration of 10 μg of DNP-OVA/ml was added to the cultures.
§§ FDC were isolated from DNP-OVA immune C57BL/6 mice.

DNP-OVA immune mice. These results demonstrate that a mere solid phase of antigen is not enough to induce B cell activation. The success of the FDC-associated antigen may be due to two not mutually exclusive mechanisms: the arrangement of the repeating immune complex units on FDC and other cell surface molecules expressed by FDC.

FDC Provide Signal(s) Independent of mIg Crosslinking. To address the above possibilities, experiments were designed in order to distinguish the antigen-specific from the antigen-independent contribution of FDC. For this, FDC were isolated from mice hyper-immune to either haptenated (DNP-OVA, DNP-KLH) or nonhaptenated (OVA, KLH) antigens, and evaluated for their ability to induce the APC function of resting B cells. As shown in Table 3, in the absence of exogenous antigen, FDC from DNP-OVA immune mice were the only group able to induce the APC function of the resting B cells. However, in the presence of 10 μg/ml of exogenous DNP-OVA, all FDC populations supported this activity (Table 3). It is unlikely that non–antigen-specific B cell–FDC interaction induced a general, mIg-independent, increase in pinocytosis, since exogenous OVA was not efficiently acquired and presented by resting B cells in these cultures (Table 3). In contrast, the activated B cells presented the exogenous OVA as well as the OVA and DNP-OVA retained by the FDC populations (Table 3). These results demonstrate that FDC can provide resting B cells with signal(s) that are independent of crosslinking mIg. However, it is not clear whether these signals are temporally or physically dissociated from those provided as a consequence of mIg crosslinking by the FDC in physiological circumstances.

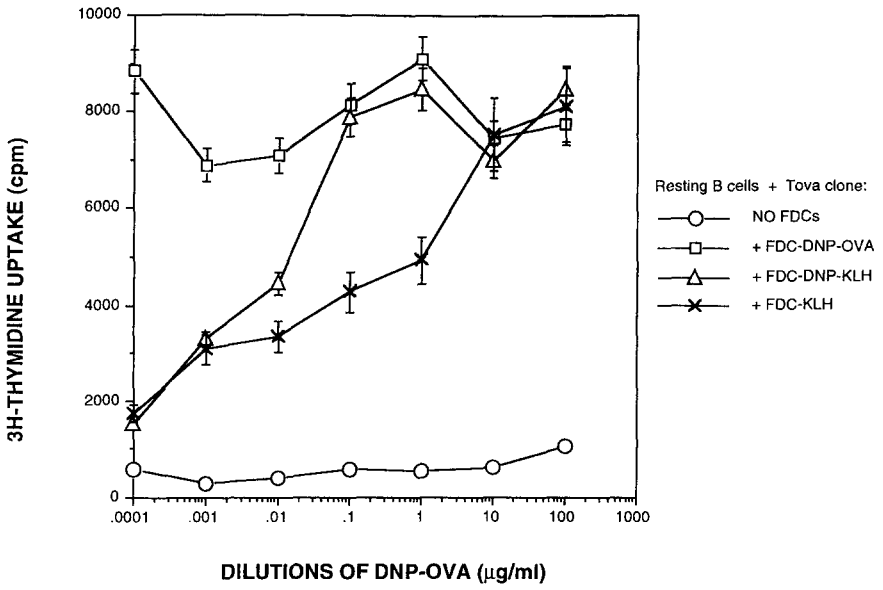


Figure 1. Limiting the amount of antigen demonstrates a difference between the FDC populations. Resting B cells were assessed for their ability to stimulate the T-ova clone in the presence or absence of FDC and 10-fold dilutions of exogenous DNP-OVA. The resting B cells and T-ova clone were cultured with antigen alone (O) or with one of three FDC populations: (□), FDC from DNP-OVA immune mice; (Δ), FDC from DNP-KLH immune mice; (X), FDC from KLH immune mice. 1 μCi of [^{3}H]thymidine was added per well at 48 h of a 66-h culture period. The resting B cells and various FDC populations were exposed to 1,000 rad before the addition of the T-ova clone.

This question was addressed in the next series of experiments. The relative efficiencies of FDC obtained from animals hyper-immunized with various antigens were compared when exogenous DNP-OVA was limiting. FDC bearing DNP-OVA, DNP-KLH, or KLH were cocultured with the resting B cells and T-ova clones in the presence of varying concentrations of DNP-OVA. At high concentrations of exogenous DNP-OVA (10–100 μg/ml), all of the FDC groups supported comparable responses (Fig. 1). However, when the concentration of antigen became limiting, only the B cells interacting with FDC bearing DNP continued to function as APC for T cell activation (Fig. 1).

Taken together, these results indicate that FDC may provide resting B cells with at least two signals. At low concentrations of exogenous DNP-OVA, or in more physiological circumstances where the only source of antigen is provided

Table 3. *Antigen Presentation Capacity of Resting B cells after Stimulation by FDC from Either DNP-OVA, DNP-KLH, OVA, or KLH-immune Mice*

Resting B cells (high density)	Thymidine incorporation*				
	Medium	DNP-OVA‡	DNP-KLH‡	OVA‡	KLH‡
			cpm		
+ T cells +	518§	1,820	692	527	544
+ T cells + FDC-DNP-OVA +	14,647	38,206	19,802	15,525	16,041
+ T cells + FDC-DNP-KLH +	1,640	30,710	1,433	1,913	2,033
+ T cells + FDC-OVA +	2,854	34,227	2,482	1,986	2,979
+ T cells + FDC-KLH +	2,401	29,460	2,145	2,319	3,263
	DNP-OVA	FDC-DNP-OVA	FDC-DNP-KLH	FDC-OVA	FDC-KLH
B cells +	176	945	1,507	993	908
T cells +	175	456	563	404	414
Activated B cells + T cells +	47,696‖	11,390	966	12,964	1,346

FDC were isolated from either DNP-OVA, DNP-KLH, OVA, or KLH-immune C57BL/6 mice.
* B cells and FDCs were irradiated with 1,000 rad before culture. [^{3}H]Thymidine was added for the last 24 h of a 76-h culture period.
‡ DNP-OVA, DNP-KLH, OVA, or KLH were added at a concentration of 10 μg/well.
§ All values represent the average of triplicate wells; all standard deviations for these values fall within 10% of the mean.
‖ Activated B cells + T cells: + OVA, 13,900 cpm; + DNP-KLH, 840 cpm.

in the form of immune complexes on FDC, the two signals are not dissociable. They are comprised of antigen-specific focusing of the FDC–B cell interaction, which in turn results in some signal "X" and the concomitant crosslinking of mIg. Signal X is invoked because resting B cells do not present DNP-OVA in the absence of an interaction with FDC, even when the antigen is added at high concentrations (Table 1). And it is then revealed in circumstances of FDC–B cell interaction that do not involve mIg, when the concentration of exogenous DNP-OVA is high (Table 3). While the nature of signal X remains to be characterized, it likely involves the facilitation of mIg-mediated endocytosis.

T Cells Need Not Interact with FDC. To assess whether the observed T cell proliferation was dependent upon T cell contact with the FDC, B cells and FDC were cocultured for 24 h and then the FDC removed. This was accomplished by vigorously pipetting the FDC–B cell clusters, followed by removal of FDC using the FDC-specific mAb, FDC-M1, bound to Dynabeads. As shown in Table 4, adding the T-ova clones 24 h after initiation of the FDC–B cell cocultures resulted in levels of thymidine incorporation comparable to those observed when all three components are added at the onset of culture. Vigorous pipetting of cultures without removing FDC resulted in slightly lower levels of T cell activation, possibly due to cell damage incurred by this procedure. Removal of FDC subsequent to pipetting further diminished T cell activation, although, as shown in Table 4, the FDC-depleted population still produced a strong antigen-specific T cell response. Thus, once the B cells have interacted with FDC, the latter are not required for efficient T cell–B cell interaction.

Phenotypic Characterization of B Cells after Interaction with FDC. In addition to the striking functional consequences of FDC–B cell interactions, it was of interest to determine whether associated changes in the B cell phenotype were induced. Toward this end, cell size, as well as the level of membrane expression of both MHC class II and B7/BB1, were assessed by flow cytometry. Expression of MHC class II antigens by cells in the FDC preparations was excluded since these cells were obtained from the I-E-negative strain, C57BL/6, and the analyses were done by gating on I-E-positive cells. Anti-κ beads were used as a positive control for B cell stimulation.

As illustrated in Fig. 2 *A*, an arbitrary gate, R2, was set to measure increases in the forward and side light scatter profiles, taken to represent B cell activation. That this is a fair representation of the proportion of blasts in the population is supported by the fact that the percentage of cells that fall into this gate increases from 5.9% with resting B cells to 33.8% upon anti-κ bead stimulation (Fig. 2 *D*). The scatter profile of resting B cells cocultured with FDC isolated from mice hyper-immune with DNP-OVA indicates that the efficiency of antigen-mediated FDC–B cell interaction in the induction of B cell blastogenesis approaches that of anti-κ beads (Fig. 2 *E*). Consistent with our other observations of interactions not involving mIg, coculture of B cells and FDC from OVA-primed animals resulted in a lower but significant induction of stimulation (Fig. 2 *B*).

Table 4. *Effect of Preincubation of Resting and Activated B Cells with FDCs on Antigen Presentation Capacity*

	Thymidine incorporation*	
	Resting B cells (high density)	Activated B cells‡ (low density)
	cpm	
Medium alone	746§	632
+ T cells	843	1,012
+ T cells + DNP-OVA	769	79,513
+ T cells + FDC-$_{D\text{-}O}$‖	63,224	66,576
+ FDC-$_{D\text{-}O}$‖	1,595	2,174
Pre-incubation:		
+ FDC-$_{D\text{-}O}$¶/ + T cells	59,074	61,033
+ FDC-$_{D\text{-}O}$**/ + T cells	48,294	59,546
+ FDC-$_{D\text{-}O}$‡‡/ + T cells	22,617	40,298
Additional control		
FDC-$_{D\text{-}O}$ + T cells	883	

* B cells and FDCs were irradiated with 1,000 rad before culture.
‡ The activated B cell preparation contained up to 90% B220$^+$ cells and <5% MØ and DC.
§ All values represent the average of triplicate wells; all standard deviations for these values fall within 10% of the mean.
‖ FDCs were isolated from DNP-OVA-immune C57BL/6 mice.
¶ B cells and FDCs were incubated together for 24 h and then the T cell clones added.
** B cells and FDCs were incubated together for 24 h and then vigorously pipeted to disrupt the cellular clusters. T cells were then added to three of these wells.
‡‡ Alternatively, after disruption of the FDC/B cell clusters, magnetic Dynabeads coated with the anti-FDC mAb, FDC-M1, were added to the wells in order to deplete the FDCs. After a 30-min incubation, the non-Dynabead-attached cells were removed and added to wells containing T cell clones.

Similar results were obtained when the induction of MHC class II expression was assessed. FDC isolated from animals immunized with either OVA or DNP-OVA supported increased MHC class II expression, with the FDC-DNP-OVA doing so more effectively (Fig. 3). Although no CD4$^+$ or CD8$^+$ T cells were detectable in these cultures (Fig. 2, *G* and *H*), the possibility that IL-4 was contributing to either blastogenesis or increased MHC class II expression could not be formally excluded. However, when these experiments were repeated using FDC-OVA or FDC-DNP-OVA populations obtained from IL-4-deficient mice, identical results were obtained (Figs. 2, *C* and *F*, and 3).

B7/BB1 is a costimulatory molecule that is important for APC function, and its expression is upregulated after B cell activation (39). We assessed the membrane expression of B7/BB1 using a chimeric form of its ligand: a fusion protein of CTLA-4 and the constant region of human IgG1. Resting B cells cocultured for only 24 h with either FDC or anti-κ beads did not detectably bind the CTLA-IgG1 construct (not

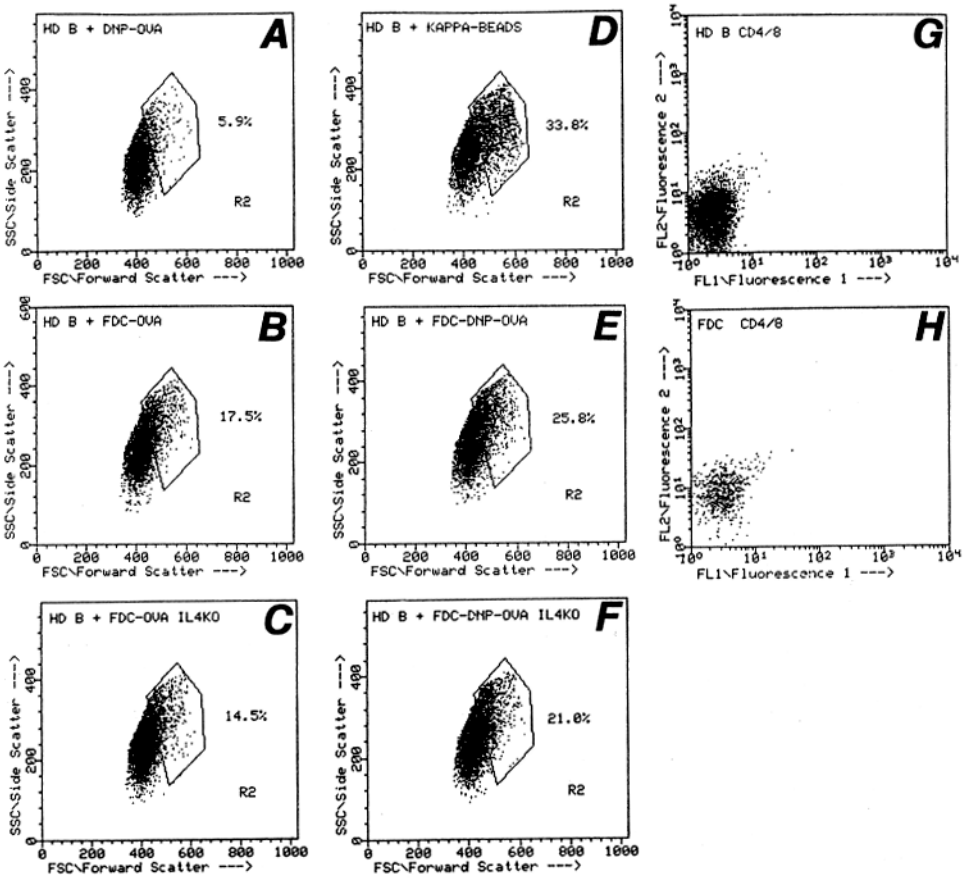


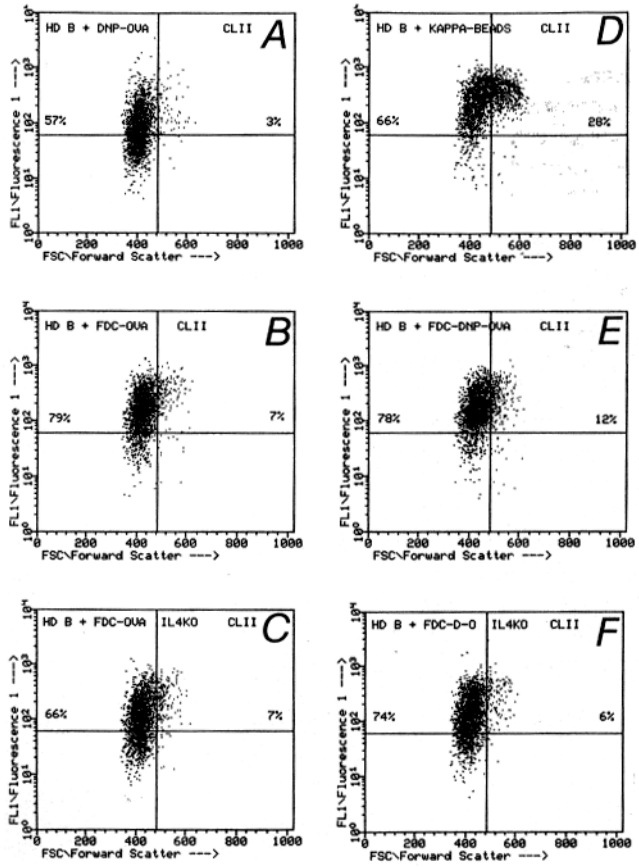
Figure 2. Analysis of resting B cell blastogenesis. *A–F* represent forward vs. side scatter profiles of the resting B cells in the presence or absence of antigen (DNP-OVA), follicular dendritic cells (FDC), or anti-κ Ig-coated Sepharose beads (κ-beads). To analyze only the B cell population, the cells were labeled for MHC class II using the mAb 14.4.4, which recognizes the IE expressed by the B cells (isolated from BALB/c) and is not expressed by the FDC population (isolated from C57BL/6). As such, a gate (R1) was set to analyze cells that were medium to highly bright in their expression of IE. A second gate (R2) was drawn on the forward and side scatter profiles in order to quantitate the number of cells that fall into the larger, more activated phenotype. (*A*) HD B cells cultured with 10 μg/ml DNP-OVA; (*B*) HD B cells cultured with FDC from OVA-immune C57BL/6 mice; (*C*) HD B cells cultured with FDC from OVA-immune, IL-4-deficient transgenic C57BL/6 mice (IL4KO); (*D*) HD B cells cultured with anti-κ Ig-coated Sepharose beads; (*E*) HD B cells cultured with FDC from DNP-OVA-immune C57BL/6 mice; (*F*) HD B cells cultured with FDC from DNP-OVA-immune, IL-4-deficient transgenic C57BL/6 mice (IL4KO). *G* and *H* demonstrate the lack of CD4- or CD8-positive T cells in the HD B cell or FDC preparations, respectively.

shown). However, within 48 h, expression of B7 was induced by either FDC-DNP-OVA or anti-κ beads (Fig. 4). Coculture with FDC-OVA induced only a marginal upregulation of B7 expression, while culture with soluble DNP-OVA had no detectable effect (Fig. 4). The induction of B7 by FDC-DNP-OVA correlates well with the ability of these FDC to induce B cell APC function. Further, the marginal effects of coculture with FDC-OVA on B7 expression support the conclusion that while non–antigen-specific FDC–B cell interaction potentiates some APC functions in resting B cells, the involvement of mIg is clearly providing essential signals.

Discussion

In many experimental systems, resting B cells are relatively poor APC (22–26). They can, however, present when provided with antigens that crosslink their mIg receptors (31). Furthermore, stimulation of resting B cells with nonmitogenic anti-Ig antibodies moves them into a state in which they can very effectively process and present soluble antigens (43). How then do B cells present antigen in vivo? Thymus-dependent antigens usually enter the lymph or bloodstream as monomeric molecules, but may become complexed with preexisting, low affinity, crossreactive serum antibodies. These complexes could then be rapidly localized onto the specialized accessory cells of B cell follicles, namely FDC. Previous studies (44) have shown that the spacing of repeating antigenic epitopes on the surface of FDC are within the optimal range required for stimulation of B cells (45). These characteristics, together with their proven ability to support B cell proliferation (35, 46–49), make the FDC a prime candidate for the cell that primes resting B cells for interaction with T cells.

The results presented in this study demonstrate that FDC stimulate resting B cells in a manner that leads to their effective presentation of antigen to T cells. As a consequence of their interaction with FDC, resting B cells undergo phenotypic changes that may explain their newly acquired capacity. These changes involve alterations in cell size, an increase in MHC class II expression, and the de novo expression of B7/BB1, a molecule shown to provide a prerequisite costimulatory signal to T cells (39, 40). While there is preliminary evidence that crosslinking of mIg by antibodies induces an increased rate of mIg internalization (B. Stockinger, personal



communication), we have not determined if the interaction with FDC has this effect. But it is evident from these studies that crosslinking sIg does not account for all of the observed effects. Although the induction of B7 appears to require the signaling through mIg, both changes in MHC class II and the acquisition of the capacity to present soluble, specific antigen added to the cultures occur in the presence of FDC carrying antigens for which the B cells are not specific.

It is of obvious importance then to define the character of the FDC-derived signals. Upregulation of MHC class II expression is known to occur after stimulation with anti-Ig antibodies (28) with an augmentation in the presence of IL-4 (50, 51). However, as we have discussed, antigen-specific receptor crosslinking does not fully explain the phenomena in our system, and an obligatory role for IL-4 was ruled out using IL-4-deficient mice. Signaling via MHC class II molecules themselves seems unlikely since FDC do not express CD4 (15). This mechanism cannot be entirely ruled out since other molecules such as CD23 expressed by FDC (52) may mediate the signal.

Figure 3. MHC class II level of expression vs. forward scatter profiles. Resting B cells were cocultured for 24 h with the same populations described in the legend to Fig. 2. Percentage of positive cells falling in the top two quadrants are shown. The mAb 14.4.4, which recognizes only IE molecules, was used in order to evaluate class II expression of the B cells (obtained from a BALB/c background). Since the FDC population was obtained from the IE-negative C57BL/6 mouse strain, their contribution to the analysis was avoided.

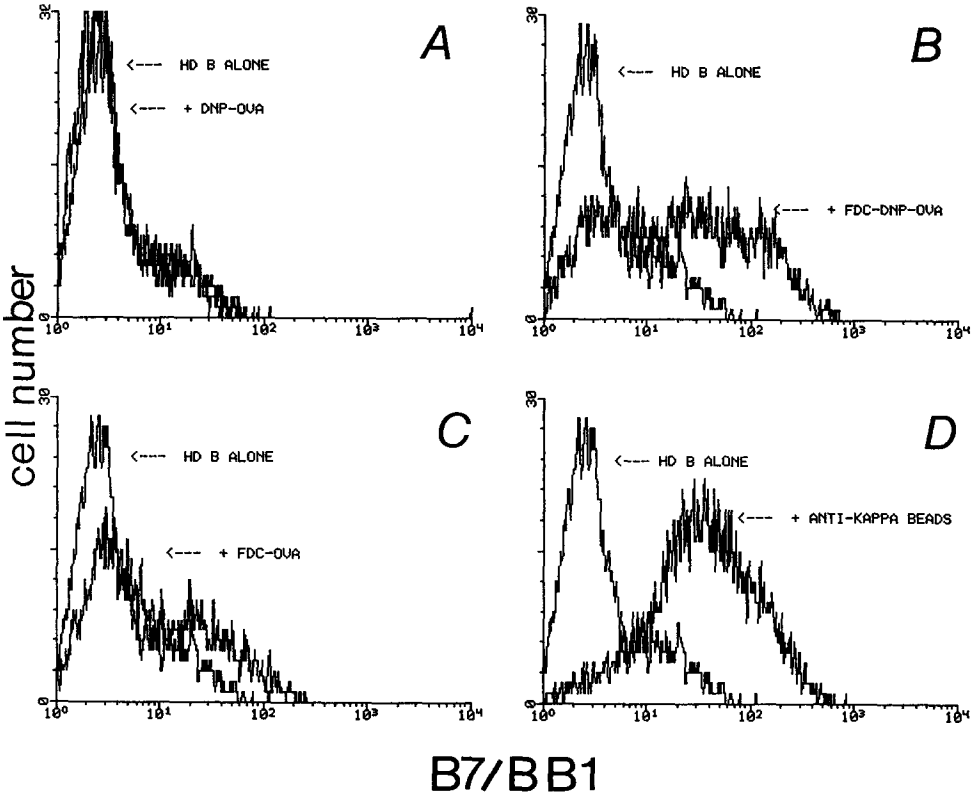


Figure 4. De novo expression of B7/BB1. The level of B7/BB1 expression on B cells after 48 h of culture. Resting B cells were cocultured in the presence or absence of DNP-OVA (10 μg/ml; *A*), FDC from mice immune to DNP-OVA (*B*) or OVA (*C*), or anti-κ beads (*D*). The level of B7/BB1 was assessed using the chimeric fusion protein made from the extracellular portion on the mouse CTLA-4 gene and the constant region of the human IgG1 molecule.

The most enticing idea involves the antigen-independent effects of the immune complexes attached to FDC. In addition to antigen and antibody, various complement components are involved that anchor the complexes into the FDC's plasma membrane via CR1/CD35 and CR2/CD21 molecules (15). CD21 (CR2), which binds C3dg, is also expressed by B cells in a trimolecular complex together with CD19 and a molecule known as TAPA-1 (53, 54). Signaling via CD21 was implicated some time ago in the progression of B cells through cell cycle (55, 56) while more recently, others have demonstrated that prior ligation of CD21 on B cells with polymeric C3dg augmented the subsequent anti-Ig-mediated proliferation (57). The effect of ligation of CD21 with membrane-bound CD23, one of its several natural ligands (58), to our knowledge, has not yet been tested. Carter and Fearon (59) more recently have extended their studies on the function of the CD19-CD21-TAPA-1 complex. They showed that antibody ligation of CD19 at the same time as IgM reduces the threshold concentration of anti-IgM required to induce B cell proliferation by 100-fold. Since the natural ligand for CD19 has not been determined to date, it is not implausible that it could be expressed by FDC. In addition to these molecules, data are emerging concerning the costimulatory role of antibodies to CD38 on mouse germinal center B cell proliferation (M. Kosco-Vilbois and M. Parkhouse, unpublished observations).

The scheme that we would propose is that FDC "prime" resting B cells for the subsequent antigen-specific, cognate interaction with a T cell. The restricting event would therefore be between these two cells, requiring that the B cell not only express the appropriate peptide/MHC complex, satisfying the T cell specificity, but in addition that it functions as an APC. One might argue that FDC-primed B cells may be able to take advantage of T cell factors through a bystander mechanism. However, the monoclonal nature of each germinal center (4), and our previous data showing that lymphokines will not replace the T cell contact required to mimic germinal center–like proliferation in vitro (35), argue against nonspecific B cell proliferation and differentiation in this microenvironment.

There are three stages of the immune response that should be considered in the context of this study in assessing the physiological relevance of our findings: (*a*) primary activation of virgin B cells; (*b*) activation of memory B cell precursors in germinal centers; and (*c*) persistent stimulation of memory B cells. The high density, resting B cells used in this study are likely to contain at least two of these populations: the virgin B cells and memory B cells. Several studies suggest that in vivo the primary activation of virgin B cells occurs outside the follicles, i.e., virgin B cells do not gain access to FDC. This is based upon the identification of the sites of primary B cell proliferation (60, 61) and analysis of the activation of virgin B cells after restriction of antigen to FDC (62–64). Neither of these observations rules out that a small number of virgin B cell reach the locale of the FDC to be "primed" for T cell interaction. Indeed, these may be the B cells that give rise to the germinal center. While Linton et al. (65) have called such memory precursors a separate lineage, it may be a stochastic process that leads to the proliferation of a subset of B cells in germinal centers. When forced to interact with FDC in vitro, it may be that all B cells can enter this pathway. It is difficult to argue that contact with FDC is important for the primary activation of all virgin B cells, but we would propose that "FDC priming" is a prelude to differentiation along the memory cell pathway, including isotype switching and somatic hypermutation. It is an open question whether recirculating memory B cells ever return to a small, dense resting state. However, if they do they would have access to FDC. It has already become clear that interaction with antigen-coated FDC is required for continued memory B cell survival (19, 66).

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