Adhesion of CD40-stimulated Germinal Center B Cells to HK Cells Employs the CD11a/CD18-CD54 Interactions

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ABSTRACT

Background: The molecular basis of follicular dendritic cells (FDC)-germinal center (GC) B cell interaction is largely unknown, although this cellular interaction is thought to be important for the whole process of GC B cell differentiation. **Methods:** Using FDC-like cells, HK, and highly purified GC B cells, we attempted to identify the molecules that play critical roles in the interactions between FDC and B cells. GC B cells were co-cultured with HK cells and soluble CD154 in the presence or absence of various function-blocking monoclonal antibodies to examine their effect on GC B cell binding to HK cells and B cell proliferation. **Results:** Anti-CD11a and anti-CD54 antibodies inhibited GC B cell binding to HK cells while anti-CD49d and anti-CD106 antibodies did not. GC B cell proliferation was not impaired by the disruption of GC B cell-HK cell adherence. **Conclusion:** Our results suggest that CD11a/CD18-CD54 interactions play an important roles in the initial binding of GC B cells to FDC and diffusible growth factors from FDC may be responsible the massive proliferation of GC B cells. **(Immune Network 2003;3(3):176-181)**

Key Words: Follicular dendritic cell, germinal center B cell, CD40, CD11a/CD18-CD54

Introduction

T cell-dependent humoral immune responses culminate in the germinal center (GC) of the secondary lymphoid organs (1). The GC is a prominent histologic area in tonsils comprised mostly of proliferating B cells with follicular dendritic cells (FDC), tingible body macrophages and a small number of T cells (2). Antigen-specific resting B cells are activated in the T cell-rich areas outside of GC of secondary lymphoid organs (3) to give rise to IgM-secreting plasma cells and B cell blasts that colonize the primary follicles (4). The subsequent GC reaction is initiated by the rapid proliferation of a few B blasts in association with FDC (1), where B cells follow the differentiation pathway from centroblasts to centrocytes, and then to either plasma cells or memory B cells (5).

FDC are major microenvironmental components of secondary lymphoid follicles, and are known to play critical roles in the survival, proliferation, selection, and differentiation of GC B cells (1,6,7). FDC display distinct morphological characteristics and retain unprocessed antigens in the form of immune complexes for a long time (8). We reported previously upon the establishment of FDC-like cells, HK, from human tonsils to overcome the practical difficulty in isolating pure FDC and to mimic the GC reaction *in vitro* (9). HK cells indeed have the functional features of FDC by delaying apoptosis, and by stimulating growth and differentiation of GC B cells. HK cells bind and prevent the apoptosis of GC B cells preferentially and have co-stimulatory effects on the proliferation of CD40-stimulated GC B cells (10, 11).

Interactions of FDC with GC B cells are thought to be important for each step of B cell differentiation. However, the molecular mechanism of FDC-B cell interactions is largely unknown. Since GC B cells undergo rapid apoptosis without proper survival stimuli and the optimal cluster formation and proliferation of B cells require the three major cellular components of GC, i.e., FDC, T, and B cells (12), we investigated interaction of soluble CD40-stimulated GC B cells with HK cells to identify the molecular mechanism of FDC-B cell interactions.

Materials and Methods

Cytokines and reagents used in this study. The culture medium was RPMI-1640 (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% fetal calf serum (Life Technologies, Grand Island, NY, USA), 2 mM glutamine, 100 U/ml Penicillin G, and 100μg/ml Streptomycin (Irvine Scientific). Soluble human trimeric sCD154 was obtained from Dr. Choi (Ochsner Medical Foundation, New Orleans, LA, USA) and used at 400 ng/ml that was determined to give optimal proliferation of GC B cells. Percoll and Ficoll were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Function-blocking anti-CD11a (clone R3.1) and anti-CD54 (clone RR1/1) antibodies were generous gifts from Dr. Choi. Anti-CD49d (clone HP2/1) and anti-CD106 (clone 1G11) were purchased from Immunotech (Westbrook, ME, USA). Isotype-matched control antibody was obtained from Sigma Chemical Co. (St. Louis, MO, USA). IL-2 was obtained from Dr. Choi Recombinant human IL-4 was prepared in our laboratory by using COS expression system. IL-10 was purchased from R&D Systems (Minneapolis, MN, USA).

Preparation of HK cells and B cells. HK cells were established as described previously (9). Tonsillar B cells were prepared as described previously (11). Tonsillar mononuclear cells were subjected to two rounds of depletion of T cells by rosetting with SRBC; the resulting cells contained more than 95% $CD19^+$ B cells as analyzed by FACScan (Becton Dickinson, Sunnyvale, CA). B cells were further separated according to their density using a discontinuous gradient of Percoll consisting of four layers 80%, 60%, 50%, and 35% Percoll solutions in a 15 ml conical tube. B cells (1×10^8) were laid at the bottom of the gradient and centrifuged for 10 min at 2000 x g at 20°C. B cells recovered at $60 \sim$ 80% Percoll interface were referred to as high-density B cells and those recovered at $35 \sim 50\%$ as low-density B cells. GC B cells were obtained from low density B cells by depleting IgD^+ and $CD44^+$ cells as described before (13). The purity of GC B cells was more than 98% by estimating $IgD-CD38^+$ cells in FACS. All the purification steps were performed at 4°C in order to prevent death of GC B cells, except centrifugation of cells using Ficoll or Percoll, which was carried out at room temperature.

Culture of GC B cells. For the culture of GC B cells, HK cells (2×10^4 cells/well) irradiated with 3000 rad were prepared 1 day before the addition of B cells in 24 multi-well plate. GC B cells were cultured under the various conditions as described in the figure legends. Fresh culture media were added on the third day in culture. IL-2 (20 U/ml), IL-4 (100 U/ml), IL-10 (50 ng/ml) and sCD154 were added after determining the optimal concentrations by the ability to induce proliferation of GC B cells.

Determination of the bound and unbound cells. Cultured B cells were very gently washed twice with HBSS to collect unbound cells. Bound B cells were procured by vigorously pipetting the remaining HK cells and B cells. Viable B cells were enumerated by trypan blue dye exclusion (13). Large HK cells were easily excluded from smaller B cells during counting.

Statistical analysis. Statistical analysis and graphic presentation employed the GraphPad Prism 4.0 package (GraphPad, San Diego, CA, USA). Statistical significance of differences was determined by Student's *t* test; $P < 0.05$ was considered significant.

Results

Adhesion of GC B cells to HK cells depends on the essential T cell signal molecule, CD154. HK cells that are derived from human tonsils exhibit functional as well as phenotypic properties of FDC and have been utilized as an FDC model for some time (13-17). We examined adhesion of GC B cells to HK cells in order to understand the molecular mechanism and functional consequences of B cell binding to FDC in the GC. Highly purified GC B cells were cultured with HK cells in the presence or absence of soluble CD154 (sCD154, CD40 ligand). CD154 is an essential survival factor for GC B cells (18). As shown in
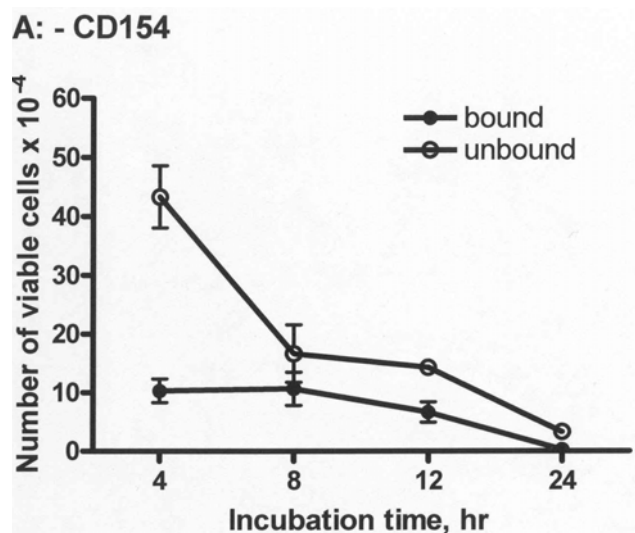

Figure 1. GC B cells require stimulation via CD40 to adhere to HK cells. GC B cells (1×10^6 cells) were co-cultured with HK cells in the absence (A) or presence (B) of sCD154. At the indicated time points, bound cells and unbound cells were harvested for viable counting. Results are presented as the mean and standard error of triplicates. Representative results out of three reproducible experiments are shown.
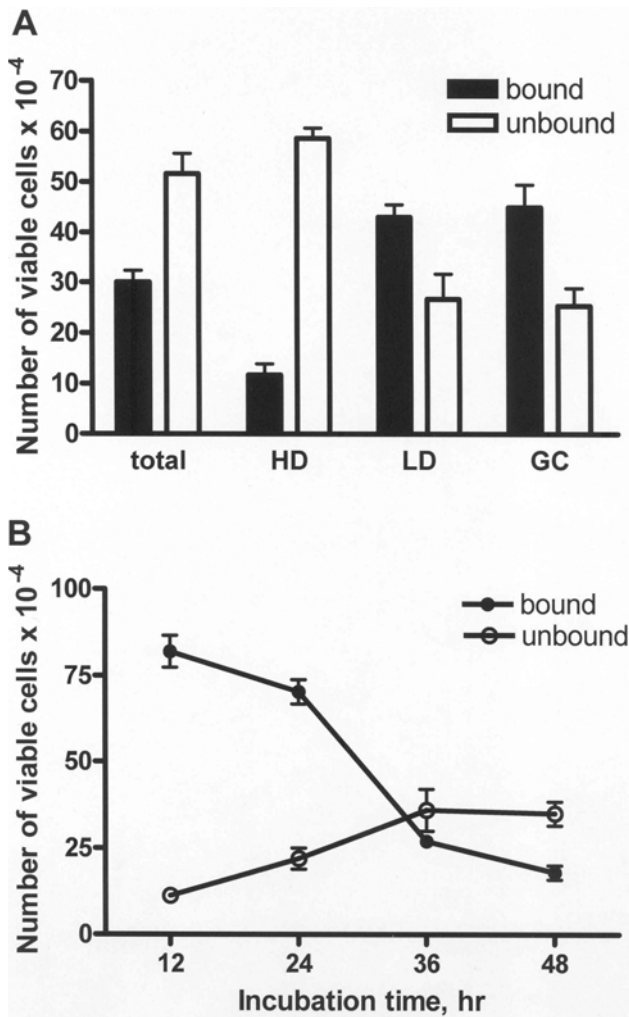
A

B

Figure 2. GC B cells exhibit unique ability to bind to HK cells. (A) Total or fractionated B cell subsets (1×10^6 cells) were cultured with HK cells in the presence of sCD154 for 12 hr. Bound and unbound B cells were harvested for viable counting. (B) GC B cells were cultured with HK cells and sCD154 up to 48 hr. At the indicated time points, bound cells and unbound cells were harvested for viable counting. Results are presented as the mean and standard error of triplicates.

Figure 1A, most of viable GC B cells did not bind to HK cells in the absence of sCD154. Only 10% of input cells bound to HK cells after 4 hr of co-culture but 45% of input cells were unbound. After this time point, either bound or unbound GC B cells rapidly underwent spontaneous apoptosis and almost no viable cells were observed at 24 hr of the culture. In the presence of sCD154, GC B cells did not undergo rapid apoptosis and about 50% of input cells were viable up to 24 hr (Figure 1B). At 4 hr of incubation, only 13% of input cells adhered to HK cells. However, since then, most of viable GC B cells bound to HK cells and the proportion of bound cells increased to 40% of input cells up to 24 hr culture. In another independent experiment, the number of bound GC B cells reached a plateau at 12 hr and then declined gradually (Figure 2B). The increase of bound cells occurred concomitantly with the decrease of unbound cells and the decrease of bound cells was partially reflected in the increase of unbound cells. These results indicate that GC B cells do not efficiently adhere to HK cells without proper stimuli such as essential survival signal coming from CD40 and that HK cells do not provide GC B cells with significant survival signal.

We next examined the cellular specificity among B cell subsets in binding to HK cells in the presence of sCD154. Tonsillar B cells were fractionated to high-density (HD) B cells that contained mostly resting B cells and low-density (LD) B cells of which GC B cells were major cellular subsets. GC B cells were isolated from LD B cells. As shown in Figure 2A, about 30% of unfractionated B cells bound to HK cells while approximately 50% were unbound. When HD B cells were compared with LD B cells in binding ability to HK cells, only 13% of them were found to bind whereas nearly 50% of LD B cells adhered to HK cells, which was similar to the binding proportion of GC B cells. These results suggest that at the given time point, GC B cells bind efficiently
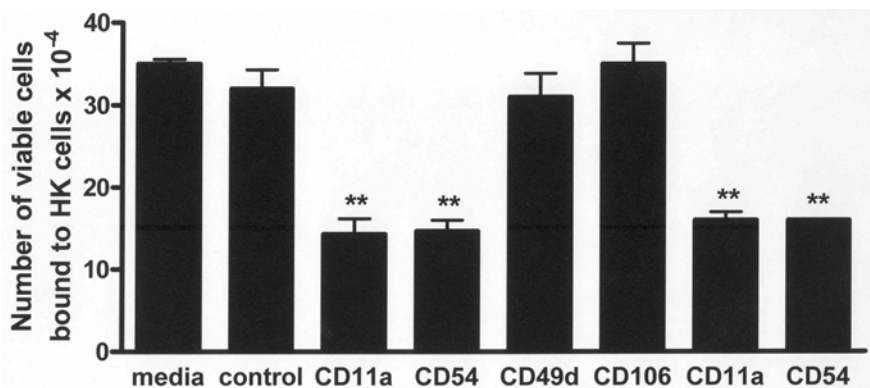

Figure 3. Anti-CD11a and anti-CD54 antibodies inhibit GC B cell binding to HK cells. GC B cells (1×10^6 cells) were cultured with of sCD154 and HK cells in the presence of indicated function-blocking antibodies (10 μg/ml) for 12 hr. Viable cells were counted after trypan blue staining. Two asterisks (**) were added to indicate statistical difference from control ($p < 0.001$). Results are presented as the mean and standard error of triplicates.
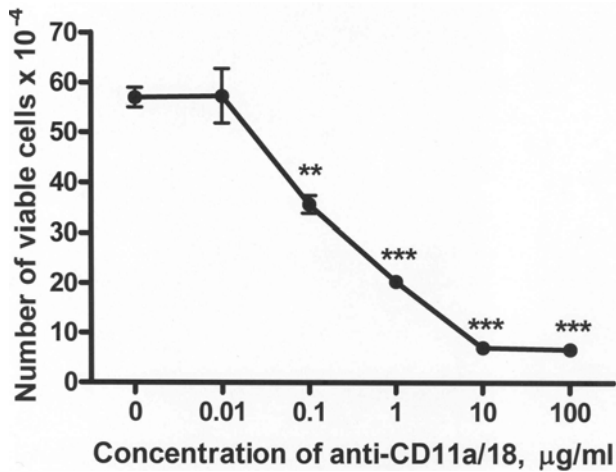
Figure 4. Anti-CD11a antibody inhibits GC B cell binding to HK cells in a dose-dependent manner. GC B cells were cultured with various concentrations of anti-CD11a antibody for 16 hr. Two or three asterisks (** or ***) were added to indicate statistical difference from control ($p < 0.001$ or $p < 0.0001$, respectively). Representative data of two reproducible experiments are presented.

but resting B cells does not adhere to HK cells, suggesting that HK cells may have been derived from FDC.

Anti-CD11a and anti-CD54 antibodies inhibit adhesion of GC B cells to HK cells. To elucidate molecular mechanisms of GC B cell binding to HK cells, function-blocking monoclonal antibodies that were raised against well-known adhesion molecules expressed on FDC and GC B cells were added to the co-culture. FDC express CD54 (ICAM-1) and CD106 (VCAM-1) while GC B cells express CD11a/18 (LFA-1) and CD49d (VLA-4) (19,20). Both anti-CD11a and anti-CD54 antibodies exhibited more than 50% inhibition of GC B cell binding to HK cells (Figure 3). In a dose-response experiment, anti-CD11a antibody inhibited the binding up to 88% when the antibody concentration was 100 g/ml (Figure 4). However, neither anti-CD49 nor anti-CD106 antibodies had inhibitory effect on the binding. Furthermore, addition of anti-CD49d with anti-CD11a and anti-CD106 with anti-CD54 did not enhance the inhibition exerted by anti-CD11a and anti-CD54, respectively. These results suggest that the interactions between GC B cells and HK cells involve CD11a/CD18-CD54 pathway and this pathway appears to be the major one.

Adhesion-blocking anti-CD11a antibody does not inhibit proliferation of GC B cells. Since the CD11a/CD18-CD54 interactions appeared to be the major pathway in GC B cell binding to HK cells, we explored whether this interaction contributes to GC reactions by studying effect of anti-CD11a antibody on the
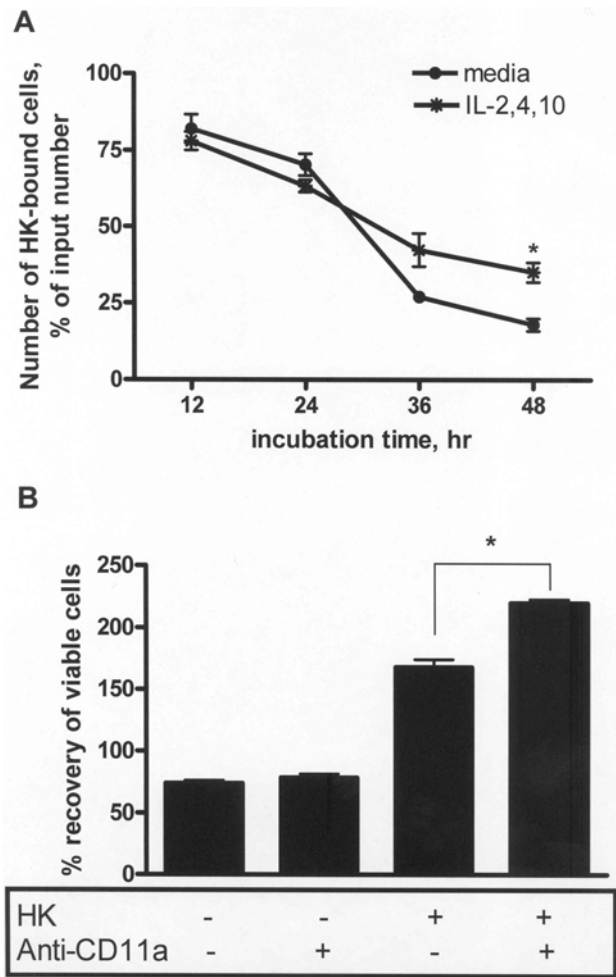

Figure 5. Disruption of GC B cell-HK cell binding does not inhibit proliferation of GC B cells. (A) GC B cells were cultured with HK cells and sCD154 in the presence or absence of IL-2, IL-4, and IL-10. (B) GC B cells (2×10^5 with HK cells, 5×10^5 without HK cells) were cultured in the presence of sCD154, IL-2, IL-4, and IL-10 for 72 hr. Recovery was calculated as a percentage of the initial number of viable cells. Anti-CD11a antibody was added at the concentration of 5μg/ml, which was the optimal concentration in most experiments. An asterisk was added to indicate statistical difference from the media controls. Results are presented as the mean and standard error of triplicates.

proliferation of GC B cells. GC is the anatomic site where massive proliferation of B cells takes place (21). GC B cells were cultured under the optimal condition that contained HK cells, sCD154, IL-2, IL-4, and IL-10 (13). Since the optimal condition included cytokines in addition to sCD154, we first examined whether the presence of cytokines affects the GC B cells adherence. As shown in Figure 5A, GC B cells bound to HK cells in a similar kinetic fashion up to 36 hr irrespective of cytokine addition, indicating the presence of cytokines did not significantly modulate the cellular interactions. At 48 hr, in

contrast, higher numbers of viable cells were bound to HK cells in the presence of cytokines than in their absence, suggesting that cytokines might affect the GC B cell binding in later stage. The requirement of HK cells for GC B cell proliferation was confirmed by the increase of recovery of viable cells from 74% to 175% (Figure 5B). However, anti-CD11a antibody did not inhibit proliferation of GC B cells that was supported by HK cells. The addition of anti-CD11a antibody to the optimal condition resulted in rather increase of viable cells after 72 hr culture ($p < 0.05$). Anti-CD11a antibody also did not inhibit GC B cell proliferation that was induced by sCD154, IL-2, IL-4, and IL-10 in the absence of HK cells (74% vs. 79%, $p = 0.277$).

Discussion

The current study revealed that GC B cells but not resting B cells adhere to an FDC-like cells, HK cells, and stimulation through CD40 is required for GC B cells to attach to HK cells. CD11a/CD18-CD54 pathway was involved in the GC B cells-HK cells binding. However, disruption of GC B cell binding to HK cells does not impair cellular proliferation supported by HK cells.

The results that unstimulated GC B cells do not bind to HK cells and GC B cells stimulated with sCD154 adhere gradually to reach maximal binding around 12 hr suggest that sCD154 induces qualitative or quantitative changes of adhesion molecules on GC B cells. Since CD11a/CD18-CD54 interactions were shown to be the major pathway in the cellular attachment of GC B cell binding to HK cells, we speculate CD11a/CD18 molecules on GC B cells undergo qualitative change during interaction with CD54 on HK cells. sCD154 may activate CD11a/CD18 molecules on GC B cells, which peaks at 12 hr and then declines gradually. As the activated CD11a/CD18 molecules return to resting state, bound GC B cells may detach from HK cells as demonstrated in Figure 2B. Resting B cells may require other signals in addition to sCD154 or longer incubation period in order to obtain sufficient activation of CD11a/CD18 molecules to bind to HK cells. The regulation of CD11a/CD18 conformation by sCD154 may take place directly or indirectly via other regulatory molecule between CD40 signaling and CD11a/CD18 conformational change. In supportive of our speculation, van der Voort et al. demonstrated that c-Met proteins were predominantly expressed on GC B cells but not on resting B cells, and ligation of CD40 by CD154 induced a strong up-regulation of c-Met proteins (22). Furthermore, stimulation of B cells with c-Met ligand, hepatocyte growth factor (HGF), augmented adhesion of B cells

to CD106 without change in the expression level of CD49d, suggesting CD49d underwent qualitative change rather than quantitatively. Since HK cells also express CD40 (9), we do not exclude the possibility that sCD154 induce conformational change of CD54 on HK cells. These possibilities will be tested by examining expression levels of CD11a/CD18 and CD54 on GC B cells and HK cells after stimulation with sCD154, respectively. Use of monoclonal antibodies that can detect conformational changes of these adhesion molecules may certify our hypotheses.

Adhesion of human B cells to FDC was demonstrated to involve both CD11a/CD18-CD54 and CD49d-CD106 pathways (20, 23). In the experiment by Koopman et al., anti-CD11a, anti-CD54, anti-CD49d, and anti-CD106 antibodies inhibited B-FDC cluster formation (20). Airas et al. also demonstrated that anti-CD18 and anti-CD49d antibodies blocked B-FDC aggregate formation (23). However, unlike anti-CD11a and anti-CD54 antibodies, anti-CD49d and anti-CD106 antibodies did not display any inhibitory effect on GC B cell binding to HK cells. This discrepancy may result from the different experimental systems that we and other previous researchers adopted. Our system employed sCD154 as B cell stimulus whereas other two research groups did not include any B cell stimuli. Furthermore, their cluster formation was maximal at 3 hr of incubation whereas our binding was maximal at around 12 hr. Our argument that discordant results may reflect different experimental systems utilized is supported by Airas et al. themselves. When they estimated activated lymphocyte binding to GCs on tissue sections, they found only anti-CD49d and anti-CD106 antibodies blocked the binding but anti-CD11a and anti-CD54 antibodies did not (23). Another reason for the failure of anti-CD49d to block GC B cell binding to HK cells may be found in the expression levels of CD106 on HK cells. HK cells do not express CD106 unless induced by proper signals, such as IL-4 (our unpublished observations).

Since CD11a/CD18-CD54 interactions appeared to be the major pathway in the cellular adhesion of GC B cells to HK cells, we expected disruption of direct cellular interaction by anti-CD11a antibody would damage HK cell-dependent GC B cell differentiation. Unlike our expectation, anti-CD11a did not impair the proliferation of GC B cells that was induced by HK cells. This result suggests that the proliferation of GC B cells does not require direct cellular attachment to HK cells or FDC but depends on diffusible growth factors secreted from them. Spontaneous apoptosis-prone GC B cells were provided with potent survival signal sCD154 in our culture condition. GC B cells require growth signals from

HK cells to proliferate since sCD154 does not stimulate their proliferation. Under *in vivo* condition, FDC may provide both survival signals and proliferation molecules, which may be obtained through direct interaction with GC B cells. In addition, it is worthwhile to mention that GC B cells in our experimental system did not receive signals coming from antigen receptors on the cell membrane. Since the experiments in this study were conducted in the absence of the essential selection signals, our results may not fully reflect the *in vivo* situation. Further experiment with freshly isolated FDC and surrogate antigens will answer to these questions.

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