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*J Immunol* 2009; 183:352-358; doi: 10.4049/jimmunol.0803183
http://www.jimmunol.org/content/183/1/352

Supplementary Material
http://www.jimmunol.org/content/suppl/2009/06/18/183.1.352.DC1

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Notch Ligands Expressed by Follicular Dendritic Cells Protect Germinal Center B Cells from Apoptosis

Sun-Ok Yoon,* Xin Zhang,* Paul Berner,* Bianca Blom, † and Yong Sung Choi1*

The Notch signaling pathway is one of the most conserved mechanisms to regulate cell fate in many tissues during development and postnatal life. In the immune system, Notch signaling regulates T and B cell development and modulates the differentiation of T and B cells. In this study, we investigated the functional roles of Notch signaling in human B cell differentiation within the germinal center (GC). Notch ligands, Delta-like 1 (Dll1) and Jagged 1 (Jg1), are expressed by follicular dendritic cells (FDC) but not by B cells in the GC, while GC-B cells express the Notch receptors, Notch1 and Notch2. The blockade of Notch signaling pathways using a γ-secretase inhibitor, DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyler ester), reduces the survival of GC-B cells in the presence of FDC/HK cells. Jg1 has a dominant effect on GC-B cell survival mediated by Notch signaling. Furthermore, Notch cooperates with another anti-apoptotic factor, BAFF/Blys produced by FDC to support GC-B cell growth. Taken together, our data shows the important role of Notch signaling provided by FDC in the survival of GC-B cells in vitro. The Journal of Immunology, 2009, 183: 352–358.

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Received for publication September 23, 2008. Accepted for publication May 4, 2009.

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Cruz Biotechnology. Other Abs used in this work were PE-conjugated anti-8D6 (16); FITC-conjugated anti-CD20, PerCP-conjugated anti-CD20, allophycocyanin-conjugated anti-CD38, and FITC- or PE-conjugated goat anti-mouse Ig (BD Biosciences); FITC- or PE-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories).

Cytokines used were IL-2 (Hoffman-La Roche), IL-4 (a gift from Schering-Plough, Union, NJ), and IL-21 (Biovision). Soluble human CD40L was provided by Dr. R. Armitage (Angen Corporation, Seattle, WA). CFSE and DMSO were obtained from Sigma-Aldrich. DAPT (N-[N-(3,5-difluorophenacetyl-l-alanyl)]-S-phenylglycine t-butyl ester) was purchased from Calbiochem.

Preparation and culture of B cell subsets

GC-B cells and naive B cells were isolated from tonsillar B cells as described previously (15, 20). GC-B cells (2 × 10⁷ cells/well) were cultured in 96-well plates containing irradiated HK cells, DII1-O9, Jg1-O9, or OP9 control (2 × 10⁴ cells/well; 5000 rad), CD40L (100 ng/ml), IL-2 (30 U/ml), and IL-4 (50 U/ml) or IL-21 (10 ng/ml) in the absence or presence of DAPT. The culture medium was IMDM (Irvine Scientific) supplemented with 10% FCS (Invitrogen), 2 mM glutamine, 100 U/ml penicillin G, and 100 µg/ml streptomycin (Irvine Scientific). Naive B cells were cultured using the same condition as GC-B cells but without HK cells.

For apoptosis assays, GC-B cells were cultured with CD40L and HK, DII1-O9, Jg1-O9, or OP9 control cells in the absence or presence of DMSO (25 µM), DAPT (25 µM), or anti-IL-6 (10 µg/ml) for 24 h. The cells were collected and stained with Annexin V-FITC and propidium iodide (PI; BD Biosciences) according to the protocol provided by the manufacturer.

For cell division experiments, GC-B cells were labeled with CFSE (5 µM in PBS) at 37°C for 10 min. FCS was added to stop staining, and then labeled cells were washed with culture medium. After culture with 25 µM DAPT or DMSO, the CFSE intensity was measured by FACSCalibur.

Flow cytometry and ELISA

Cells were stained with PerCP-conjugated anti-CD20 and allophycocyanin-conjugated anti-CD38 as described previously (15). In brief, cells were incubated with the appropriate concentration of mAbs for 15 min at 4°C. After washing with PBS containing 0.2% BSA and 0.1% sodium azide, cells were fixed with 1% paraformaldehyde and analyzed by flow cytometry. The amount of IgG, IgM, and IgA in the culture supernatants was measured by ELISA as described previously (25).

PCR analysis

Total cellular RNA was isolated using RNeasy protect kit (Qiagen). Total RNA was DNase I (Promega) treated and reverse transcribed with Promega ImPromII reverse transcriptase kit. For RT-PCR, cDNA was amplified in a 25-µl reaction mixture containing 200 µM each dNTP, 500 nM primers, and 2.5 U of Taq polymerase. Amplification of each cDNA sample was conducted under the following conditions: denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 2 min. Human G3PDH was used to ensure equal sample loading. Amplified PCR products were resolved on 1.5% agarose gels and visualized by ethidium bromide staining. The following primer sequences were used: DII1 (forward: 5'-AGG AGA CCA TGA ACA CTT GAG-3'); Jg1 (forward: 5'-AGT CAC TGG CAC GGT TGT AG-3'); reverse: 5'-TCC TCG TAT CTG TCC ACC TG3-3') (26).

Notch1 (forward: 5'-CAG CTG CAC TTC ATG TAC GTG-3'); reverse: 5'-GCC AGA CAC AGC CGG ATG CAC C-3') (27), Notch2 (forward: 5'-CCA CGC ACT CGG GCG CTA-3'); reverse: 5'-GGA GTA ATA AGG CGG AGC GCG-3') (27), Notch3 (forward: 5'-AAG CGG CTA AAG CGG CAT GAG GAG-3'); reverse: 5'-GCA TCG GCT GTG ACA GCT GTG-3'); Notch4 (forward: 5'-TGG GTA TCT CGA CCA GTG TG-3'); reverse: 5'-CAG TCG CAT AGT AAA CCC AGG-3') (27), GPDH (forward: 5'-GCC AAA AAC CTG CAC GCC TG-3'); reverse: 5'-CCG AAC AGT TTC CCG GAG GG-3') (28).

Quantitative real-time PCR reactions were performed in a 10-µl volume containing 25 ng of cDNA, QuantiTect SYBR Green PCR Master Mix (Qiagen), and 2.5 µM of each gene-specific primer. The DNA Engine Opticon system with PTCT-200 DNA Engineycler and CDP-3200 Opticon Detector (MJ Research) were used with the following program: 95°C for 15 min, followed by 40 cycles of 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min. After cycling, the specificity of amplification was validated by generating a melting curve using slow denaturation of the PCR products. The primer sequences used are as follows; Deltex1 (forward: 5'-CTC GCC GCA AGA CCA AGA AGA A-3'); reverse: 5'-TGC CAT CCT TGT TGC CA-3'), Hes1 (forward: 5'-TGG ATG CGG AGT CTA CGA TG-3'); reverse: 5'-TAA GGC CCC TCT CCA CTG TC-3'), and β₂-microglobulin (forward: 5'-TGA GTG CTC TGT CCA TGT A-3'); reverse: 5'-TCT GGG TCC CAC CTC TAA GTT G-3'). Ct values were analyzed with the ΔΔCt method using β₂-microglobulin expression for normalization.

Statistical analysis

Statistical analysis and graphic presentation were conducted with GraphPad Prism 4.0. Results are presented as means of triplicate assays plus SEM. The statistical significance of differences was determined by Student’s t test; p < 0.05 was considered significant.

Results

FDC express Notch ligands and GC-B cells express Notch receptors

Since it was not known whether Notch signaling plays a functional role in human B cell differentiation in the GC, we first determined whether Notch ligands are expressed in tonsillar tissue. As shown in Fig. 1, DII1 and Jg1 are expressed in the GC whereas Jg2 is expressed only outside the GC. To identify the cellular source of DII1 and Jg1 in the GC, FDC-specific 8D6 mAb (16, 30) and B cell-specific anti-CD20 mAb were used to co-stain with anti-DII1 mAb or anti-Jg1 mAb, respectively. Both anti-DII1 mAb and anti-Jg1 mAb (green) co-stained with anti-8D6 mAb (red; co-staining, yellow; Fig. 1, A and B), but not with anti-CD20 mAb (supplementary Fig. S1, A and B), suggesting that 8D6-positive FDC, but not B cells, produce Notch ligands, DII1 and Jg1. The stainings were specific because anti-DII1 and anti-Jg1 Abs stained DII1 and Jg1 expressing OP9 cells, respectively (supplementary Fig. S1C and S1D) and any specific signals were not detected on tonsillar tissue sections when stained with corresponding control Abs (Fig. 1). At the same time, a FDC line, HK cells, expressed DII1 and Jg1 expressing OP9 cells, respectively (supplementary Fig. S1C and S1D), but not Jg2 (Fig. 1D), suggesting that both primary FDC and HK, a cell line derived from FDC express the identical type of Notch ligands.

The expression of Notch ligands by FDC suggests that Notch signaling may have a biological function in the GC-B cells because GC-B cells require FDC/HK for survival, proliferation, and differentiation. Thus, the expression of Notch receptors was examined in GC-B cells. mRNAs specific for Notch1 and Notch2 were expressed in ex vivo GC-B cells but not Notch4 (Fig. 1E). Notch3 expression was detectable but negligible. The same Notch receptors were expressed in tonsillar naive B cells and human peripheral blood B cells as reported previously (31).

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Blockade of Notch signaling reduces GC-B cell growth
Since Notch is activated through binding to its ligand on adjacent cells (1), we determined whether Notch ligands on FDC/HK cells have an influence on GC-B cell growth and differentiation. GC-B cells were cultured with CD40L, IL-2, and IL-4 in the presence of HK cells to induce cellular growth and expansion, as described in Materials and Methods (32). The effects of the Notch signaling blockade were investigated using a well-accepted γ-secretase inhibitor (GSI), DAPT that inhibits activation of the Notch signaling pathway by blocking ligand-induced cleavage of Notch (33).

**FIGURE 1.** Expression of Notch ligands and Notch receptors in the GC. A–C, Cryosections of tonsils were double-stained with anti-Dll1 (green fluorescence, A), anti-Jg1 (green fluorescence, B), anti-Jg2 (green fluorescence, C), or isotype controls and anti-8D6 (red fluorescence, A–C). D and E, RT-PCR analysis of Notch ligand and receptor expression. Tonsillar B cells (D, lane 1) and HK cells (D, lane 2). Peripheral blood B cells (E, lane 1), tonsillar naive B cells (E, lane 2), and tonsillar GC-B cells (E, lane 3). G3PDH was used as a control for sample loading.

**FIGURE 2.** DAPT inhibits GC-B cell growth in a dose-dependent manner. GC-B cells (2 × 10^5 cells/well) were cultured in 24-well plates with irradiated HK cells (2 × 10^5 cells/well, 5000 rad) and CD40L, IL-2 and IL-4 (A) or IL-21 (B–D) with the indicated amount of DAPT for the indicated times. As controls, medium or 100 μM DMSO was added into the culture in parallel. At the end of the culture, viable cells were counted (A and B), Ab concentrations in the culture supernatants quantified (C), and the percentage of plasmablasts determined by phenotype (i.e., CD20^{lo} CD38^{hi}) (D).
When various amounts of DAPT were added in culture, GC-B cell growth was inhibited in a dose-dependent manner (Fig. 2A). A similar inhibition was observed when GC-B cells were cultured with IL-21, which induces differentiation of GC-B cells to Ab-secreting cells (Fig. 2B) (34). The addition of DAPT to the culture reduced Ab production of all isotypes (IgA, IgM, and IgG). The extent of the reduction was proportional to the amount of DAPT added (Fig. 2C). The reduction in Ab production by DAPT appeared to be the consequence of reduced GC-B cell growth because the percentage of CD20lowCD38high plasmablasts generated remained the same regardless of DAPT dose (Fig. 2D). This data suggests that Notch signaling is required for GC-B cell proliferation that precedes differentiation.

To confirm that DAPT specifically blocked Notch signaling activated by Notch ligands from FDC/HK cells, the inhibitory effect of DAPT was examined on naive B cells that do not require FDC/HK cells for survival and growth (35). DAPT had no inhibitory effect on the survival or proliferation of naive B cells whether cytokines were added or not (Fig. 3A). In addition, DAPT did not exhibit any cytotoxic effects on HK cells in culture (Fig. 3B), ruling out the possibility that inhibition of GC-B cell growth resulted indirectly from the cytotoxic effect of DAPT on HK cells. Since GSI is known to inhibit a diverse array of signaling events besides Notch signaling (36), we further determined whether DAPT affected specific transcriptional activation by Notch receptors. Quantitative real-time PCR measuring transcription of downstream targets of Notch signaling, Hes1 and Deltex1 (2) revealed that DAPT reduced both Notch-activated transcripts by more than 70% in GC-B cells cultured for 24 h (Fig. 3C).

Notch signaling blockade affects GC-B cell survival

Ag-activated B cells entering the GC appear to undergo a sequential process of survival, proliferation, and differentiation (12). Since it had not been determined which of these cellular processes were mediated by Notch signaling, we cultured GC-B cells with CD40L and HK cells for 24 h to examine the effect of Notch signaling on cell survival. Although less than 10% of seeded GC-B cells survived after 24 h in the absence of CD40L and HK cells, more than 70% of GC-B cells survived in the presence of CD40L and HK cells, which is greater than CD40L or HK cells alone (Fig. 4). The blockade of Notch signaling by DAPT decreased Annexin V− GC-B cells by 25% compared with that of control (i.e., GC-B cells cultured with DMSO). Inhibition by DAPT is specific because the addition of a neutralizing Ab against IL-6, a PC differentiation factor produced by HK cells (22), did not affect GC-B cell survival. The result is reproducible in similar experiments using three different donors as summarized in Table I, suggesting that Notch signaling from HK cells protects GC-B cells from apoptosis.

Since GC-B cells surviving apoptosis undergo proliferation and differentiation in order, we determined whether Notch signaling also has a direct effect on the cellular proliferation of GC-B cells. GC-B cells were labeled with CFSE and cultured with DMSO or DAPT in the presence of HK cells, CD40L, IL-2, and IL-4 to analyze the effect of Notch signaling on cell cycle progression. There was no difference in cell division profiles between the culture with DAPT and DMSO (supplementary Fig. S2). A similar

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Figure 3. DAPT effect on GC-B cells is specific to Notch signaling. DAPT does not affect the viability of either naive B cells (A) or HK cells (B). A. Tonsillar naive B cells (2 × 10⁵ cells/24-well) were cultured with 25 μM DAPT or DMSO for 3 days in the presence of medium, IL-2 plus IL-4 plus CD40L, or IL-21 plus CD40L. Viable cells were counted by trypan blue exclusion assay. B. HK cells (0.5 or 1 × 10⁵ cells/6-well, as indicated) were cultured with 25 μM DAPT or DMSO for 3 days. Cells were harvested at the end of the culture and recovered live cells are presented as percentages, compared with the control culture containing DMSO (100%). C. Quantitative real-time PCR analysis on the expression of Hes1 and Deltex1, downstream targets of Notch in GC-B cells after culturing with 25 μM DAPT or DMSO for 24 h. The relative fold changes of each transcript are shown in comparison to the level of the transcript in DMSO-treated GC-B cells, which is assigned the value 1.

Figure 4. DAPT causes apoptosis of GC-B cells. Annexin V-FITC and PI binding were measured by flow cytometry after culturing GC-B cells for 24 h as indicated. Typical dot plots are shown.
result was observed when GC-B cells were cultured with IL-21, which induces more vigorous GC-B cell proliferation than IL-2 and IL-4. This data suggests that Notch signaling is required for the survival of GC-B cells but has no direct effect on proliferation and differentiation of the surviving cells.

**Jg1 has a dominant effect on GC-B cell survival**

To assess the effect of each Notch ligand on GC-B cells, we cultured GC-B cells in the presence of OP9 stromal cells transduced with retroviral vectors containing either humanDll1 or Jg1 cDNAs (24). HK and OP9 cells were used as controls. The number of GC-B cells recovered at day 4 in the presence of HK cells was 2 fold higher than that in the control OP9 culture, indicating that FDC/HK produce survival and growth factors essential for GC-B cell growth. The recovery of GC-B cells cultured with Jg1-OP9 was reproducibly greater than that of control OP9 (p < 0.01, 163 ± 7.3% vs 100 ± 7.3%) while the recovery with Dll1-OP9 was similar to that with control OP9 (113 ± 10.6% vs 100 ± 7.3%) (Fig. 5A), suggesting that Jg1 is the dominant functional ligand. The increase in cell recovery by Jg1 was specific to Notch signaling because DAPT abrogated increased cell recovery (Fig. 5B). The greater recovery of GC-B cells in culture with Jg1-OP9 is attributed to the anti-apoptotic effect of Jg1 because less GC-B cells underwent apoptosis when cocultured with Jg1-OP9 cells compared with OP9 cells or Dll1-OP9 (Fig. 5C). The specificity of Notch signaling by Jg1 was confirmed by measuring the expression levels of Notch target genes (Hes1 and Deltex1). As shown in Fig. 5D, a marked increase in the expression of Hes1 and Deltex1 was observed in GC-B cells cultured with Jg1-OP9 compared with that with control OP9 cells. In contrast, a modest increase in the expression of Hes1 and Deltex1 was observed in Dll1 culture.

**FIGURE 5.** Jg1 has a dominant effect on GC-B cell survival. GC-B cells (2 × 10^5 cells/well) were cultured in 24-well plates with irradiated HK, Dll1-OP9, Jg1-OP9, or OP9 control cell lines (2 × 10^5 cells/well, 5000 rad) in the presence of CD40L, IL-2, and IL-4 (A) with 25 μM DAPT or DMSO (B). After 4 days of culture, viable cells were counted, and the increased cell recovery was presented as percentages compared with the control OP9 culture (100%). C, Annexin V-FITC and PI binding were measured by flow cytometry after culturing GC-B cells in the presence of HK, Dll1-OP9, Jg1-OP9, or OP9 control cell lines and CD40L with 25 μM DAPT or DMSO for 24 h. PI^− dead cells were excluded from analysis and apoptotic Annexin V^+ cells shown as percentage. D, Real-time PCR analysis of Hes1 and Deltex1 was performed on GC-B cells after 24 h of coculture with Dll1-OP9, Jg1-OP9, or control OP9 cell lines in the presence of CD40L, IL-2, and IL-4. The relative fold changes of each transcript are shown in comparison to the level of the transcript in GC-B cells cultured with control OP9 cell line, which is assigned the value 1. Significant differences are indicated (**, p < 0.01; *, p < 0.05; NS, not significant).
Anti-BAFFR further decreases GC-B cell growth in combination with DAPT

BAFF is an anti-apoptotic factor for B cells (37–39). Since FDC/HK cells produce BAFF that protects GC-B cells from apoptosis (40), we examined whether Notch could cooperate with BAFF to support GC-B cell growth. Whereas blocking BAFF signaling with anti-BAFFR decreased the recovery of GC-B cells by 10%, the combination of DAPT and anti-BAFFR decreased GC-B cell recovery by 55–65%, which was greater than DAPT alone (35–50%) (Fig. 6). This additive effect of DAPT and anti-BAFFR was not affected by T cell cytokines added to the culture (i.e., IL-2 plus IL-4 or IL-21), which induced the differentiation of GC-B cells, suggesting that both Notch and BAFF signaling are required for survival and growth before differentiation.

Discussion

We report that Notch signaling pathways play important functional roles in the proliferation and differentiation of Ag-activated B cells in the GC. We first identified the cellular source of Notch ligands and receptors in tonsillar tissue. Notch ligands, Dll1 and Jg1, are expressed by the FDC network in the GC of tonsillar tissue while GC-B cells do not express Notch ligands but receptors, Notch1 and Notch2. Since an FDC line, HK, express the same ligands as primary FDC, we are able to investigate the functional roles of Notch signaling in our in vitro experimental model. GC-B cells can be cultured on HK cells with CD40L and T cell cytokines for longer than 14 days in our culture system (23) whereas more than 90% of GC-B cells undergo spontaneous apoptosis within 24 h without FDC and T cell help (17, 19–21). Thus, our experimental model allows us to investigate the effect of Notch signaling on GC-B cell differentiation.

Ag-activated B cells entering the GC appear to undergo a sequential process of survival, proliferation, and differentiation to generate memory B cells and plasma cells (12). Our experimental results show that the initial survival of GC-B cells is a critical checkpoint that precedes proliferation and differentiation. The blockade of Notch signaling by specific inhibitor DAPT decreases the survival of GC-B cells but does not directly decrease proliferation and differentiation of surviving GC-B cells. Furthermore, we find that Jg1 but not Dll1 is the functional ligand for GC-B cell survival using Jg1- or Dll1-expressing OP9 stromal cells. Since GC-B cells express both Notch1 and Notch2, it is not conclusive which Notch receptor Jg1 interacts with to deliver survival signals to GC-B cells. Experiments using Notch ligand expressing cell lines also confirm that FDC/HK cells produce other factors required for GC-B cell survival and growth besides Notch ligands because the recovery of GC-B cells with HK cells is consistently higher than that with Jg1-OP9. Notch ligands expressed by FDC/HK may cooperate with other FDC factors to support GC-B cell growth. Indeed, GC-B cell recovery is decreased further when Notch signaling is blocked together with known anti-apoptotic factor BAFF produced by FDC/HK cells (40). Therefore, it is of interest to investigate how FDC signaling molecules are integrated to regulate the survival and growth of Ag-activated B cells in the GC.

The functional role of Notch signaling in murine B cell proliferation and differentiation has been reported recently (8, 9). However, the observations made in mouse B cells differ from our data with human GC-B cells in several aspects. First, Notch signaling enhanced the proliferation of the cells upon anti-CD40 stimulation through cell division in mouse B cells instead of cell survival (8, 9). This discrepancy may be explained by the difference in B cell subsets, purified human GC-B cells vs murine spleen B cells. The former cells die in culture without FDC whereas the latter survive without FDC. Second, only Jg1 has a positive effect in enhancing GC-B cell survival although human FDC in the GC expressed both Dll1 and Jg1. In contrast, Dll1 is the only Notch ligand detected in the FDC area of follicles in mouse spleen to enhance B cell proliferation (8, 9). Last, Notch signaling plays important roles in generation of plasma cells in the murine system (8, 9). Two Notch ligands, Jg1 and Dll1, have opposite effects on plasma cell generation through the same receptor Notch1, which was inducible upon activation with LPS (9). However, Notch signaling does not directly affect PC generation in our GC-B cell culture (i.e., 3–4 day short-term culture), as evidence by the following observations: 1) there is no decrease in percentage of plasmablasts generated from GC-B cells when Notch signaling is blocked by DAPT under culture conditions that induce PC differentiation (i.e., CD40L and IL-21), as assessed by CD20 and CD38 expression; and 2) there is no difference in plasmablast generation when GC-B cells are cultured with Jg1 or Dll1 expressing cell lines or control OP9 cell line (data not shown).

Notch signaling occurs when Notch-expressing cells interact with adjoining cells expressing Notch ligands (1, 2). Although different types of cells in the GC may express Notch and Notch ligands, our results show that presentation of Jg1 by FDC to GC-B cells rescue GC-B cells from spontaneous apoptosis using in vitro culture system. This interaction is likely to occur in vivo because FDC are found in close contact with B cells within the GC (10).

Our observations may have significant implication in treating B cell lymphomas. The majority of B cell lymphomas originates from the GC (41) and remains dependent on the GC microenvironment-like GC-B cells (42). Since it has recently been speculated that stromal microenvironments may contribute to the development of drug resistance in B cell lymphomas during treatment with rituximab (43), targeting the Notch signaling pathway deserves consideration as a candidate for treatment of B cell lymphomas in combination with the current therapy.

Acknowledgments

We thank Dr. Aiken and Dr. Li for critical reading. Human tonsils were provided by Dr. J. L. Guarisco (Ochsner Clinic Foundation).

Disclosures

The authors have no financial conflict of interest.

References


Supplemental Legends

Figure S1-A and B. B cells in the GC do not express either Dll1 or Jg1. Cryosections of tonsils were double-stained with anti-Dll1 (red fluorescence, A) or anti-Jg1 (red fluorescence, B) and anti-CD20 (green fluorescence, A and B). Figure S1-C and D. Anti-Jg1 and anti-Dll1 antibodies are specific. Jg1 expressing OP9 cells (C) and Dll1 expressing OP9 cells (D) were stained with anti-Jg1 Ab and anti-Dll1 Ab, respectively. At the same time, isotype control Abs were used in the place of anti-Jg1 Ab and anti-Dll1 Ab for negative controls.

Figure S2. DAPT does not affect GC-B cell proliferation. GC-B cells were labeled with CFSE (5 µM) and then cultured for 4 days with 25 µM of DMSO or DAPT in the presence of HK cells, CD40L, IL-2 and IL-4 (A) or IL-21 (B). CFSE profiles of harvested cells at day 1 and day 4 were compared by FACS analysis.
Supplementary Figure 2