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Establishment of Lymphotoxin β Receptor Signaling-Dependent Cell Lines with Follicular Dendritic Cell Phenotypes from Mouse Lymph Nodes

Yumiko Nishikawa,* Masaki Hikida,† Masaki Magari,* Naoki Kanayama,* Masaharu Mori,§ Hiroshi Kitamura,‡ Tomohiro Kurosaki,† and Hitoshi Ohmori2*1

Follicular dendritic cells (FDCs) have been shown to play a crucial role in the positive selection of high-affinity B cells that are generated by somatic hypermutation in germinal center (GC). Because of technical difficulties in preparing and maintaining pure FDCs, a role for FDCs in this complicated process has not been fully elucidated. In this study, we established a cell line designated as pFL that retained major FDC phenotypes from a three-dimensional culture of mouse lymph node cells. pFL cells proliferated slowly in response to an agonistic anti-lymphotoxin β receptor mAb and TNF-α. A more rapidly growing clone, named FL-Y, which has similar requirements for growth was isolated from a long-term culture of pFL. Analysis of surface markers in these two cell lines by immunostaining, flow cytometry, and DNA microarray revealed the expression of genes, including those of CD21, FcγRIIB, lymphotoxin β receptor, ICAM-1, VCAM-1, IL-6, and C4, which have been shown to be characteristic of FDCs. In addition, B cell-activating factor was expressed in these two cell lines. At the pFL or FL-Y:B cell ratio of 1:100, the cell lines markedly sustained B cell survival and Ab production during 2 wk of culture, while most B cells collapsed within 1 wk in the absence of the FDC-like cells. Interestingly, expression of typical GC markers, Fas and GL-7, was notably augmented in B cells that were cocultured with Th cells on these two cell lines. Thus, pFL and FL-Y cells may be useful for providing insight into the functional role for FDCs in GC.

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1 Department of Biotechnology, Graduate School of Natural Science and Technology, Okayama University, Tsurumi-Naka, Okayama, Japan; 2 Laboratory for Lymphocyte Differentiation, RIKEN Research Center for Allergy and Immunology, Tsurumi-ku, Yokohama, Kanagawa, Japan; 3 Laboratory of Immunogenomics, RIKEN Research Center for Allergy and Immunology, Tsurumi-ku, Yokohama, Kanagawa, Japan; and 4 Faculty of Health and Welfare Sciences, Okayama Prefectural University, Soja, Okayama, Japan

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2 Address correspondence and reprint requests to Dr. Hitoshi Ohmori, Department of Biotechnology, Okayama University, 3-1-1 Tsushima-Naka, Okayama 700-8530, Japan. E-mail address: hit2224@cc.okayama-u.ac.jp
3 Abbreviations used in this paper: GC, germinal center; FDC, follicular dendritic cell; BAFF, B cell-activating factor; BMDC, bone marrow-derived DC; CGG, chicken γ-globulin; Con A-sup, Con A-stimulated rat spleen cell culture; HS, horse serum; IC, immune complex; KLH, keyhole limpet hemocyanin; LN, lymph node; LT, lymphotoxin; NIP, 4-hydroxy-3-nitrophenylacyctyl; NP, 4-hydroxy-3-nitrophenylacetyl; TNP, 2,4,6-trinitrophenyl.

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mouse lymphoid tissues. For this purpose, we took advantage of three-dimensional culture of mouse lymph node (LN) cells that were included in collagen gel matrix, and succeeded in growing a FDC-like cell line named pFL. In addition, a more stably growing clone designated as FL-Y was isolated from a long-term culture of pFL. In this study, we characterized pFL and FL-Y cells, focusing on their ability to support survival and differentiation of B cells in vitro. Result suggests that these two cell lines provide useful means to gain insight into biological functions of FDCs.

Materials and Methods

Experimental animals
Male BALB/c mice were purchased from Charles River Laboratories. Quasimonoclonal mice (H-2b/b, 17.2.25 VH/JH) were used throughout present experiments. All mice were treated in accordance with the guidelines approved by the Committee of Laboratory Animal Care, Okayama University.

Abs and related reagents
Abs used in the present study were obtained from the following sources: anti-CD21 (7G6), FITC anti-CD5 (145-2C11), FITC GL-7, PE anti-CD138 (28-9 G5), PE-Cy5 anti-CD93 (1H10), PE-Cy5 anti-CD20 (RA-3-B20), biotinylated mouse anti-hamster IgG (G70-204, G94-56), and hamster anti-mouse LTβR mAb (AF-H6) (BD Pharmingen); biotinylated anti-B20 (RA3-6B2), anti-B20 (HM-40.4-3), and -VCAM-1 (I-429) (eBioscience); HRP streptavidin, HRP goat anti-mouse IgM, HRP goat anti-mouse IgG, and HRP mouse anti-rat IgG (H + L) (Zymed Laboratories); biotinylated F4/80 (A3-1 (F4-80)) (Serotec); mouse anti-C4 (16D2) that reacts with the same epitope as 14.4.4.8 (Takara Bio); donkey anti-goat IgG, anti-mouse IgG, and anti-rat IgG (whole, fractionated) (Bethyl Laboratories); purified goat IgG (Sigma-Aldrich); and biotinylation kit (American Qualex). Magnetic microbeads were purchased from Dynabeads (Dyna Biotech). Anti-FCR-YII/III mAb (2.4G2) was purified from the ascites of nude mice in our laboratory.

Cytokines and other reagents
IL-4, TNF-α, TGFB-1, and GM-CSF were purchased from PeproTech. Other materials were obtained from the following sources: horse serum (HS) (In Vitrogen Life Technologies); FCS (Sanko Junyaku); type I collagen solution, Cellmatrix I-A (Nitta Gelatin); collagenase, DNase I, keyhole limpet hemocyanin (KLH), OVA, chicken γ-globulin (CGG), BSA, 4-hydroxy-3-iodo-5-nitrophénylacétique acid (4-Iod-E3), 4-hydroxy-3-iodo-5-nitrophénylacétique acid (4-Iod-E4), CSE (Dojindo); CFSE (Dojindo); rat anti-mouse B cell-activating factor (BAFF) IgG (R&D Systems); biotinylated rabbit anti-rat IgG F(αb)2 (Rockland); rabbit FITC anti-goat IgG (H + L) (Bethyl Laboratories); purified goat IgG (Sigma-Aldrich); and biotinylation kit (American Qualex). Magnetic microbeads were purchased from Dynabeads (Dyna Biotech). Anti-FCR-YII/III mAb (2.4G2) was purified from the ascites of nude mice in our laboratory.

Establishment of a FDC-like cell line, pFL, from mouse LN
BALB/c mice were immunized with 20 μl of saline containing 20 μg of TNP-KLH and 0.4 μg of alum in each hind footpad. Popliteal LNs were collected on day 7 after immunization, and fragmented into pieces with the diameter of ~1 mm. The LN fragments were embedded in the collagen gel matrix, as follows. The collagen gel solution was made by mixing 8 vol of Cellmatrix I-A (1 vol of 10× MEM, and 1 vol of 0.08 N NaOH solution containing 200 mM HEPES, 2.6×10^5 g/ml TNP-KLH, and 50 μg/ml TNP-KLH. The LN fragments included in the gel matrix were cultured for 2 wk with changing one-half of the culture medium every 3–4 days. Then gel discs were removed from the wells, and digested with 0.5 mg/ml collagenase dissolved in 10% HS-containing DMEM/RPMI (HS/DMEM/RPMI) at 37°C for 10 min. The released LN fragments were subsequently treated with 50 μg/ml DNase I in the same medium for 10 min at 37°C, followed by gently pipetting. These treatments led to the release of dissociated cells from the LN fragments. The released cell preparation contained adherent cells with dendrites that formed cluster with B220+ cells, which were purified by panning with anti-B220-conjugated magnetic beads. Starting from 24 LNs, 5–7×10^6 clustered adherent cells were obtained at this stage. These cells were suspended at 1.25–2.5×10^6 cells/ml in HS/DMEM/RPMI containing 1×10^−7 M 2-ME, plated into a 48-well plate at 0.4 ml/well, and cultured with 10 μg/ml TNP-KLH for 1 wk. Then the cells were stimulated with 4×10^5/well mitomycin C-treated LN cells (referred to as feeder cells, hereafter) in 0.4 ml of HS/DMEM/RPMI containing 5 ng/ml each TNF-α, GM-CSF, IL-4, and TGFB-1 and 10 μg/ml TNP-KLH. Feeder cells were prepared by rupturing LN cells (2×10^6 cells/ml) from TNP-KLH-primed BALB/c mice with 25 μg/ml mitomycin C for 30 min at 37°C. TGF-B1 was usually added, as it has been reported to inhibit Fas-mediated apoptosis in a human FDC-like cell line (38). IL-4 was sometimes replaced by 10% (v/v) Con A-sup. The medium in each well containing feeder cells, indicated cytokines, and the Ag was renewed every 7 days. After 5–6 passages, the adherent cells increased to the total number of ~10^6 (a 14–17-fold increase). The resultant cell line was designated as pFL. The established pFL cells were routinely expanded in HS-DMEM/RPMI supplemented with feeder cells and cytokines indicated above, and could be stored frozen for >6 mo.

Isolation of a FDC-like clone from cultured pFL cells
To isolate a pFL-derived clone that can grow more rapidly, pFL cells were plated at 3×10^4 cells/well in 24 wells in a 48-well plate. The cells were maintained in medium containing 10% HS-containing (v/v) DMEM, 10% (v/v) MEM, and 0.1% (v/v) HS and 50 μg/ml TNP-KLH. Feeder cells were prepared by rupturing LN cells (referred to as feeder cells, hereafter) in 0.4 ml of HS-DMEM/RPMI containing 5 ng/ml each TNF-α, MC-CSF, and 10% Con A-sup. After removing the medium, 0.4 ml of the culture medium containing 1×10^5/ml QCF B cells with or without 1×10^6/ml OVA-specific Th cell clone, DO11.10 (39), was added to each well that was coated with adherent pFL or FL-Y cells. The cells were cultured for 7–13 days in the presence of 0.01–1.0 μg/ml NP-OVA. Thus, the FDC-like cell line FL-Y. FL-Y cells were routinely maintained in the same culture medium as that used for cloning.

Culture of murine lymphocytes on pFL or FL-Y cells
pFL or FL-Y cells were seeded into a 48-well plate at 4×10^4 cells/well and incubated for 24 h in 0.4 ml of FCS-DMEM/RPMI containing 5 ng/ml each TNF-α, TGFB-1, and GM-CSF and 10% Con A-sup. After removing the medium, 0.4 ml of the culture medium containing 1×10^5/ml QCF B cells with or without 1×10^6/ml OVA-specific Th clone, DO11.10 (39), was added to each well that was coated with adherent pFL or FL-Y cells. The cells were cultured for 7–13 days in the presence of 0.01–1.0 μg/ml NP-OVA. Thus, the FDC-like cell line FL-Y. FL-Y cells were prepared by deleting T cells using anti-Thy-1.2-conjugated magnetic beads from nonadherent poptelite LN cells of QCF, mice that had been immunized with 20 μg of NP-CCR and 0.4 μg of alum in each hind footpad. At indicated time points, lymphocytes were harvested and examined for the viability and GC marker expression in the B cells. Culture supernatants were assayed for the level of secreted anti-NP Abs. In some experiments, TNP-KLH-primed LN cells from BALB/c mice were cultured on pFL or FL-Y cells with or without 10 μg/ml TNP-KLH in the same fashion.

Proliferation of QCF, B cells was assessed using the CFSE-labeled cells, as described (40). QCF, B cells were washed and suspended in PBS at 10^5 cells/ml, and labeled with 5 μM CFSE at 37°C for 10 min. Excess CFSE was quenched with FCS and washed twice with MEM. The labeled cells were then cultured on pFL cells or FL-Y cells with or without 10 μg/ml TNP-KLH in the same fashion.

In some experiments, isolated GC B cells were cultured on pFL cells. LN cells from TNP-KLH-primed BALB/c mice were collected on day 7 after immunization. GC B cells were purified from the LN cells by sorting GL-7+ B220− cells with FACSS Aria (BD Biosciences). More than 95% of the resultant cells possessed GC marker. GC B cells thus obtained (1×10^5 cells) were cultured in triplicate with or without pFL (8×10^5 cells) in 48-well plates in the presence of 1 μg/ml anti-CD40 and 10 ng/ml IL-4. Cultured cells were harvested and enumerated on day 4.

ELISA of secreted Abs
Anti-NP Abs secreted from NP-OVA-stimulated QCF, B cells were assessed by ELISA using 96-well plates coated with NP-BSA, as described by Aydar et al. (27). This procedure is effective in estimating the level of anti-NP Abs in the presence of the inducing Ag, NP-OVA, because anti-NP Abs usually bind to NIP ~10-fold more strongly than NP, thereby enabling...
to trap the Abs onto the solid phase (27, 41). IgM and IgG Abs bound to the plates were assayed with HRP-conjugated goat IgG Abs to each class. Bound HRP activity was measured using 5 mM ABTS/5 mM H$_2$O$_2$.

**Phenotypic analysis of pFL and FL-Y cells**

pFL and FL-Y cells were seeded on Lab-Tek chamber slides (Nalge Nunc International) at 3–5 x 10$^4$/well and incubated for 24 h. The slides were washed with PBS and fixed in 3% paraformaldehyde. Endogenous peroxidase activity was blocked by immersing the slides in 0.1% H$_2$O$_2$. After blocking with 1% BSA, the cells were labeled, respectively, with biotinylated anti-B220, anti-CD21, anti-C4, F4/80, or 2.4G2. After washing, the slides were reacted with streptavidin-HRP. When anti-C4 was used as the first Ab, the slide was labeled with biotinylated rabbit anti-rat IgG F(ab')$_2$ in combination with streptavidin-HRP. Bound HRP activity was visualized using 3-aminio-9-ethyl-calbazole.

Surface markers on FL-Y cells were analyzed by flow cytometry using FACSCalibur and CellQuest software (BD Biosciences), as described previously (37). FL-Y cells were harvested from culture plates by washing with 0.02% EDTA-containing PBS (pH 8.0).

**IC binding to pFL cells**

ICs were prepared by incubating 200 µg/ml NP-BSA-biotin with 500 µg/ml mouse anti-NP IgG1 mAb (G1-5) overnight at 4°C. To detect IC deposition on pFL, the cells adherent to Lab-Tek chamber slides were treated with the 10-fold diluted IC solution for 4 h, washed, and fixed with 3% paraformaldehyde. After blocking endogenous HRP activity with 0.1% H$_2$O$_2$, bound ICs were labeled with streptavidin-HRP and visualized using 3-aminio-9-ethyl-calbazole.

**Microarray analysis**

Total RNA samples were prepared from each 5 x 10$^5$ cells of bone marrow-derived DCs (BMDCs), pFL, and FL-Y using TRIZol reagent (Invitrogen Life Technologies). According to the manufacturer’s indication, BMDCs were obtained from the culture of murine BM cells that were depleted of T and B cells and erythrocytes in the presence of each 10 ng/ml GM-CSF and IL-4, as described (42). cDNA synthesis, aRNA amplification, biotinylation, and fragmentation were performed with a One-Cycle Target Labeling Kit (Affymetrix). A total of 20 µg of labeled samples was added to the hybridization mixture, and hybridized with Mouse Genome 430 2.0 GeneChips (Affymetrix) at 45°C for 16 h, as described in the manufacturer’s instructions. Washing and streptavidin-PE staining were conducted using a GeneChip Fluidics Station (Affymetrix). Subsequently, the chips were scanned using a GeneChip Scanner 3000 (Affymetrix). At least two biological replicates per chip were analyzed. The intensity for each probe set was calculated using the MASS method of the GCOS software package (Affymetrix) at the default setting. Per chip normalization was performed using a median correction program in the GeneSpring software package (Agilent). The data of probe sets were excluded when the values were judged as absent by the GCOS program in at least one sample in the replicates. Functional annotation of genes on the Mouse Genome 430 2.0 array was performed using information from the NetAffx analysis center (www.affymetrix.com/analysis/index.affy). The raw data of the array analyses are available from the Gene Expression Omnibus of the National Center for Biotechnology Information (accession numbers of the data are GSM101418, GSM101419, GSM101420, GSM101421, GSM101422, and GSM101423).

**RT-PCR and quantitative real-time PCR analyses**

Total RNA samples were extracted from FL-Y cells with TRIzol, as described above. Each cDNA was generated using Superscript II (Invitrogen Life Technologies) reverse transcriptase and oligo(dT) nucleotides. The resulting cDNA was used in quantitative real-time PCR using iQ 5 Real Time PCR Detection System and iQ SYBR Green Supermix (Bio-Rad), as

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**FIGURE 1.** Establishment of an FDC-like cell line, pFL, from three-dimensional culture of mouse LN fragments. A, Scheme for three-dimensional culture of LN fragments. Poptileal LN from TNP-KLH-immunized mice were fragmented and included in the collagen gel matrix. After culture in HS-containing medium for 14 days, the LN cells were released from the gel matrix by treating with collagenase/DNase I. B, Microscopic observation of adherent cells clustered with lymphocytes after being released from the gel matrix. C, Requirements for growth of the adherent cells that were harvested from the gel matrix. Adherent cells clustered with B cells were purified from the cells that were released from the three-dimensional culture by panning with anti-B220-conjugated magnetic beads. The purified cells were stimulated with or without feeder cells and TNP-KLH in the presence of indicated cytokines every 7 days. Where indicated, following cytokines were added: 4, IL-4; G, GM-CSF; T, TNF-α; A, all of these three cytokines. Number of viable adherent cells was counted on day 17 of the culture. Each column represents the mean of duplicate wells. Five to six passages of the adherent cell culture under the optimal condition (see Materials and Methods) led to the establishment of a FDC-like cell line, pFL. D, Adherent cell cultures were conducted in the same fashion as in C, except that feeder cells and TNP-KLH were replaced by an agonistic anti-LTβR mAb (L) and/or TNF-α (T). Each column represents the mean ± SD from triplicate wells. Significantly different from the control at p = 0.023 (**) and p = 0.002 (**). E, LTα and LTβ expression in feeder cells. Feeder cells that were stimulated with TNP-KLH for 0 or 20 h were examined for the gene expression by RT-PCR. NIH 3T3 cells were used as a negative control.
described by the manufacturer’s protocol. PCR primers used for these analyses are as follows: LTβR, 5'-GCCCTTGAGCATTTGCT-3’ and 5'-GCCAGCTGACATGATAC-3’; FcγRIIIB, 5'-ATGGGAATCCTGTGCAGTTCCTA-3’ and 5'-CCCACTGAGAATTTAGC-3’; BAEFF, 5'-CAGCGACACGGCGACTATA-3’ and 5'-TAGGCTTGTGACTC-3’; and LTβ, 5'-TCCAAAATGCTCAGGAATCTACG-3’ and 5'-GATCTGTGTTAGAATGCCGAG-3’ (PromerBank). All real-time PCR were performed in triplicate.

LTα and LTβ gene expression in feeder cells (TNP-KLH-primed LN cells) was analyzed by RT-PCR. LTα, LTβ, and β-actin cDNA were amplified by AmpliTaq Gold DNA polymerase (Applied Biosystems) using specific primer pairs described above. PCR was conducted, as follows: denaturation at 94°C for 1 min, annealing at 58°C for 30 s, and extension at 72°C for 1 min. Amplified cDNA was visualized on polyacrylamide gel and stained with SYBR Green I Nucleic Acid Gel stain (Cambrex Bio Science Rockland).

Statistical analysis

Student’s t test was used to compare results obtained from each triplicate experiment.

Results

Establishment of a FDC-like cell line, pFL, from three-dimensional culture of mouse LN fragments

To establish a cell line with FDC phenotypes from murine lymphoid tissues, we took advantage of three-dimensional culture of LN fragments in collagen gel matrix using culture medium containing HS. The culture in collagen gel matrix may help maintain the LN architecture that will provide a microenvironment sustaining optimal growth and differentiation of constituent cells (43–46). In addition, the use of HS instead of FCS has been reported to be beneficial in avoiding the outgrowth of fibroblasts that will otherwise overwhelm the growth of desired cells (46). Thus, we embedded popliteal LN fragments prepared from TNP-KLH-immunized mice in collagen gel matrix, and cultured in the medium containing HS and TNP-KLH (Fig. 1A; for details, see Materials and Methods). After 2 wk, the cells included in the gel were released by the treatment with collagenase and DNase I, and further cultured with TNP-KLH for 5–7 days. Microscopic observation of the culture revealed the occurrence of many adherent cells that formed cluster with lymphocytes (Fig. 1B). Such clustered adherent cells were not obtained from the LN fragments cultured in the absence of the gel matrix.

When the cells released from the gel matrix were restimulated with TNP-KLH, B cells were found to proliferate actively concomitant with a slight increase in the number of B cell-binding adherent cells (data not shown). Because FDCs have been shown to develop in the B cell follicle in response to LTβR- and TNFR-mediated signals (13–24), we hypothesized that if the adherent cells were FDC-like, the cells might be maintained and hopefully expanded in the culture when they were stimulated with LTα/β2 and TNF-α. Thus, we tested whether the adherent cells recovered from the gel culture could proliferate when the cells were cultured with mitomycin C-treated C57BL/6-LP-KLH-primed LN cells (feeder cells) as a source of LTα/β2 and other growth factors. To investigate optimal conditions for growing FDC-like lines, the adherent cells clustered with B cells were first purified by panning with anti-B220-conjugated magnetic beads, and cultured with feeder cells in the presence of various cytokines. The purified adherent cells grew poorly in the absence of feeder cells. The growth was enhanced slightly by the addition of feeder cells (columns 1 and 2 in Fig. 1C), and further stimulated in the presence of TNP-KLH, the priming Ag of feeder cells (column 3 in Fig. 1C). The cell growth was further enhanced by the addition of IL-4, TNF-α, or the combination of IL-4, GM-CSF, and TNF-α in the presence of both feeder cells and TNP-KLH (columns 4–7 in Fig. 1C), but not in their absence (data not shown). Although GM-CSF alone did not significantly improve the cell growth, the cells developed more dendrites and formed clusters with lymphocytes more efficiently in its presence (data not shown). In cultured human FDCs, it has been reported that GM-CSF was found to augment the cell viability and ICAM expression (31), suggesting beneficial effects of this cytokine. Thus, we usually added GM-CSF to the culture. Interestingly, an agonistic anti-LTβR mAb plus TNF-α partially replaced the effects of feeder cells (Fig. 1D). In addition, it was confirmed by RT-PCR that feeder cells expressed both LTα and LTβ (Fig. 1E). Thus, it was suggested that feeder cells were effective, at least

![FIGURE 2. Phenotypic analysis of pFL cells by immunostaining.](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org)
in part, as an LTα1β2 source (13–24). Because the cell growth was enhanced most reproducibly by the combination of IL-4, GM-CSF, and TNF-α in the presence of feeder cells and TNP-KLH, the culture was continued with these supplements being replaced every 7 days. Starting from 24 popliteal LNs, 5 × 10^4 purified adherent cells were obtained after the three-dimensional culture,
which were expanded to \( \sim 10^6 \) cells after 5–6 passages. The resulting cell line was designated as pFL, which could be stored frozen for >6 mo.

**Analysis of phenotypic marker expression on pFL cells**

pFL cells thus obtained were examined for the expression of surface markers to investigate whether the cell line retains characteristic phenotypes of FDCs. Immunostaining with each specific Ab revealed that pFL cells were positive for CD21, FcyRII/III, and C4, which also have been recognized as FDC-M2 (36), all of which have been reported to be expressed on primary FDCs (Fig. 2A) (11, 47). pFL cells were, however, negative for FDC-M1, a typical FDC marker (data not shown). More than 95% of cultured pFL cells were positive for CD21 and FcyRII/III, thus confirming the purity of the cell line. In contrast, the cells were negative for F4/80, which is a marker of the macrophage lineage (48).

NP-BSA was found to deposit on pFL cells when it was incubated with the cells in the presence of an anti-NP IgG1 mAb, but not in its absence, suggesting the ability of the cells to bind ICs (Fig. 2B). In addition, pFL cells formed clusters with B cells when TNP-KLH-primed LN cells were cultured on pFL cells (Fig. 2C).

Collectively, these results strongly suggest that pFL cells retain intrinsic properties of FDCs.

**Augmentation of cell viability, GC marker expression, and Ab production in B cells by pFL cells**

FDCs have been reported to support the viability of GC B cells that are otherwise apt to undergo apoptosis in this microenvironment (9, 11). Thus, we investigated whether pFL cells can support the viability of B cells during culture. To examine this, we used B cells from QCF1 mice that bear the knockin 17.2.25 VH gene that is known to constitute NP-specific BCR when the encoded H chains associate with \( \lambda \) L chains and some \( \kappa \) L chains (49). Thus, 30–40% of total B cells in the NP-CGG-primed LN cells showed specificity for NP, as assessed by the binding of NP-BSA (data not shown). In this experiment, B and T cells were cultured at low density (see Materials and Methods) to minimize supportive effects of autocrine trophic factors. When B cells from NP-CGG-primed QCF1 mice were cultured with OVA-specific Th clone, DO11.10 in the presence of pFL cells, the proportion and the absolute number of viable B cells after 7 days’ culture increased in the presence of a cognate Ag, NP-OVA, in a dose-dependent fashion. Thus, it is suggested that pFL cells can support B cells in an Ag-independent fashion in the absence of Th cells or NP-OVA (data not shown). Taken together, results show that pFL cells retain characteristic functions of FDCs that may be responsible for promoting GC reaction.

**Isolation of a more rapidly growing clone from pFL cells**

As described above, we established culture conditions to prepare a FDC-like cell line, pFL, from the primary culture of LN fragments. Although the cell line thus induced is valuable to investigate FDC functions in vitro, the preparation procedure is a time-consuming and laborious process. Thus, we next tried to isolate a pFL-derived clone that can be proliferated more stably and rapidly. When pFL cells were cultured in the resident medium supplemented with TNF-\( \alpha \) alone, two colonies were found to grow after 30–50 days, one of which was named FL-Y (Fig. 6A). FL-Y cells grew slowly in the presence of TNF-\( \alpha \) alone, but not in its absence. Interestingly, more rapid growth was induced in the presence of an agonistic anti-LTbird mAb, which was further accelerated when TNF-\( \alpha \) was supplemented in addition to anti-LTbird (Fig. 6B). The decline of the growth curve in the anti-LTbird/TNF-\( \alpha \)-stimulated cells on day 8 was due to overgrowth of the cells. If appropriately

Consistent with the long-term maintenance of B cell viability by pFL cells, it was found that Ag-specific IgM and IgG production in QCF1 B cells that were cocultured with DO11.10 Th cells was induced, and sustained from days 7 to 13 of the culture in the presence of pFL cells, while the Ab response in the absence of pFL cells was <1/15 of that elicited in their presence (Fig. 4). It should be noted that the B cell-supporting activities were clearly observed at the pFL:B cell ratio of 1:100 that may mimic the situation in GC, thus suggesting the strong competence of this cell line.

Furthermore, pFL cells markedly augmented expression of GC markers, GL-7, and Fas in B cells that were cultured with Th cell and Ag. On day 0 of the culture, no GL-7\( ^{\text{+}} \)B cells were observed, while a significant proportion of B cells became positive for the GC markers after culture for 7 days in the presence of pFL cells, but not in their absence (Fig. 5A). It was found that the number of GL-7\( ^{\text{+}} \)Fas\( ^{\text{+}} \)B cells markedly increased during culture strictly depending on pFL cells and the dose of NP-OVA (Fig. 5B).

In addition, purified GC B cells that were stimulated with anti-CD40 plus IL-4 increased in number by \( \sim 4 \)-fold after culture for 4 days only in the presence of pFL cells (Fig. 5C). GC B cells did not increase when anti-CD40 and IL-4 were not added to the culture (data not shown). Thus, pFL cells are considered to be potent in supporting GC B cell proliferation. The Ab response (Fig. 4) and GC marker expression (Fig. 5) were not induced in the absence of Th cells or NP-OVA (data not shown). Taken together, results show that pFL cells retain characteristic functions of FDCs that may be responsible for promoting GC reaction.

![FIGURE 4. Long-term maintenance of Ab production by pFL cells. Culture supernatants were collected on days 7 and 13 from the coculture of QCF1 B cells and DO11.10 cells that were conducted in Fig. 3. NP-specific IgM (□) and IgG (■) in each sample were assayed as anti-NIP Abs (see Materials and Methods). Each column represents the mean \( \pm \) SD from triplicate wells. Significantly different from each control at \( p < 0.05 \) (•) and \( p < 0.01 \) (**).](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org)}
diluted, the cells could be maintained in the culture for more than several months. Although signals mediated by TNFR and/or LTβR/H9252R have been shown to be required for maturation and maintenance of FDCs in vivo (13–24), FDC-like cell lines whose growth is dependent on these stimuli have not been described. In addition, flow cytometric analysis revealed that FL-Y cells are positive for LTβR/H9252R, VCAM-1, FcγRII/III, CD21, and BAFF (Fig. 6C). Quan-

titative real-time RT-PCR confirmed the expression of LTβR/H9252R, FcγRIIb, and BAFF in FL-Y cells, which was apparently marginal in flow cytometric analysis (Fig. 6D), thus indicating that FL-Y cells retained major phenotypic markers of FDCs. In addition, it was found that BAFF and FcγRIIb transcripts were increased in response to LTβR stimulation in FL-Y cells. BAFF and FcγRIIb, but not LTβR, were expressed at higher levels in pFL cells than FL-Y cells (Fig. 6D). In contrast, FL-Y cells as well as pFL cells were negative for FDC-M1 (Fig. 6C). It is possible that FDC-M1 expression was unstable after the cells were transferred to in vitro
culture. Because biological functions of FDC-M1 have not been defined, it remains unclear how the function of the cell line is affected by the phenotypic defect.

Characterization of gene expression profile in pFL and FL-Y cells by microarray analysis

To confirm the character of pFL and FL-Y cells as FDC-like lines, gene expression profile of these cell lines was examined in comparison with that of BMDCs by microarray analysis. In the analysis of FL-Y cells, unstimulated and anti-LTβR-stimulated cell preparations were used. pFL and FL-Y cells were shown to share almost the same gene expression pattern, thus indicating that FL-Y was a direct descendant of pFL (Fig. 7A). However, several genes, including C4 and Cxcl13, were expressed at lower levels in FL-Y cells than pFL cells. Because the gene expression profile of BMDCs was distinct from that of pFL or FL-Y lines, it was clearly shown that our cell lines do not belong to the DC lineage. In addition to analyses of cell surface markers (Figs. 2 and 6), the microarray analysis further supported that the cell lines were closely related to FDCs. For instance, consistent with previous reports (8, 50), expression levels of class II Ags (H-2Aa and H-2Ea) were high in BMDCs, but much lower in pFL and FL-Y cells (Fig. 7, A and C). In contrast, among the genes expressed at higher levels in our FDC-like lines compared with BMDCs (listed in Fig. 7B), those marked with asterisks have been shown to be expressed in FDC preparations described in previous reports from other laboratories (51–53). These included the genes of CXCL12, CXCL13, procollagen type Iα, biglycan, VCAM-1, C4, CXCL1, connective tissue growth factor, insulin-like growth factor-binding protein 3, secreted acidic cysteine-rich glycoprotein, and prostacyclin synthase, thus further confirming the FDC-like characters of pFL and FL-Y cells. FDCs present in GCs have been shown to express FcyRIib by immunohistochemical analysis (26). We revealed that pFL and FL-Y cells were reactive with 2.4G2 that recognizes both FcγRII and FcγRIII (Figs. 2A and 6C). It was confirmed by microarray analysis and real-time RT-PCR that the Fcγ2b gene was expressed in these two cell lines (Figs. 6D and 7A).

B cell-supporting activity of FL-Y cells

FL-Y cells were found to support B cells in terms of sustaining viability, GC marker induction, and differentiation to CD138+ plasma cells as efficiently as the parental line, pFL (Fig. 8A). As assessed by flow cytometry, real-time RT-PCR, and DNA microarray analysis, both pFL and FL-Y cell lines were shown to express BAFF (Fig. 6, C and D, and Fig. 7, A and B). Immunohistochemical analysis of human tonsil sections has revealed recently BAFF expression in FDCs within GCs (54), but, to our knowledge, this is the first report describing that murine FDC-like cells express BAFF. Because enhancement of B cell viability by FL-Y cells during culture was significantly reduced by the addition of neutralizing anti-BAFF Abs, but not of negative control Abs (Fig. 8B),...
BAFF may be responsible, at least in part, for supporting B cells by FL-Y. Collectively, FL-Y cells may be useful as a FDC-like line to examine biological role for FDCs in GC reaction at cellular and molecular levels.

**Discussion**

Normal development of FDCs has been shown to be essential for the establishment of proper segregation of T/B cell areas in secondary lymphoid organs and the formation of GCs within the B cell follicle (8–23). Although accumulating evidence suggests a pivotal role of FDCs in B cell differentiation and selection in GCs, molecular mechanisms underlying these complicated processes have not been fully elucidated. This is because FDCs have been difficult to examine due to problems in isolating cells to significant homogeneity and in maintaining or proliferating them in vitro. Thus, it is of great importance to develop a procedure for isolating primary FDCs more easily or to establish cell lines that retain major FDC functions as sufficiently as possible.

Primary FDCs prepared from lymphoid organs in irradiated mice have been used in many in vitro experiments reported to date (25–27, 34). B cell viability, proliferation, and Ab production have been reported to be markedly enhanced when B cells were cocultured with the FDC preparations at high FDC:B cell ratio, such as 1:2–1.5, which may be, however, too high compared with the ratio in GCs (1:100 or lower) (29). This may be because active FDCs were rare in these FDC preparations, or primary FDCs were not sufficiently functional under the culture conditions used. In contrast, a human cell line, HK, has been established from tonsil, and widely used as a FDC-like cell line (30). HK cells retain some properties characteristic of FDCs, including LTβR-induced up-regulation of cell adhesion molecules (ICAM-1 and VCAM-1) and cytokines (IL-6, IL-15) (55), TNF-induced NF-κB activation (56), and supporting GC B cell survival and proliferation (38, 55–61), while it has been reported that HK cells lost a representative surface marker of FDCs, CD21 shorty after isolation from human tonsil (30).

However, murine FDC-like cell lines that retain major physiological functions of FDCs have not been established. In the present work, we succeeded in establishing culture conditions to obtain FDC-like cell lines from mouse LN. The use of three-dimensional primary culture of mouse LN fragments in HS-containing culture medium is considered to be critical for successful isolation of the cell lines. It has been reported that a mouse stromal cell line that can support long-term hematopoiesis was established from mouse spleen fragments by the same procedure (46). pFL cells could be reproducibly established from the three-dimensional culture, and maintained in the culture without changing the major phenotypes for at least 3–4 mo. In addition, the cells could be stored frozen.

Furthermore, we isolated a more rapidly growing clone, FL-Y, from a long-term culture of pFL cells. Microarray analysis revealed that pFL and FL-Y cells expressed a panel of genes that have been reported to be expressed in some previous FDC preparations (51–53), thus confirming the FDC-like characters of these cell lines. pFL and FL-Y cells were positive for CD21 (Figs. 2 and 6). However, CD19 that is associated with CD21 in B cells was not shown). Interestingly, FL-Y cells can be continuously expanded in the TNF-α-supplemented culture (Fig. 6B). More rapid growth was induced by the addition of an agonistic anti-LTβR or feeder cells. Although LTα1β2 and/or TNF-α signals have been shown to be critical for the maintenance of FDC network in vivo (13–16), to our knowledge, this is the first report that showed the effectiveness of these stimuli in proliferating FDC lineage cells. Thus, it is likely that LT and TNF signals may be involved not only in the maintenance, but also in the proliferation of FDCs in vivo during development of FDC network.

It has been shown that LTβR- and TNFR-mediated signals control the maintenance and gene expression in FDCs. For instance, IC-bearing, FDC-M1-positive cells rapidly disappeared from the B cell follicle in the mouse spleen after injection of LTβR-Ig fusion protein that blocks LTβR-mediated signal (13). In human FDC-like cell lines, LTα1β2- or TNF-α-induced up-regulation of VCAM-1, ICAM-1, IL-6, and IL-15 has been observed (55). We also observed in the microarray analysis that expression of several genes, including, for instance, BAFF, FcγRIIB, and ICAM-1, was increased in response to anti-LTβR stimulation in FL-Y cells (Fig. 7A), thus further confirming the responsiveness of this cell line to LTβR-mediated signals.

Our FDC-like lines, pFL and FL-Y, showed strong supporting effects on the viability and Ab production of B cells when lymphocytes were cocultured with these FDC-like cells that were added to the culture at 1/100 of total B cells, thus suggesting that our cell lines retain more sufficient functions than primary FDCs described in previous reports (25–27, 34). pFL and FL-Y cells...
preferentially augmented B cell maintenance and proliferation (Figs. 3 and 8A), while T cell proliferation was rather suppressed under the same culture conditions (our unpublished data). Although FDCs in human tonsil GCs have been shown to express BAFF as assessed by a specific mAb (54), BAFF expression in murine counterparts has not been reported. In the present work, we found that both pFL and FL-Y cells expressed BAFF (Figs. 6C and 7, A and B). Enhancement of B cell viability by pFL and FL-Y cells may be, at least in part, due to the expression of BAFF in these cells because the effect was significantly neutralized by anti-BAFF Abs (Fig. 8B). It has been proposed that soluble trophic factors released from FDCs, instead of ICs trapped on FDCs, might be more important in the selection process of high-affinity B cells (29). Our results imply that FDC-derived BAFF plays a role in the differentiation of B cells in GCs.

Another interesting observation is that expression of GL-7 and Fas that are characteristic markers in GC B cells was markedly enhanced in B cells that were cultured in the presence of our FDC-like cell lines (Figs. 5 and 8). Thus, these FDC-like cell lines may be useful to examine functional roles of FDCs in GC reaction.

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Disclosures
The authors have no financial conflict of interest.

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