Functionally Active Epstein-Barr Virus-transformed Follicular Dendritic Cell-like Cell Lines

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Summary

Follicular dendritic cells (FDC) are unique nonlymphoid cells found only in germinal centers. FDC can be distinguished from other accessory cells based on a characteristic set of cell surface markers. It is known that FDC are able to rescue germinal center B cells from apoptosis. To investigate the role of FDC in the process of selection and maturation of B cells during germinal center reactions, we tried to establish factor-independent immortalized FDC-like cell lines. Because freshly isolated FDC express the Epstein-Barr Virus (EBV) receptor CD21, we attempted EBV transformation on isolated FDC. After incubation of FDC-enriched cell populations with EBV, cell lines were obtained consisting of slowly duplicating very large cells. These cell lines have a fibroblast-like morphology but could be clearly distinguished from several human fibroblast cell lines by displaying a different phenotype including intercellular adhesion molecule 1, CD40, and CD75 expression. Detection of the EBV-encoded proteins latent membrane protein 1 and Epstein-Barr virus nuclear antigen 2 in our FDC-like cell lines implicated successful EBV transformation. FDC-like cells are able to bind nonautologous B cells and preserve the latter from apoptosis. The binding of B cells to FDC-like cells is dependent on adhesion via lymphocyte function-associated antigen 1/intercellular adhesion molecule 1 and closely resembles the pattern of emperipolesis as described by others. These data demonstrate that FDC can be successfully infected by EBV and that the cell lines obtained share phenotypic and functional characteristics with freshly isolated FDC.

Germinal centers of lymphoid follicles are microenvironments where mature B lymphocytes localize, proliferate, and differentiate into memory B cells or into Ig-producing cells. Within germinal centers follicular dendritic cells (FDC)[1] are found in close contact with B cells (reviewed in references 1–4). FDC are unique nonlymphoid cells only found in germinal centers. Their long dendritic processes form a fine network that is intimately associated with B lymphocytes. FDC can be distinguished from other accessory cells in secondary lymphoid tissues based on several features, including lack of phagocytotic activity, and a characteristic set of cell surface markers, such as receptors for complement C3 fragments and adhesion molecules (5–12). It was recently shown that FDC are able to rescue germinal center B cells from apoptosis by a cell–cell contact-dependent mechanism (13). To facilitate the study of the role of FDC in the process of B cell selection and maturation during germinal center reactions it would be important to establish FDC cell lines for use in in vitro experiments. Although Tsunoda et al. (14) have reported that long-term culture of FDC was feasible, these cells did not divide in vitro. In addition, several other investigators have reported on malignant FDC-like tumors but they were not able to establish cell lines from these patients (15–17). Recently, Clark et al. (18) reported that they were able to culture FDC-like cells in vitro. These cells were not immortalized or transformed and the proliferation of these cells was dependent on GM-CSF. Alternatively, in this article we report on the establishment of immortalized follicular dendritic cell-like cell lines (FLCL) obtained by EBV transformation of FDC-enriched cell populations. These cell lines proliferate independent of growth factors and share phenotypic and functional properties with freshly isolated FDC.

[1] *Abbreviations used in this paper:* EBNA-2, Epstein-Barr virus nuclear antigen 2; FDC, follicular dendritic cell(s); FLCL, FDC-like cell line(s); ICAM-1, intercellular adhesion molecule 1; IMDM/g, IMDM with gentamicin; LMP-1, latent membrane protein 1; SAC, *Staphylococcus aureus* Cowan I; VCAM, vascular cell adhesion molecule 1.

Materials and Methods

Isolation of FDC. FDC were isolated as described by Parmentier et al. (8). In brief, tonsils, freshly obtained from children under-

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going routine tonsillectomy, were cut into small pieces. These pieces were incubated twice for 30 min at 37°C under continuous shaking in 30 ml of IMDM plus 90 μg/ml gentamicin (IMDM/g) containing 200 U/ml collagenase type IV (Worthington Biochemical Corp., Freehold, NJ) and 10 U/ml DNase I (Boehringer Mannheim GmbH, Mannheim, Germany). Next, the cell suspensions were cooled on ice and centrifuged for 10 min at 600 *g*. The pellets were resuspended in IMDM/g and subjected to 1 *g* sedimentation (30 min, 0°C) in discontinuous BSA gradients consisting of layers of 1.5%, 2.5%, and 5% BSA in HBSS. Cells at the interfaces between 2.5 and 5% BSA were collected, washed, and layered on Percoll gradients (Pharmacia, Uppsala, Sweden), consisting of layers with densities of 1,070, 1,060, and 1,030 mg/ml, and centrifuged for 20 min at 1,200 *g*. Cells with densities of <1,060 mg/ml were collected. These FDC-enriched cell suspensions were used for further experiments and contained 5–20% DRC-1-positive cells.

EBV Transformation. EBV containing supernatant from the marmoset B95.8 cell line (19) was added (10% vol/vol) to 5 × 10^6 cells from FDC-enriched cell suspensions and incubated at 37°C and 5% CO_2. After 24 h the cells were harvested, washed, and brought into culture in IMDM/g plus 10% FCS in six-well culture plates (Costar, Cambridge, MA). After 1 wk nonadhering EBV-transformed B cells were removed by repeated thorough washing with IMDM/g. In some experiments the FDC-enriched cell populations were depleted of LFA-1–positive cells before transformation. This depletion was done by incubation with anti–LFA-1 antibodies (Table 1) and subsequent incubation with Dynabeads coated with goat anti-mouse-Ig (Dynal, Oslo, Norway).

Immunophenotyping. FDC-like cells were harvested by incubating with 0.25% trypsin solution (GIBCO, Paisley, Scotland) for 5–10 min at 37°C. Immunophenotyping of surface markers was done by indirect staining with a number of mAbs (Table 1) and a FITC-conjugated $F(ab')_2$-rabbit anti-mouse Ig (Zymed, San Francisco, CA). 5 × 10^3 cells per sample were analyzed using a FACScan 4® cytofluorometer (Becton Dickinson & Co., Mountain View, CA). For cytoplasmic markers, immunophenotyping was done by indirect staining of cytospin preparations; 2 × 10^4 cells were spun onto glass slides using a cyto-centrifuge (Shandon Inc., Pittsburgh, PA). Cytospin preparations were incubated for 60 min with mAbs (Table 1) or isotype-matched control Igs followed by FITC-conjugated or PE-conjugated $F(ab')_2$-rabbit anti-mouse Ig (Zymed) for fluorescence microscopy or horseradish peroxidase–conjugated rabbit anti-mouse Ig (Dakopatts, Glostrup, Denmark). Peroxidase activity was visualized by incubation with the substrate 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO) in acetate buffer pH 4.9 for 30 min at room temperature. Cells in these preparations were counterstained with hematoxylin.

The reexpression of HLA-DR and vascular cell adhesion molecule 1 (VCAM-1) was studied after preincubation with 100 U/ml recombinant human (rh) IL-4 (DNAX, Palo Alto, CA) for 24 h followed by FACScan® or cytospin labeling. As controls in these experiments, the human fibroblastic cell lines HFB-VH, HSF-7 (foreskin derived), HFC, Hengelmolen (skin derived), and NN310 (tonsil derived) were used.

Cloning of FDC-like Cell Lines. After at least 3 wk of culture and immunophenotyping, FLCL were cloned by limiting dilution. The cells were harvested and subsequently cultured at 10, 3, or 1 cell per well in 96-well plates in IMDM/g plus 10% FCS or in IMDM/g plus 10% FCS with the addition of 100 U/ml GM-CSF (Genzyme Corp., Boston, MA), supernatant from cultures of FLCL (10% vol/vol) or a mixture of supernatants from JY and Raji cells (i.e., 25% vol/vol) JY supernatant and 25% vol/vol Raji supernatant) (20). Wells showing cell growth were counted after 2–4 wk.

Adherence of B Cells to Cultured FDC Lines. FLCL or fibroblasts were harvested, resuspended, and seeded into six-well culture plates (Costar) in IMDM/g plus 10% FCS. 2.5 –5 × 10^3 FLCL or 2 × 10^4 fibroblasts were seeded into each well. Low-density and high-density B cell fractions were obtained by centrifugation (15 min, 1,200 *g*, 4°C) of purified tonsillar B cells on a Percoll (Pharmacia, Uppsala, Sweden) gradient, consisting of four density layers (1,077/1,067/1,056/1,043 mg/ml). Cells at the 1,043/1,056 interface (low-density cells) and at the 1,067/1,077 interface (high-density cells) were used. The low-density B cell fraction mainly consisted of germinal center B cells (±70% $CD38^+$, ±20% $sIgD^+$ and $CD39^+$); the high-density B cell fraction was enriched for mantle zone B cells (50% $sIgD^+$ and $CD39^+$, 40% $CD38^+$). For blocking experiments, the B cells were incubated for 30 min with anti–LFA-1 antibodies before culture. After 24 h of culture of the FLCL or fibroblasts, 0.5–2 × 10^6 freshly isolated tonsillar low-density or high-density B cells were added to the culture wells and incubated at 37°C for another 18 h. Subsequently the cultures were stained for 1 h at 37°C with 25 μg/ml of the fluorescent dye Hoechst 33258 (Sigma Chemical Co.) as described previously (13). Next, a cover glass (24 × 24 mm) was lowered into the well and excess culture medium was removed. The cell cultures were examined directly in their culture wells using a Leitz Orthoplan fluorescence microscope (E. Leitz, Wetzland, Germany) with Ploem Opak illumination. An NPL 25×/0.75 and a NPL 50×/1.00 oil immersion objective were routinely used.

Influence of Cultured FDC-like Cell Lines or FLCL Culture Supernatant on B Cell Proliferation. FLCL were harvested, resuspended, and seeded at 100–500 cells per well in 96-well culture plates (Costar) in IMDM/g plus 10% FCS and cultured for 24 h at 37°C. Next, 2 × 10^4 freshly isolated tonsillar B lymphocytes were added in the presence or absence of immobilized anti-IgM antibodies (Immunobeads, 5 μg/ml; Bio-rad Laboratories, Richmond, CA) and 100 U/ml rhIL-2 (generous gift of Eurocetus BV., Amsterdam, The Netherlands) or 100 U/ml rhIL-4 (DNAX) or in the presence of *Staphylococcus aureus* Cowan strain I (SAC, 1:10,000, Pansorbin, Calbiochem Corp., La Jolla, CA) and 100 U/ml rhIL-2. After 48 h of culture at 37°C, 0.3 μCi [^{3}H]thymidine (Amersham International, Amersham, UK) per well was added to the cultures and they were further incubated for 18 h. Cells were harvested onto glass fiber filters using a cell harvester (Titertek, Skatron, Lierbyen, Norway). Incorporation of [^{3}H]thymidine was assessed using an LKB-Wallac liquid scintillation counter (Bromma, Sweden) and the data were expressed as counts per minute. To study the effect of FLCL supernatant on B cell proliferation, FLCL culture supernatants were collected after 3 d of culturing FLCL in tissue culture flasks. The influence on B cell proliferation was assessed by stimulation of B cells, as described above, in the presence or absence of 50% vol/vol of FLCL culture supernatant.

Results

Infection of FDC with EBV Results in Continuous Proliferation of FDC. After infection with EBV, large adherent and slowly duplicating cells were obtained (Fig. 1). These cell cultures also contained EBV-transformed B cells, which could be largely removed by extensive washing of the culture wells. Depletion of LFA-1–positive cells before EBV infection, however, reduced the number of accompanying EBV-transformed B cells to a minimum.

After about 3 wk of culture, the cells were immunopheno-

typed and tested for the expression of EBV-associated antigens. In contrast to freshly isolated FDC, all EBV-transformed FDC-like cell lines expressed the EBV-encoded antigens latent membrane protein 1 (LMP-1), Epstein-Barr virus nuclear antigen 2 (EBNA-2) (Fig. 2), and the protein BCL-2 (data not shown), which is upregulated after EBV transformation (21), indicating the establishment of stable EBV-transformed FDC. The staining pattern of EBNA-2 closely resembles the pattern of EBNA-2 expression in EBV-transformed B cell lines as described by Young et al. (22), i.e., a characteristic granular labeling of the nuclei with unstained nucleoli. The expression of Ki-67 could only be detected in a small percentage of FLCL (data not shown).

The phenotype of the proliferating cells was studied by FACScan® (for membrane markers) or staining of cytospin preparations (for intracellular markers) using a panel of mAbs (Table 1). FLCL demonstrated high intercellular adhesion molecule 1 (ICAM-1) and gp93 expression and expression of CD40 and CD75 (Fig. 3). CD40 is a 44–48-kD integral membrane protein sharing structural homology with NGF-R, TNF-R,

Table 1. *Antibodies Used*

mAb	Marker/Isotype	Source
T cell associated		
OKT3	CD3/IgG2a	Ortho, Raritan, NJ
B cell associated		
B1	CD20/IgG2a	Coulter Corp., Hialeah, FL
BU32	CD21(CR2,C3dr)/IgG1	Binding Site, Birmingham, UK
IOB1a	CD21(CR2,C3dr)/IgG1	Immunotech, Luminy, France
MHM6	CD23/IgG1	Dakopatts, Glostrup, Denmark
S2C6	CD40/IgG1	Gift from Dr. S. Pauli, Stockholm, Sweden
EBU141	CD75/IgM	Gift from Dr. M. Gramatzki, Erlangen, Germany
Adhesion molecules		
CLB-LFA1/2	CD11a(LFA-1,α1)/IgG1	Gift from Dr. R. van Lier, CLB, Amsterdam
HP 2/1	CD49d(VLA-4,α4)/IgG1	Immunotech, Luminy, France
R/R-1	CD54(ICAM-1)/IgG1	Gift from Dr. T. Springer, Boston, MA
4B9	VCAM-1/IgG1	Gift from Dr. J. Harlan, Seattle, WA
Myeloid associated		
CLB44	CD11b(CR3,C3bi)/IgM	CLB, Amsterdam, The Netherlands
LeuM3	CD14/IgG2b	Becton Dickinson, Mountain View, CA
2E1	CDw32(FcγRII)/IgG2a	Immunotech, Luminy, France
D3	CD35(CR1,C3b)/IgG1	Gift from Prof. M. Daha, Leiden, The Netherlands
CLB-FcRgr	CD16(FcγRIII)/IgG2a	CLB, Amsterdam, The Netherlands
FDC associated		
12B1	FDC(gp93)/IgG2a	Immunotech, Luminy, France
DRC-1	FDC/IgM	Dakopatts, Glostrup, Denmark
BU30*	FDC/IgG	Gift from Dr. G. Johnson, Birmingham, UK
Nonlineage		
2D1	CD45(HLe-1)/IgG1	Becton Dickinson, Mountain View, CA
JC/70A	CD31/IgG1	Dakopatts, Glostrup, Denmark
F8/86	Factor VIII/IgG1	Dakopatts, Glostrup, Denmark
B8.12.2	HLA-DR/IgG2b	Immunotech, Luminy, France
124	BCL-2/IgG1	Gift from Dr. D.Y. Mason, Oxford, UK
Ki67	Nuclear antigen/IgG1	Dakopatts, Glostrup, Denmark
Cs1-4	LMP-1/IgG1	Dakopatts, Glostrup, Denmark
PE2	EBNA-2/IgG1	Dakopatts, Glostrup, Denmark
MAS-516	Human fibroblast/IgM	Sera-Lab, Crawley Down, Sussex, UK

* Not FDC specific. Also, some reaction with follicular mantle B cells and smooth muscle cells but not reactive with human fibroblasts.

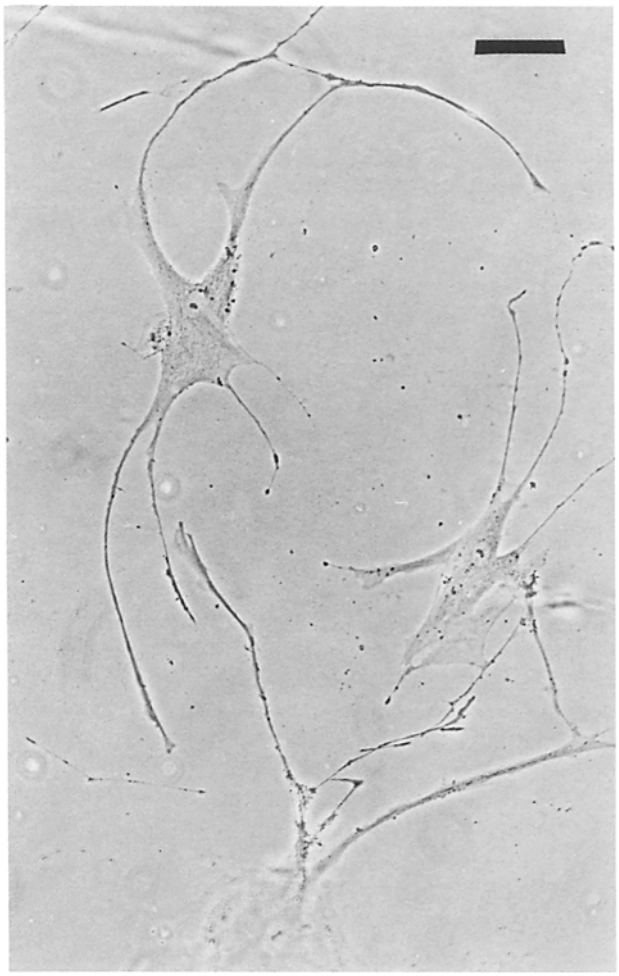

Figure 1. Photomicrograph of cultured FDC-like cells. Note long spiny processes and nuclei with clear nucleoli. ×120; bar, 0.1 mm.

CD27, and Fas antigen (23, 24) and is important in the proliferation and differentiation of B cells (25). CD75 is a cell surface carbohydrate antigen on a glycolipid and its formation is dependent of a α2-6 sialyltransferase (26–29). It is highly expressed on activated B cells. The functions of these molecules on FDC are presently unknown. Furthermore, FLCL did not express CD3, CD20, LFA-1, or VLA-4, indicating that these cell lines are not T cell or B cell derived. Also, CD45 is not found on FLCL, in line with recent reports describing that freshly isolated FDC do not express CD45 or contain CD45 mRNA (11). This supports the idea that the FLCL are not bone marrow derived. These FLCL did not express endothelial cell markers CD31 or factor VIII (von Willebrand factor).

Although FLCL shared some morphological details with human fibroblasts, the phenotype of FLCL indicated that these cells are FDC derived. The labeling for FDC-related markers DRC-1 and BU30 showed that these markers are present on freshly isolated FDC but disappear after 2–3 wk in culture. These data are in line with reports from other groups who also could not detect DRC-1 expression after prolonged culture of FDC (14, 18, 30). The FLCL did not express CD21 and HLA-DR, previously reported to be present on freshly isolated human FDC but lost after short periods of culture (14). VCAM-1 expression, also present on freshly isolated FDC (18, 31), could not be found on cultured FLCL. Incubation of FLCL for 24 h with 100 U/ml rhIL-4, resulted in upregulation of VCAM-1 expression which is in line with the data of Clark et al. (18), but not in reexpression of HLA-DR (Fig. 4). In contrast to Clark et al. (18) and to Tsunoda et al. (14), we did not find upregulation of HLA-DR after incubation of FLCL with 100 U/ml IFN-γ (data not shown).

Cloning of FDC-like Cell Lines. FLCL were cloned by culturing in limiting dilution; 2–3 wk after cloning, the number of wells with growing FLCL were scored. Cloning in culture medium or in the presence of GM-CSF or FLCL-conditioned medium resulted in poor cloning efficiency. In contrast, high cloning efficiency could be seen when cloning was performed in the presence of Raji/JY-conditioned medium. The comparative data of three experiments with different tonsils are given in Table 2. In subsequent experiments cloning was performed only in the presence of RAJI/JY supernatant. To date, using this procedure, FDC-like cell lines from at least 25 different tonsil donors have successfully been cloned and these clones are now growing in vitro for periods up to 8 mo. The highest proliferation rate is maintained when these clones remain cultured in the presence of Raji/JY-supernatant. After cloning, individual FLCL clones were tested for phenotypic and functional characteristics. The phenotype of individual clones was the same as was reported for the parental cell lines as is summarized in Table 3.

Functional Capacities of Cloned, EBV-transformed FDC-like Cell Lines. Freshly isolated nonautologous tonsil low-density or high-density B cells rapidly adhered to cloned FLCL upon co-culture (Fig. 5). Our observations suggest an intimate cellular contact between FLCL and B cells. This is in line with previous observations done by our group and by others for in vitro culture of freshly isolated FDC and B cells (13, 14, 32). The percentage of adherent B cells was considerably higher with low-density B cells in comparison with high-density B cells. The binding of B cells to FLCL could be prevented by the addition of antibodies against the adhesion molecule LFA-1 (Table 4). Staining of these cultures after 18 h with Hoechst 33258 revealed that cells adhering to cloned FLCL were viable, whereas B cells co-cultured with fibroblasts hardly made cell–cell contact to the latter, and as a consequence, most B cells displayed an apoptotic appearance (Table 4).

When freshly isolated FDC-enriched cell suspensions (±10% FDC, 10% T cells and 80% B cells) were stimulated with immobilized anti-IgM or SAC and IL-2, B cell proliferation was found to be decreased compared with FDC-depleted cell suspensions (Table 5). These data suggest an inhibiting effect of FDC on B cell proliferation. In line with these results, it was also found that addition of 100–500 cloned FLCL to cultures of in vitro–stimulated nonautologous tonsillar B lymphocytes resulted in decreased proliferation (Table 6). The presence of 500 human fibroblasts had no effect on B cell proliferation. Only with 10^4 human fibroblasts, was a minor inhibiting effect on B cell proliferation observed. To deter-

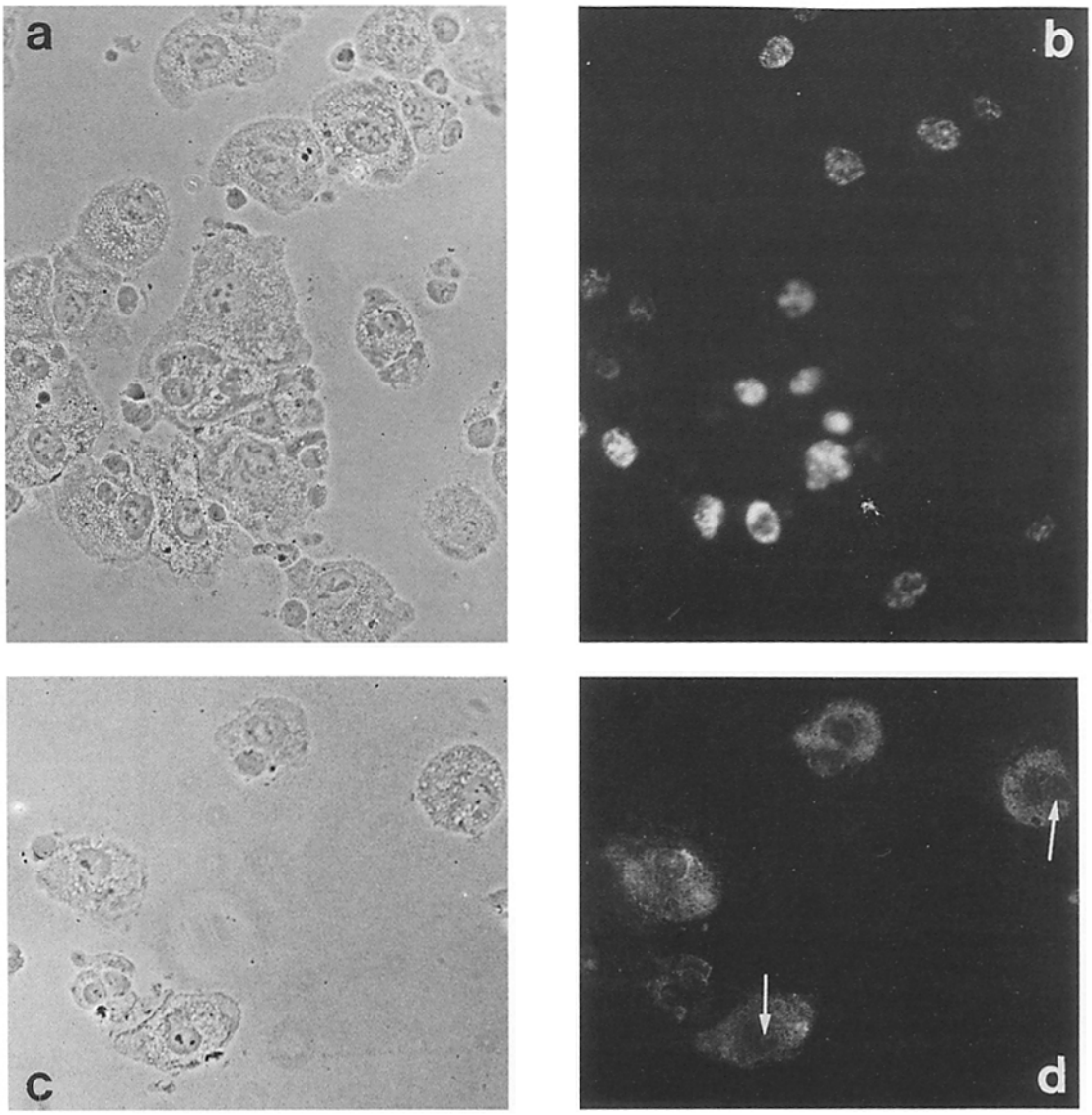

Figure 2. Cytospin preparations of FLCL stained with antibodies against (*a* and *b*) EBNA-2 or (*c* and *d*) LMP-1. EBNA-2 staining: (*a*) phase-contrast picture; (*b*) fluorescence picture showing staining of most nuclei (note nonstained nucleoli). LMP-1 staining: (*c*) phase contrast picture; (*d*) fluorescence picture showing staining of LMP-1–positive cells; note nonstained nuclei (*arrows*). ×320.

mine whether or not the observed inhibition of proliferation was cell–cell contact dependent or dependent on a soluble factor, we have investigated the influence of FLCL-culture supernatant on B cell proliferation. Under these conditions an inhibition of B cell proliferation was observed (Table 7), suggesting that part of the influence of FLCL on B cell proliferation is mediated by soluble factors. These results are in line with data from Freedman et al. (33) who also described inhibition of B cell proliferation by FDC or FDC-culture supernatants. In contrast, the presence of human fibroblast culture supernatant hardly influenced B cell proliferation.

Discussion

FDC are thought to play a crucial role in the development of high-affinity B cells and memory B cell formation during germinal center reactions. To study the exact nature of the functions of FDC in these processes we have attempted to immortalize FDC by EBV transformation of FDC-enriched cell suspensions.

As far as we are aware, EBV infection of FDC has not been demonstrated before. Binding of EBV to CD21 (complement/EBV receptor) on B cells is mediated by the viral envelope glycoprotein, gp350/220 (34) through a peptide that is highly homologous to a portion of the C3dg ligand (35). Because freshly isolated FDC express high amounts of CD21, FDC might very well be susceptible to infection by EBV. Expression in FLCL of the EBV-encoded proteins LMP-1, EBNA-2, and the protein BCL-2 upregulated by LMP-1 (21) after incubation with EBV-containing supernatant confirmed that FDC were effectively infected by EBV.

Recently it was described by others that human epithelial

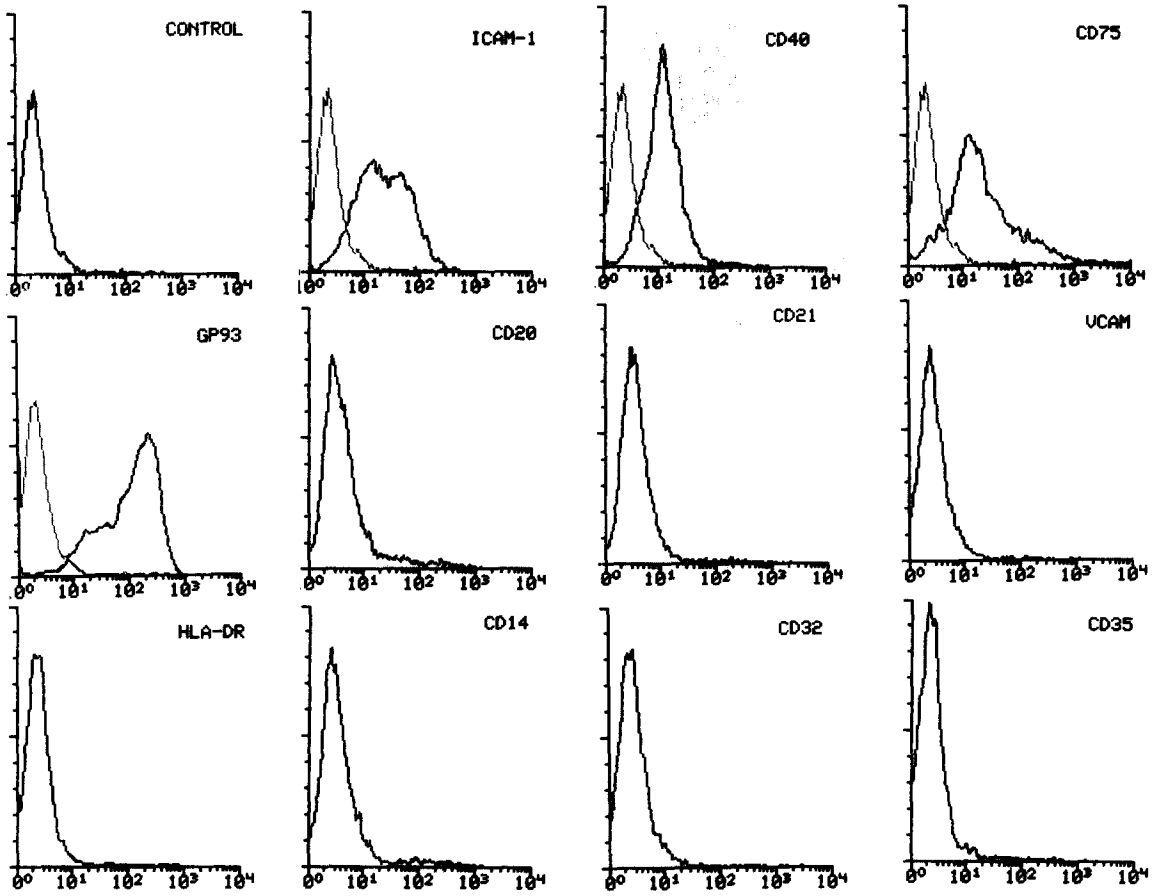

Figure 3. Histogram plots from FACScan® analysis of representative FLCL. Conjugate control is printed in the top left graph and as a dotted line in panels with positive staining. Histograms are plotted as log fluorescence intensity (*x*-axis) vs. relative cell number (*y*-axis).

Table 2. *Cloning of FCD-like Cell Lines*

		Addition			
Experiment	Cells/well	Medium	GM-CSF	Raji/JY-sup	FLCL-sup
1	1	0/24	0/24	5/24	0/24
	3	1/24	1/24	7/24	0/24
	10	7/24	3/24	21/24	7/24
2	1	0/24	0/24	3/24	0/24
	3	0/24	0/24	10/24	1/24
	10	3/24	1/24	10/24	3/24
3	1	0/24	1/24	0/24	0/24
	3	1/24	0/24	5/24	1/24
	10	6/24	2/24	8/24	7/24

Data are represented as number of wells with growth per number of wells seeded.

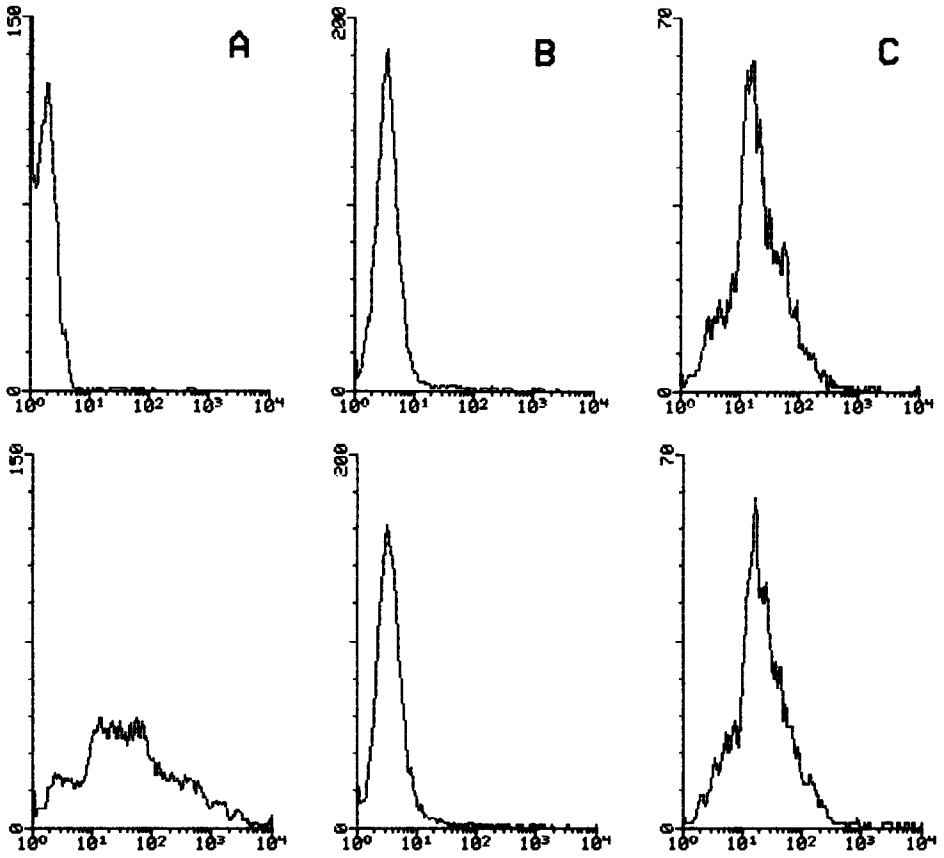

Figure 4. Histogram plots from Facscan analysis after incubation with rhIL4 of (*a*) FLCL labeled with antibodies against VCAM-1, (*b*) human fibroblasts labeled with antibodies against VCAM-1, or (*c*) FLCL labeled with antibodies against HLA-DR. Conjugate control is printed in the top graphs. Histograms are plotted as log fluorescence intensity (*x*-axis) vs. relative cell number (*y*-axis).

tumor cell lines either expressing CD21 (36) or transfected with CD21 cDNA (37) could be infected by EBV and that occasionally replication of the virus was observed. Whether or not virus replication occurs in EBV-infected FLCL is presently unknown.

The immortalized FLCL share morphologic characteristics with fibroblasts. They have dendritic processes and they are adherent to plastic. However, FLCL could be clearly distinguished from fibroblasts by several characteristic features. First, it appeared that FLCL proliferate very slowly (their duplication time is ~3–7 d, whereas human fibroblastic cell lines used as controls proliferate very rapidly. This slow proliferation rate can correlate with the low percentage of Ki-67–positive cells observed (±10%). Ki-67 is only present from late G1 till M phase and is not present in the G0 phase (38), indicating that only a small percentage of the cells is in cycle. FLCL appeared to display a unique FDC-like phenotype (Table 3) clearly different from that of five different established human fibroblastic cell lines. The FLCL phenotype includes expression of the adhesion molecule ICAM-1 (CD54) and of the B cell–related antigens CD40 and CD75. ICAM-1, CD40, and CD75, according to our observations and those of others (18, 39), are expressed by freshly isolated FDC but not by fibroblasts. Markers specific for T cells (CD3, CD11a, CD49d), markers specific for B cells (CD20, CD11a, CD49d), and the leukocyte common antigen CD45 were not expressed indicating that our FLCL are not lymphocyte-derived cells. Markers for endothelial cells (CD31, factor VIII) were not seen on these cells, indicating that they are not endothelial cell related. Typical FDC markers like DRC-1 were only expressed during the first 2 wk of culture. This phenomenon was also described by other investigators (14, 18). Furthermore, expression of VCAM-1 not found on FLCL could be induced on FLCL by incubation with rhIL-4 (Fig. 3), in line with the report of Clark et al. (18). In contrast to Clark et al., however, we could not induce expression of HLA-DR after incubation with IFN-γ.

Second, it could be demonstrated that FLCL display functional properties that are characteristic for human FDC. It appeared that both low-density as well as high-density B cells adhere very rapidly to FLCL upon co-culture, whereas B cells hardly adhere to human fibroblasts in our experiments. In general, the tendency of low-density B cells to adhere to FLCL was two to three times higher than of high-density B cells. We observed that the adherence of B cells to FLCL was very intimate and closely resembled the pattern of emperipolesis described by others for both in vivo as well as in vitro studies (30, 32, 40) for the interaction of B cells and FDC. Our observations however, do not allow definite conclusions about emperipolesis. The binding of B cells to FLCL was dependent on interaction between the adhesion molecules LFA-1 on the B cells and ICAM-1 on the FLCL, because binding

Table 3. *Immunophenotyping of Cultured FDC-like Cells*

Antibody	Marker	FDC	FLCL	HFB	Antibody	Marker	FDC	FLCL	HFB
T cell associated					FDC associated				
OKT3	CD3	−	−	−	12B1	FDC	+ +	+ +	±
B cell associated					DRC-1*	FDC	+ +	± / −	−
B1	CD20	−	−	−	BU30	FDC	+ +	−	−
BU32	CD21	+ +	−	−					
IOB1a	CD21	+ +	−	−	Nonlineage				
MHM6	CD23	+ / −	−	nt	2D1*	CD45	−	−	−
S2C6	CD40	+	+	−	JC/70A*	CD31	−	−	−
EBU141	CD75	+	+	−	F8/86*	Factor VIII	−	−	−
Adhesion molecules					B8.12.2	HLA-DR	+ +	−	−
CLB-LFA1/2	CD11a	−	−	−	124*	BCL-2	−	+ +	−
HP 2/1	CD49d	−	−	−	Ki67‡		−§	+§	+ +§
R/R-1	CD54	+ +	+ +	−	MAS516	αHuFB	+ +	+	+ +
4B9	VCAM-1	+ +	−	−	Cs1-4*	LMP-1	−	+ +	−
Myeloid associated					PE2*	EBNA-2	−	+ +	−
CLB44	CD11b	+	−	−					
LeuM3	CD14	+	−	−					
CLB5D2	CD16	−	−	−					
2E1	CDw32	−	−	−					
D3	CD35	+ +	−	−					

Rating scale: −, negative; +, positive; + +, strongly positive (arbitrary). Abbreviation: HFB, human fibroblasts.
* Determined on acetone fixed cytospins.
‡ Determined on acetone/methanol (1:1) fixed cytospins.
§ Only for Ki67 expression: −, negative; +, ⩽10% positive; + +, ⩾10% positive.

Table 4. *Clustering of B Cells to FLCL*

	Percentage of B cells in cluster (percent cells apoptotic)			Percentage of B cells single (percent cells apoptotic)		
	FLCL	HFB	FLCL + αLFA	FLCL	HFB	FLCL + αLFA
Expt. 1*						
LD B	39 ± 4 (5 ± 3)	2 ± 2 (0)	2 ± 1 (0)	61 ± 4 (85 ± 3)	98 ± 2 (82 ± 8)	98 ± 1 (85 ± 3)
HD B	14 ± 5 (2 ± 4)	2 ± 2 (0)	3 ± 2 (0)	86 ± 5 (74 ± 9)	98 ± 2 (76 ± 3)	97 ± 2 (71 ± 12)
Expt. 2*						
LD B	57 ± 16 (3 ± 4)	7 ± 4 (0)	6 ± 1 (0)	43 ± 16 (87 ± 6)	93 ± 4 (83 ± 2)	94 ± 1 (82 ± 4)
HD B	36 ± 8 (1 ± 2)	2 ± 2 (0)	3 ± 1 (0)	64 ± 8 (66 ± 10)	98 ± 2 (56 ± 18)	97 ± 1 (69 ± 15)

Data are represented as mean ± SD for four micrographs taken after 18 h of culture and 1 h of Hoechst 33258 staining; each micrograph depicts at least 150 lymphocytes.
* Two representative experiments with different donors. Abbreviations: LD B, low density B cells; HD B, high density B cells; HFB, human fibroblasts.

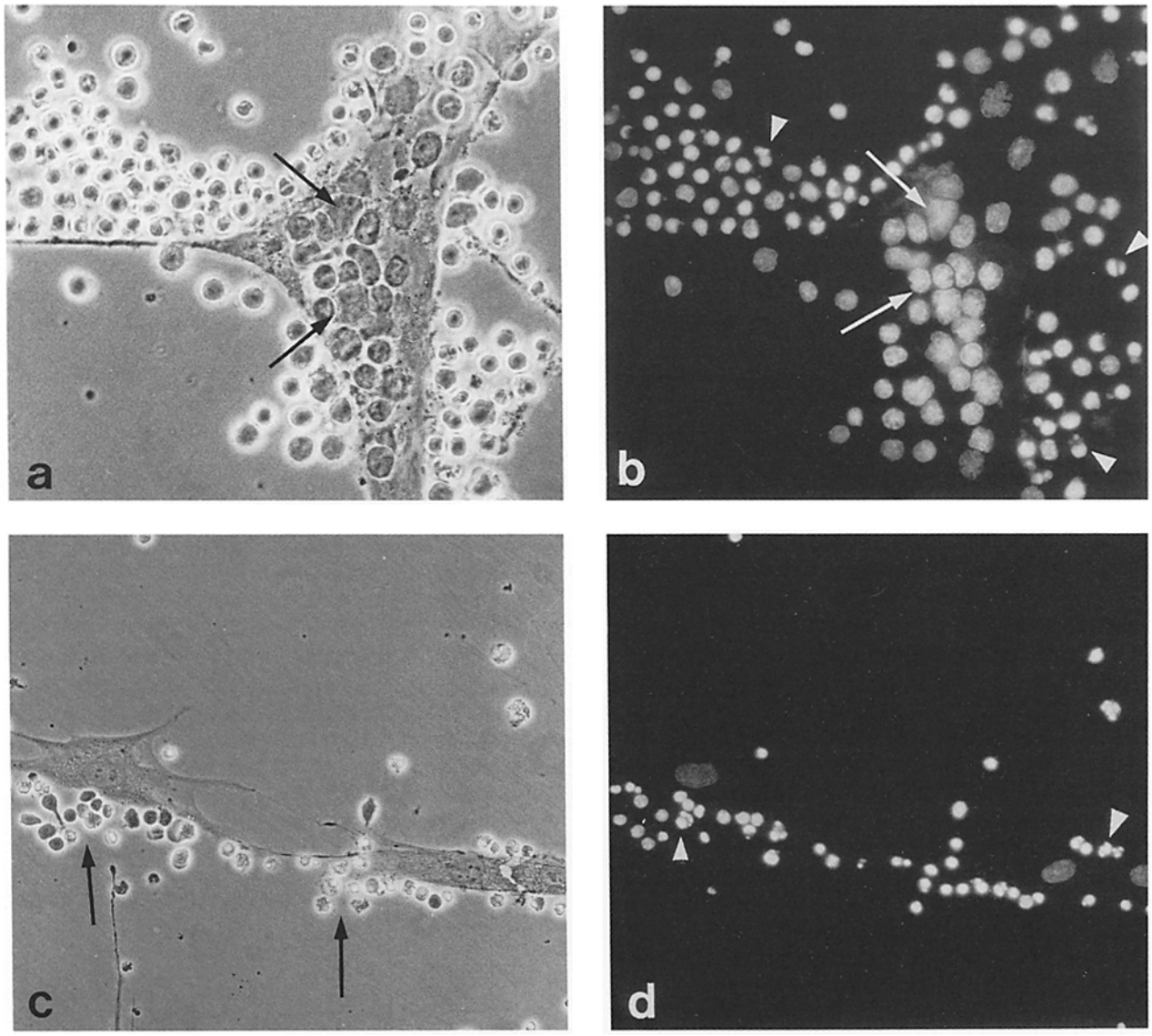

Figure 5. Cultures of nonautologous tonsil B cells with (*a* and *b*) FLCL or (*c* and *d*) human fibroblasts. Cultures were stained with Hoechst 33258. (*a*) Micrograph showing B cells adhering very closely to FLCL (*arrows*). (*b*) Fluorescence picture showing that adhering cells are viable (*arrows*), whereas nonadhering cells remain loosely accumulated against the outline of the FLCL and are mainly apoptotic as concluded from the bright and dense staining of their sometimes fragmented nuclei (*arrowheads*). (*c*) Micrograph showing that B cells loosely accumulate against the outline of human fibroblasts (*arrows*). (*d*) Fluorescence picture showing that most B cells became apoptotic (*arrowheads*). (*a* and *b*) ×640; (*c* and *d*) ×320.

of B cells to FLCL could be prevented with antibodies against LFA-1 (Table 4). This is in line with other reports (13, 31). Staining of cultures of FLCL and B cells with Hoechst 33258 revealed that B cells binding to FLCL were prevented from apoptosis, whereas the vast majority of nonbinding cells displayed an apoptotic appearance (84% of the nonbinding low-density B cells and 69% of the high-density B cells) (Fig. 5 and Table 4). This has also been described for B cells adhering to freshly isolated FDC (13). The difference found in apoptotic cells between low-density and high-density B cells is in line with experiments done by other groups in which it was demonstrated that germinal center B cells, in contrast to mantle zone B cells, die rapidly by apoptosis upon culture in vitro (41, 42). Furthermore it was observed that B cell proliferation in vitro was inhibited if B cells were stimulated in the presence of FLCL and not in the presence of HFB (Table 6). Part of the inhibitory effect of FLCL may be caused by soluble factors as the presence of FLCL-derived culture supernatants often resulted in decreased proliferation of B cells (Table 7). The nature of a putative inhibitory factor is presently unknown but experiments to reveal this are in progress.

The data on the inhibitory effect of FLCL and FLCL-culture

Table 5. *Effect of FDC on B Cell Proliferation*

	Expt. 1	Expt. 2
	cpm	
α-μ + IL-2 stimulation		
FDC enriched	2,862 ± 399	12,456 ± 2,777
FDC depleted	7,204 ± 286	24,989 ± 3,573
SAC + IL-2 stimulation		
FDC enriched	15,734 ± 692	11,860 ± 596
FDC depleted	22,328 ± 1,113	31,752 ± 2,295

FDC-enriched fractions (containing ± 10% DRC-1 positive cells) were depleted from FDC by labeling with BU30 mAb followed by goat anti-mouse Ig-coated Dynabeads. The resulting population contained ≤1% DRC-1 positive cells. 2 × 10^4 cells/well were stimulated with immobilized anti-IgM (α-μ) or SAC in the presence of 100 U/ml IL-2. Results are shown from two independent experiments using cells derived from different donors. Data are mean ± SD from triplicate wells.

supernatants are in line with previous experiments from our group using freshly isolated FDC (Table 5) and with data of Freedman et al. (33), who also showed that freshly isolated FDC or FDC culture supernatants inhibit B cell proliferation.

Previous studies in both the murine and human system, however, have suggested that FDC-enriched cell preparations may enhance B cell proliferation in vitro (32, 43–45). These contrasting results may be explained as follows: in vivo, FDC mainly are present in the light zone of germinal centers. In the dark zone of germinal centers, B cell proliferate very rapidly while only few FDC with low ICAM-1 expression are present. In the basal part of the light zone FDC form a dense network of high ICAM-1–positive cells (3). During germinal center reactions, it is in this latter area that the rapid proliferation of B cells is downregulated in close contact with FDC or in the close vicinity of FDC, suggesting an inhibitory effect of high ICAM-1 expressing FDC (which may be caused by soluble factors) on B cell proliferation. The present results therefore may reflect the actions of high ICAM-1–positive FDC in the basal light zone. Alternatively, the method for preparation of FLCL used in this study may have selected for FDC that can have a downregulatory effect on B cell proliferation.

With these FLCL we now have a helpful tool to unravel the functions of FDC in the processes of B cell differentiation and selection of high-affinity antigen-specific B cells.

Table 6. *Effect of FLCL on B Cell Proliferation*

	Expt. 1	Expt. 2	Expt. 3
		cpm	
α-μ + IL-2			
–	3,031 ± 611	1,281 ± 127	1,856 ± 381
FLCL 361	547 ± 82	603 ± 53	
FLCL 369	1,457 ± 123	890 ± 243	
FLCL 413			1,576 ± 129
FLCL 386			1,446 ± 32
HFB-VH (500/well)	2,001 ± 441	1,352 ± 118	1,917 ± 191
HFB-VH (10^4/well)			1,572 ± 233
SAC + IL-2			
–	10,252 ± 348	4,806 ± 89	6,984 ± 401
FLCL 361	536 ± 80	569 ± 14	
FLCL 369	2,491 ± 167	1,925 ± 337	
FLCL 413			3,504 ± 270
FLCL 386			3,120 ± 390
HFB-VH (500/well)	8,584 ± 652	5,031 ± 528	8,639 ± 1,028
HFB-VH (10^4/well)			4,991 ± 658

Tonsillar B cells were stimulated in the presence or absence of 500 FLCL or HFB-VH per well. 2 × 10^4 cells/well were stimulated with immobilized anti-IgM (α-μ) or SAC in the presence of 100 U/ml IL-2. Results are shown from three independent experiments using cells derived from different donors. Data are mean ± SD from triplicate wells.

Table 7. *Effect of Supernatant of Cultured FLCL on B Cell Proliferation*

	Expt. 1	Expt. 2	Expt. 3
		cpm	
α-μ + IL-2			
–	1,516 ± 265	2,328 ± 218	2,660 ± 188
+ sup 324	352 ± 107	1,396 ± 315	1,134 ± 430
+ sup 325	1,134 ± 389	3,028 ± 599	
+ HFB supernatant			2,943 ± 229
SAC + IL-2			
–	5,506 ± 306	7,156 ± 1,301	3,454 ± 322
+ sup 324	1,308 ± 198	2,682 ± 145	844 ± 198
+ sup 325	2,180 ± 548	3,964 ± 247	
+ HFB supernatant			2,918 ± 72

B cells are stimulated in presence or absence of 50% FLCL or human fibroblast (HFB) culture supernatant. 2 × 10^4 cells/well were stimulated with immobilized anti-IgM (α-μ) or SAC in the presence of 100 U/ml IL-2. Results are shown from three independent experiments using cells derived from different donors. Data are cpm ± SD from triplicate wells.

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References

1. Gray, D., and T. Leanderson. 1990. Expansion, selection and maintenance of memory B-cell clones. *Curr. Top. Microbiol. Immunol.* 159:1.
2. MacLennan, I.C.M., and D. Gray. 1986. Antigen-driven selection of virgin and memory B cells. *Immunol. Rev.* 91:61.
3. MacLennan, I.C.M., Y.J. Liu, and G.D. Johnson. 1992. Maturation and dispersal of B cell clones during T cell dependent antibody responses. *Immunol. Rev.* 126:143.
4. MacLennan, I.C.M., Y.J. Liu, S. Oldfield, J. Zhang, and P.J.L. Lane. 1990. The evolution of B-cell clones. *Curr. Top. Microbiol. Immunol.* 159:37.
5. Gerdes, J., H. Stein, D.Y. Mason, and A. Ziegler. 1983. Human dendritic reticulum cells of lymphoid follicles: their antigenic profile and their identification as multinucleated giant cells. *Virchows Arch. B Cell Pathol.* 42:161.
6. Heinen, E., C. Lilet Leclercq, D.Y. Mason, H. Stein, J. Boniver, D. Radoux, C. Kinet Denoël, and L.J. Simar. 1984. Isolation of follicular dendritic cells from human tonsils and adenoids. II. Immunocytochemical characterization. *Eur. J. Immunol.* 14: 267.
7. Johnson, G.D., D.L. Hardie, N.R. Ling, and I.C.M. MacLennan. 1986. Human follicular dendritic cells (FDC): a study with monoclonal antibodies (MoAb). *Clin. Exp. Immunol.* 64:205.
8. Parmentier, H.K., J.A. Linden van der, J. Krijnen, D.F. Wichen van, L.H.P.M. Rademakers, A.C. Bloem, and H.J. Schuurman. 1991. Human follicular dendritic cells: isolation and characteristics in situ and in suspension. *Scand. J. Immunol.* 33:441.
9. Petrasch, S., C. Perez-Alvarez, J. Schmitz, M.H. Kosco, and G. Brittinger. 1990. Antigenic phenotyping of human follicular dendritic cells isolated from non-malignant and malignant lymphatic tissue. *Eur. J. Immunol.* 20:1013.
10. Schnizlein, C.T., M.H. Kosco, A.K. Szakal, and J.G. Tew. 1985. Follicular dendritic cells in suspension: identification, enrichment and initial characterization indicating immune complex trapping and lack of adherence and phagocytic activity. *J. Immunol.* 134:1360.
11. Schriever, F., G. Freeman, and L.M. Nadler. 1991. Follicular dendritic cells contain a unique gene repertoire demonstrated by single-cell polymerase chain reaction. *Blood.* 77:787.

12. Sellheyer, K., R. Schwarting, and H. Stein. 1989. Isolation and antigenic profile of follicular dendritic cells. *Clin. Exp. Immunol.* 78:431.
13. Lindhout, E., M.L.C.M. Mevissen, J. Kwekkeboom, J.M. Tager, and C. Groot de. 1993. Direct evidence that human follicular dendritic cells rescue germinal center B cells from death by apoptosis. *Clin. Exp. Immunol.* 91:330.
14. Tsunoda, R., M. Nakayama, K. Onozaki, E. Heinen, N. Cormann, C. Kinet Denoël, and M. Kojima. 1990. Isolation and long-term cultivation of human tonsil follicular dendritic cells. *Virchows Arch. B Cell Pathol.* 59:95.
15. Hollowood, K., C. Pease, A.M. McKay, and C.D.M. Fletcher. 1991. Sarcomatiod tumours of lymph nodes showing follicular dendritic cell differentiation. *J. Pathol.* 163:205.
16. Weiss, L.M., G.J. Berry, R.F. Dorfman, P.M. Banks, E. Kaiserling, J. Curtis, J. Rosai, and R.A. Warnke. 1990. Spindle cell neoplasms of lymph nodes of probable reticulum cell lineage. *Am. J. Surg. Pathol.* 14:405.
17. Pallesen, G., and O. Myhre-Jensen. 1987. Immunophenotyping of neoplastic cells in follicular dendritic cell sarcoma. *Leukemia.* 1:549.
18. Clark, E.A., K.H. Grabstein, and G.L. Shu. 1992. Cultured human follicular dendritic cells. Growth characteristics and interactions with B lymphocytes. *J. Immunol.* 148:3327.
19. Miller, G., and M. Lipman. 1973. Release of infectious Epstein-Barr virus by transformed marmoset leucocytes. *Proc. Natl. Acad. Sci. USA.* 70:190.
20. Mevissen, M.L.C.M., J. Kwekkeboom, E. Goormachtig, E. Lindhout, and C. de Groot. 1993. Improvement of EBV transformation- and cloning-efficiency of human B cells using culture supernatants from lymphoblastoid cell lines. *Hum. Antib. Hybrid.* 4:66.
21. Henderson, S., M. Rowe, C.D. Gregory, D. Croom-Carter, F. Wang, R. Longnecker, E. Kieff, and A.B. Rickinson. 1991. Induction of BCL-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. *Cell.* 65:1107.
22. Young, L.S., C. Alfieri, K. Hennesy, H. Evans, C. O'Hara, K.C. Anderson, J. Ritz, R.S. Shapiro, A.B. Rickinson, E. Kieff, and J.I. Cohen. 1989. Expression of Epstein-Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease. *N. Engl. J. Med.* 321:1080.
23. Stamenkovic, I., E.A. Clark, and B. Seed. 1989. A B lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1403.
24. Itoh, N., S. Yonehara, A. Ishii, M. Yonehara, S.-I. Mizushima, M. Sameshima, A. Hase, Y. Seto, and S. Nagata. 1991. The polypeptide encoded by the cDNA for human cell surface antigen FAS can mediate apoptosis. *Cell.* 66:233.
25. Alés-Martínez, J.E., E. Cuende, C. Martínez-A., R.M.E. Parkhouse, L. Pezzi, and D.W. Scott. 1991. Signalling in B cells. *Immunol. Today.* 12:201.
26. Stamenkovic, I., H.C. Asheim, A. Deggerdal, H.K. Blomhoff, E.B. Smeland, and S. Funderud. 1990. The B cell antigen CD75 is a cell surface sialyltransferase. *J. Exp. Med.* 172:641.
27. Munro, S., B.J.E.G. Bast, K.J. Colley, and T.F. Tedder. 1992. The B lymphocyte surface antigen CD75 is not an α-2,6-sialyltransferase but is a carbohydrate antigen, the production of which requires the enzyme. *Cell.* 68:1003.
28. Stamenkovic, I., D. Sgroi, and A. Aruffo. 1992. CD22 binds to α-2,6-sialyltransferase-dependent epitopes on COS cells. *Cell.* 68:1003.
29. Bast, B.J.E.G., L.J. Zhou, G. Freeman, K.J. Colley, T.J. Ernst, J.M. Munro, and T.F. Tedder. 1992. The HB-6, CDw75, and CD76 differentiation antigens are unique cell-surface carbohydrate determinants generated by the β-galactoside α-2,6-sialyltransferase. *J. Cell Biol.* 116:423.
30. Tsunoda, R., M. Nakayama, E. Heinen, K. Miyake, K. Suzuki, N. Sugai, and M. Kojima. 1992. Emperipolesis of lymphoid cells by human follicular dendritic cells in vitro. *Virchows Arch. B Cell Pathol.* 62:69.
31. Koopman, G., H.K. Parmentier, H.-J. Schuurman, W. Newman, C.J.L.M. Meijer, and S.T. Pals. 1991. Adhesion of human B cells to follicular dendritic cells involve both the lymphocyte function–associated antigen 1/intercellular adhesion molecule 1 and very late antigen 4/vascular cell adhesion molecule 1 pathways. *J. Exp. Med.* 173:1297.
32. Petrasch, S., M.H. Kosco, C. Perez-Alvarez, J. Schmitz, and G. Brittinger. 1991. Proliferation of germinal center B lymphocytes in vitro by direct membrane contact with follicular dendritic cells. *Immunobiology.* 183:451.
33. Freedman, A.S., J.M. Munro, P. Schow, J. Daley, N. Lee, J. Svahn, L. Eliseo, and L.M. Nadler. 1992. Follicular dendritic cells inhibit human B-lymphocyte proliferation. *Blood.* 80:1248.
34. Tanner, J.E., J. Weis, D.T. Fearon, Y. Whang, and E. Kieff. 1987. Epstein-Barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping and endocytosis. *Cell.* 50:203.
35. Nemerow, G.R., R.A. Houghton, M.D. Moore, and N.R. Cooper. 1989. Identification of an epitope in the major envelope protein of Epstein-Barr virus that mediates viral binding to the B lymphocyte EBV receptor (CR2). *Cell.* 56:369.
36. Birkenbach, M., X. Tong, L.E. Bradbury, T.F. Tedder, and E. Kieff. 1992. Characterization of an Epstein-Barr virus receptor on human epithelial cells. *J. Exp. Med.* 176:1405.
37. Li, Q.X., L.S. Young, G. Niedobitek, C.W. Dawson, M. Birkenbach, F. Wang, and A.B. Rickinson. 1992. Epstein-Barr virus infection and replication in a human epithelial cell system. *Nature (Lond.).* 356:347.
38. Gerdes, J., H. Lemke, H. Baisch, H.H. Wacker, U. Schwab, and H. Stein. 1984. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J. Immunol.* 133:1710.
39. Möller, P., and B. Mielke. 1989. Leucocyte Typing IV: White Cell Differentiation Antigens. Oxford University Press, Oxford. 175-177.
40. Cormann, N., E. Heinen, C. Kinet Denoël, R. Tsunoda, and L.J. Simar. 1988. Isolation of follicular dendritic cells from human tonsils and adenoids: in vitro culture. *Adv. Exp. Med. Biol.* 237:171.
41. Liu, Y.J., D.E. Joshua, G.T. Williams, C.A. Smith, J. Gordon, and I.C.M. MacLennan. 1989. Mechanism of antigen-driven selection in germinal centres. *Nature (Lond.).* 342:929.
42. Mangeney, M., Y. Richard, D. Coulaud, T. Tursz, and J. Wiles. 1991. CD77: an antigen of germinal center B cells entering apoptosis. *Eur. J. Immunol.* 21:1131.
43. Cormann, N., F. Lesage, E. Heinen, N. Schaaf Lafontaine, C. Kinet Denoël, and L.J. Simar. 1986. Isolation of follicular dendritic cells from human tonsils and adenoids. V. Effect on lymphocyte proliferation and differentiation. *Immunol. Lett.* 14:29.
44. Kosco, M.H., E. Pflugfelder, and D. Gray. 1992. Follicular dendritic cell-dependent adhesion and proliferation of B cells in vitro. *J. Immunol.* 148:2331.
45. Burton, G.F., D.H. Conrad, A.K. Szakal, and J.G. Tew. 1993. Follicular dendritic cells and B cell costimulation. *J. Immunol.* 150:31.