Follicular Dendritic Cells Are Related to Bone Marrow Stromal Cell Progenitors and to Myofibroblasts[1]

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Follicular dendritic cells (FDC) are involved in the presentation of native Ags to B cells during the secondary immune response. Some authors consider FDC to be hemopoietic cells, whereas others believe them to be mesenchymal cells. The low proportion of FDC in the lymphoid follicle, together with technical difficulties in their isolation, make these cells difficult to study. We show that Fibroblast Medium can be used successfully to isolate and maintain FDC lines. In this culture medium, we obtained 18 FDC lines from human tonsils, which proliferated for as long as 18 wk and showed a stable Ag phenotype as detected by flow cytometry and RT-PCR. FDC lines were CD45-negative and expressed Ags associated to FDC (CD21, CD23, CD35, CD40, CD73, BAFF, ICAM-1, and VCAM-1) and Ags specific for FDC (DRC-1, CNA.42, and HJ2). These cell lines were also able to bind B cells and secrete CXCL13, functional activities characteristic of FDC. Nevertheless, the additional expression of STRO-1, together with CD10, CD13, CD29, CD34, CD63, CD73, CD90, ICAM-1, VCAM-1, HLA-DR, alkaline phosphatase, and α-smooth muscle actin (α-SM actin) indicated that FDC are closely related to bone marrow stromal cell progenitors. The expression of α-SM actin also relates FDC with myofibroblasts. Like myofibroblasts, FDC lines expressed stress fibers containing α-SM actin and were able to contract collagen gels under the effect of TGFβ1 and platelet-derived growth factor. These findings suggest that FDC are a specialized form of myofibroblast and derive from bone marrow stromal cell progenitors. ***The Journal of Immunology,*** **2006, 177: 280–289.**

Follicular dendritic cells (FDC)[3] (for review, see Refs. 1 and 2) are located in lymphoid follicles within the microenvironment of germinal centers. These cells retain native Ags in the form of immune complexes on their membrane for months, and present these Ags to B cells during the secondary response. FDC rescue bound B cells from apoptosis, and induce the differentiation of B cells into long-term memory B cell clones (3). Furthermore, FDC are involved in the pathogenesis of AIDS (4), of transmissible spongiform encephalopathies (5), and arthritis (6). The origin and cell lineage of FDC are controversial. Whereas their immune functions and expression of Ags associated with hemopoietic cells suggest that they belong to the hemopoietic lineage (7), their spindle-shaped morphology "in vitro," lack of CD45, and presence on FDC of Ags expressed by fibroblasts (8) indicate that FDC may be mesenchymal (nonhemopoietic) cells. Based on studies with mouse radiation chimeras, Humphrey et al. (9) concluded that FDC were not derived from the bone marrow, but came from a local mesenchymal precursor. However, Kapasi et al. (7), using mice homozygous for the SCID (*prkdc*^{scid}) mutation, which lack T, B lymphocytes, and FDC, demonstrated that after reconstitution with bone marrow from donor mice, the FDC of the reconstituted mice expressed the donor phenotype. These authors concluded that FDC precursors came from bone marrow. Studies with mice deficient in members of the TNF family such as TNF, lymphotoxin α (LTα) or LTβ, and their receptors such as TNF receptor type I (55 kDa) and LTβ receptor, which lack FDC, have provided important insights into the development of FDC (10).

To study the origin, phenotype, or functions of FDC, most authors obtain these cells from human tonsils and from mouse lymph nodes. However, FDC have been difficult to investigate because of problems with isolating cells to significant homogeneity or maintaining them in culture. The isolation of FDC for in vitro studies is difficult because they constitute only ~1% of the cells in lymphoid tissue, and form tight clusters that entrap B lymphocytes within their dendritic processes. The most common method is enzymatic digestion of the tissues followed by gradient centrifugation through Percoll. This produces an enriched population of FDC that is further purified by cell sorting with mAbs against CD14 (11) or against an FDC-specific Ag (12). These methods to obtain fresh FDC have several problems. It is difficult to dissociate bound B cells from FDC in vivo (11), and the presence of contaminating B cells may lead to spurious results when studying the FDC phenotype. The use of an anti-CD14 for cell sorting can also contaminate the FDC preparation with macrophages. When an FDC-specific mAb is used to avoid macrophage contamination, the number of purified FDC is normally insufficient to perform extensive experiments. To solve this problem, some authors have enriched and purified FDC by culture. Nevertheless, obtaining an FDC line is

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[3] Abbreviations used in this paper: FDC, follicular dendritic cell; α-SM actin, α-smooth muscle actin; BMSCP, bone marrow stromal cell progenitor; LT, lymphotoxin; PDGF, platelet-derived growth factor; DSC, decidual stromal cell; PRL, prolactin; ALP, alkaline phosphatase; BAFF, B cell-activating factor belonging to the TNF family.

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not a common achievement, and in the resulting cells, the expression of some Ags declines steadily (13–15). In this article, we show that Fibroblast Medium is a suitable culture medium to obtain FDC lines that maintain a stable antigenic phenotype. We observed that FDC were related to bone marrow stromal cell progenitors (BMSCP) (16, 17), and expressed stress fibers containing α-smooth muscle actin (α-SM actin) and exhibited cell contractility like myofibroblasts. The generation of FDC lines is very important because it will greatly facilitate the study of FDC.

Materials and Methods

Samples

The tonsil samples were obtained from patients with tonsillitis at the Hospital Universitario de San Cecilio (Granada, Spain). Patients (3–10 years old) were in complete remission before the intervention. Informed consent was obtained from the parents or guardians of each patient. This study was approved by the Comité Etico y de Investigación (Research and Ethics Committee) of the Hospital Universitario de San Cecilio.

Monoclonal Abs

The mAbs used in this study are shown in Table I.

Cytokines

TGFβ1, platelet-derived growth factor (PDGF), TNF, and LT$\alpha_1\beta_2$ were purchased from Sigma-Aldrich.

Tumor cell lines

Ramos, a human B lymphoma line, JEG-3, a human extravillous trophoblast choriocarcinoma cell line, and Meth-A, a mouse fibrosarcoma cell line, were cultured in RPMI 1640 medium (Sigma-Aldrich) with 100 U/ml penicillin, 50 μg/ml gentamicin, and 10% FCS. These tumor cells were used when they were in the log phase of growth.

Fibroblast Medium

According to the information provided by the manufacturer (Sigma-Aldrich), Fibroblast Medium consists of fibroblast basal medium (a modified version of the culture medium MCDB 105), and a supplement containing FCS (2%, final concentration), and unspecified amounts of basic fibroblast growth factor, heparin, epidermal growth factor, and hydrocortisone. Although Fibroblast Medium is no longer available from Sigma-Aldrich, with Fibroblast Growth Medium (Promocell), another low-protein medium, we obtained cell lines phenotypically similar to those reported in this work.

Isolation and culture of FDC lines

To establish FDC lines, we used the method described by Kim et al. (15) with modifications. Briefly, human tonsils freshly obtained from routine tonsillectomy were thoroughly washed in PBS solution and cut into small pieces that were finely minced between two scalpels in a small volume of RPMI 1640 medium with 100 U/ml penicillin and 50 μg/ml gentamicin. The suspension was put in a solution of 0.25% trypsin and 0.5 mM EDTA (Sigma-Aldrich) for 15 min at 37°C, and the reaction was stopped by adding cold RPMI 1640 with 20% FCS. The suspension was filtered through gauze and centrifuged at 425 $\times$ *g* for 10 min. The supernatant was discarded, and the cell pellet was suspended in RPMI 1640 and centrifuged on Ficoll-Paque (Pharmacia Biotech) for 20 min at 600 $\times$ *g*. Cells were collected from the interface, suspended in PBS, and washed. This suspension was incubated in culture flasks for 1 h at 37°C in complete RPMI 1640 with 10% FCS to allow macrophages and granulocytes to adhere to the flask. The supernatant cells were washed and incubated in Fibroblast Medium with 100 U/ml penicillin and 50 μg/ml gentamicin. After overnight incubation to allow adherent cells to attach to the flask, lymphocytes in the supernatant were discarded. Fibroblast Medium was then replaced and changed twice a week. After 2–4 wk, adherent cells were morphologically uniform and covered the whole surface of the 25-cm^2 culture flask. Cell viability was determined by trypan blue exclusion before any experiments were done. Only preparations with >95% viable cells were used.

Isolation of fresh FDC

To obtain a suspension of fresh cells containing FDC, we used a protocol similar to that reported by Montes et al. (18) for fresh decidual stromal cells (DSC). The tonsil was washed in PBS and minced between two scalpels in a small volume of RPMI 1640 with 10% FCS. The cell suspension was filtered through sterile gauze, washed by centrifugation, and suspended in culture medium. This suspension was centrifuged at 650 $\times$ *g* for 30 min over a discontinuous gradient of 20 and 30% Percoll (Pharmacia Fine Chemicals). Cells were collected from the 20%/30% interphase and

Table I. *mAbs used in this study*

mAb	Specificity	Ig Subclass	Conjugated with	Obtained from
Isotype control		IgG1	FITC or PE	Sigma-Aldrich
Isotype control		IgG2a	FITC or PE	Caltag
Isotype control		IgM	Unconjugated	Sigma-Aldrich
Isotype control		IgM	FITC	Caltag
OKT3	CD3	IgG2a	PE	Ortho-Diagnostic System
Anti-CALLA	CD10	IgG1	PE	Dako Cytomation
CD13	CD13	IgG1	FITC	Caltag
CD14	CD14	IgG2a	PE	Sigma-Aldrich
CD15	CD15	IgM	FITC	Caltag
CD21	CD21 (short isoform)	IgG1	PE	Caltag
DRC-1	CD21 (long isoform)	IgM		DakoCytomation
BU38	CD23	IgG1	FITC	The Binding Site
CD29	CD29	IgG1	FITC	Caltag
CD34	CD34	IgG1	FITC	Caltag
Anti-human CR1	CD35	IgG1	FITC	Biosource
CD40	CD40	IgG1	FITC	Caltag
CD45	CD45	IgG1	FITC	Sigma-Aldrich
ICAM-1	CD54	IgG2a	FITC	Caltag
CD63	CD63	IgG1	FITC	Caltag
CD73	CD73	IgG1	PE	BD Biosciences
Anti-human Thy-1	CD90	IgG1	FITC	Serotec
VCAM-1	CD106	IgG2a	FITC	R&D Systems
BAFF	CD257	IgG1	FITC	R&D Systems
Anti-α-SM actin	α-SM actin	IgG2a	FITC	Sigma-Aldrich
B4-78	Bone/liver/kidney isoenzyme of ALP	IgG1		Developmental Studies Hybridoma Bank
Anti-human FDC	CNA.42	IgM		DakoCytomation
OKDR	HLADR	IgG2a	FITC	Ortho-Diagnostic System
Anti-human FDC	HJ2	IgM	FITC	Sigma-Aldrich
STRO-1	STRO-1	IgM		Developmental Studies Hybridoma Bank

Table II. *Primer sequences used for RT-PCR*

mRNA	Oligonucleotide Primers	PCR Product Size (bp)
CD10	5′-AACATGGATGCCACCACTGAG-3′	525
	5′-CACATATGCTGTACAAGCCTC-3′	
CD13	5′-AAGCTCAACTACACCCTCAGC-3′	600
	5′-GGGTGTGTCATAATGACCAGC-3′	
α-SM actin	5′-ACTGTGTTATGTAGCTCTGGAC-3′	465
	5′-ACAATGGAAGGCCCGGCTTC-3′	
ALP	5′-GGACAAGTTCCCCTTCGTGG-3′	362
	5′-GCATGAGCTGGTAGGCGATG-3′	
Osteocalcin	5′-AGGCGCTACCTGTATCAATGG-3′	155
	5′-TAGACCGGGCCGTAGAAGC-3′	
CD34	5′-ACAACCTTGAAGCCTAGCCTG-3′	348
	5′-CAAGACCAGCAGTAGACACTG-3′	
CD21	5′-GGAGAGAGCACCATCCGTTG-3′	CD21 long isoform: 834
	5′-GCAGCGAGTCACAGGAGGAG-3′	CD21 short isoform: 657
CD45	5′-ACCAGGGGTTGAAAAGTTTCAG-3′	343
	5′-GGGATTCCAGGTAATTACTCC-3′	
PRL	5′-GGGTTCATTACCAAGGCCATC-3′	276
	5′-TTCAGGATGAACCTGGCTGAC-3′	
HLA-DRB	5′-CCTACTGCAGACACAACTACG-3′	154
	5′-TCAATGCTGCCTGGATAGAAAC-3′	

washed in PBS. Cell viability was determined by trypan blue exclusion. Only preparations with >95% viable cells were used. Five tonsil samples were studied independently by flow cytometry.

Treatment of cultured FDC with cytokines

Cultured FDC were preincubated in Fibroblast Medium for 1, 3, or 7 days with TNF or $LT\alpha_1\beta_2$ or both at concentrations of 10 or 100 ng/ml, and then their Ag phenotype was analyzed by flow cytometry.

Analysis of culture supernatants

To determine the concentration of prolactin (PRL) and osteocalcin, supernatants from confluent FDC cultures were collected, concentrated 10-fold in a Miniplus concentrator (Amicon). Osteoprotegerin and CXCL13 were analyzed in nonconcentrated supernatant. The presence of PRL was studied with an electrochemiluminescence immunoassay (Roche). Osteocalcin was investigated with an ^{125}I RIA kit (DiaSorin), osteoprotegerin was detected with an ELISA from Immundiagnostik. CXCL13 (BLC/BCA-1) was measured with an ELISA from R&D Systems.

Flow cytometry analysis

FDC were detached from the culture flask by treatment with 0.04% EDTA at 37°C. The cells were centrifuged, the supernatant was discarded, and the pellet was suspended in PBS at 10^6 cells/ml. For direct staining, 100 μl of the cell suspension was incubated with 10 μl of the appropriate mAb for 30 min at 4°C in the dark. Cells were washed, suspended in 0.5 ml of PBS, and immediately analyzed in a flow cytometer (Ortho-Cytoron; Ortho Diagnostic Systems). To identify dead cells, we incubated FDC with propidium iodide (Sigma-Aldrich). The percentage of cells that were Ab-positive was calculated by comparison with the appropriate isotype control (Table I). For double labeling, we followed the same procedure except that a second mAb with a fluorescent marker different from that of the first mAb was also added. For indirect labeling, FITC-labeled goat anti-mouse Ig was added after the first mAb. For intracytoplasmic labeling, FDC were fixed with 4% paraformaldehyde for 20 min at 4°C and permeabilized with cold acetone for 10 min before the mAb was added. Fresh FDC were gated based on side scatter and CD10 Ag expression.

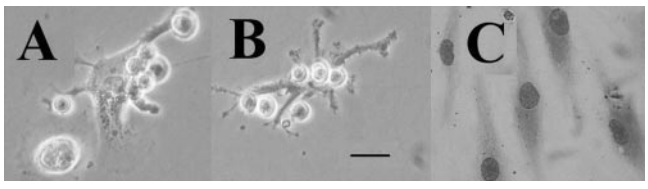

FIGURE 1. Fresh and cultured FDC. Fresh FDC cells with clustered lymphoid cells 24 h after isolation (*A* and *B*). FDC cultured in Fibroblast Medium showing fibroblast morphology (H&E staining) (*C*). Bar, 50 μm.

PCR primers

Primers used in this study are shown in Table II. They were designed according to sequences available from GenBank (⟨www.ncbi.nlm.nih.gov/GenBank⟩) and synthesized by Genset. To prevent the amplification of contaminant genomic DNA, sense and antisense primers were designed, when possible, from sequences located far apart on different exons, and tested in PCR with RNA used in cDNA synthesis.

RT-PCR

Total RNA from cells was extracted by the Ultrasec RNA isolation method according to the manufacturer's protocol (Biotecx Laboratories). A single-strand cDNA copy was made from total RNA using random hexamers (Amersham Biosciences) and Moloney murine leukemia virus H minus RNase reverse transcriptase (Promega). After heating to 65°C for 5 min and quickly cooling to 4°C in a thermal cycler (Geneamp PCR System 9600; PerkinElmer/Cetus) for denaturation, reverse transcription was performed for 1 h at 37°C. Starting with the equivalent of 75 ng of RNA, amplification was conducted in a total volume of 12.5 μl of the amplification mix, 10 mM Tris(trishydroxylmethylaminomethane)-Cl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, 0.2 mM deoxy-NTPs, 5% glycerol, 0.25 mM each primer, and 0.02 U/ml TaqDNA polymerase (Promega). After incubation for 5 min at 96°C, each cycle consisted of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s, for a total of 32 cycles. The PCR products were size-separated on ethidium bromide-stained 2% agarose gels, and a 100-bp DNA ladder was included in each run.

Immunofluorescence microscopy

FDC were plated onto slides in Fibroblast Medium. After 24 h cells were washed with PBS, fixed with 4% paraformaldehyde for 20 min at 4°C, and permeabilized with cold acetone for 10 min before the addition of fluoresceinated anti-α-SM actin (Table I). Preparations were examined with a Leica confocal microscope (Leica Microsystems).

Gel contraction assay

Cellular collagen gel contraction assays were performed as previously described (19). A sterile solution of purified, pepsin-solubilized bovine dermal collagen (Vitrogen; Cohesion Technologies) was prepared according to the manufacturer's instructions and combined with 25×10^4 FDC. The collagen/cell mixture (100 μl/well) was dispensed into culture plates and allowed to polymerize at 37°C for 30 min. Immediately after polymerization, 2 ml of Fibroblast Medium with or without the appropriate cytokine was added to each well. After incubation for 24 h, the height (*l*) and diameter (*d*) of each gel were measured with a microscope micrometer, and

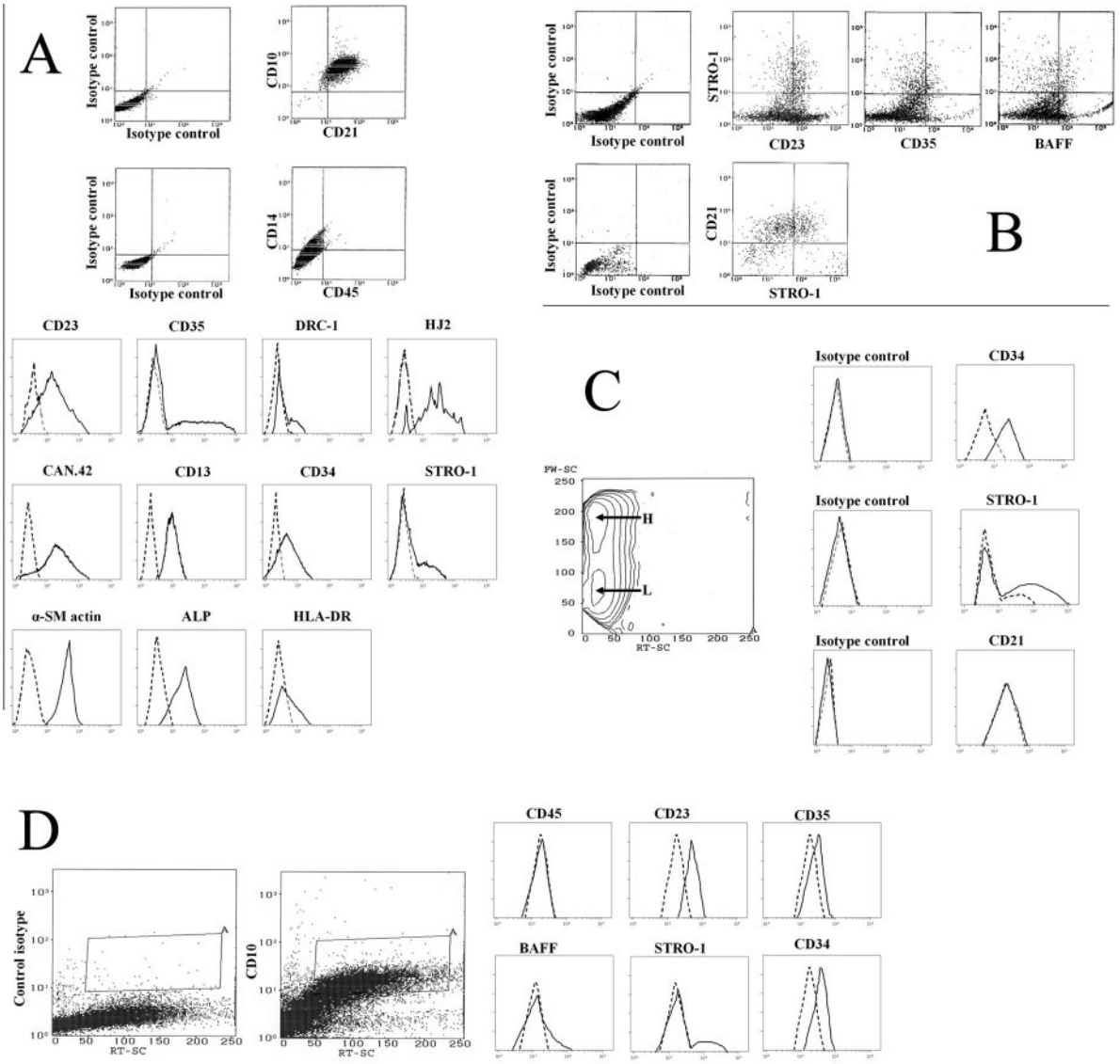

FIGURE 2. Flow cytometry analysis of Ag expression by FDC. *A*, Expression of Ags by FDC cultured in Fibroblast Medium. Isotype control (- - -); mAb (—). *B*, Coexpression of STRO-1 with FDC-associated Ags by FDC cultured in Fibroblast Medium. *C*, Differential expression of Ags by FDC of different sizes cultured in Fibroblast Medium. For this analysis, a high (H) and a low (L) forward-scatter gate were set. Cells gated in H (- - -; larger cells); cells gated in L (—; smaller cells). *D*, Expression of Ags by fresh FDC. Cells were gated based on CD10 Ag expression vs side scatter. Isotype control (- - -); mAb (—).

the volume (V) of each gel was calculated with the following formula: $V = 1/24 \times \pi \times l \times (3 \times d^2 + l^2)$.

The mean of the measurements ($n = 3$ for each sample) taken at each concentration point was used to estimate gel volume. The data are presented as the percent gel contraction of cytokine-treated FDC compared with cells cultured in the absence of cytokine, calculated with the following formula: cell contractility (percent) = 100 × (V with no cytokine – V with cytokine)/V with no cytokine.

As a negative control for cell contractility in gel, we used Ramos cells. These cells are unable to adhere to plastic or gel surfaces. They are therefore unable to contract gels, because this activity depends on adhesion to the gel matrix.

Binding assay

The method used was based on that of Clark et al. (14) with some modifications. Ramos cells as test binding cells, and Meth-A cells as control binding cells, were labeled by culturing at 10^5 cells/ml for 20 h at 37°C with 10 μCi/ml [^{3}H]thymidine (Amersham Biosciences), washed, and suspended at 5×10^5 cells/ml in complete RPMI 1640. Cultured FDC as test binder cells, and JEG-3 cells as control binder cells, were treated with 0.05% trypsin and 0.5 mM EDTA, resuspended in complete Fibroblast Medium and RPMI 1640, respectively, and then added to 96-well microtiter plates in quadruplicate for each experimental variable at 10^4 cells/well. After 20 h at 37°C, the plates were carefully washed with PBS, and 200 μl of [^{3}H]thymidine-labeled cells was added to each well. The plates were centrifuged at $5 \times g$ for 4 min, incubated for 6 h at 37°C, and then washed four times in PBS at room temperature. Two hundred microliters of trypsin was added per well, and samples were harvested and counted in a scintillation counter. The percentage of binding was calculated using the following formula: binding (percent) = 100 × mean experimental cpm/mean total cpm added.

Statistical analysis

Quantitative results were performed in triplicate or quadruplicate ± SD. Student's t test was used to compare results of the binding assay.

Results

Ag phenotype of FDC obtained in Fibroblast Medium

After 24 h of culture, adherent cells with attached lymphoid cells appeared (Fig. 1). During the first week of culture, lymphoid cells died and only a population of adherent cells with morphological features of fibroblasts survived (Fig. 1). With Fibroblast Medium,

we obtained 18 FDC lines which, with weekly passages, proliferated for 7–18 wk. After this period, the cells started to die. In these long-term cultures, proliferating cells overgrew other possible contaminant cells. Fig. 2*A* and Table III show the Ag phenotype (determined by flow cytometry) of these FDC, and demonstrate the presence of a population in which almost all cells were $CD13^+$, $CD10^+$, $CD73^+$, and α-SM $actin^+$. Ags CD21, CD29, CD63, alkaline phosphatase (ALP), and CNA.42 were also expressed by most of these cultured cells. Ags CD14, DRC-1, CD23, CD34, CD35, CD40, CD90, ICAM-1, VCAM-1, B cell-activating factor belonging to the TNF family (BAFF), HJ2, HLA-DR, and STRO-1 were also detected in these cells. Some FDC lines, however, did not express CD14, DRC-1, and HLA-DR at any time during culture. None of FDC lines expressed CD3, CD15, and CD45.

The absence of CD45 and the detection of Ags associated with FDC (CD21, CD23, CD35, CD40, CD73, ICAM-1, VCAM-1, and BAFF) (20–23), and Ags specific for FDC (DRC-1, CAN.42, and HJ2) (12, 24, 25) relate the cell lines with FDC. In contrast, the expression of STRO-1, an Ag specific for BMSCP (16, 17), relates the cell lines with these stromal cell progenitors, and this relationship is further strengthened by the expression of Ags associated to the BMSCP (CD10, CD13, CD29, CD34, CD63, CD73, ICAM-1, VCAM-1, α-SM actin, ALP, and HLA-DR, and the lack of CD3,CD15, and CD45) (16, 17, 26). Furthermore, the Ag phenotype of FDC lines is also related to that of other mesenchymal cells such as DSC (18, 27, 28), osteoblasts (29), and adipocytes (30). The expression of α-SM actin suggests that FDC lines are also related to myofibroblasts (31).

The fibroblastic morphology of the cultured cells, the expression by almost all of them of CD10, CD13, CD73, and α-SM actin, and the absence of CD3, CD15, CD45—all features characteristics of BMSCP—suggest that the cell lines constitute a homogeneous population. However, the detection of BMSCP-associated Ags (e.g., STRO-1) or FDC-associated Ags (e.g., CD23, CD35, BAFF, DRC-1) in some but not all of the cells shows the presence of subpopulations (Table III). The coexpression by some but not all the cells of STRO-1 with CD21, CD23, CD35, or BAFF (Fig. 2*B*) confirms the relationship between "BMSCP traits" and "FDC traits" and suggests the presence of cells in different stages of differentiation between BMSCP and mature FDC. The presence of subpopulations was confirmed by the detection in the cell lines of large and small cells (Fig. 2*C*), which expressed the same Ag phenotype (Table III) but differed in the intensity of expression of STRO-1 and CD34. Cells selected in a low forward-scatter gate (small cells) expressed these Ags more intensely than cells selected in a high forward-scatter gate (large cells) (Fig. 2*C*).

Table III. *Ag expression on cultured FDC (*n = *18)*

Antigen	Flow Cytometric Reactions (% of positive cells)
CD3	<1
CD10	95–99
CD13	94–97
CD14	<1–45
CD15	<1
CD21	69–99
DRC-1	1–37
CD23	8–74
CD29	74–99
CD34	21–52
CD35	15–32
CD40	24–65
CD45	<1
CD54 (ICAM-1)	30–85
CD63	75–92
CD73	95–99
CD90	42–80
CD106 (VCAM-1)	17–66
CD257 (BAFF)	7–24
α-SM actin	95–99
Bone/liver/kidney isoenzyme of ALP	79–99
CAN.42	67–93
HLA-DR	<1–23
HJ2	47–89
STRO-1	3–43

Ag phenotype of fresh FDC

Fresh FDC are difficult to study because these cells represent only 1% of the cells in the germ center. However, because we found that cultured DSC are closely related to FDC, to study fresh FDC we used a method similar to that used for fresh DSC (18). Flow cytometric analysis of Percoll-isolated low-density tonsil cells, gated by CD10 expression and side scatter, revealed the presence of cells with a phenotype similar to that of cultured FDC. $CD10^+$ cells were negative for CD45, but coexpressed CD23, CD34, CD35, BAFF, and STRO-1 (Fig. 2*D*).

Stability of the Ag phenotype of FDC lines obtained in Fibroblast Medium

We obtained an FDC line in RPMI 1640 with 20% FCS. Although stability of the surface Ag phenotype was greater than in other studies (13–15), most Ags, except for CD10, CD13, and α-SM actin, started to show down-modulation after 7 wk of culture. However, the FDC lines obtained in Fibroblast Medium maintained a stable Ag phenotype throughout the culture process (Fig. 3). Because previous attempts to maintain FDC with a stable Ag phenotype in culture were unsuccessful (13–15), the use of Fibroblast Medium appears to be suitable option to enrich and purify FDC for their study.

Detection of mRNAs in FDC lines

The expression of Ag mRNAs was studied by RT-PCR in four FDC lines obtained in Fibroblast Medium. CD10, CD13, α-SM actin, ALP, osteocalcin, CD34, CD21, and HLA-DRB mRNAs were detected in all FDC lines, whereas CD45 and PRL mRNA were not found in any of the four FDC lines (Fig. 4). The short CD21 isoform mRNA was detected in all the FDC studied; one cell line also expressed the long CD21 isoform mRNA (Fig. 4). This long isoform corresponds to DRC-1, an Ag specific for FDC (24) that was detected by flow cytometry to be weakly expressed in some but not all FDC lines (Fig. 2*A*, Table III).

Although FDC lines showed phenotypic characteristics similar to those of BMSCP, DSC, and osteoblasts (Table IV), we found some differences. FDC lines did not secrete PRL into the culture supernatant (Table IV) or express PRL mRNA (Fig. 4), although DSC produced PRL in vivo and in vitro (32, 33). Like osteoblasts and BMSCP, FDC lines expressed ALP and osteocalcin mRNA (Fig. 4), expressed ALP protein (Fig. 2*A*, Table III), and secreted osteoprotegerin (Table IV), but unlike osteoblasts and BMSCP, FDC lines did not secrete osteocalcin into the supernatant (Table IV).

FDC lines show morphological and functional features of myofibroblasts

We have shown that FDC express α-SM actin protein and mRNA (Figs. 2*A* and 4). This protein is considered a marker of myofibroblasts (31), cells with contractile activity involved in wound retraction, inflammation, and fibrosis (34). Studies with immunofluorescence microscopy, using a fluoresceinated mAb against

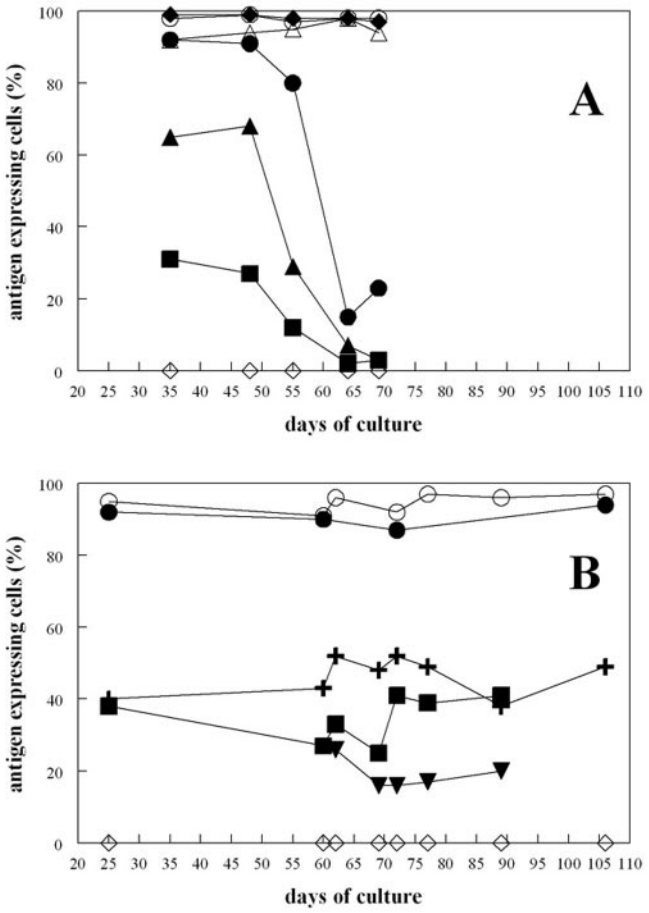

FIGURE 3. Variation over time of Ag expression by cultured FDC. Flow cytometric analysis of the variation over time of the expression of CD10 (○), CD13 (△), CD21 (●), CD23 (+), CD34 (■), CD35 (▼), CD45 (◇), α-SM actin (◆), and HJ2 (▲) by an FDC line obtained and cultured in RPMI 1640 containing 20% FCS (*A*) and by an FDC line obtained and cultured in Fibroblast Medium (*B*).

α-SM actin, showed a diffuse pattern of staining in the cytoplasm of untreated FDC, which revealed the presence of unpolymerized α-SM actin (Fig. 5*A*). PDGF or TGFβ1 increased the presence of α-SM actin in the stress fibers (Fig. 5*B*,*C*). Because the expression of α-SM actin in stress fibers increases the contractile activity of fibroblastic cells in vitro (35), we determined whether FDC were able to contract collagen gels in vitro under the effect of TGFβ1 and PDGF, two cytokines able to induce cell contractility in fibroblast cells (36, 37). We found that both cytokines induced cell contractility in FDC in a dose-response manner. Neither of these cytokines, however, showed any effect in the Ramos cells used as a negative control (Fig. 6).

Adherence of a B lineage cell to FDC lines

Binding of B cells is a specific feature of FDC (38) that was exhibited by the FDC lines obtained in our laboratory. When cells of the Ramos human B cell line were incubated with the FDC lines, these B cells strongly adhered to the FDC. FDC bound a significantly higher proportion of Ramos cells than Meth A cells (control binding cells). In contrast, JEG-3 cells (control binder cells) bound significantly fewer Ramos cells than did the FDC, and we found no significant differences between the adhesion of Ramos cells and Meth A cells to JEG-3 (Fig. 7).

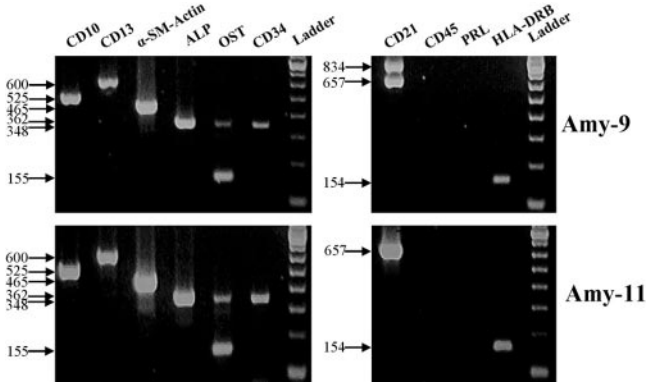

FIGURE 4. mRNA expression by FDC cultured in Fibroblast Medium. Expression of different mRNAs by two different FDC lines (Amy-9 and Amy-11) studied with RT-PCR.

Treatment of FDC lines with TNF and LTα$_1$β$_2$

Because TNF and LTα$_1$β$_2$ are cytokines responsible for the development of the germinal center and FDC in the mouse (10), we treated the cultured FDC with these cytokines. As reported previously (39–41), TNF and LTα$_1$β$_2$ increased the expression of VCAM-1 and ICAM-1 (Fig. 8*A*) and the secretion of CXCL13 (Fig. 8*B*). Although VCAM-1 seems to be a marker for the maturation of FDC in the germinal center (40), we did not observe significant modifications in the expression of other Ags that indicated complete maturation of FDC (Fig. 8). This suggests that, in addition to the effects of TNF and LTα$_1$β$_2$, other interactions are required for the differentiation of FDC. Experimental evidence in mice and humans showed that cell-cell interactions with B cells may be involved (42, 43).

Discussion

In previous studies, we isolated different DSC lines (cells of the decidua, the maternal tissue in close contact with the fetal trophoblast), and maintained them in culture (18). These cells exhibited a consistent Ag phenotype related to that of the BMSCP (28) and, like myofibroblasts, expressed α-SM actin and showed cell contractility in vitro (27, 44, 45). The fact that the DSC lines also expressed Ags associated with FDC (18, 27) prompted us to establish FDC lines and compare them with DSC lines. The present study shows that the FDC lines obtained in Fibroblast Medium have an Ag phenotype very similar to that of DSC lines, and are also related to BMSCP and myofibroblasts.

The origin, cell lineage, and differentiation of FDC are still controversial, because technical difficulties in isolating these cells have hindered their study. However, we show that Fibroblast Medium is a suitable aid to the isolation of FDC, as we obtained 18 FDC lines that showed a stable Ag phenotype (Figs. 2 and 4, Table III). FDC lines were able to bind B cells (Fig. 7), and secrete CXCL13 (Fig. 8*B*)—typical functions of FDC (38, 41)—and their Ag phenotype ($CD21^+$, $CD23^+$, $CD35^+$, $CD45^-$, $CD40^+$, ICAM-1^+, $CD73^+$, VCAM-1^+, $BAFF^+$) was compatible with that reported in previous studies of FDC (20–23). The expression by the FDC lines of DRC-1, CNA.42, and HJ2, Ags specific for FDC (12, 24, 25), further confirms their identity. Other Ags such as α-SM actin, CD29, CD63, and HLA-DR have also been reported to be expressed by FDC (20, 21, 46, 47). Furthermore, $CD10^+$ cells that coexpressed CD23, CD34, CD35, BAFF, and STRO-1, but were negative for CD45 (a phenotype compatible with that of cultured FDC), were found in fresh tonsil preparations (Fig. 2*D*). In previous reports, however, the detection of CD10 was inconsistent (21,

Table IV. *Comparison of phenotype markers and functional characteristics expressed by FDC lines with those of other mesenchymal cells*

Phenotype Marker	BMSCP	Adipocytes	Osteoblasts	DSC	FDC
CD3	−	−	−	−	−
CD10	+	+	+	+	+
CD13	+	+	+	+	+
CD14	−	−	−	−	+
CD15	−	−	−	−	−
CD21	ND[a]	ND	+[b]	+	+
CD23	ND	ND	+	+	+
CD29	+	+	+	+	+
CD34	+	+	+	+	+
CD35	ND	ND	ND	+	+
CD45	−	−	−	−	−
CD54	+	+	+	+	+
CD63	+	ND	ND	+	+
CD73	+	+	+/(−)[c]	+[b]	+
CD90	+	+	+	+	+
CD106	+	+	+	+	+
DRC-1	ND	ND	+	+	+
α-SM actin	+	+	+	+	+
ALP	+	+	+	+	+
CAN.42	ND	ND	ND	+	+
HJ2	ND	ND	+	+	+
HLA-DR	+	−	+	+	+
Osteocalcin	−/(+)[c]	+	+	−/mRNA+[b]	−/mRNA+
Osteoprotegerin	+	ND	+	+[b]	+
Prolactin	ND	ND	ND	−/(+)[c]/mRNA+	−/mRNA−
STRO-1	+	−/+[d]	+	+	+
Myofibroblast ultrastructure	+	+	ND	+	+
Cell contractility	ND	ND	ND	+	+
Hemopoietic cells-supportive activity	CFU-GM BFU-E CFU-Mix	Myeloid cells	Osteoclasts	Decidual NK cells	B cells
References	16, 17, 26, 31, 51, 54, 57, 58	30, 31, 51, 54, 59, 60, 61	29, 31, 51, 56, 62, 63, 64, 65, 66	18, 27, 28, 31, 33, 44	This study, 22, 31, 38, 52

[a] ND, Not done.
[b] Our unpublished result.
[c] Under differentiation conditions.
[d] Negative in Ref. 30, positive in Ref. 64.

48), and CD14, an Ag expressed weakly or not at all by our FDC lines (Table III), was strongly detected in other studies of FDC (21). Moreover, CD13 and CD34, expressed by cultured FDC in our results (Figs. 2*A* and 4, Table III), were not detected on FDC in two previous reports (11, 49). These discrepancies may be attributed to the fact that most of these studies were conducted with fresh nondividing FDC, probably end-differentiated cells (isolated in some reports with anti-CD14 mAbs; Ref. 9), whereas in the present study we selected proliferating FDC, probably precursor cells. This possibility is supported by the expression by fresh FDC and FDC lines of STRO-1 (Fig. 2, Table III), an Ag which identifies clonogenic BMSCP (16, 17). Although STRO-1 has not been previously studied in FDC, some authors detected BST-1, an Ag also found on BMSCP, on FDC lines (15, 46). The absence of CD45, and the presence of other Ags detected on our FDC lines such as CD10, CD13, CD29, CD34, ICAM-1, CD63, CD73, CD90, VCAM-1, HLA-DR, ALP, and α-SM actin, further support the relationship between FDC and BMSCP (16, 17, 26) (Table IV). However, STRO-1 was expressed in only a proportion of the cultured cells, as with FDC-associated Ags (Fig. 2, Table III). The coexpression of STRO-1 with CD21, CD23, CD35, or BAFF by some but not all the cells (Fig. 2*B*) confirmed the relationship between BMSCP and FDC, and suggested the presence of cells in different stages of differentiation. The detection of subpopulations of FDC—smaller cells that expressed STRO-1 and CD34 more intensely than larger cells (Fig. 2*C*)—appears to confirm this possibility. In this connection, it has been shown that STRO-1 and CD34 are differentiation Ags that are progressively down-modulated as the cells differentiate (17, 30). Other Ags such as CD14 and DRC-1, however, may be up-modulated by FDC during maturation. These two Ags were expressed only weakly or not at all by FDC lines in this study (Table III) and in early reports (14, 15), but were intensely expressed by fresh mature FDC (21). The fact that the DSC lines, which are similar in phenotype to FDC lines and are also related to the BMSCP, are actually precursors of DSC (18, 28), suggests that DSC and FDC lines are in an intermediate stage of differentiation between BMSCP and mature DSC and FDC, respectively. When we tried to induce differentiation in FDC lines with TNF and $LT\alpha_1\beta_2$—cytokines involved in the development of murine FDC (10), as previously reported (39, 40, 41)—we detected an increase in the expression of ICAM-1 and VCAM-1 (Fig. 8*A*) and in the secretion of CXCL13 (Fig. 8*B*), but no significant changes in the other Ags expressed by FDC lines. Although VCAM1 seems to be a marker for the maturation of FDCs in germinal centers (40), differentiation did not appear to be complete in the presence of TNF and $LT\alpha_1\beta_2$, because possible maturation Ags (e.g., DRC-1 or CD14), which were highly expressed in fresh FDC, were not up-modulated in our FDC lines (results not shown). In vivo experiments show that FDC differentiation requires the presence of B cells (42, 43). Additional experiments are therefore necessary to determine whether differentiation of FDC lines can be achieved in the presence of B cells or through the activation of Ags involved in FDC-B cell interaction.

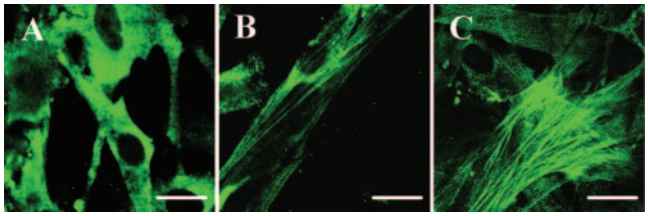

FIGURE 5. Expression of α-SM actin by FDC cultured in Fibroblast Medium. Immunofluorescence detection of α-SM actin in the cytoplasm of cultured FDC. A representative experiment of five FDC lines studied. α-SM actin was detected with a fluoresceinated mAb. A diffuse pattern of staining with almost no stress fiber was predominant (<5% of cells with stained stress fibers) in untreated FDC (*A*). PDGF (*B*) and TGFβ1 (*C*) increased the number of cells in which stress fibers were stained (42–63% for PDGF and 56–74% for TGFβ1). Bar, 50 μm.

STRO-1$^+$ bone marrow cells include fibroblast colony-forming cells and are similar if not identical, to mesenchymal stem cells (26, 50), as shown by the fact that they give rise to fibroblasts, adipocytes, osteoblasts, chondrocytes, and hemopoietic supportive stromal cells (51). Interestingly, osteoblast lines obtained under culture conditions similar to those used for DSC and FDC lines (29), and in vitro-differentiated adipocytes (30), show a phenotype similar to that of BMSCP, FDC, and DSC (Table IV). The expression of CD10, CD13, CD29, CD34, CD73, CD90, ICAM-1, VCAM-1, STRO-1, α-SM actin, and ALP; the absence of CD3, CD15, and CD45; and hemopoietic cell-supportive activity are common features to all these cell types (Table IV). FDC and DSC lines express osteogenic markers such as ALP (mRNA$^+$protein$^+$) and osteocalcin (mRNA$^+$protein$^-$) (Fig. 4) (28). Furthermore, like BMSCP and osteoblasts, FDC lines and DSC lines secrete osteoprotegerin (52) (Table IV). Although we cannot rule out that the in vitro conditions favor the persistence of the osteogenic markers, the fact that the osteogenenic pathway predominates in the in vitro differentiation of clonal mesenchymal progenitor cells (53) suggests a possible intrinsic commitment in all these cells. Nevertheless, DSC and FDC lines did not secrete osteocalcin in vitro, whereas the osteoblast lines did (29). DSC lines secrete PRL (18), which was not detected in the FDC lines (Fig. 4); these latter cells expressed CD14 (Fig. 2*A*, Table III), which was not found in any of the cell types listed in Table IV. These differences are probably related to the specific differentiation pathways of each cell type.

Although early experiments with mouse chimeras led to controversial conclusions (9), more recent results have proved that FDC derive from bone marrow (7). This, together with the expression of Ags associated with hemopoietic cells by FDC, led some authors to suggest that these cells belonged to the hemopoietic lineage (7). However, the relation between FDC and BMSCP reconciles the bone marrow origin of FDC and their mesenchymal (nonhemopoietic) characteristics. The close similarity of DSC lines, FDC lines, osteoblast lines, and adipocytes to BMSCP (Table IV) suggests that these progenitors emigrate from the bone marrow to the endometrium, secondary lymphoid organs, bone, and adipose tissue to differentiate into their respective lineages. The presence of cells with an Ag phenotype and multilineage potential similar to those of BMSCP in peripheral tissues appears to support this hypothesis (54). Further corroboration of the nonhemopoietic origin of the FDC cells comes from the absence of WASP protein, an adaptor protein that is expressed only in cells of hemopoietic origin (55).

A hotly debated subject regarding FDC is their expression of MHC class II Ags. Although many reports have shown that FDC express these Ags, others demonstrated their absence on these cells (20, 21). Denzer et al. (47) have observed that the expression of MHC class II Ags by FDC is due to the passive acquisition of exosomes, possibly derived from B cells. Nevertheless, our results showed that FDC lines express their own HLA-DR, because the long-term culture of these cells in the absence of B cells makes passive acquisition unlikely. Furthermore, the presence of HLA-DR mRNA in the FDC lines (Fig. 4) supports that these cells express these Ags. Our results, however, do not contradict with those of Denzer et al. (47), because MHC class II Ags expressed by FDC precursor may be down-modulated as these cells differentiate. FDC may then acquire exosomes passively from nearby B cells.

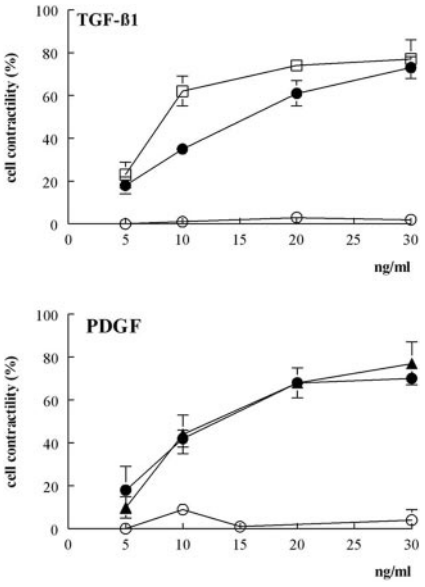

FIGURE 6. Contractility of FDC cultured in Fibroblast Medium. Induction of contractility in FDC lines by TGFβ1 and PDGF, determined by the collagen gel contraction assay. Each curve (□, ●, ▲) represents the contractile activity of different FDC lines. As a negative control for gel contraction we used Ramos cells (○). The mean of measurements (n = 3 for each sample) taken at each concentration point was used to estimate contractility. The data are presented as the percent gel contraction ± SD of cytokine-treated DSC compared with that of cells cultured in the absence of cytokine.

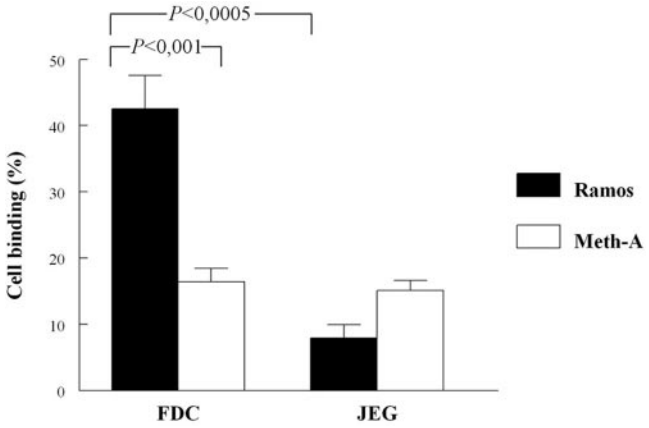

FIGURE 7. B cell binding by FDC cultured in Fibroblast Medium. Binding of Ramos B cells and control Meth A cells to cultured FDC and to control JEG cells. The data are presented as the percent cell binding ± SD (n = 4 for each sample). Ramos B cell binding to FDC was significantly higher than to control JEG cell, whereas FDC bound a significantly higher percentage of Ramos B cells than of control Meth A cells.

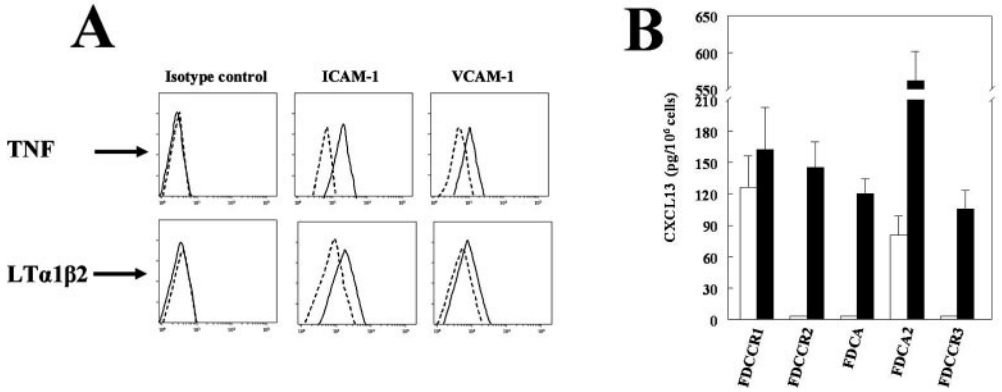

FIGURE 8. Effects of TNF and LT$\alpha_1\beta_2$ on FDC cultured in Fibroblast Medium. *A*, Flow cytometric analysis of the expression of ICAM and VCAM-1 by untreated cultured FDC (- - -) and cultured FDC treated with 10 ng/ml TNF or LT$\alpha_1\beta_2$ for 72 h (—). *B*, Secretion of CLCX13 by untreated cultured FDC (□) and by cultured FDC treated with 10 ng/ml LT$\alpha1\beta2$ for 72 h (■). Results from five different FDC lines are shown.

α-SM actin has been considered a marker of myofibroblasts (31)—cells with contractile activity involved in wound retraction (34). Although the presence of α-SM actin in FDC was reported previously (46), our results confirm the expression of α-SM actin protein and mRNA in FDC lines (Fig. 4). We also show that these cells, like myofibroblasts, express stress fibers containing α-SM actin (Fig. 5), and that FDC contracted collagen gels under the effect of TFG-β1 and PDGF (Fig. 6)—cytokines reported to induce myofibroblast contraction (36, 37). The facts that α-SM actin is also detected in BMSCP, osteoblasts, adipocytes, and DSC (16, 27, 30, 56) and that contractile activity has also been proved in DSC (44, 45) are further evidence of the relatedness of these cells (Table IV). The contractile function demonstrated in FDC may favor their activity in the stimulation of B cells during the secondary response (3). Contractile forces may help to increase the surface of the FDC membrane and thus maintain dendritic morphology, facilitating the interaction between FDC and B cells. As demonstrated for DSC (44, 45), Th1 and Th2 cytokines probably modulate FDC contractility and therefore their interaction with B cells.

Although the present work documents the contractile activity of FDC and their relation with BMSCP and other mesenchymal cells, the in vitro differentiation of FDC lines remains to be elucidated to shed light on the changes in their Ag phenotype and functions during differentiation.

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Disclosures

The authors have no financial conflict of interest.

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