Brief Definitive Report

Follicular Dendritic Cells Specifically Express the Long CR2/CD21 Isoform

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Summary

This paper describes an antibody (mAb 7D6) that specifically recognizes human follicular dendritic cells (FDCs). By expression cloning, a cDNA clone encoding for the long human CR2/CD21 isoform (CD21L) that contains an additional exon (10a) was isolated. We demonstrated that FDCs selectively express CD21L, while B cells selectively express the short CR2/CD21 lacking exon 10a (CD21S). By screening mouse Ltk$^-$ cells transfected with the CD21L cDNA, we further showed that the other two anti–human FDC mAbs DRC-1 and KiM4 also recognize CD21L. Thus, CD21L represents the first characterized human FDC-specific molecule, which may confer unique functions of FDCs in germinal center development.

Secondary lymphoid organs such as lymph nodes, spleen, Peyer's patches, and tonsils represent the major sites where immune responses to antigens occur. These lymphoid organs are divided into a T cell compartment (T cell zone or extrafollicular area) and a B cell compartment (B lymphoid follicle). While T cell zones contain interdigitating dendritic cells that play key roles in presenting antigen to naive T cells (1), B cell follicles contain follicular dendritic cells (FDC) that have the capacity to retain native antigen–antibody immune complexes for long periods of time (2, 3). During T cell–dependent humoral (antibody) immune responses, antigen-specific B cells undergo rapid clonal expansion within the FDC networks of B cell follicles, leading to the formation of germinal centers (GC) (4). During the course of clonal expansion, somatic hypermutation in immunoglobulin variable region genes (5, 6), and isotype switch in immunoglobulin constant region genes occur (7). After antigen-driven affinity selection (8), high affinity germinal center B cells will differentiate into either plasma cells or memory B cells (9–11). The functions of FDC in the GC reaction were proposed because of their selective localization and their ability to retain immune complexes. Both in vivo and in vitro experiments have suggested that FDC play important roles in GC B cell proliferation, survival, and differentiation (12–15). However, little is known at the molecular level of how FDCs contribute to GC development. Several monoclonal antibodies have been generated against FDCs (16, 17); however, the nature and function of the antigens recognized by these monoclonal antibodies are unknown. Here, we describe an antibody (mAb 7D6) that specifically recognizes human FDC. By expression cloning, using mAb 7D6, a cDNA clone encoding for the long isoform of CD21L (CD21L) that contains an additional exon (10a) was isolated. We show that FDC selectively express CD21L, while B cells selectively express the short CD21 (CD21S) lacking exon 10a. By screening mouse L cells transfected with the CD21L cDNA, we further demonstrate that the other two anti–human FDC mAbs, DRC-1 and KiM4, also recognize CD21.

Materials and Methods

Isolation of FDC from Human Tonsils by Percoll Gradient. Tonsils obtained from children undergoing tonsillectomy were cut into small pieces and digested for 12 min at 37°C with an enzyme cocktail in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) containing collagenase IV (1 mg/ml; Sigma Chemical Co., St. Louis, MO) and deoxyribonuclease I (50 kU/ml; Sigma Chemical Co.). The released cells were collected and a new stock of enzyme solution was added to the remaining tissue fragments for another 12 min. The cells, collected after two successive rounds of enzymatic digestion, were pooled and centrifuged through Ficoll-Hypaque (Eurobio, Paris, France) for 20 min at 400 *g* to remove red and dead cells. After two washes, cells were layered on a 1.5% BSA (Pentex® Path-o-cyte 5; Miles Inc., Kankakee, IL) gradient and centrifuged at 10 *g* for 10 min at 4°C. The FDC-lymphocyte clusters were recovered from the pellet. This BSA gradient process was repeated two to three times. The resulting cell population contains 15–30% FDC that form tight clusters with lymphocytes (13).

Isolation of a Highly Purified Single FDC Suspension by FACS® Sorting of CD14$^+$CD21$^+$ Large Tonsillar Cells. Since human B cells, T cells, fibroblasts, endothelial cells, and epithelial cells express no or low levels of CD14, and human T cells, fibroblasts, endothelial cells, and epithelial cells express no or low levels of CD21, CD14^{high}CD21^{high} FDC were isolated by FACS® sorting of enriched FDC preparations by Percoll gradient. After cell sorting, the resulting population contained >98% pure single FDC (Fig. 3). These highly purified FDCs may have been damaged inasmuch as they displayed cytoplasm losses and were unable to support B cell growth in vitro. However, these cells were used for PCR assays.

Purification of Tonsillar B Cells, Follicular Mantle B Cells, and GC B Cells. Briefly, tonsils were finely minced and the resulting cell suspension was subjected to two rounds of T cell depletion using first rosetting with sheep red blood cells, and then depletion with anti-CD3 magnetic beads. The resulting purified cells contained >97% CD19$^+$ B cells and <1% T cells and monocytes. To isolate IgD$^+$CD38$^-$ follicular mantle B cells and IgD$^-$CD38$^+$ germinal center B cells, total tonsillar B cells (10^7/ml) were incubated with anti–IgD-FITC and anti–CD38-PE in PBS containing 2% BSA (PBS–BSA) for 30 min. Cells were washed twice and suspended in PBS at 3×10^6/ml. The two B cell subpopulations were then purified by cell sorting. Two rounds of cell sorting were carried out to obtain >98% purity.

Generating FDC-specific mAb 7D6. BALB/c mice were immunized with 5×10^6 enriched human tonsillar FDC intraperitoneally three times at 3-wk intervals. The final boost was carried out 3 d before fusion. Using polyethylene glycol 1500 (Boehringer Mannheim GmbH, Mannheim, Germany), 50×10^6 splenic cells were fused with NS1 myeloma cells. Hybridomas were cultured in complete medium supplemented with 20% vol/vol FCS, hypoxanthine and azaserine, oxaloacetic acid, pyruvate, and insulin (OPI; Sigma Chemical Co.). Hybridomas were selected by immunohistological staining of the culture supernatants of the FDC networks on tonsillar tissue sections. Ascites was produced in BALB/c mice, and mAb 7D6 (IgG1) was purified by high-pressure liquid chromatography with an anion-exchange column (DEAE 5PW; Waters Chromatography Div., Milford, MA).

Immunoenzymatic Staining. Frozen sections from human tonsils, spleen, and thymus were washed in PBS for 5 min. The sections were incubated with mouse IgG1 mAb 7D6, anti-CD21, and anti-CD54, respectively, for 60 min. After washing for 5 min in PBS, the sections were incubated with sheep anti–mouse IgG1 for 30 min in PBS containing 10% human serum, and then with alkaline phosphatase coupled to mouse antibodies specific for alkaline phosphatase (APAAP complexes; Dako, Roskilde, Denmark). After a final washing, alkaline phosphatase was developed by Fast red substrate (Sigma Chemical Co.) which gives a red color. Cytospin preparations of FDC clusters were fixed in acetone for 10 min at 4°C. The slides were washed in PBS and incubated for 1 h with the anti-FDC mAb 7D6. After washing, the cytospin slides were incubated for 30 min with anti–mouse IgG1. The binding of antibody was revealed using APAAP method and developed by Fast red substrate.

cDNA Library Construction and Screening. Poly(A)$^+$ RNA was purified from a B lymphoblastic cell line IM9 established from a bone marrow sample of a myeloma patient. This cell line stained weakly (variable; ⩾28% positive cells) with mAb 7D6. cDNA library construction was as described (18) using the Superscript Reverse Transcriptase cDNA Synthesis Kit (GIBCO BRL). Double-stranded cDNA was size-fractionated using a Chromaspin-1000 column (Clontech, Palo Alto, CA) and ligated into the BstxI/NotI-digested pJFE14 expression vector (19).

A cDNA clone encoding the 7D6 antigen was isolated by a method similar to that described (18) except that cell sorting (FACS®), rather than panning, was used to enrich COS7 cells transiently expressing the 7D6 antigen (20). After a 1 h incubation with 50 μg/ml isotype IgG1 antibody to block binding to FcγR, COS7 cells were stained with 10 μg/ml biotinylated mAb 7D6. Cells which bound mAb 7D6 were detected with streptavidin–phycoerythrin (Becton Dickinson, Milpitas, CA). Plasmid DNA recovered from sorted cells was transformed into *Escherichia coli* DH10B for expansion and then reintroduced into COS7 cells. A cDNA clone (p7D6) with a 4 kb insert was identified which encoded the antigen recognized by mAb 7D6. The sequence of the cDNA insert was determined in part manually as described (18), and in part on an automated sequencer (Applied Biosystems, Foster City, CA) using Taq Dye Deoxy Terminator cycle sequencing.

Expression of the 7D6 Antigen. The 7D6 cDNA clone was expressed transiently in COS7 cells (18). Mouse Ltk$^-$ cells (L cells) stably expressing the 7D6 antigen were generated by cotransfection with a neomycin-resistance plasmid by the calcium phosphate method (GIBCO BRL). Cells which survived in 1 mg/ml G418 were selected for 7D6 expression by FACS® and also were positive for CD21 (CALTAG Labs., South San Francisco, CA).

PCR Assay to Detect the Expression of Short and Long CD21 Isoforms. mRNA was extracted from 10^4 FDC purified by FACS® sorting according to their high expression of CD21 and CD14 antigens. cDNA was obtained by reverse transcription (Superscript Reverse Transcriptase Kit; GIBCO BRL). PCR assay was performed using a 5′ primer UHCR2-1704 (GGAGAGAGCACCATCCGTTG), a 3′ primer ULCR2-2363 (GGGCAGCGAGTCACAGGAGGAG) (see Fig. 2), and a taq polymerase (Perkin-Elmer Corp., Norwalk, CT) in a thermal cycler. The first cycle of denaturation was at 94°C for 3 min, and then 35 cycles including 1 min of denaturation at 94°C, 2 min for primer annealing at 60°C, and 3 min of extension at 72°C. Complete extension was achieved for 10 min at 72°C. PCR products were loaded on a 1% low melting point gel for purification (WIZARD PCR DNA Purification System; Promega Biotec, Madison, WI). These products were ligated and cloned in the PCR^{tm}II vector with TA cloning kit (Invitrogen, San Diego, CA). Plasmids were extracted from individual bacterial colonies and both strands were sequenced on an automated DNA sequencer (Applied Biosystems) using PCR II vector primers (21 M13, and M13RP).

Results and Discussion

mAb 7D6 Selectively Stains FDC. mAb 7D6 was selected because it specifically stains FDC networks on tonsillar and splenic sections (Fig. 1, *A* and *C*). The reactivity on FDC networks was further confirmed by staining of isolated FDCs (Fig. 1, *G* and *H*). 7D6 antibody did not give any specific staining on sections from fetal thymus (Fig. 1 *E*) or fetal liver (not shown). There was no positive staining of mAb 7D6 on total cell suspensions of bone marrow and peripheral blood by FACS® analysis (not shown).

7D6 cDNA Encodes the CD21L. After screening over 50 cell lines including Burkitt's lymphoma cells, B lymphoblastoid cells, T cells, myeloma cells, monocytic cells, and erythroblastoid cells, we found that Raji (Burkitt's lymphoma cell), UD123, UD261, IM9 (lymphoblastoid B cells), and K562 (erythroblastoid) expressed low, but significant, levels of 7D6 antigen (7–28% of cells were positive). Accordingly, we isolated a 4-kb cDNA clone from the IM9 cDNA library by FACS® sorting. Transfection of COS7

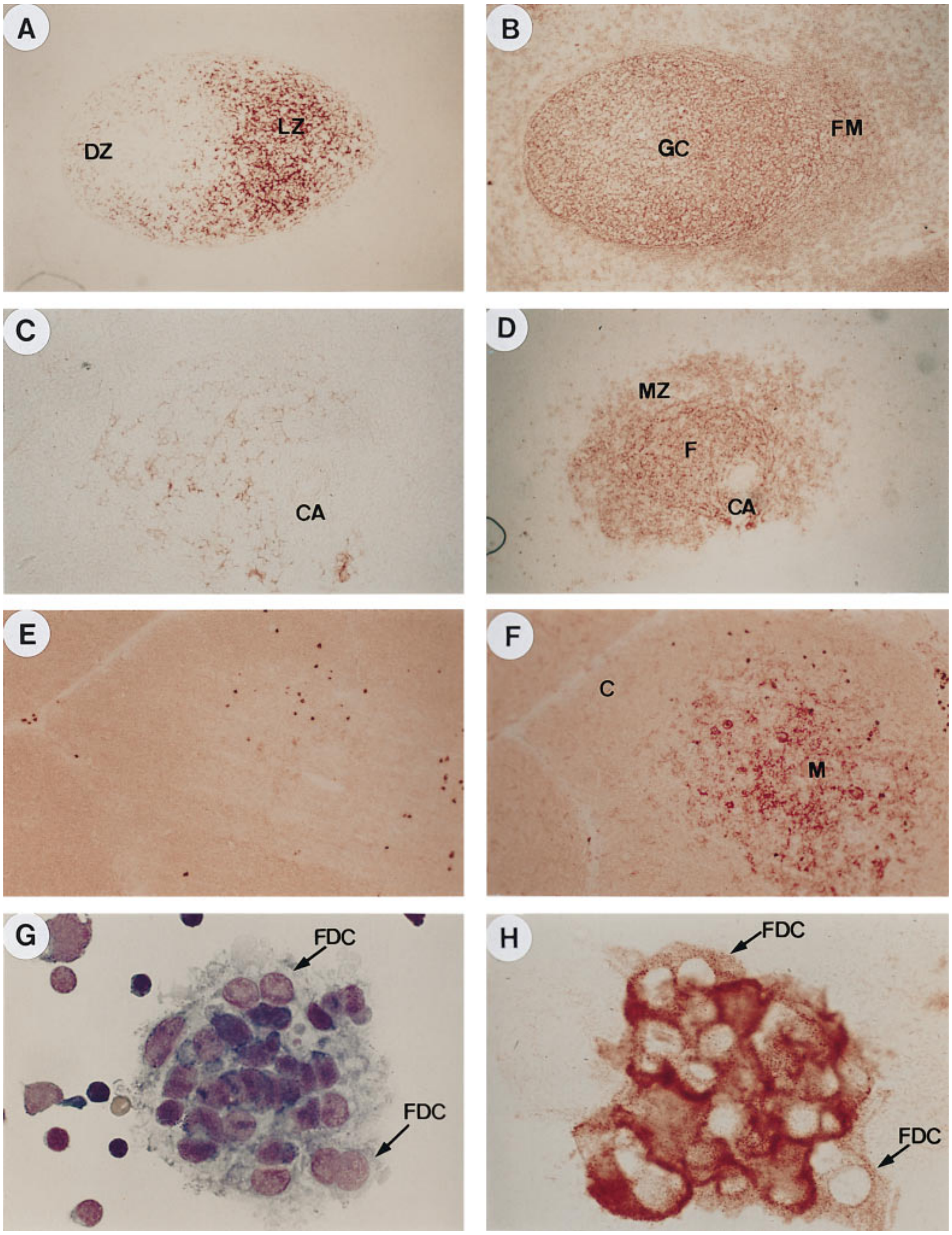

Figure 1. mAb 7D6 specifically stains FDC networks within tonsillar and splenic GCs or FDCs in isolated form. (*A*) Red mAb 7D6 staining of FDC networks within a GC of human tonsil (*DZ*, dark zone; *LZ*, light zone; ×200). (*B*) Red anti-CD21 staining of FDC networks and B lymphocytes within the follicular mantle (*FM*) and extrafollicular area (*A* and *B* show the same secondary follicle on two serial sections). (*C*) mAb 7D6 staining of FDC networks within a primary follicle of human spleen (*CA*, central arteriole; ×200). (*D*) anti-CD21 staining of FDC networks as well as follicular B cells and marginal zone (*MZ*) B cells (*C* and *D* show the same splenic white pulp on two serial sections). (*E*) Negative mAb 7D6 staining on human thymus (×200). (*F*) Positive staining of anti-ICAM1/CD54 on human thymus (*E* and *F* show the same thymic area on two serial sections) (*C*, cortex. *M*, medullar). (*G*) Giemsa staining of isolated FDC-lymphocyte clusters. FDC can be recognized as cells containing one or two big round nuclei with decondensed chromatin and clear nucleoli (×1000). (*H*) mAb 7D6 staining of isolated FDCs.

and L cells with p7D6 cDNA resulted in expression of the 7D6 antigen (not shown). The sequence of the p7D6 cDNA insert matched the sequence of long CR2/CD21 isolated from the Raji cell line (21), with several polymorphisms as described (22), and two additional ones: position 1979 [AGT (Ser) → A AT (Asn)] and position 2075 [CGT (Arg) → CAT (His)] (The sequence of the 7D6-reactive isoform of CD21 is available upon request). Consistent with this finding, all the anti-CR2/CD21 mAbs available from the Fifth International Leukocyte Typing Workshop that had been shown to stain both B cells and FDCs, stained p7D6 cDNA transfected Cos7 cells or L cells (not shown). However, mAb 7D6 is specific for FDC, and does not recognize CD21 expressed on other cells. Two CD21 isoforms have been described. A "long" form (CD21L) has an extracellular domain with 16 short consensus repeats (SCR) (Fig. 2), and is encoded by p7D6 and the clone described earlier (23). A short CD21 isoform was reported with an extracellular domain containing only 15 SCRs, the missing SCR (SCR10a) of 59 amino acids being encoded by 177 bp (Fig. 2) (23). Whether the mAb 7D6 epitope is encoded by SCR10a, or is a conformational determinant induced elsewhere in the molecule by the presence of SCR10a, is not known.

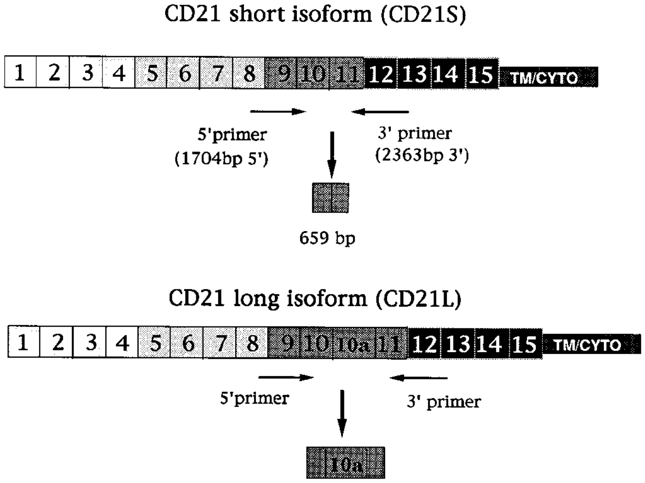

Figure 2. Diagrams of CD21S and CD21L and their detection by PCR assay. This figure is made according to Ahearn and Fearon (30). Boxes represent the short consensus repeats (SCRs). 15 (CD21S) and 16 (CD21L) SCRs are grouped into four long homologous repeats indicated by four different intensities of background. SCR10a is present in CD21L but not in CD21S. The PCR was performed using: (*a*) a 5′ primer starting at bp 1,704 within the CD21S cDNA sequence, covering part of the coding region of SCR10 (5′ GGAGAGAGCACCATCCGTTG); and (*b*) a 3′ primer starting at position 2363 within the coding region of SCR11 (3′ GGGCAGCGAGTCACAGGAGGAG). Accordingly, the predicted PCR products derived from CD21S and CD21L mRNA should be 659 and 736 bp, respectively.

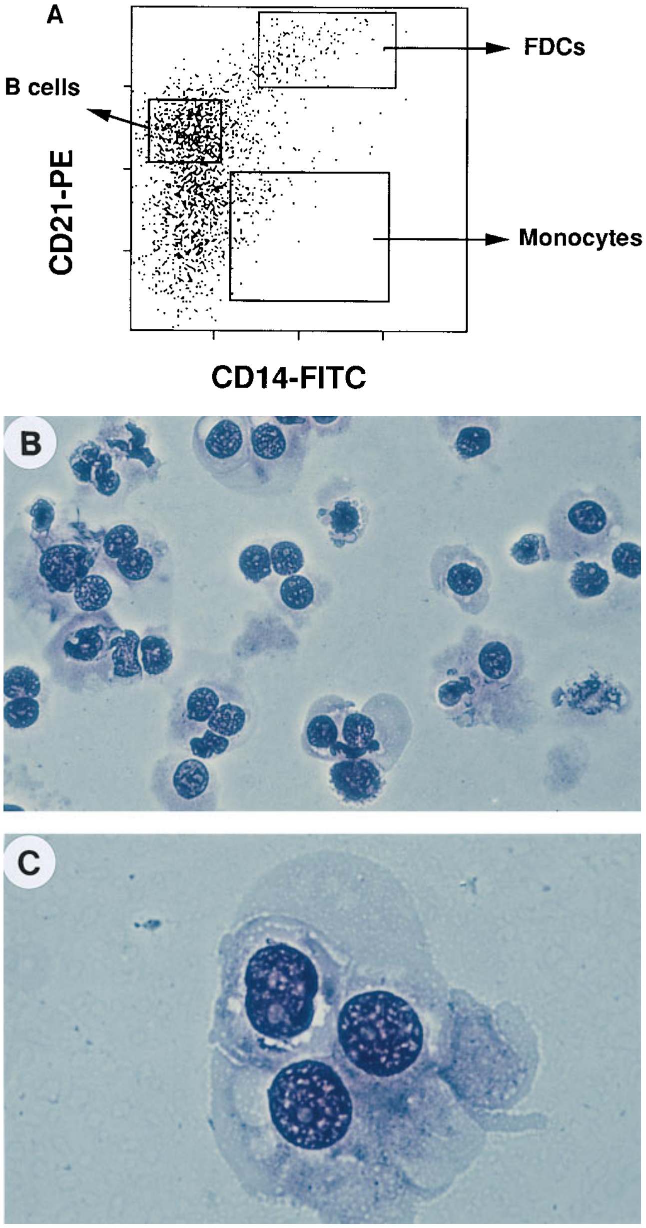

Figure 3. Isolation of highly purified FDC by FACS® sorting. (*A*) Low density tonsillar cells were stained by anti–CD21-PE and anti–CD14-FITC (detailed in Materials and Methods). FDCs were sorted according to their CD21^{++}CD14^{+} phenotype. B cells and monocytes could be recognized as CD21^{+}CD14^{-} and CD21^{-}CD14^{+} cells, respectively. (*B* and *C*) Giemsa staining of FACS® sorted FDC (×400 and ×1,000).

Human B Cells Selectively Express the CD21S; FDC Selectively Express CD21L. The pattern of mAb 7D6 staining suggests that FDC specifically express CD21L, while B cells specifically express CD21S. To directly test this hypothesis, a PCR assay using a 5′ primer starting from basepair 1704 and a 3′ primer starting from basepair 2363 of the short CR2/CD21 sequence, was carried out on RNA from 10^4 highly purified FDC (Fig. 3) in parallel with follicular mantle B cells (FM) and GC B cells isolated by FACS® sorting. Fig. 4 shows that a single large PCR product was generated from FDC, and a single smaller PCR product was generated from both FM and from GC B cells of the same donor. Further, sequencing analysis of these two PCR products shows that the FDC-derived large PCR product is 836 bp containing the 177-bp insertion that encodes the SCR10a. The B cell–derived PCR product is 659 bp, which does not contain the 177-bp insert (Fig. 5).

mAbs DRC-1 and KiM4 have been widely used as human FDC-specific antibodies, but the target antigen(s) have not been characterized (16, 17). Interestingly, both DRC-1 and KiM4 strongly and specifically stain COS7 cells as well as L cells transfected with CD21L cDNA (not shown). This indicates that 7D6, DRC-1, and KiM4 specifically recognize the CR2/CD21L that is selectively expressed by FDCs. The weak staining of DRC-1 and KiM4 on tonsillar B cells may be explained by the cross-reaction of these two antibodies to CD21S expressed on B cells.

In conclusion, the present study demonstrates that FDC express the SCR10a-containing CD21L, and this long form of CD21 appears to be a specific cell surface marker for FDC. In contrast, B cells express the short form of CD21. The low frequency of isolation of CD21L cDNAs from a tonsillar cDNA library reported earlier was probably due to the fact that FDC are considerably less abundant in tonsil than B cells (23). Thus, FDC and B cells exhibit a cell type–specific splicing mechanism for CD21 expression.

CR2/CD21 has been shown to play key roles in B cell activation and humoral immune responses. Monoclonal

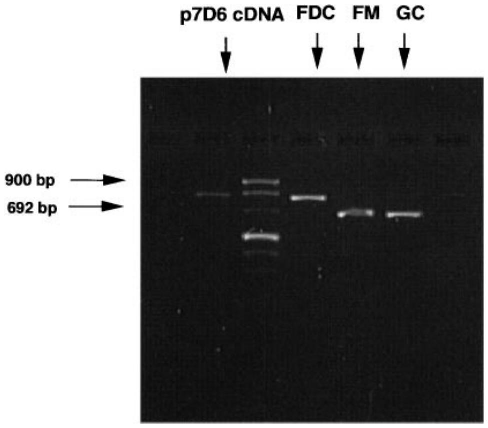

Figure 4. Detection of CD21S and CD21L mRNA by PCR among CD21^{++}CD14^{+} FDCs, IgD^{+}CD38^{-} FM, and IgD^{-}CD38^{+} GC B cells. The method for FDC isolation is detailed in Materials and Methods and in Fig. 3. PCR primers used are indicated in the legend for Fig. 2. p7D6 cDNA was used as a positive control.

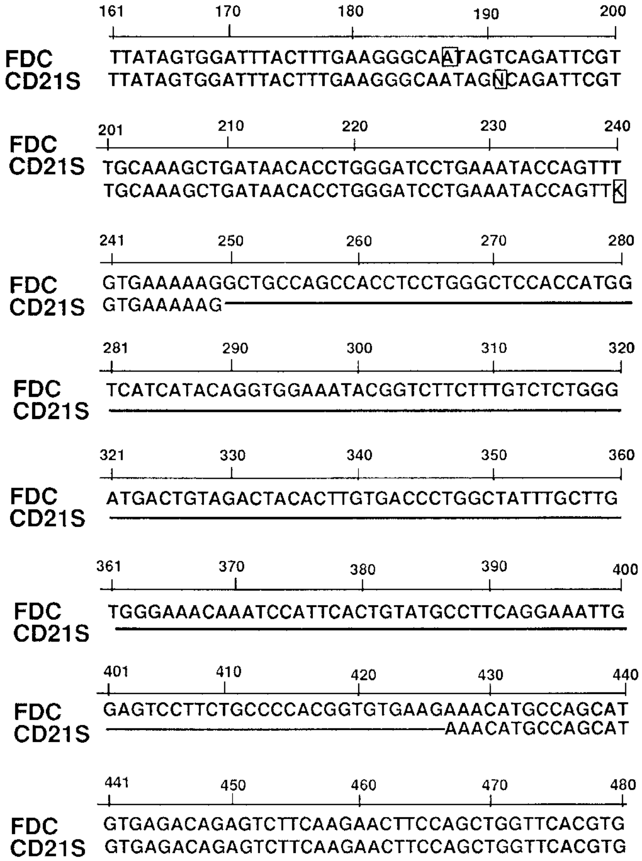

Figure 5. Partial sequence of the FDC-derived PCR product contains the 177 bp that encode for the SCR10a. The FDC-derived PCR product contains 836 bp. The sequence between positions 161 and 249 of FDC-derived PCR product encodes for a part of SCR10 that can be found in both CD21L and CD21S cDNA sequences. The 177 bp from position 249 to 426 can be found in CD21L and p7D6 cDNA sequences, but not in the CD21S cDNA sequence (*dash line*). The sequence from position 426 to 480 encodes for part of SCR11 that can be found in both CD21L and CD21S cDNA.

anti-CD21 and a recombinant CD21-Ig fusion protein suppressed IgG responses to T cell–dependent antigens in mice (24, 25). CD21-deficient mice exhibit deficiencies in a B cell (B-1a) compartment, and in their ability to generate a T-dependent antibody response and GC reaction (26). Remarkably, antigen (hen egg lysozyme; HEL) attached to C3d (HEL-C3d) was 1,000–10,000-fold more immunogenic than HEL alone when these antigens were administered to mice (27). Several possible mechanisms have been proposed for the biological functions of CR2/CD21: (*a*) long-term retaining and presenting native antigens in the form of immune complexes on FDC, (*b*) binding the B cell activation antigen CD23/FcεRII (28), and (*c*) serving as a co-receptor for B cell activation within the TAPA-1/CD19/CD21 complex (29).

The differential expression of CD21L and CD21S, respectively, on FDCs and B cells, may suggest their different functions. For example, understanding the functional significance of the additional SCR10a exon may provide a clue for explaining the mechanisms by which FDCs retain native antigen and costimulate GC B cells. The availability of anti-CD21L antibodies specific for FDCs will facilitate their purification after studies of their developmental pathway and their functional characterization.

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