ISOLATED HUMAN FOLLICULAR DENDRITIC CELLS DISPLAY A UNIQUE ANTIGENIC PHENOTYPE

By FOLKE SCHRIEVER, ARNOLD S. FREEDMAN, GORDON FREEMAN, ELLEN MESSNER, GRACE LEE, JOHN DALEY, AND LEE M. NADLER

From the Division of Tumor Immunology, Dana-Farber Cancer Institute; and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115

Lymphoid follicles are primary sites for the humoral immune response. Within the complex microenvironment of the lymphoid follicle, B cells receive a multitude of signals that influence their migration and differentiation. In the germinal center, follicular dendritic cells (FDCs) are physically associated with B cells by forming a dense three-dimensional network. These cells play a central role by trapping and delivering immune complexes to B cells.

Although morphologically similar, FDCs can be distinguished from the lymphoid dendritic cells (interdigitating reticulum cells), which were first described by Steinman et al. (1, 2). Whereas FDCs are localized to primary and secondary follicles, lymphoid dendritic cells are found in T cell-rich interfollicular areas. The latter cells are functionally distinct in that they are potent stimulators of T cells in autologous and allogeneic mixed lymphocyte reactions (3–6). Consistent with their function to fix immune complexes, FDCs express all three receptors for the third complement component (CR1, CR2, and CR3), whereas lymphoid dendritic cells do not (6).

The cellular origin of FDCs is still unknown. Attempts have been made to define the lineage of these cells by in situ and in vitro staining with mAbs directed against lineage-restricted and -associated antigens (7–9). These investigations demonstrated that FDCs express CR1, CR2, and CR3, as well as the marker DRC-1. However, the expression of other B and myeloid markers has been more controversial. Since germinal center B cells are in such close proximity to FDCs, it is not possible to conclusively distinguish in situ whether staining is localized to FDCs or to B cells. Similarly, partially enriched FDCs form dense clusters with lymphoid cells in vitro (7) and, therefore, it has been difficult to precisely determine their antigenic phenotype. In the present study, unclustered FDCs were isolated and phenotypically characterized. These cells display a unique phenotype in that they expressed both B cell and myeloid markers, as well as many adhesion molecules. Using their antigenic and morphologic characteristics, FDCs were isolated to homogeneity by flow cyto-
metric cell sorting. These highly purified FDCs did not express IL-6 mRNA. It may now become possible to further clarify the role of these cells in cell-cell interaction and cytokine production within the microenvironment of the germinal center.

Materials and Methods

**Antibodies.** Antibodies used in the present study are listed Table I. Many of the reagents were provided by the Fourth International Workshop on Human Leukocyte Differentiation Antigens. Other antibodies were either produced in our laboratory, obtained through companies (listed in Table I), or were generous gifts of the following investigators: Dr. A. Albino (J143; Memorial Sloan-Kettering Cancer Center, New York); Dr. C. Damsky (B1E5; UCSF, San Francisco); Dr. C. Dinarello (rabbit anti-IL-6; Tufts University, Boston); Dr. S. Gillis (Immunex, Seattle); Dr. J. Griffin (904, Mol, Mo2, AML-2-23, 322, My7, IV-3 D3, My9, L4F3, 12-8; Dana-Farber Cancer Institute, Boston); Dr. M. Hemler (TS2/7; Dana-Farber Cancer Institute); Dr. R. Hynes (rabbit anti-VLA-β1; Massachusetts Institute of Technology, Cambridge); Dr. J. Johnson (63D3; Institut für Immunologie, München, FRG); Dr. T. Kishimoto (rabbit anti-rhIL-6; Institute for Molecular and Cellular Biology, Osaka, Japan); Dr. D. Mason (R4/23; John Radcliffe Hospital, Oxford, England); Dr. C. Morimoto (2H1, 4B4, GAP P3, 2H4, 8F2; Dana-Farber Cancer Institute); Dr. J. Ritz (J2, J5, 2F12, 10F12, S6F1, NKH-1; Dana-Farber Cancer Institute); Dr. C. Rudd (AIA5; Dana-Farber Cancer Institute); Dr. A. Sonnenberg (GOH3; Central Laboratory for the Netherlands, Amsterdam); Dr. T. Springer (RR.1; Center for Blood Research, Boston); Dr. A. Wardlow (rabbit anti-CD18; Center for Blood Research). Biotinylated Bl (CD20) and B2 (CD21) were obtained from Coulter Immunology (Hialeah, FL). mAb 543 (CD35) was produced from a hybridoma cell line (American Type Culture Collection, Rockville, MD). The working concentration of the antibodies was confirmed by positive control stainings of reactive cell populations. Biotinylation of mAbs and preparation of phycoerythrin-streptavidin conjugations were done according to Daley (10). In all experiments mouse and rat lgs of the corresponding isotype and preimmune rabbit serum, respectively, were used as negative controls.

**Immunophenotyping.** Reactivity of a large panel of antibodies with FDCs was determined by immunoperoxidase staining on cytospin preparations of tonsillar low density cells. Cells were spun onto microscopic slides with a cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, PA), air dried, and fixed with acetone for 3 min. Mouse, rat, and rabbit antibody binding was detected using affinity-purified peroxidase-conjugated antiserum of rabbit anti-mouse Ig, (P260; Dako Corp., Glostrup, Denmark), rabbit anti-rat Ig (P162; Dako Corp.), and swine anti-rabbit Ig (P399; Dako Corp.), respectively. Antibody reactivity was visualized with 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO) (0.25 mg/ml in 0.1 M acetate buffer, pH 5.0) with 0.003% H2O2. Nonspecific antibody binding to Fc receptors was inhibited by diluting the antibodies in 10% human serum in PBS. Cells were counterstained with Meyer's hematoxylin. To quantitate the percentage of FDC clusters among total FDCs, immunoperoxidase staining with mAbs against CR1, CR2, and CR3 was used. Briefly, in all cell preparations equal numbers of FDCs were counted over a representative microscopic field. Clustered FDCs were defined as cells that contained one or more lymphocytes trapped in their cytoplasmic processes. In some experiments specific antibody binding to FDCs was confirmed by double labeling FDCs using a combination of immunoperoxidase and immunofluorescence. Briefly, after incubation with antibodies and peroxidase-coupled antiserum, cytospin preparations were labeled with biotinylated mAbs against CD21 (mAb B2) and CD35 (mAb 543), followed by incubation with streptavidin/phycoerythrin complex. Immunoperoxidase staining was subsequently developed as described above, which did not affect the immunofluorescence staining. Antibody binding to the cell surface of viable FDCs in suspension was visualized by using fluoresceinated goat anti-mouse Ig (Tago Inc., Burlingame, CA).

**Cytochemical Analyses.** Specific and nonspecific esterase staining was performed using naphthol AS-D chloroacetate esterase and α-naphthylacetate esterase (Sigma Chemical Co.), respectively, as substrate. Cytoplasmic peroxidase was detected with the substrate shown above.

**Phagocytosis Assay.** To test FDCs for their ability to phagocytize, low density cell prepara-
**Antibodies Used for the Present Study**

| Antibody | Source | CD/specificity | CD/specificity | Source |
|----------|--------|----------------|----------------|--------|
| CD1a T6A, T6 | Coulter | CD35 | 543 | ATCC |
| CD1b T099, T074, T070 | WS | CD37 | HD28, G28-1 HH1 | WS |
| CD1c T042 | WS | CD38 | 5D2; GR7A4; | |
| CD2 T11(2); T11(3) | | CD39 | G28-8; AC2 | WS |
| CD3 T3 | WS | CD40 | G28-5; EA-5 | WS |
| CD4 T4 | Coulter | CDw10 | Gp IIb/IIIa | Dako |
| CD5 T1 | Coulter | CD10 | 2H4 | |
| CD6 2H1 | | CD45 | GAP P3 | |
| CD7 T159, T160, T161 | WS | CD45R | 2H4 | |
| CD8 T8 | Coulter | NK | NKH-1 | |
| CD9 J2 | | B5 | | |
| CD10 J5 | | B7 | | |
| CD11a 2F12 | | Surface IgG | | |
| CD11b 904; M01 | | Surface IgD | | |
| CD11c p150/95 | Dako | Surface IgM | | |
| CD13 My7 | | Surface λ | | |
| CD14 AM1-2-23, 322, M02 | | Surface λ | | |
| CD16 Leu-11b | BD | ICAM-1 | RR.1 | |
| CD18 101712; S617 | BS | VLA 1 | TS2/7 | |
| CD19 B4, B-C3; J3-119; CLB-CD19 | Coulter | VLA 5 | J143 | |
| CD20 B1; 1F5; 2H7; B-ly1 | Coulter | VLA 4 | 8F2 | |
| CD21 B2; BU-35; 21A/5; BU-36 | WS | DRC-1 | R4/23 | Dako |
| CD22 HD6; HD39; HD239; G28-7 | WS | PCA-1 | Rabbit serum | |
| CD23 Blast 2; H107; M-L47; BU-38; 3-5; | WS | Factor VIII | Dako |
| CD24 LC66; 32D12; ML5 | WS | epithelial membrane | HB8 antigen | Dako |
| CD25 Tac | | 63D3 | | ATCC |
| CDw29 4B4; A1A5 | | HLA class I | W6.32 | |
| CD30 RSC1 | Dako | HLA class II | 5/7; 6; 332 | |
| CD32 IV-3 D3 | | | | |
| CD33 My9; L4F3 | | | | |
| CD34 12-8 | | | | |

* Antibodies were provided by other investigators (see Materials and Methods) or from the following companies: ATCC, American Type Culture Collection, Rockville, MD; BD, Becton Dickinson & Co., Mountain View, CA; Coulter Immunology, Hialeah, FL; Dako Corp., Santa Barbara, CA; WS, 4th International Workshop on Human Leukocyte Differentiation Antigens.

**Isolation and Purification of Follicular Dendritic Cells.** Human tonsils were obtained immediately after nonurgent tonsillectomy, placed in a Petri dish, and gently teased apart using forceps, knife, and stainless steel mesh. Tissue was digested with RPMI media (Gibco Laboratories, Grand Island, NY) containing 2 mg/ml collagenase (type IV; Worthington Biochemical Corp., Freehold, NJ), 5 mM EDTA (pH 7.4), 10% FCS, 4 mM glutamine, 10 U/ml penicillin, 10 U/ml streptomycin, 1 mM sodium pyruvate, and 0.1 mg/ml DNase (type I; Sigma Chemical Co.). To investigate cluster formation of FDCs with lymphocytes under different conditions,
equal small tonsil pieces were digested separately for 1 h on ice, at room temperature and at 37°C, or at room temperature in the presence of 2, 10, and 50 mM EDTA (pH 7.4). Cell suspensions were centrifuged at 1,800 rpm for 25 min over a discontinuous gradient of 15% (1.02 g/ml density) and 35% (1.04 g/ml density) Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden), and low density cell preparations enriched for FDCs were collected from the 15%/35% interphase.

Final FDC purification was achieved by labeling low density cell suspensions with mAb 322 (CD14), subsequent staining with fluoresceinated goat anti-mouse Ig (Tago Inc.), and sorting with an EPICS cell sorter (Coulter Electronics Inc., Hialeah, FL). FDCs were sorted using parameters of log fluorescence and log 90° light scatter, and gate windows were set to exclude cell clusters. Cells were sorted twice to reach the highest purity possible. Both antibodies were ultracentrifuged at 4°C with 15,000 rpm for 30 min immediately before use to eliminate protein aggregations. Nonspecific binding to cell surface Fc receptors was blocked by diluting the antibodies in 10% human serum in media. Purity of the sorted FDC preparations was determined by re-analysis with flow cytometry. For two-color analyses, sorted FDCs were labeled with biotinylated mAbs against BI (CD20) or B2 (CD21), stained with streptavidin/phycoerythrin complex, and analyzed by FACS.

**RNA Purification and Polymerase Chain Reaction.** SRBC receptor negative cells from human tonsil were stimulated for 10 h with anti-Ig beads and highly purified FDCs were obtained by cell sorting. RNA from these cells was extracted by a modification of the acid guanidinium thiocyanate-phenol-chloroform isolation procedure designed for small quantities of cells (11). Since the number of highly purified FDCs after the sort was very small (10⁵), gene expression of these cells was analyzed using the very sensitive technique of polymerase chain reaction (PCR) (12). RNA (250-500 ng) was reverse transcribed using 200 U of Moloney murine leukemia virus reverse transcriptase and 0.8 μg oligo-dT as primer for 1 h at 37°C. One third of the resulting cDNA was analyzed for IL-6 or aldolase A expression by amplifying with 5 U of Thermus aquaticus (Taq) polymerase (Perkin-Elmer Corp., Norwalk, CT) and primers (50 pmol each) during 40 cycles of PCR (12) on a PHC-1 Programmable Dri Block (Techne, Inc., Princeton, NJ). The sense primer for IL-6 consisted of nucleotides 200-223 and the antisense primer consisted of nucleotides 542-517 (13). The sense primer for aldolase consisted of a 21 nucleotide-long sequence and the antisense primer was a 23 nucleotide-long sequence (14) (both primers as well as an oligonucleotide probe were a generous gift of Dr. Daniel Bergsagel, Dana-Farber Cancer Institute, Boston). Every cycle included denaturation at 94°C, annealing of primers and fragments at 37°C, and primer extension at 72°C. 16 μl of the product DNA obtained by PCR was applied to a 3% NuSieve GTG/1% Sea-Kem GTG agarose gel. Gels were run in tris acetate EDTA buffer containing ethidium bromide at 50 V for 3-5 h and Southern transferred onto a Zeta-Probe membrane (Bio-Rad Laboratories, Richmond, CA). Hybridization was performed using ³²P-labeled cDNA for IL-6 (kindly provided by Dr. Steven Clark, Genetics Institute, Cambridge, MA) and a ³²P-labeled 23-base oligonucleotide probe specific for the amplified aldolase A fragment (14).

**Results**

**Morphological Identification of FDCs.** Using characteristic morphological features of FDCs, it was possible to distinguish them from other dendritic-shaped cells, i.e., macrophages and interdigitating reticulum cells. As seen in Fig. 1, FDCs had four fundamental morphologic characteristics. They were very large in size (70-100 μm) (Fig. 1 A) and often contained two nuclei (Fig. 1, A and B). In addition, FDCs formed intense clusters with lymphocytes (Fig. 1, C and D) by a network of fine processes (Fig. 1 C, arrows). Although Wright Giemsa staining demonstrated large cell size, binuclear appearance, and acidophilic cytoplasm, it was impossible to definitively conclude by cytochemical staining alone that a cell was an FDC. Therefore, the reactivity of mAbs directed against CRs (CR1, CR2, CR3) was used to provide additional evidence that cells were FDCs. All anti-CR mAbs bound homogeneously to the cell surface and the cytoplasm of FDCs and, therefore, the prominent cytologic
features of these cells could be revealed by those antibodies (Fig. 1, B and C). Similarly, mAbs directed against other antigens expressed on FDCs, including anti-CD14 (Fig. 1 A), demonstrated the identical staining pattern seen with anti-CR mAbs. Fig. 1 D depicts an example for a negative control antibody. Antisurface IgD bound to clustered B cells (arrows) but lacked reactivity with the FDC.

Inhibition of Lymphocyte Adherence to FDCs. During preparation of single cell suspensions and density sedimentation, lymphocytes consistently adhered to FDCs, forming tight clusters. Attempts to disrupt these aggregations by vigorous vortexing or by incubating the cells with EDTA or cold media did not diminish the number of bound lymphocytes. This cluster formation presented a major problem for further phenotypic analyses since it was extremely difficult to distinguish mAb staining of lymphocytes from that of FDCs.

Therefore, in an attempt to block the formation of cell clusters, tonsils were manipulated under a number of different conditions. Table II summarizes the results of two representative experiments investigating if aggregate formation of FDCs is temperature dependent and if it can be influenced by chelation of Ca$^{2+}$ and Mg$^{2+}$. FDCs were identified by their characteristic morphology and reactivity with anti-CR mAbs. As seen in Table II, by lowering the incubation temperature during tonsillar digestion, the percentage of clusters could be decreased consistently. In addition, cluster formation was inhibited progressively by increasing concentrations of EDTA, indicating that the aggregation of FDCs with lymphocytes is Ca$^{2+}$ and Mg$^{2+}$ dependent. Furthermore, it was examined if adherence of lymphocytes to FDCs could
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Table II

Inhibition of Cluster Formation of FDCs with Lymphocytes

| Conditions during digestion of tonsil | Room temperature | EDTA 2 mM | EDTA 10 mM | EDTA 50 mM |
|--------------------------------------|------------------|-----------|------------|------------|
| Exp. | FDCs | 37°C | 4°C | % |  |
| 1 | Single cells | 30 | 49 | 74 | 55 | 65 | 70 |
| | Clusters | 70 | 51 | 26 | 45 | 35 | 30 |
| 2 | Single cells | 53 | 73 | 90 | 66 | 74 | 87 |
| | Clusters | 47 | 27 | 10 | 34 | 26 | 13 |

Single and clustered FDCs were determined by immunoperoxidase stainings of low density cell preparations.

be blocked with mAbs directed against adhesion molecules (anti-leukocyte function-associated antigen 1 [anti-LFA-1] and anti-intercellular adhesion molecule 1 [anti-ICAM-1]) or against CD14 and class I. Tonsils were digested in the presence of each of these antibodies, however, no significant decrease in cluster formation was observed.

Antigen Expression of Follicular Dendritic Cells. Using conditions that significantly decrease cluster formation, it was possible to precisely examine the expression of cellular antigens on FDCs. These studies were performed with the objective to define lineage and to detect functionally relevant cell surface molecules. Single cell suspensions prepared in the presence of EDTA contained <0.1% FDCs (Table III). By centrifuging over a low density gradient, FDCs were enriched to 4–6% (Table III). In these cell preparations, 50–70% of FDCs showed no adherent lymphocytes. Staining with lineage-restricted mAbs directed against B cells (anti-CD20, anti-CD24) and T cells (anti-CD2) demonstrated that the overwhelming majority (95%) of the remaining clustered lymphocytes were B cells, whereas <5% were T cells. By immunoperoxidase staining of single unclustered FDCs, mAb binding to these cells could be confidently determined. To exclude that the binding pattern of some of the antibodies was due to the expression or lack of certain epitopes, some of the markers were tested with a series of different mAbs. In addition to immunoperoxidase staining of fixed cytospin preparations, viable cells in suspension were also ex-

Table III

Purification Steps and Enrichment of FDCs from Human Tonsil

| Preparation | FDCs % |
|-------------|--------|
| Physical isolation | |
| Single cell suspension | <0.1* |
| Preparation of low density cells (35% Percoll gradient) | 4-6* |
| Antigen-specific purification | |
| Sort for highly CD14 positive cells by FACS | 70% |
| Sort of cells obtained from above sort | 98.8% |

* Determined by immunoperoxidase staining.
† Assessed by FACS.
amined for expression of cell surface antigens. All FDC+ mAbs (Table IV) demonstrated reactivity with the cytoplasm of FDCs as well as with their cell surface.

FDCs demonstrated a unique lineage-restricted antigen pattern by coexpressing B and myeloid markers. As seen in Table IV, FDCs were stained with mAbs directed against the B lineage-restricted CD19 and CD21 antigens and mAbs against myeloid-restricted antigens CD14, CD11b, and 63D3. However, other B cell lineage-restricted molecules, including CD20, CD22, and sIg (IgM, IgD, IgG, κ and λ), were negative (Table V). In addition, several myeloid markers, including CD13 and CD33, were not expressed (Table V). Antigens found on both B and some myeloid cells, including HLA class II, CD23, CD37, and CDw40, were also expressed on FDCs, whereas CD24 was not. In contrast to the finding that some of the B and myeloid antigens were found on FDCs, all T lineage-restricted antigens, including CD3, CD6, and CD8, were negative. In addition, anti-NKH1, which uniquely stains resting and activated NK cells, did not bind to FDCs.

An interesting observation was that although FDCs shared antigenic properties with B and myeloid cells, they did not express the leukocyte common antigen (CD45 and CD45R). This molecule is a marker for most hematopoietic cells and is found on virtually all B, T, myeloid, and NK cells. Stein and colleagues (7) have previously

| Table IV |
| Positive Reactivity of Antibodies with FDCs |

| Antigens | Example antibody |
|----------|------------------|
| Lineage-restricted antigens: | | |
| Myeloid lineage | | |
| CD14 | Mo2 |
| CR3 (CD11b) | Mo1 |
| 63D3 | 63D3 |
| B cell lineage | | |
| CD19 | B4 |
| CR2 (CD21) | B2 |
| Complement receptors, adhesion molecules, and Fc receptors: | | |
| Complement receptors | | |
| CR1 CD35 (C3bR) | 543 |
| CR2 CD21 (C3dR) | B2 |
| CR3 CD11b (C3biR) | Mo1 |
| Adhesion molecules | | |
| VLA 3 | J143 |
| VLA 4 | 8F2 |
| VLA 5 | BIE5 |
| VLA 6 | GOH3 |
| VLA β chain | 4B4 |
| ICAM-1 | RR1 |
| Fc receptors | | |
| Low affinity IgE Fc receptor (CD23) | Blast 2 |
| Not lineage restricted: | | |
| DRC-1 | R4/23 |
| CD37 | G28-1 |
| CDw40 | G28-5 |
| HLA Class I | W6/32 |
| HLA Class II | 6/7 |
described an antigen, termed DRC-1, that is found on all FDCs as well as on a subset of B cells. We observed that DRC-1 was strongly expressed on all FDCs, confirming the identity of these cells. In addition, some low density B cells were positive for DRC-1. Anti-CD30 directed against Reed-Sternberg cells was also unreactive with FDCs. This observation is of interest since the Reed-Steinberg cells have a density

| Lineage-restricted antigens | Example antibody | Adhesion molecules and Fc receptors | Example antibody |
|-----------------------------|------------------|------------------------------------|------------------|
| T cell lineage              |                  |                                    |                  |
| CD1a                        | T6               |                                    | T62             |
| CD1b                        | T009             | p150/95 (CD11c)                    | T009            |
| CD2                         | T11              | β chain of CD11a, CD11b and CD11c  | T11             |
| CD3                         | T3               | gpIb/IIa (CDw41)                   | T3              |
| CD6                         | 2H1              | VLA 1                              | 2H1             |
| CD7                         | T159             | 40-kD FcR (CD32)                   | T159            |
| CD8                         | T8               | Low affinity FcR                    | T8              |
| B cell lineage              |                  |                                    |                  |
| CD20                        | B1               |                                    | B1              |
| CD22                        | G28-7, HD39      |                                    | G28-7, HD39     |
| B5                          | B5               |                                    | B5              |
| B7                          | B7               |                                    | B7              |
| Surface IgG                 |                  |                                    |                  |
| Surface IgD                 |                  |                                    |                  |
| Surface IgM                 |                  |                                    |                  |
| Surface κ                   |                  |                                    |                  |
| Surface λ                   |                  |                                    |                  |
| PCA-1                       |                  |                                    |                  |
| Myeloid lineage             |                  |                                    |                  |
| CD13                        | My7              |                                    | My7             |
| CD33                        | My9              |                                    | My9             |
| CD34                        | I2-8             |                                    | I2-8            |
| CD38                        | SD2              |                                    | SD2             |
| CD39                        | G28-8            |                                    | G28-8           |
| NK lineage                  |                  |                                    |                  |
| NKH-1                       |                  |                                    |                  |
| Lymphoid lineage            |                  |                                    |                  |
| CD45 (LCA)                  | T200             |                                    | T200            |
| CD45R (LCAr)                | 2H4              |                                    | 2H4             |
| Not lineage restricted      |                  |                                    |                  |
| CD1c                        | T024             |                                    | T024            |
| CD4                         | T4               |                                    | T4              |
| CD5                         | T1               |                                    | T1              |
| CD9                         | J2               |                                    | J2              |
| CD10                        | J5               |                                    | J5              |
| CD24                        | HB8              |                                    | HB8             |
| CD25                        | IL-2R            |                                    | IL-2R           |
| CD30                        | RSCI             |                                    | RSCI            |
| Other lineages              |                  |                                    |                  |
| Factor VIII                 |                  |                                    |                  |
| (endothelial)               |                  |                                    |                  |
| EMA                         |                  |                                    |                  |
and morphology similar to FDCs (15). Evidence that FDCs were not of epithelial or endothelial origin is derived from their lack of reactivity with mAbs against the epithelial membrane antigen (EMA) and factor VIII, respectively. Anti-CALLA has been previously shown to stain a population of cells in the germinal center (16) which to date has not been identified. Isolated FDCs were common acute lymphoblastic leukemia antigen (CALLA) negative. These data provided support for the idea that FDCs, although expressing B and myeloid markers, represent a unique cell type.

Since FDCs fix and retain immune complexes, we examined these cells for the expression of complement and Fc receptors. As stated above, FDCs demonstrated very strong reactivity with mAbs against the three receptors for the third complement component, CR1, CR2, and CR3 (Table IV). In addition, the low affinity IgE Fc receptor (CD23) was strongly positive on FDCs, which was of interest since this receptor is expressed on activated B cells and monocytes. However, it was surprising to observe that the low affinity and the 40-kD Fc receptors (CD16 and CDw32) were not found on FDCs.

To investigate if FDCs have cell surface structures that might regulate their adhesion properties in the germinal center microenvironment, mAbs against intercellular and cell-matrix adhesion molecules were examined. As seen in Tables IV and V, the majority of molecules belonging to the three subgroups of the integrin superfamily were tested. FDCs expressed most of the adhesion proteins of the VLA family, including VLA-3, -4, -5, -6, and their common β chain, whereas VLA-1 was negative. The second subgroup of the integrins includes LFA-1, Mol, and p150/95, which form a group of heterodimer proteins with a homologous α chain, CD11a, CD11b, and CD11c, and a common β chain (CD18). In contrast to the broad expression with VLA proteins, only the α chain of Mol (CD11b) was positive. A very interesting observation was that antibodies against the corresponding β chain of this molecule did not bind to FDCs, whereas reactivity was seen with other lymphocytes. This negative result is based upon lack of staining with two anti-CD18 mAbs and a heteroantisera against CD18. Although LFA-1 was not expressed on FDCs, its natural ligand ICAM-1 was strongly positive. GpIIb/IIIa, which is a member of the third subgroup of integrins, was also not detected on FDCs. However, this observation was not surprising since this molecule is selectively expressed on platelets.

**Phagocytic Activity and Cytochemical Staining.** To determine if FDCs not only express some monocyte antigens but also behave functionally like monocytes, the ability of these cells to phagocytize opsonized human RBCs was examined. FDCs incubated with RBCs for 3 h at 37°C failed to ingest RBCs, whereas control monocytes demonstrated avid phagocytosis. The cytochemical pattern of FDCs, however, was similar to that of monocytes. FDCs were weakly stained for monocyte-associated nonspecific esterase, whereas they did not contain granulocyte-restricted specific esterase and peroxidase.

**Isolation of Homogeneous Populations of FDCs.** To obtain homogeneous cell populations that would allow to study the gene expression of these cells, an isolation procedure based on the unique antigenic phenotype of FDCs was performed. Using CD14 as a marker for flow cytometric cell sorting, FDCs were further purified from low density cells (4–6% FDCs). This antigen was selected because it was strongly positive on all FDCs of the 45 tonsil preparations examined by in situ staining. In con-
contrast, mAbs against CD14 showed a significantly weaker binding to monocytes, macrophages, and granulocytes and were completely negative on lymphocytes.

Using flow cytometry, FDCs could be detected by their very strong staining with anti-CD14. Moreover, by examining both parameters of fluorescence intensity and log 90° light scatter, a well-demarcated FDC population could be identified (Fig. 2A). As seen in Fig. 2A, brightly stained CD14+ FDCs (4–7%) could be distinguished from a population (4–5%) expressing lower levels of CD14 (Fig. 2A). By flow cytometry cell sorting with parameters specific for FDCs, an enriched cell population (~70%) was obtained (Table III). To isolate a homogeneous FDC preparation, these partially purified cells were resorted using the identical parameters. As seen in Table III and depicted in Fig. 2B, this procedure resulted in a 98.8% pure cell preparation. As shown in Fig. 3, the highly purified cells had a large cytoplasm and some of the cells contained two nuclei, which is a morphology identical to the one of FDCs in tonsillar low density cell preparations. This also demonstrated that the sorted cells were FDCs. In an attempt to confirm the purity and antigenic phenotype of this homogeneous FDC preparation, cells were labeled with biotinylated anti-CD21 (Fig. 2C) or anti-CD20 (Fig. 2D) mAbs and stained with streptavidin-phycoerythrin. As seen in Fig. 2C, these cells uniformly expressed CD21, whereas they were totally unreactive with anti-CD20 (Fig. 2D). This demonstrates that the FDC preparation did not contain monocytes or B cells. Moreover, these findings confirm the in situ observation that FDCs coexpress CD14 and CD21 yet lack CD20.

Analysis of IL-6 Expression in FDCs. To further examine the role of FDCs within the lymphoid follicle, it was of interest to examine whether these cells produce IL-6. This cytokine was selected since it has been shown to be important in terminal B cell differentiation (17). To test if FDCs express the IL-6 protein, immunostaining with rabbit antiserum to IL-6 was performed. The antibody strongly stained the cytoplasm of activated tonsillar B lymphocytes but was unreactive with FDCs (data

![Cell sorting of FDCs to homogeneity and double labeling of highly purified FDCs.](image-url)

**Figure 2.** Cell sorting of FDCs to homogeneity and double labeling of highly purified FDCs. (A) Staining profile of tonsillar low density cell preparation with anti-CD14. (B) 98.8% pure FDC preparation obtained after cell sorting of highly CD14+ low density cells shown in A. (C) Co-expression of CD14 and CD21 on highly purified FDCs. (D) Lack of reactivity of homogeneous FDCs with anti-CD20.
not shown). In an attempt to determine whether FDCs expressed IL-6 mRNA, PCR-amplified cDNA from highly purified FDCs was examined. Activated B cells were used as a positive control cell preparation (18). Because of its abundant and uniform cellular expression, aldolase A was selected as a positive control. Fig. 4 shows the ethidium bromide staining pattern and the corresponding Southern blot analysis of PCR product DNA obtained for IL-6 and aldolase A. Activated B cells were IL-6+ (Fig. 4, A and B, lane 3), whereas FDCs were negative (Fig. 4, A and B, lane 4). As seen in Fig. 4, A and B (lanes 1 and 2), both activated B cells and FDCs expressed aldolase A PCR product DNA.

**Figure 3.** Morphology of highly purified FDCs with Wright Giemsa stain.

**Figure 4.** IL-6 and aldolase A gene expression of highly purified FDCs. cDNA produced by PCR from activated tonsillar B cells (lanes 1 and 3) and highly purified FDCs (lanes 2 and 4). cDNA was amplified using primers specific for aldolase A (lanes 1 and 2) and for IL-6 (lanes 3 and 4). (A) Electrophoretic analysis of the PCR products. (B) Southern analysis of the gel using a 32P-labeled oligonucleotide probe for aldolase A (lanes 1 and 2) and a 32P-labeled cDNA probe for IL-6 (lanes 3 and 4). The molecular sizes of a Hae III digest of φX174 are given on the right side of the figure.
Discussion

In the present report, FDCs were purified in an attempt to examine the expression of cellular antigens that define lineage and function. In addition, these cells were isolated to homogeneity in order to study their gene expression. FDCs were identified by their characteristic morphology and by their expression of receptors for the third complement component, the myeloid-restricted antigen CD14, and the FDC antigen DRC-1. Unclustered FDCs displayed a unique antigenic phenotype since they expressed several B and myeloid lineage-restricted antigens, but lacked T and NK cell antigens as well as the leukocyte common antigen. FDCs expressed adhesion molecules, including most of the VLA proteins, ICAM-1, and CD11b. FDCs could be isolated to homogeneity by their intense staining with anti-CD14 using flow cytometric cell sorting. These highly purified FDCs expressed CD14 and CD21 but lacked CD20. This antigen pattern and characteristic morphology confirmed that these cells were, in fact, homogeneous FDC preparations. Analysis of the cDNA from these highly purified cells demonstrated no mRNA for IL-6. The study suggests that FDCs may belong to a unique cellular population.

Immunostaining of FDC clusters, both in situ and in suspension, have provoked controversy. Gerdes et al. (7) observed that human FDC clusters were positive for CRs, class I and II molecules, and monocyte markers, and therefore concluded that these cells were most closely related to monocytes or macrophages. Humphrey et al. (19) challenged these findings by the observation that murine FDCs do not express Iα and macrophage markers and that they do not originate from the bone marrow. In the present study FDCs could be separated from adherent B cells, and therefore, it was possible to more precisely demonstrate the expression of lineage-restricted antigens. FDCs had an antigenic phenotype uncommon to any of the known hematopoietic cells. They shared several myeloid (CD14, CD11b, 63D3) and B (CD19, CD21) lineage-restricted antigens and did not express any of the known T or NK cell markers. However, FDCs lacked several other important B- (sIg, CD20, CD22, B5, B7) and myeloid (CD13, CD33, CD34, CD38, CD39)-restricted antigens. Therefore, the expression of B- and myeloid-restricted antigens may not be sufficient to define the lineage of these cells. Cytochemical staining and lack of phagocytosis further suggests that these cells only partially resemble myeloid cells. The finding that FDCs were not positive for the common leukocyte marker CD45 supports the hypothesis that human FDCs do not originate from bone marrow-derived hematopoietic cells. Moreover, FDCs do not appear to be of epithelial or endothelial origin. Therefore, our results are consistent with the hypothesis that FDCs may not belong to a classical hematopoietic cell lineage.

Since FDCs create a three-dimensional network with B cells within the germinal center and in tissue culture, we attempted to examine those cellular antigens that might be involved in interactions between these cells. The ability to bind and present antigen-antibody-complement complexes to B lymphocytes appears to be an important function of the FDCs (19–22). FDCs displayed a unique antigenic pattern by expressing all three receptors for the third complement component. In addition, one major feature of the FDCs appears to be their ability to bind to germinal center B lymphocytes. FDCs expressed the cell-cell adhesion molecules ICAM-1 and CD11b, which is consistent with previous in situ observations (23, 7). Our results imply that the binding of FDCs to B cells is a dynamic, temperature-dependent process that
is regulated by \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \). These conditions are crucial for the function of cell-cell adhesion molecules, as shown for binding of LFA-1 on lymphocytes to ICAM-1 (24). Although cluster formation could not be inhibited by using mAbs against these molecules, it cannot be excluded that these receptors, in addition with other adhesive proteins, might indeed regulate B-FDC adhesion.

The observation that FDCs were stained with mAbs against VLA proteins demonstrates that FDCs apparently express receptors for laminin, fibronectin, and collagen. These proteins belong to a group of heterodimers with unique \( \alpha \) chains and a common \( \beta \) chain (25), and some of them are found on a large variety of cell types (26). One of their major functions seems to be to mediate the adhesion of leukocytes to extracellular matrices (27). VLA-4 differs from the other members of the VLA family since it may have cell-cell adhesion functions (28). The fact that FDCs were positive for nearly all of the VLA proteins suggests that these receptors might play a role for FDC function.

FDCs and lymphoid dendritic cells (interdigitating reticulum cells) are morphologically similar, low density cells. Both cell types provide in vivo and in vitro a complex microenvironment for lymphoid cells by forming extensive networks with cellular processes (29-31). This report, however, demonstrated that FDCs could be identified as a unique cell type with phenotypic characteristics that were distinct from those of lymphoid dendritic cells. In contrast to lymphoid dendritic cells, which contain mostly T cells in their in vitro aggregations (31), FDCs had B cells bound within their processes. In addition, the immunophenotypic profile of tonsillar FDCs differs significantly from that of isolated lymphoid dendritic cells. The latter cells were negative for most of the markers that are expressed on FDCs. As reported by Hart and McKenzie (6), lymphoid dendritic cells were not stained, for example, by mAbs against the complement receptors CR1, CR2, and CR3, the myeloid markers CD14 and 63133, and the FDC antigen DRC-1. Moreover, lymphoid dendritic cells, in contrast to FDCs, did express the common leukocyte antigens CD45 and CD45R. This observation supports the notion that these cells might represent distinct cell entities with different cellular origins. This hypothesis is supported by the finding that lymphoid dendritic cells originate from the bone marrow (1), in contrast to FDCs, as observed by Humphrey et al. (19).

Several observations deserve further comment. Other investigators have reported that FDC clusters express surface Ig and CALLA (7), whereas in this study, singly dispersed FDCs were negative for these markers. It was of great interest that CD11b was clearly expressed on FDCs, but its corresponding \( \beta \) chain (CD18) could not be detected. A possible explanation for this observation might be that the level of CD18 was too low to be detected by in situ staining technique. It was also surprising to note that although FDCs fix immune complexes, they did not express the 40-kD Fc receptor and the low affinity Fc receptor. However, FDCs did have low affinity Fc IgE receptors (CD23). The importance of this Fc receptor to FDC function is still unknown.

Finally, isolation of homogeneous populations of FDCs will facilitate functional characterization of these cells. It should now be possible to determine whether these cells produce cytokines important for B cell differentiation. Although highly purified FDCs do not seem to produce IL-6, these cells might secrete this or other cytokines after appropriate stimulation. In addition to the examination of the cytokine produc-
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It will be important to study the structures that regulate FDC function and that are involved in cell-cell adhesion. In the future, it will also be necessary to confirm the antigenic phenotype of the FDCs by detecting the expression of the corresponding genes.

Summary

In the present study, follicular dendritic cells (FDCs) were purified to homogeneity in order to define the lineage and function of these cells. FDCs were identified by their characteristic morphology and by their expression of receptors for the third complement component, the myeloid-restricted antigen CD14, and the FDC antigen DRC-1. Unclustered FDCs displayed a unique antigenic phenotype since they expressed several B- and myeloid lineage-restricted antigens, but lacked T and NK cell antigens as well as the leukocyte common antigen. FDCs expressed adhesion molecules, including most of the VLA proteins, intercellular adhesion molecule 1 (ICAM-1), and CD11b. FDCs could be isolated to homogeneity by their intense staining with anti-CD14 using flow cytometric cell sorting. These highly purified FDCs expressed CD14 and CD21 but lacked CD20. This antigen pattern and characteristic morphology confirmed that these cells were, in fact, homogeneous FDC preparations. Analysis of polymerase chain reaction-amplified cDNA from highly purified FDCs showed no transcripts for IL-6. The isolation of homogeneous FDC populations will be important for the analysis of the functional role of FDCs within the lymphoid follicle.

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Note added in proof: FDCs were also tested for reactivity with the blind panel antibodies of the Fourth International Workshop on Human Leukocyte Differentiation Antigens. The corresponding antigens were revealed after submission of the manuscript. In contrast to the findings presented in this study, FDCs were stained by the blind panel antibodies against surface κ and surface IgM.

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