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Human Blood Dendritic Cell-Like B Cells Isolated by the 5G9 Monoclonal Antibody Reactive with a Novel 220-kDa Antigen

Rui-Kun Zhong,* Albert D. Donnenberg, † Hai-Fan Zhang, † Simon Watkins,* Jie-Hua Zhou,* and Edward D. Ball†*†

We developed a murine IgG1 mAb, 5G9, following immunization of a BALB/c mouse with Daudi cells. By immunoprecipitation, 5G9 reacted with a 220-kDa Ag on Daudi cells, which reduced to four subunits (55, 65, 80, and 85 kDa). mAb 5G9 bound to 40–60% of peripheral blood B cells, weakly reacted with monocytes and granulocytes, and did not bind to erythrocytes, platelets, T cells, or NK cells. mAb 5G9 brightly stained scattered cells in human tonsil sections, which appeared to be dendritic cells (DC) by morphology. mAb 5G9 also stained scattered cells in cytospin slides of monocyte-derived DC with long, thin, beaded membrane processes, morphologically distinct from other monocyte-derived DC. Positive selection of blood mononuclear cells with mAb 5G9 and sheep anti-mouse IgG Dynabeads demonstrated an enriched population of DC. By flow cytometry analysis, these cells were CD19, CD20, CD40, CD44, CD83, CD86, IgD, and HLA-Dr positive and either CD40L and L chain positive. They did not express CD3, CD4, CD5, CD10, CD11b, CD13, CD25, CD56, CD14, CD33, or CD64. Isolated 5G9+ B cells were potent APCs in alllogeneic MLR, compared with 5G9− PBMC, 5G9− B cells, monocytes, and monocytes cultured in IL-4 and GM-CSF for 24 h. mAb 5G9 defines a novel peripheral blood cell with B cell phenotype and DC morphology and function: DC-like B cells. The significance of this cell in immune responses requires further study. The Journal of Immunology, 1999, 163: 1354–1362.

D endritic cells (DC)† are a system of bone marrow-de
duced and potent APCs specialized for initiating primary T cell immune responses (1). Recent investigation indicates that DC are of diverse origin, with at least two types of precursors (myeloid and lymphoid). These cells have been classified, on the basis of their tissue location, as interdigitating reticulo
cellular system present in lymphoid organs, as veiled cells present in afferent lymph, as blood DC in the circulation, as Langerhans cells in the epidermis, and as dermal DC in the dermis of the skin (1, 2). The diversity of DC in different locations, the limited knowledge of the interrelationship of different DC populations, the dramatic change in the morphology, function and phenotype at the different developmental stages of DC, and the low frequency of DC in the blood have all made DC somewhat difficult to study.

Most current studies of human DC have focused on myeloid DC. However, Galy et al. (3) recently found that a subpopulation of CD34+lin−CD45RA+CD10− cells gives rise only to T and B lymphocytes, NK cells, and DC. Dilution analysis demonstrated the existence of multipotential B/NK/DC progenitor clones in the CD34high lin−CD10+ adult bone marrow cell popu
lation, indicating that nonprimitive progenitors for lymphoid cells and for DC can be distinct from those of myeloid, implying that the DC lineage is developmentally more closely related to the lymphoid than the myeloid lineage. Serreze et al. (4) studied the APCs that contribute to the development and activation of diabetogenic T cells in the nonobese diabetic (NOD) mouse model of insulin
dependent diabetes mellitus (IDDM). They utilized a functionally inactivated Ig μ allele to generate a “speed congenic” stock of B cell-deficient NOD mice. They found that these mice were free of overt IDDM and insulin resistance, although with normal numbers of T cells, implying that B cells play an unrecognized role (i.e., functioning as potent APCs), which is essential for the initial development and/or activation of T cells autoreactive with pancreatic β cells in NOD mice. Bjorck’s recent study demonstrated that murine CD19+ pro-B cells develop into DC with T cell stimula
tory properties when cultured with murine IL-1β, IL-3, IL-7, TNF-α, stem cell factor (SCF), and human Flt-3 ligand (5). The above studies suggested a close relationship between DC and B cells and B cell precursors having B/DC bipotential lineage capacity.

For decades, immunologists have reported the conversion of B lymphocytic tumors to cells with properties of macrophages. Borrello and Phipps recently discussed the identification of normal B/macrophage (B/MΦ) cells, indicating that B/MΦ cells are the normal counterpart of the well-described endpoint of CD5+ B cell lymphoma lineage switch to macrophages. These B/MΦ cells are morphologically distinct from classical macrophages, having unique surface characteristics that suggest their B cell origin (6). The identification of B/MΦ imply that switching can occur be
tween subsets of distantly related cells that derived from separate lineages with specialized functions.

In an attempt to generate novel anti-B cell mAbs, we discovered a hybridoma that reacted with B lymphoma cells and a subpopu
lation of tonsillar cells. Further work revealed the mAb to define what appears to be a novel Ag on a subset of B cells. In this paper, we describe the production of this unique mAb reactive with a 220
kDa Ag expressed on a B cell subpopulation that has the properties of a DC and functions as a potent stimulator of allogeneic T cells.

Materials and Methods

Production of mAb 5G9

Dauidi cells (American Type Culture Collection, ATCC, Manassas, VA) were washed in serum-free RPMI 1640, incubated at 37°C for 2 h, then washed 2× in PBS. Immunizations were conducted by injecting 1×10^6 Dauidi cells i.p. into a 7- to 8-week-old female BALB/c mouse (Charles River Laboratory, Wilmington, MA) once a week for 4 wk. Four days after the last injection, the mouse splenocytes were dissected and fused with P3X63Ag8.653 myeloma cells (ATCC) according to the procedure described by Kohler and Milstein (7). Supernatants were tested for the presence of Abs against Dauidi cells and the absence of reactivity with HL-60 cells by an ELISA. The hybridoma clone producing mAb 5G9 was subcloned twice by the limiting dilution method. The subtype of mAb 5G9 was determined by the mAb Check Kit for isotyping of mouse mAb in culture supernatant (Sterogene Bioseparations, Arcadia, CA) following the manufacturer's instructions.

Ab purification

Hybridoma cells were injected i.p. into BALB/c mice. Ascites was collected and centrifuged at 8000 × g for 15 min. Supernatant was removed and filtered through a 0.45-μm filter. The protein was precipitated by adding a saturated ammonium sulfate solution to a final concentration of 45% saturation. The precipitate was dialyzed in PBS (pH 8.0) at 4°C for 24 h. Ab was purified by a protein A-Sepharose column (Pharmacia, Piscataway, NJ), and the purity was determined by SDS-PAGE.

Immunoprecipitation

Dauidi cells (2×10^5) were cultured for 1 h in 5 ml of methionine- and cysteine-free medium. Labeling was initiated by adding 300 μCi of [35S]methionine-cysteine, and cells were collected after 3 h of culture. Labeled Dauidi cells were lysed by suspension in 500 μl ice-cold lysis buffer (50 mM Tris-HCl, 1% Nonidet P-40, 50 mg/ml BSA, with a mixture of protease inhibitors (5 mM EDTA, 100 μg/ml PMSF, and 5 μg/ml apro tinin). Lysates were mixed with 100 μl of normal mouse (NM) serum and precleared with killed Staphylococcus aureus cells that had been washed in lysis buffer. The precleared lysate was divided into 100-μl aliquots. MAb 5G9, SCC1-1 (a mouse IgG2a mAb reactive with the transmembrane Ag of Daudi cells) and the ferrin receptor used for positive control; Ref. 8), and NM serum (negative control) were added to the aliquots of lysate and mixed for 1 h at 4°C. Rabbit anti-mouse IgG Ab was then added and mixed for another hour. The aliquots of lysate were adsorbed to 50 μl of packed, washed protein A-Sepharose, and each sample was divided into two parts, one kept under nonreducing conditions, the other exposed to DTT. The samples were analyzed by SDS-PAGE.

Cell isolation procedure

Leukocyte-enriched leukopaks (obtained from Central Blood Bank, Pittsburgh, PA) were treated with Gey's solution to lyse erythrocytes and washed 4× at 210 × g with PBS to remove platelets. PBMC were cultured overnight in RPMI 1640 medium containing 10% heat-inactivated FCS, 10 mM glutamine, and penicillin/streptomycin. A DC-enriched population of monocytes was obtained as previously described with some modifications (9, 10). Briefly, the mononuclear cells were suspended in IMDM (Life Technologies, Grand Island, NY) medium containing 0.2% BSA (Sigma, Louis, MO) at 1× 10^7/ml, and incubated with 2–3 subsequent 45-min periods at 37°C in 150 × 15-mm petri dishes (Becton Dickinson Labware, Lincoln, NB). The cell lines were removed adherent to petri dishes. Nonadherent cells were either isolated immediately or after culturing 1–5 days in RPMI 1640 complete medium with 1000 U/ml IL-4. Cells were resuspended in 5G9 culture supernatant at 2–4× 10^7/ml, incubated for 30 min at 4°C, washed 3×, resuspended in RPMI 1640, 2% FCS and mixed with sheep anti-mouse IgG Dynabeads M-450 continuously for 60 min at 4°C. 5G9+ cells were obtained by washing 3× the cell suspension against Dynal MPC. The cells attached to magnetic beads were suspended in RPMI 1640 10% FCS and incubated at 37°C overnight. About 30–50% of isolated 5G9+ cells were detached from the Dynabeads and washed three times against Dynal MPC.

Cells and cell lines

Peripheral blood samples from adult patients with chronic lymphocytic leukemia (CLL) obtained with informed consent under the auspices of the Institutional Review Board of the respective institution. MoNDCL cells were isolated by Ficol-Hypaque centrifugation. The cell lines are listed in Table I. Cell lines were cultured in RPMI 1640 (Life Technologies) containing 10% FBS (HyClone, Logan, UT) in a humidified atmosphere of 5% CO2 and air. Cells in log phase were freshly harvested from culture, washed three times, and resuspended in RPMI 1640 medium before use.

Flow cytometry and mAb

PE-labeled mouse anti-human mAbs CD3, CD4, CD5, CD10, CD11b, CD13, CD14, CD19, CD20, CD21, CD22, CD33, CD38, and CD45 HLA-Dr were purchased from Becton Dickinson (San Jose, CA); CD83-PE and CD19-energy-coupled dye (ECD) from Immunotech (Westbrook, ME); and CD80-FITC and CD86-PE from PharMingen (San Diego, CA). The samples were washed twice with IFA medium (PBS supplemented with 4% FCS and 0.1% sodium azide), and the cells were resuspended in IFA medium containing 2% human AB serum and 2% goat serum (to block FcγR). The lack of reaction of control tubes that were stained with mouse IgG1-FITC/mouse IgG1-PE or goat anti-mouse IgG-FITC demonstrated that nonspecific binding was well blocked. Indirect immunofluorescence staining was conducted after washing the cells twice with PBS containing 1% BSA and 0.1% sodium azide. Cells were incubated with mAb 5G9 for 30 min at 4°C; washed twice, incubated with FITC-labeled goat F(ab′)2 anti-mouse IgG (HL) (Caltag Laboratories, Burlingame, CA), and washed before analysis on FACSscan system (Becton Dickinson Immunochemistry System) or a Coulter XL (4-color studies; Beckman Coulter, Miami, FL). Double staining involved labeling with mAb and goat F(ab′)2 anti-mouse IgG followed by incubation 10 min with 10% mouse serum at 4°C before adding a PE-conjugated mAb.

Light microscopy

The cytospin slides of peripheral blood, cultured moDC, and purified 5G9+ cells were stained with Wright-Giemsa by using an Ames HEMA-TEK Slide Stainer (Curtis Matheson Scientific, Houston, TX). Tissue was fixed in 10% neutral buffered formalin and embedded in paraffin; and 4-micron sections were cut and stained with hematoxylin-eosin (H-E) for microscopic examination.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Cell Line</th>
<th>% Positive</th>
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<tbody>
<tr>
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</tr>
<tr>
<td></td>
<td>Raji</td>
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</tr>
<tr>
<td></td>
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<td>Molt</td>
<td>—</td>
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<tr>
<td></td>
<td>CEM</td>
<td>—</td>
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<td></td>
<td>Jurkat (T)</td>
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<tr>
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<td>HL60</td>
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<td>NB4</td>
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</tr>
<tr>
<td></td>
<td>KG1a</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>K562</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>U937</td>
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<tr>
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<td>RPMI8226</td>
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<tr>
<td>CLL</td>
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</table>

*Negative reaction is defined as <2% of positive cells.

* n = 4
Stimulators for the MLR were total PBMC, freshly isolated 5G9+ cells, 5G9- cells, 5G9- B cells, and monocytes derived from the separation procedures, cultured in IL-4/GM-CSF for 24 h. 5G9- B cells were obtained by negative selection of 5G9- PBMC population with a mixture of mAbs CD3, 3G8 (CD16), 251 (CD33) and sheep anti-mouse IgG Dynabeads M-450. These cells were irradiated (3000 rad) and added in graded doses to 2 × 10^3 allogeneic or syngeneic (autologous) mononuclear cells in 96-well U-bottom tissue culture plates in final volume of 0.2 ml. The medium used for MLR was AIM-V medium (Life Technologies) containing 10% inactivated human AB serum. Proliferation was measured by the uptake of [3H]thymidine (1 μCi/well) added 8 h before the end of culture (37°C, 5% CO₂) on the 5th day and counted in a 1205 Betaplate counter.

In alternative experiments, 5G9+ cells isolated by beads were cultured in RPMI 1640 with 10% FCS supplemented with IL-4 (1000 U/ml), or soluble CD40 ligand (CD40L) from a stable transfected cell supernatant (three parts RPMI one part CD40L supernatant) (kindly provided by Dr. Thomas Kipps, University of California San Diego), or IL-4 plus CD40L supernatant (IL-4/CD40L 5G9+ cells) for 4 days. The cultured 5G9+ cells were then used as MLR stimulators to compare with MoDC cultured in IL-4/GM-CSF medium for 5 days and 5G9+ cells freshly isolated from PBMC that were cultured for 5 days in medium supplemented with IL-4 (IL-4/PBMC 5G9+ cells).

**Immunohistochemistry**

Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used for immunohistochemical analysis (biotin-avidin-peroxidase-AEC) of 5G9, CD19, or CD21 expression on cytopsin slides for PBMC, DC, MoDC, 5G9+ cells, or tissue section slides, following the manufacturer’s instruction. The slides or sections were counterstained with hematoxylin. For double staining, cytopsin slides were first incubated with mAb B4 (anti-CD19), washed, and incubated with alkaline phosphatase-conjugated goat F(ab’)2, anti-mouse IgG (Caltag), then developed with Vector blue alkaline phosphatase substrate kit III following the manufacture’s instruction. After blocking with goat serum and blocking serum of ABC kit, the slides were incubated with mAb 5G9, and Vectastain ABC kit was used for developing second color (red). Control slides were stained with double staining procedure without mAb CD19 or 5G9 or both.

**Scanning electron microscopy**

Cytospin glass discs were fixed in 1% glutaraldehyde and dehydrated in a concentration gradient of ethanol. Cells were then critical-point dried through CO₂ to maintain cellular ultrastructure and attached to carbon planchettes with colloidal graphite, coated with a 250 Å layer of gold in a sputter-coater, and studied in a scanning electron microscope.

**Statistical analysis**

The significance level was determined by the student t test when applicable.

**Results**

**mAb characterization**

mAb 5G9 was obtained by fusing murine myeloma cells with BALB/c mouse spleen lymphocytes immunized with Daudi cells. The subtype of mAb 5G9 was determined to be a murine IgG1. By Western blot and immunoprecipitation, it reacted with a 220-kDa Ag on Daudi cells, which reduced to four subunits with apparent m.w. of 55, 65, 80, and 85 kDa (Fig. 1).

5G9+ B cells reacted with 40–60% peripheral blood high HLA-Dr-expressing B cells (Fig. 2). mAb 5G9 did not react with peripheral blood erythrocytes, platelets (data not shown), T cells (CD3+), and NK (CD56+ cells) and reacted weakly with monocytes and granulocytes (n = 8). Double-staining 5G9+ cells in resting PBMC with CD83 and CD86 showed no double-positive cells (Fig. 2). This mAb reacted with lymphoma cell lines and cells from CLL patients (four of four), but did not react with AML and ALL cell lines or AML and ALL patient-derived cells (Table I).

**Immunohistochemical study**

Immunohistochemical study of 5G9 using Vectastain ABC demonstrated that the mAb stained scattered cells on human tonsil sections, which appeared morphologically to be DCs (Fig. 3, A and B). On cytopsin slides of MoDC cultured with IL-4, GM-CSF, and TNF-α for 4–10 days, mAb 5G9 strongly reacted with cells with typical dendritic morphology that demonstrated exceptionally long, fine, and often beaded membrane processes, morphologically different from those of other MoDC (Fig. 3, C and D). These cells comprised about 2.5 ± 1.7% of MoDC cells (n = 6, range 1.25–6.1%), and were double stained by mAb CD19 and CD20 by flow cytometry (data not shown).

**FIGURE 1.** Molecular weight of the 5G9 Ag on Daudi cells. The pre-cleared lysate of [35S]methionine-labeled Daudi cells was divided into 100-μl aliquots. Various clones of mAb 5G9 and positive (SCCL1, a mouse IgG mAb reactive with the transferrin receptor) and negative (NM serum) control mAb were added to the aliquots of lysate. The samples under nonreducing and reducing conditions were analyzed by SDS-PAGE.

**FIGURE 2.** The reaction of mAb 5G9 to PBMCs. As assessed by two color flow cytometry analysis, mAb 5G9 reacted with 40–60% of peripheral blood CD19+, HLA-Dr+B cells. 5G9-positive cells were CD3, CD56, CD14, CD83, and CD86 negative.
Isolation of 5G9\(^+\) cells

5G9\(^+\) cells were successfully isolated by positive selection with mAb 5G9 and goat anti-mouse IgG magnetic beads as described in Materials and Methods above. From five experiments, where the average starting PBMC was 5.7 \(\times\) 10\(^8\) (range = 2.8–8.2 \(\times\) 10\(^8\)), 4.3 \(\times\) 10\(^6\) (range = 3–5.6 \(\times\) 10\(^6\)) 5G9\(^+\) cells were obtained after overnight culture. Immunohistochemical staining of cytospin slides of 5G9\(^+\) cells with mAb 5G9 (Fig. 3E) or double staining (Fig. 3F) with mAb 5G9 (red) and CD19 (blue) (F) (top right corner, double-stained control slide without mAb CD19; bottom right corner, double stained control slide without mAb 5G9) showed isolated cells with DC morphology that were double positive for 5G9 and CD19. G. PBMC cultured with IL-4 (1000 U/ml) for 4 days demonstrating intense DC differentiation in selected cells.

Isolation of peripheral blood DC

Based on these findings, human blood mononuclear cells were enriched for DC by metrizamide density gradient centrifugation as described in Materials and Methods to determine whether this DC population expressed detectable levels of 5G9 Ag. mAb 5G9 reacted with a fraction of metrizamide low density mononuclear cells representing \(\sim 17 \pm 4\%\) \((n = 4)\) of the DC-enriched fraction of cells. This fraction was not the lineage-negative, CD83-positive

FIGURE 3. Immunohistochemical study (peroxidase-AEC) of 5G9 + cells in tonsil and peripheral blood. A and B. Human tonsil sections stained with mAb 5G9: (A) 100\(\times\) magnification, (B) 400\(\times\) magnification. C and D. MoDC cytospin slides stained with mAb 5G9: (C) 100\(\times\), (D) 1000\(\times\). mAb 5G9 reacted strongly with cells displaying typical DC morphology with long, thin, and beaded processes distinct from other MoDC. E and F. Immunohistochemical staining of isolated 5G9\(^+\) cells with mAb 5G9 (E) or double staining with mAb 5G9 (red) and CD19 (blue) (F) (top right corner, double-stained control slide without mAb CD19; bottom right corner, double stained control slide without mAb 5G9) showed isolated cells with DC morphology that were double positive for 5G9 and CD19. G. PBMC cultured with IL-4 (1000 U/ml) for 4 days demonstrating intense DC differentiation in selected cells.
DC described previously (10). 5G9 cells were CD19, CD20, CD40, HLA-Dr positive, and CD83 dim (Fig. 4).

Flow cytometry analysis of 5G9 cells
Flow cytometry analysis of magnetic bead-isolated 5G9 cells showed a population of small cells in the low light scatter gate and a population of larger cells in the high light scatter gate. Both populations showed the same phenotype (CD19+, CD20+, CD3–, CD14–, CD56–, and CD83 dim). The difference was that the cells with high light scatter had higher percentages of positive cells and higher mean fluorescence intensity (MFI) for HLA-Dr, CD83, and CD86, suggesting that a higher DC composition existed in the cells with high light scatter (Fig. 5, A and B; Table II). The expression of DC morphology and of CD83 and CD86 on these cells of enlarged cell size may have occurred during the separation procedure or after the binding of 5G9 to these cells. Previous studies proved that, regardless of the culture period (0–72 h), B cells did not express detectable levels of CD83 as determined by flow cytometry analysis (10). Therefore, the expression of CD83 on 5G9 cells after isolation by mAb 5G9 and magnetic beads (and following 24-h culture) may have been induced by the binding of mAb 5G9 to B cells. Four color flow cytometry analysis of mAb 5G9 and magnetic bead-purified cells showed that these cells expressed CD83-PE, CD19-ECD, HLA-Dr-PC5, and 5G9-FITC simultaneously (Fig. 6). Three populations (R4, R5, and R6) were defined based on CD19 and 5G9 expression of normal PBMC control (Fig. 6B) in the same experiment (R4: 5G92, CD191; R5: 5G91, CD192; R6: 5G9+, CD192+). We can see from the four-color FACS data, the major population of isolated cells fell in R4, a smaller portion in R5 and fewer in R6. The separation procedure selectively obtained higher 5G9 expressing cells.

The specificity of mAb 5G9 to DC-like B cells was compared with mAb against CD19 (B4) and CD21. The three mAb were used

**Table II. The phenotypic difference between high light scatter and low light scatter gates of bead-isolated 5G9 cells**

<table>
<thead>
<tr>
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<th>High Light Scatter Gate</th>
<th>Low Light Scatter Gate</th>
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<tbody>
<tr>
<td>% Positive</td>
<td>MFI</td>
<td>% Positive</td>
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<tr>
<td>HLA-Dr</td>
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<td>CD83</td>
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<td>230</td>
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<tr>
<td>CD86</td>
<td>81</td>
<td>1200</td>
</tr>
</tbody>
</table>

**FIGURE 4.** The reaction of mAb 5G9 with metrizamide low density, CD83-positive DCs. MAb 5G9 reacted with a fraction of metrizamide low density mononuclear cells, which represented about 17 ± 4% (n = 4) of the population. These cells were CD19+, CD20+, and CD3–, CD14–, CD56–, and CD83 dim.

**FIGURE 5.** Flow cytometry analysis of magnetic bead-isolated 5G9+ cells. FL-1, cells were incubated with mAb 5G9, then goat anti-mouse IgG-FITC; FL-2, PE-conjugated Abs as labeled in each panel. A group of small cells in the low light scatter gate (B) (gated through R2) and a group of larger cells in the high light scatter gate (A) (gated through R1) were demonstrated on forward and side scatter. Both groups of cells possessed the same phenotype, with CD19, CD20, CD83, CD86, CD40, IgD, HLA-Dr-positive, either κ- or η-L chain positive, and CD3, CD14, CD33, CD56 negative. The difference was that the high light scatter gate cells had higher fluorescence intensity, particularly for HLA-Dr, CD83, and CD86.
as described in Materials and Methods to isolate B cells from the same donor’s PBMC after being cultured in RPMI 1640, 10% FCS, and 1000 U/ml IL-4 for 4 days. The results showed that there were 89% of CD83$^+$ cells in 5G9-isolated fraction and 51% and 47%, respectively, in the mAb CD19 and CD21 isolated fractions. In the mAb CD21 isolated cell population, 50% of the cells were 5G9$^+$; only 5G9$^+$ cells were double positive for CD83 (Fig. 7).

When observed under the phase contrast microscope that DC-like 5G9$^+$ B cells cultured at 37°C had the ability to extend and retract cell membrane processes constantly, and had marked cell motility (data not shown). Scanning electron microscope study of 5G9$^+$ cells revealed a characteristic “porcupine like” DC morphology (Fig. 8).

**Allogeneic MLR stimulation**

The ability of 5G9$^+$ B cells to induce the proliferation of allogeneic T cells was compared with that of other leukocyte populations, such as total PBMC, resting monocytes, monocytes cultured in IL-4 and GM-CSF for 24 h, 5G9-B cells, and 5G9$^-$ blood PBMC. Because of the cell isolation procedure for obtaining 5G9$^-$ cells, adherent monocytes were cultured in GM-CSF/IL-4 for 5 days. To study the APC function of freshly isolated 5G9$^+$ cells, monocytes cultured for 24 h from the same donor were used in MLR as stimulator to compare with 5G9$^-$ cells after isolation. The first day after isolation, 5G9$^+$ B cells were consistently found to be the most potent allogeneic MLR stimulators (Fig. 9A). At a stimulator:responder ratio of 1:20, the stimulating activity of the 5G9$^+$ B cell population was 2.4-fold higher than the 5G9$^-$ B cells ($n = 5; p < 0.026$), 1.5-fold higher than monocytes cultured in IL-4 and GM-CSF for 24 h ($n = 6; p < 0.01$), and 4.1-, 3.5-, 3.4-fold higher than resting monocytes, 5G9-PBMC, and total PBMC, respectively.

Alternatively, 5G9$^+$ cells isolated by beads for use as MLR stimulators were either isolated and then cultured in medium supplemented with IL-4/CD40L for 4 days (IL-4/CD40L-5G9$^+$ cells), or isolated from PBMC after PBMC were cultured for 4 days in medium with IL-4 for MLR assay (IL-4/PBMC-5G9$^+$ cells). At this time, monocytes were cultured in IL-4/GM-CSF for 5 days.

**FIGURE 6.** Four color flow cytometry analysis of mAb 5G9 and magnetic bead purified cells (A). Events were gated on CD83 and 5G9 positivity (R2 and R3), respectively. Three populations (R4, R5, and R6) were defined based on CD19 and 5G9 expression of normal PBMC control (B) in the same experiment (R4: 5G9$^{2+}$, CD19$^{+}$; R5: 5G9$^{2+}$, CD19$^{2+}$; R6: 5G9$^{+}$, CD19$^{2+}$). The major population of isolated cells falls in R4, a part of R5, and few in R6, suggesting that the separation procedure selectively obtained higher 5G9-expressing cells. CD83 and HLA-Dr expression was assessed on these populations (lower panels). All populations expressed CD83-PE, CD19-ECD, HLA-Dr-PC5, and 5G9-FITC simultaneously.

**FIGURE 7.** CD83 expression on mAb 5G9, CD19, and CD21 isolated cells. PBMC were cultured in RPMI 1640 with 10% FCS and 1000 U/ml IL-4 for 4 days. 5G9$^+$, CD19$^+$, or CD21$^+$ cells were isolated as described in Materials and Methods. Left panel, mAb 5G9-isolated cells; middle panel, CD19-isolated cells; right panel, CD21-isolated cells. Upper panel, mouse IgG1 and goat anti-mouse IgG-FITC and mouse IgG1-PE control; lower panel, 5G9, goat anti-mouse IgG-FITC and CD83-PE.

**FIGURE 8.** Scanning electron microscope study of 5G9$^+$ cells. Typical DC-like B-Cell demonstrated “porcupine like” morphology.
The results from three experiments showed that, compared with MoDC, the APC function of 5G\(^+\) cells was significantly reduced after 5 days in culture, whether cultured in medium with IL-4, IL-4/GM-CSF, or isolated from PBMC cultured in IL-4 (Fig. 9B). At a stimulator:responder ratio of 1:20, the stimulating activity of MoDC was 1.87-fold higher than the IL-4/CD40L-5G\(^+\) cells \((n = 3; p < 0.048)\), 1.79 fold higher than IL-4/PBMC-5G\(^+\) cells \((n = 3; p < 0.043)\).

**Discussion**

A novel peripheral blood cell with B cell phenotype plus DC morphology and function, herein called B-DC, was identified and characterized using new mAb 5G9. The mAb 5G9-positive fraction of DC-like cells exhibits unique long, thin, and often beaded processes and was shown to be CD19, CD20, CD22, CD40, HLA-Dr\(^{high}\) positive, as well as IgD positive and either \(\kappa\) or \(\lambda\) chain positive. CD83 and CD86 were expressed only after positive selection with 5G9. They did not express CD3, CD4, CD5, CD10, CD25, CD13, CD14, CD15, CD16, CD33, CD56, or CD64. The 5G\(^+\) cells were either \(\kappa\) or \(\lambda\) positive. This provides the definitive proof that the cells detected are B cells and not another cell lineage capable of binding cytophilic Abs.

Functional studies showed the freshly isolated 5G\(^+\) cells to be the most potent APCs in allogeneic MLR compared with 5G\(^-\) PBMC cells, 5G\(^+\) B cells, 5G\(^+\) blood mononuclear cells, monocytes cultured in IL-4, and GM-CSF for 24 h (mo-G4). 5G\(^+\) cells consistently were the most potent allogeneic MLR stimulators (data from one of five experiments). At the stimulator:responder ratio of 1:20, the stimulating activity of the 5G\(^+\) B cell population was 2.4-fold higher than the 5G\(^-\) B cells \((n = 5; p < 0.026)\), 1.5-fold higher than MoDC cultured in IL-4 and GM-CSF for 24 h \((n = 6; p < 0.01)\), 1.6-fold higher than monocytes cultured in GM-CSF for 24 h \((n = 6; p < 0.01)\), and 4.1-, 3.5-, and 3.4-fold higher than resting monocytes, 5G\(^+\) PBMC, and total PBMC, respectively.

**FIGURE 9.** MLR stimulation function of 5G\(^+\) cells. A. The ability of 5G\(^+\) cells to induce the proliferation of allogeneic T cells was compared with total blood mononuclear cells, monocytes, 5G\(^+\) B cells, 5G\(^+\) blood mononuclear cells, monocytes cultured in IL-4, and GM-CSF for 24 h (mo-G4). 5G\(^+\) cells consistently were the most potent allogeneic MLR stimulators (data from one of five experiments). At the stimulator:responder ratio of 1:20, the stimulating activity of the 5G\(^+\) B cell population was 2.4-fold higher than the 5G\(^-\) B cells \((n = 5; p < 0.026)\), 1.5-fold higher than MoDC cultured in IL-4 and GM-CSF for 24 h \((n = 6; p < 0.01)\), 1.6-fold higher than monocytes cultured in GM-CSF for 24 h \((n = 6; p < 0.01)\), and 4.1-, 3.5-, and 3.4-fold higher than resting monocytes, 5G\(^+\) PBMC, and total PBMC, respectively. B. Comparison of T cell stimulation by MoDC and purified 5G\(^+\) cells cultured for 5 days in IL-4 and CD40L.

The morphology of 5G\(^+\) cells in such cultures demonstrated short dendrites resembling hairy cells. In PBMC or MoDC culture supplemented with IL-4 or IL-4/GM-CSF, 5G\(^+\) cells grew with good viability and expressed long dendrites for up to 15 days. Purified 5G\(^+\) cells could also be cultured in MoDC culture supernatant for up to 10 days. The increased B-DC in isolated 5G\(^+\) cells from cultured total PBMC suggests that the growth factors secreted by monocytes, MoDC, or other cells in the culture may induce the differentiation of DC-like B cells. We plan to screen a spectrum of growth factors to find the best growth factor or combination of growth factors for B-DC growth.
The percentage of 5G9+ DC-like cells in human peripheral blood was studied by flow cytometry analysis of 5G9+ cells in the high light scatter gate and low light scatter gate. The higher HLA-DR, CD83, and CD86 expression of 5G9+ cells in the high light scatter gate and the relatively larger cell size of DC-like cells shown in immunohistochemical and Wright-Giemsa of cytosin slides of 5G9+ cells suggested that cells with DC-like morphology were confined to the high light scatter gate. Typically, 5G9+, CD19+, CD3−, CD14−, and CD56− cells in the high light scatter gate represented 0.29 ± 0.06 (n = 6) of total mononuclear cells and about 3% of a B cell subpopulation. Therefore, 5G9+ DC-like B cells represented a trace population of PBMCs. Total PBMC were cultured for 4 days in RPMI 1640, 10% FCS, and 1000 U/ml IL-4. Flow cytometry analysis of isolated 5G9+ cells from these cultured cells revealed the overlapping of the percentage of CD19+ cells (90%) and percentage of cells with DC morphology studied by immunohistochemical and Wright-Giemsa stain of cytosin slides (64%), thus indicating the B cell phenotype of the DC-like cells. We also confirmed the presence of CD19 on 5G9+ cells of DC-like morphology by double immunohistochemistry staining (Fig. 3F). The marked increase in percentage of DC-like B cells in the 5G9+ fraction of cultured PBMC compared with uncultured cells (64% vs 4.7%) implies the existence of both B-DC and progenitors of B-DC in PBMC. Upon stimulation by IL-4 or growth factors secreted by other cells during the culture period, the progenitors developed into DC-like cells.

Compared with freshly isolated CD19+ and CD21+ cells, only cells isolated with mAb 5G9 were double positive for CD83, further suggesting the correlation of 5G9 Ag and DC-like cells. The high percentage of cells reactive with mouse IgG1 and goat anti-mouse IgG-FITC in the CD19-isolated fraction (Fig. 7) implies that the CD19 mAb remained on the cell surface after the isolation procedure. The low percentage of CD21 expression on isolated CD21+ cell fraction suggests that, after isolation, CD21 Ag was lost from the B cell surface. The negative reaction of isolated 5G9+ cells with goat anti-mouse IgG-FITC suggests that 5G9 Ag/Ab complexes were shed or completely internalized after overnight culture. The positive reaction with goat anti-mouse IgG-FITC after incubating with freshly added mAb 5G9 suggests that 5G9 Ag may recycle to the cell surface or that new Ag may be synthesized.

Morphologically, 5G9+ B-DC are most similar to human tonsil follicular dendritic cells (FDC) isolated by Hart et al. (11). Both 5G9+ B-DC and FDC have long, fine, and often beaded membrane processes, which suggests the close relationship between the two cell types. The beaded structures on the processes of FDC were described by Hart et al. as “consisting of a series of interconnected immune complex coated bodies, termed ‘iccosomes.’” The function of “iccosomes” of FDC was demonstrated to mediate the delivery of Ag to germinal center B cells and macrophages (12, 13). The similar “iccosomes” structure was observed on the processes of 5G9+ B-DC, implying a common function of these cells. However, FDC were lineage negative. The lineage relationship of DCs with other hemopoietic cells and within the broader class of DC is not well understood. DC in different tissue sites and having slightly different characteristics all play a specialized role in maintaining self tolerance by the endocytosis and presentation of Ags within their environment. Recent evidence now suggests a possible lineage relationship between T cells and lymphoid DCs. Like B-DC, the discovery of T cell-derived DC also appears to conflict with the view that DCs have an exclusive common origin with myeloid cells. One possibility is that DCs mature in different tissue sites from bone marrow-derived precursors and develop region-specific characteristics that could reflect lineage differences.

Although pioneering studies of DC by Steinman and Cohn identified mouse spleen DC in 1973 and established lymphoid tissue-derived DC as potent stimulators of primary immune response (2, 14–17), most current studies of DC are concentrated on myeloid lineage-derived DC. One generally recognized DC feature is its lineage negativity. A recent working definition of blood DC by Hart (18) emphasized a cell-surface Ag phenotype distinguishing it from other leukocytes, notably monocytes/macrophages and B lymphocytes. The most current separation procedures of blood DC are also based on the lineage-negative feature of DC. The finding in this study of DC-like cells with B cell surface Ag phenotype appears to conflict with current definitions of DC. However, the DC ontogeny has remained controversial since the discovery of DC. The DC-like B cells described in the current study possess consensus DC properties and characteristics. The distinctive morphology, the potent ability to stimulate allogeneic T cell proliferation, marked cell motility, and active membrane extension/retraction, and the expression of certain DC-associated Ags such as CD83 and HLA-DRh high lead to the conclusion that B-DC are previously unrecognized members of the DC family.

Galy et al. recently found that a subpopulation of CD34+ lin CD45RA−CD10+ cells gives rise only to lymphoid T, B, NK, and DC. Limiting dilution analysis demonstrates the existence of multipotential B/NK/DC progenitor clones in the CD34+lin CD10+ adult bone marrow cell population, indicating that nonprimitive progenitors for lymphoid cells and for DC can be distinct from those of myeloid DC. The implication is that at least one DC subset is developmentally more closely related to lymphoid lineage than to the myeloid lineage (3). A subset of B cells capable of assuming dendritic morphology after stimulation with phorbol esters and attachment to a surface was reported by Corradi and Lipsky in 1987 (19). Serreze (4) studied the APCs that contribute to the development and activation of diabeticogenic T cells in the NOD mouse model of IDDM. They utilized a functionally inactivated Ig µ allele to generate a “speed congenic” stock of B cell-deficient NOD mice. These mice were free of overt IDDM and were insulin resistant, even with normal numbers of T cells. The implication is that B cells play an unrecognized role, perhaps as potent APCs, which is essential for initial development and/or activation of β cell autoreactive T cells in NOD mice. Cerny’s study (20) concluded that mature B cells and/or B cell products are required for the development of mature FDC in the mouse lymph node. A novel member of the ubiquitin family was isolated from human DC by Bates (26). Expression of this 777-bp mRNA was restricted to DC and B cells, with strong expression in
mature B cells. Comparative analysis and the expression pattern of this gene suggested a function in Ag processing and presentation. Vesel recently reported three patients with low grade B cell lymphoproliferative disease who developed subsequent DC tumors, and indicated the clear association between these tumors and low grade B cell malignancies (27). Bjorck’s recent study demonstrated that murine CD19+ pro-B cells have the B/DC bipotential lineage capacity (5). The above studies suggested a close relationship between DC and B cells and the possibility that B cells can be morphologically and functionally differentiated to DC. The morphological and functional similarity of 5G9+ B/DC and FDC, and the fact that both 5G9+ B/DC and FDC have beaded “icososomes” on the long fine processes, suggests that the study of circulating B/DC may shed new light on the mysterious origin of FDC.

When Ag binds to the Ab molecules on the surface of a virgin or a memory B cell, it initiates a complicated series of events, culminating in B cell proliferation and maturation to produce either memory cells or active Ab-producing plasma cells, apparently distinct from DC. This classic pathway of B cell development, and the separation of circulating DC-like cells from the blood B cell fraction by this study, suggest that an alternative outcome may develop during B cell development. A fraction of B cells, which might be a fraction of memory B cells or differentiate simultaneously with memory B cells during primary immunization, may develop into DC. With Ag-specific Igs on their surface and class II HLA expression, these cells may be very efficient Ag-specific professional APCs, and function as positive feedback cells to stimulate further T cell proliferation and subsequent Ab-specific B cell proliferation, and thus produce a more profound immune response. A suggested hemopoietic differentiation pathway for DC-like B cells or B-DC is shown in Fig. 10. The exact differentiation pathway of B-DC is unknown at present. However, the proposed pathway suggests that B-DC might develop from B cell precursors, lymphoid DC precursors, memory B cells, or naive B cells.

The long hairy appearance of DC-like B cells raises the possibility that they are the normal counterparts of the hairy cell leukemia (HCL). Although definitive evidence of the B cell origin of HCL is now available (28–30), the exact position of these cells in B cell development remains controversial. Phenotyping and gene expression studies of DC-like B cells compared with HCL may yield clues to the origin of hairy cells.

Almost every fraction of blood cells has been examined for biologic therapy, including T cells, NK cells, DC, monocytes/macrophages, and granulocytes. Few efforts have been conducted with B cells. As we demonstrate in this study, B cells or DC-like B cells may be the next candidates for clinical trials as APCs.

References


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