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Generation of Dendritic Cells *In Vitro* From Peripheral Blood Mononuclear Cells With Granulocyte-Macrophage–Colony-Stimulating Factor, Interleukin-4, and Tumor Necrosis Factor-α for Use in Cancer Immunotherapy

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Objective
The purpose of the study was to characterize the requirements in terms of precursors, developmental pathways, and media for the generation of large numbers of mature dendritic cells (DC) under conditions acceptable for use in adjuvant, active immunotherapy strategies for surgically treated malignancies.

Summary Background Data
Although limited previously by the small numbers accessible, DC-based immunotherapies for malignancy have become more realistic with the development of methods for efficiently generating larger numbers of DC from peripheral blood mononuclear cells (PBMC) *in vitro,* but these methods rely on clinically unacceptable culture conditions (such as inclusion of fetal bovine serum), necessitating the development of methods for generating functionally equivalent DC in serum-free conditions.

Methods
Plastic-adherent PBMC (from healthy donors and patients with cancer) were incubated for 7 days with granulocyte-macrophage–colony-stimulating factor (GM–CSF) and interleukin-4 (IL-4) with and without tumor necrosis factor-α (TNF-α) in fetal bovine serum-containing and serum-free media and were analyzed by Wright's stain for morphology, flow cytometry for phenotype, and mixed lymphocyte reaction for allostimulatory function.

Results
Growth in either serum-containing or serum-free media supplemented with GM–CSF and IL-4 yielded a similarly heterogeneous population of cells, 6% to 10% of which had the morphology (large cells with thin projections), immunophenotype (including CD83+), and function of mature DC. Tumor necrosis factor-α significantly augmented the number of these mature DC, whereas preculture depletion of CD14+ PBMC virtually eliminated them.

Conclusions

Generation of mature DC in the authors' serum-free clinically applicable conditions is similar to serum-containing conditions and requires CD14+ precursors, differentiation through a CD14−CD83− immature stage under the influence of GM−CSF and IL-4, and maturation into a CD83+ DC under the influence of TNF-α.

To improve on the modest absolute survival benefits of standard adjuvant therapies for surgically resected breast, colon, and pancreatic cancer[1−3] and the lack of benefit for other solid tumor malignancies, new strategies such as immunotherapy are being developed. Immunologic abrogation of tumors occurs primarily by the recognition of tumor-associated antigens such as carcinoembryonic antigen by cytotoxic T lymphocytes capable of lysing cells that express the specific antigen.[4] Dendritic cells (DC), the primary stimulators of T-cell-initiated immune responses[5−10] *in vivo,* prime naive T cells to specific antigens in a human leukocyte antigen (HLA)-restricted fashion. Thus, injections of DC pulsed with HLA-restricted peptides are considered the most promising method of stimulating potent *in vivo* cytotoxic T-lymphocyte responses against tumors expressing the peptides. In animal models[11−15] and one human study,[16] this has led to demonstrable tumor regressions and prevention of new tumor development. Thus, there is considerable excitement in using DC to induce active immunity in patients with cancer.[17]

To use DC in active immunotherapy strategies, large numbers of mature DC must be produced under conditions acceptable for clinical use. Although DC have been identified throughout the body,[18−20] previous methods for directly isolating DC (density gradient centrifugations, plastic adherence, sheep erythrocyte rosetting, and various panning techniques[21−25]) have yielded small numbers, limiting their clinical application. More recently, simple procedures for generating large numbers of dendritic-like cells have been developed based on culturing peripheral blood mononuclear cells (PBMC) or their precursors in serum-containing media supplemented with granulocyte-macrophage−colony-stimulating factor (GM−CSF) and interleukin-4 (IL-4) or TNF-α.[5,26−33] These cultures have been shown to contain DC by their characteristic constellation of morphologic, immunophenotypic, and functional characteristics, including the expression of high levels of HLA class I, HLA class II, the absence of the lineage specific markers CD3, CD14, CD16, CD19, and the ability to stimulate potent allogeneic T-cell responses in mixed lymphocyte reactions (MLR). These procedures, however, rely on the addition of fetal bovine serum, which is undesirable clinically because of the risk of allergic reaction or stimulation of immune responses against unintended antigens.

Because of our interest in optimizing the activity and yield of DC for therapeutic uses, we sought to determine the required precursors and maturational pathways that, although only recently evaluated in serum-containing conditions,[34,35] are not known in serum-free conditions. To characterize mature DC, we have used morphology, allostimulatory activity, and phenotype emphasizing the presence of CD83, a 45,000-MW glycoprotein in the immunoglobulin superfamily reported by Zhou and Tedder and others,[36−38] to be a specific marker for mature DC whether directly isolated from blood and generated in GM−CSF, IL-4, and TNF-α. The data collected for these experiments serve as the basis for our ongoing clinical trials with DC-based active immunotherapy for carcinoembryonic antigen-expressing malignancies.

METHODS

Dendritic Cell Cultures

Peripheral blood mononuclear cells were isolated from either leukocyte-enriched buffy coats prepared from donated units of peripheral blood (American Red Cross, Durham, NC) or from heparinized peripheral blood by density gradient centrifugation on lymphocyte separation medium (Organon Teknika, Durham, NC). Heparinized peripheral blood was obtained from healthy donors after informed consent according to protocols approved by the Human Use Committee of Duke University Medical Center. For serum-containing conditions, 1.5×10^7 mononuclear cells were plated in 3-mL RPMI-1640 (Life Technologies, Grand Island, NY)/200-mmol L-glutamine (Life Technologies), 50-mmol 2-mercaptoethanol (Sigma Chemical, St. Louis, MO), 20 μg/mL gentamicin (Sigma Chemical), 10% fetal bovine serum (FCS, Life Technologies). After 2 hours of incubation at 37 C, the nonadherent cells were removed with a gentle rinse and discarded. The adherent cells were cultured in RPMI-1640/50-mmol 2-mercaptoethanol/20 μg/mL gentamicin/10% FCS supplemented with cytokines. After 5 to 7 days' incubation at 37 C in 5% carbon dioxide, the cells were washed vigorously from the plate and analyzed. Cytokines used

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in these experiments included rh-GM–CSF (Schering, Kenilworth, NJ); rh-IL-4 and rh-TNF-α (Life Technologies); rh-IL-1α (10, 50, 100 units/mL), IL-1β (20, 100, 500 units/mL); c-kit ligand (20, 100, 500 units/mL), rh-IL-3 (25, 125, 625 units/mL), rh-M–CSF (20, 100, 500 units/mL), rh-IL-2 (10, 50, 250 units/mL) (all from R& D Systems, Minneapolis, MN). In serum-free cultures, cells were grown exactly as described for serum-containing cultures, except AIM V (Life Technologies) was substituted for RPMI-1640, and no FCS was added.

Peripheral blood mononuclear cells were depleted of CD14+ cells by incubation in anti-CD14 (Leu M3; Becton Dickinson, San Jose, CA) followed by the addition of rat antimouse-conjugated ferrous beads and application to a mini-MACS column (Miltenyi Biotec, Sunnyvale, CA) after column instructions. The CD14-depleted PBMC were characterized by fluorescence-activated cell sorter (FACS) analysis for the residual CD14 cell content by staining with a different CD14 antibody (TUK4; Dako, Carpenteria, CA) shown previously not to be blocked by Leu M3. CD14-depleted PBMC were cultured as above.

Monoclonal Antibodies, Immunofluorescence Staining, and Fluorescence-Activated Cell Sorter Analysis

The following murine antihuman monoclonal antibodies were used for immunofluorescence staining: anti-CD3 (Leu-4; Becton Dickinson), anti-CD4 (Leu-3a; Becton Dickinson), anti-CD11c (Dako, Carpenteria, CA), anti-CD14 (Becton Dickinson), anti-CD15 (Dako), anti-CD16 (Becton Dickinson), anti-CD18 (Dako), anti-CD19 (Dako), anti-CD54 (Becton Dickinson), anti-CD80 (Becton Dickinson), anti-CD86 (PharMingen, San Diego, CA), anti-CD83 (HB15),[36–38] anti-HLA A,B,C (PharMingen), and anti-HLA-DR (Becton Dickinson). In two color analyses, CD83 was identified by indirect staining with PE-conjugated F(ab')$_2$ goat antimouse immunoglobulin-G (Dako) and the second antigen was identified by fluorescein isothiocyanate-conjugated antibodies. (The exceptions were CD54 and CD80 for which only PE-conjugated antibodies were available and, therefore, the indirect staining of CD83 was performed with fluorescein isothiocyanate-conjugated F(ab')$_2$ goat antimouse immunoglobulin-G (Dako), and the second-antigen was identified directly with PE-conjugated monoclonal antibody.)

Immunofluorescence staining was performed by washing cells with medium supplemented with 2% FCS followed by incubation on ice for 20 minutes with the appropriate antibodies. Labeled cells were washed with PBS containing 2% FCS and fixed with 1% paraformaldehyde (Sigma Chemical). Greater than 10,000 events were collected on a FACScan or FACStar^{PLUS} using a 488 argon laser for fluorescence excitation (Becton Dickinson). Data were analyzed using CellQuest software (Becton Dickinson) on a Macintosh computer (Apple Computer, Inc., Cupertino, CA). In all experiments, isotypically stained cells were used to set cursors so that the results of $<1\%$ of the cells were considered positive.

Wright–Giemsa Staining

Cytospins were prepared by cytocentrifuging 1×10^5 cells onto glass slides at 900 revolutions per minute for 3 minutes. Cytospins were air-dried and stained with Wright–Giemsa stain and examined by light microscopy.

Electron Microscopy

For transmission electron microscopy, cells were pelleted by centrifugation and fixed with 2% glutaraldehyde in 150-nM sodium cacodylate buffer plus 2.5-mM calcium chloride, pH 7.2. The pellet was washed with buffer, embedded in 1% agar, postfixed for 1 hour on ice with 2% osmium tetroxide plus 1% potassium ferrocyanide, and washed with cacodylate buffer followed by 200-mM sodium acetate, pH 5.2. Samples were stained *en bloc* for 1 hour with 1% uranyl acetate in sodium acetate buffer. After dehydration with ethanol, the pellet was infiltrated with and embedded in EMBED 812 (EM Sciences, Fort Washington, PA). Thin sections of 90 nm were cut on a Reichart Ultracut E microtome and stained with uranyl acetate, followed by Sato lead, washed and examined with a Philips EM300 electron microscope (Philips, Eindhoven, The Netherlands).

Mixed Lymphocyte Reactions

Allogeneic responder PBMC (1.5×10^5) obtained from healthy donors were cultured in RPMI-1640 supplemented with 10% FCS or 10% human antibody serum in 96-well U-bottom tissue culture plates. Irradiated (3500 rads) DC preparations were added in graded doses of 150 to 15,000 cells in a total volume of 200 μL. Cell proliferation after 96 hours was quantified by adding 1 μCi (37 kBq) of [*methyl*^{3}H] thymidine (NEN–DuPont, Boston, MA) to each well. After 16 hours, the cells were harvested onto filters, and radioactivity was measured in a scintillation counter with results presented as the mean cycles per minute for triplicate cultures.

RESULTS

Peripheral Blood Mononuclear Cells Grown in the Presence of Granulocyte-Macrophage–Colony-Stimulating Factor and Interleukin-4 Generate Heterogeneous Populations of Cells

To compare the generation of DC in serum-free media with the better characterized serum-containing media, DC

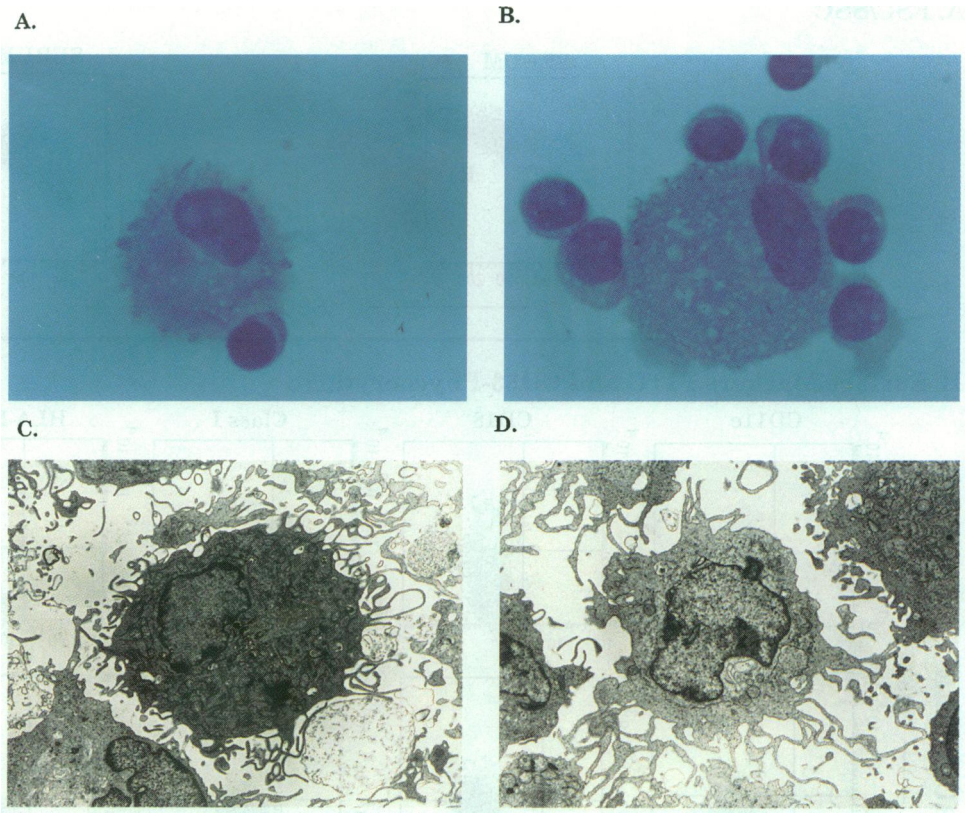

Figure 1. Dendritic cells generated from peripheral blood mononuclear cells in serum-free conditions were examined with light microscopy (B) or electron microscopy (D) and compared to dendritic cells generated in serum-containing cultures (A and C, respectively).

were generated from PBMC in both types of media supplemented with GM–CSF and IL-4. At the end of 5 to 7 days of culture, nonadherent and moderately adherent cells were removed with vigorous washing and examined for morphology and immunophenotype. In both serum-free and serum-containing cultures, large cells with dendritic processes and large eccentric nuclei, consistent morphologically with DC, readily were identified (Figs. 1A–1D). Numerous small cells with a lymphocytic morphology also were identified. By flow cytometric analysis, a heterogeneous population of cells based on light scatter properties and surface immunophenotype also was seen (Figs. 2A and 2B, representative of eight experiments). The large cells within these cultures (Region R1 in Figs. 2A and 2B) expressed CD11c, CD18, HLA class I, HLA-DR and failed to express CD14, CD15, CD3, CD16, and CD19 (data not shown). A minority of the large cells (3%–15%) also expressed CD83, CD86, and low levels of CD80. The small cell populations in both serum-containing and serum-free cultures (R2 in Figs. 2A and 2B) primarily were made up of CD3+ and CD19+ lymphocytes (data not shown). Cells grown in serum-free conditions tended to be more adherent to the culture dishes than did cells grown in serum containing conditions and contained a population of cells that was strongly adherent. In addition, the expression of CD83 was nonsignificantly lower in serum-free cultures (Fig. 2B).

Large Cells that Express CD83 in the Peripheral Blood Mononuclear Cells Cultures Are the Most Potent Stimulators in the Mixed Lymphocyte Reaction

To determine whether the large cells that expressed CD83 (large CD83+ cells) possessed the functional properties of DC, cells from the PBMC cultures were sorted on the basis of size and CD83 expression and tested in an allogeneic MLR. The large CD83+ cells derived from both serum-containing or serum-free cultures were the most potent stimulators of the MLR. In particular, the large CD83+ cells were more potent stimulators than were large CD83– cells, small CD83– cells, small CD83+ cells, and PBMC (Fig. 3).

Tumor Necrosis Factor-α Increases the Yield of Large CD83+ Cells from Peripheral Blood Mononuclear Cells Cultured with Granulocyte-Macrophage–Colony-Stimulating Factor and Interleukin-4

To improve the yield of large CD83+ cells from PBMC, the effects of adding additional cytokines to GM–CSF and IL-4 in serum-containing and serum-free cul-

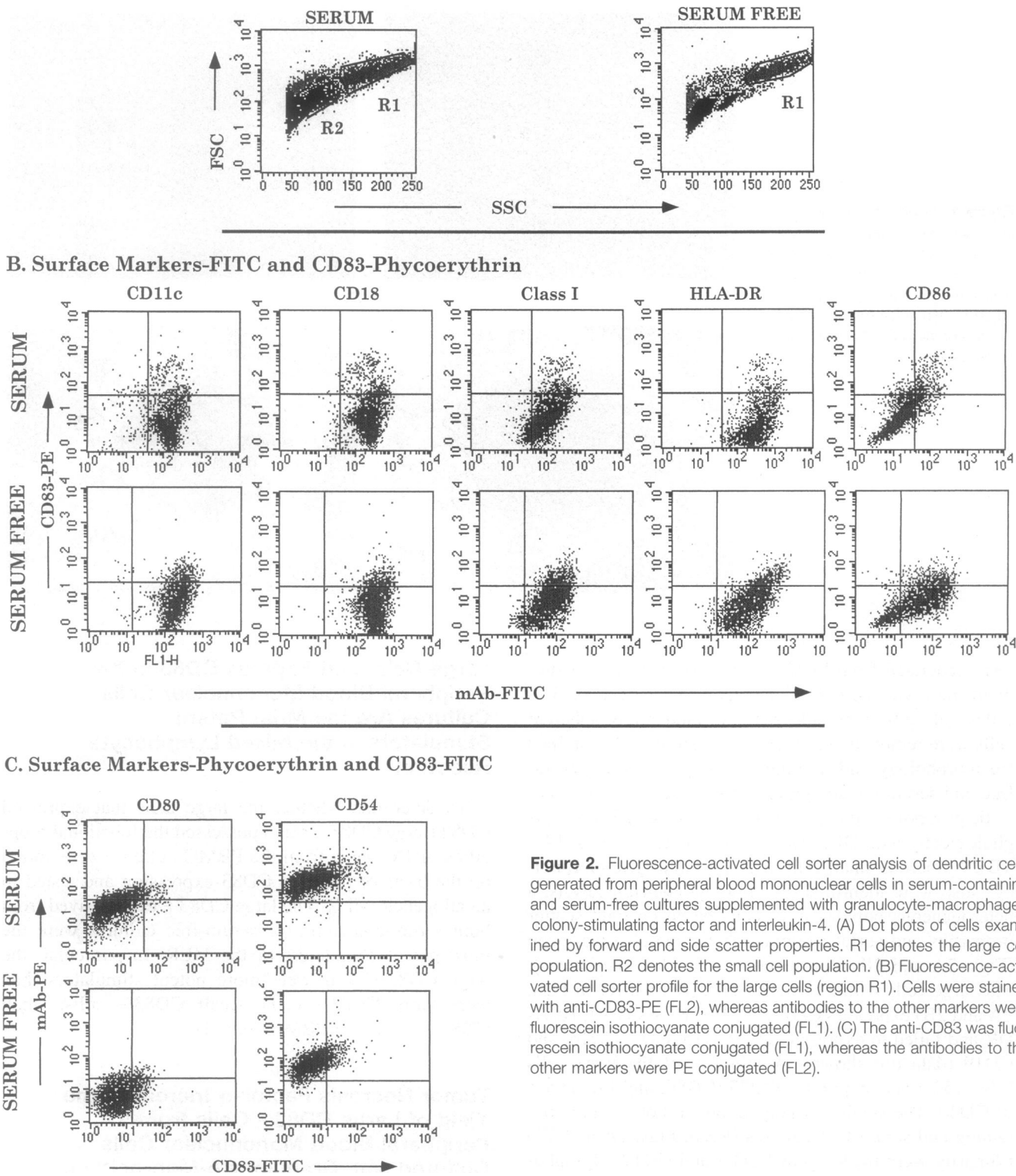

Figure 2. Fluorescence-activated cell sorter analysis of dendritic cells generated from peripheral blood mononuclear cells in serum-containing and serum-free cultures supplemented with granulocyte-macrophage–colony-stimulating factor and interleukin-4. (A) Dot plots of cells examined by forward and side scatter properties. R1 denotes the large cell population. R2 denotes the small cell population. (B) Fluorescence-activated cell sorter profile for the large cells (region R1). Cells were stained with anti-CD83-PE (FL2), whereas antibodies to the other markers were fluorescein isothiocyanate conjugated (FL1). (C) The anti-CD83 was fluorescein isothiocyanate conjugated (FL1), whereas the antibodies to the other markers were PE conjugated (FL2).

tures were evaluated. Cultures containing GM–CSF and IL-4 were supplemented with various cytokines including rh-IL-1α, rh-IL-1β, rh-IL-2, rh-IL-3, rh-M–CSF, c-kit ligand, and rh-TNF-α on the first day of culture. Only TNF-α increased the yield of large CD83+ cells (Fig. 4), whereas no increase in yield was noted with any other cytokines (data not shown). In all cases, the increase in the level of expression of CD83 was more pronounced

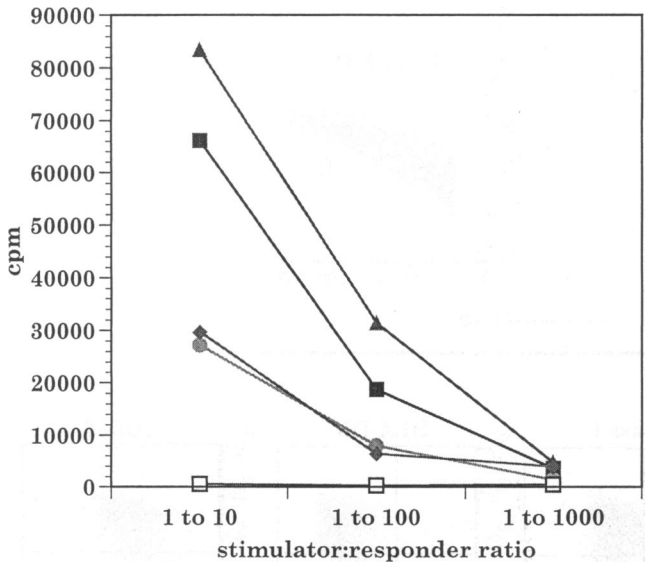

Figure 3. The proliferative response of allogeneic peripheral blood mononuclear cells to large CD83+ cells (▲) sorted from the heterogeneous cell population obtained after 7 days in serum-free cultures containing granulocyte-macrophage–colony-stimulating factor and interleukin-4 compared to large CD83− cells sorted from serum-free cultures (♦), large CD83+ cells grown in serum-containing cultures (■), large CD83− cells grown in serum-containing cultures (●), and uncultured peripheral blood mononuclear cells (□). The data are representative of four individual experiments.

in serum-containing cultures than in serum-free cultures (Figs. 5A and 5B), and no effect of TNF-α was noted on the immunophenotype of the small cells in these cultures (data not shown). There was a dose-response effect of TNF-α with a plateau between 50 and 100 ng/mL. The mean number of large CD83+ cells generated under various conditions is described in Figure 4. The differences in the yield of large CD83+ cells generated under serum-free conditions or serum-containing conditions were not statistically significant, although there was a trend toward recovery of fewer large CD83+ cells in serum-free conditions.

Cells cultured with TNF-α added to GM–CSF and IL-4 typically possessed more potent allostimulatory function than did cells cultured in serum-free media with GM–CSF and IL-4 alone (Fig. 6). No significant differences in allostimulatory function were noted between cells generated in serum-free and serum-containing conditions (Fig. 6).

Tumor Necrosis Factor-α Increases the Yield of Large CD83+ Cells by Increasing the Expression of CD83 on Large CD83− Cells

Potentially, TNF-α could improve the yield of large CD83+ cells either by promoting the proliferation of DC precursors or by inducing further differentiation of immature DC or both. To determine which of these mechanisms was operative, PBMC grown in serum-free cultures with GM–CSF and IL-4 were supplemented with TNF-α and examined for CD83 expression at progressive timepoints. In serum-containing media, maximal expression of CD83 occurred within 12 hours (Fig. 7A). In serum-free conditions, maximal expression occurred within 12 to 24 hours (Fig. 7B). Irradiating the cells before adding TNF-α had no significant effect. These observations indicate that TNF-α increased the yield of large CD83+ cells primarily by promoting the expression of CD83 rather than by promoting cell proliferation.

Depletion of CD14+ from Peripheral Blood Mononuclear Cells Reduces the Yield of Large CD83+ Cells

Recently, directly isolated CD14+ peripheral blood cells cultured in GM–CSF, IL-4, and TNF-α were shown to differentiate into CD83+ DC.[28] To define whether CD14+ cells within cultured PBMC were required for generating most of the large CD83+ cells, PBMC were depleted of CD14+ cells by immunoabsorption and cultured in serum-containing and serum-free media for 5 to

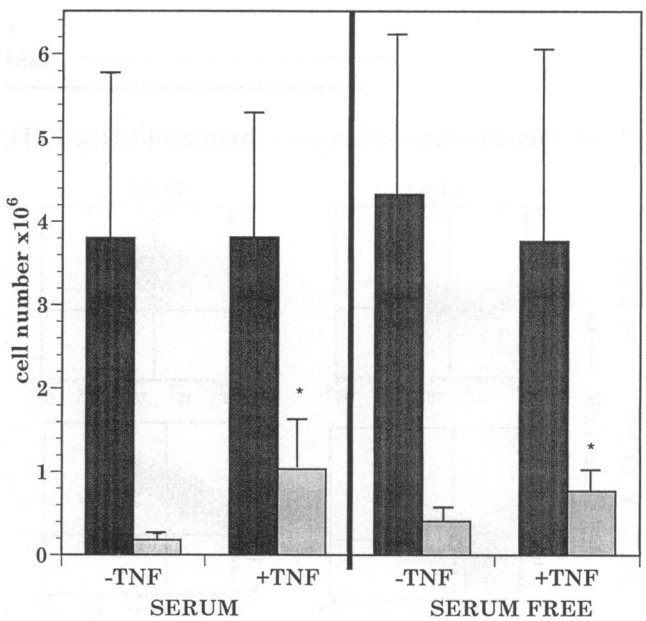

Figure 4. The yield of total cells (black bars) and large CD83+ cells (open bars) was determined in cultures of 15 × 10^6 peripheral blood mononuclear cells grown in serum-free and serum-containing cultures, with and without the addition of tumor-necrosis factor (TNF-α). The data are presented as the means and standard deviations of eight individual experiments. * denotes $p < 0.05$ between cells grown with and without TNF-α. The actual cell numbers (×10^6) for the large CD83 positive cells were: 0.18 ± 0.08 (+serum), 0.4 ± 0.27 (serum-free), 1.03 ± 0.64 (+serum, +TNF-α), 0.77 ± 0.28 (serum-free, +TNF-α).

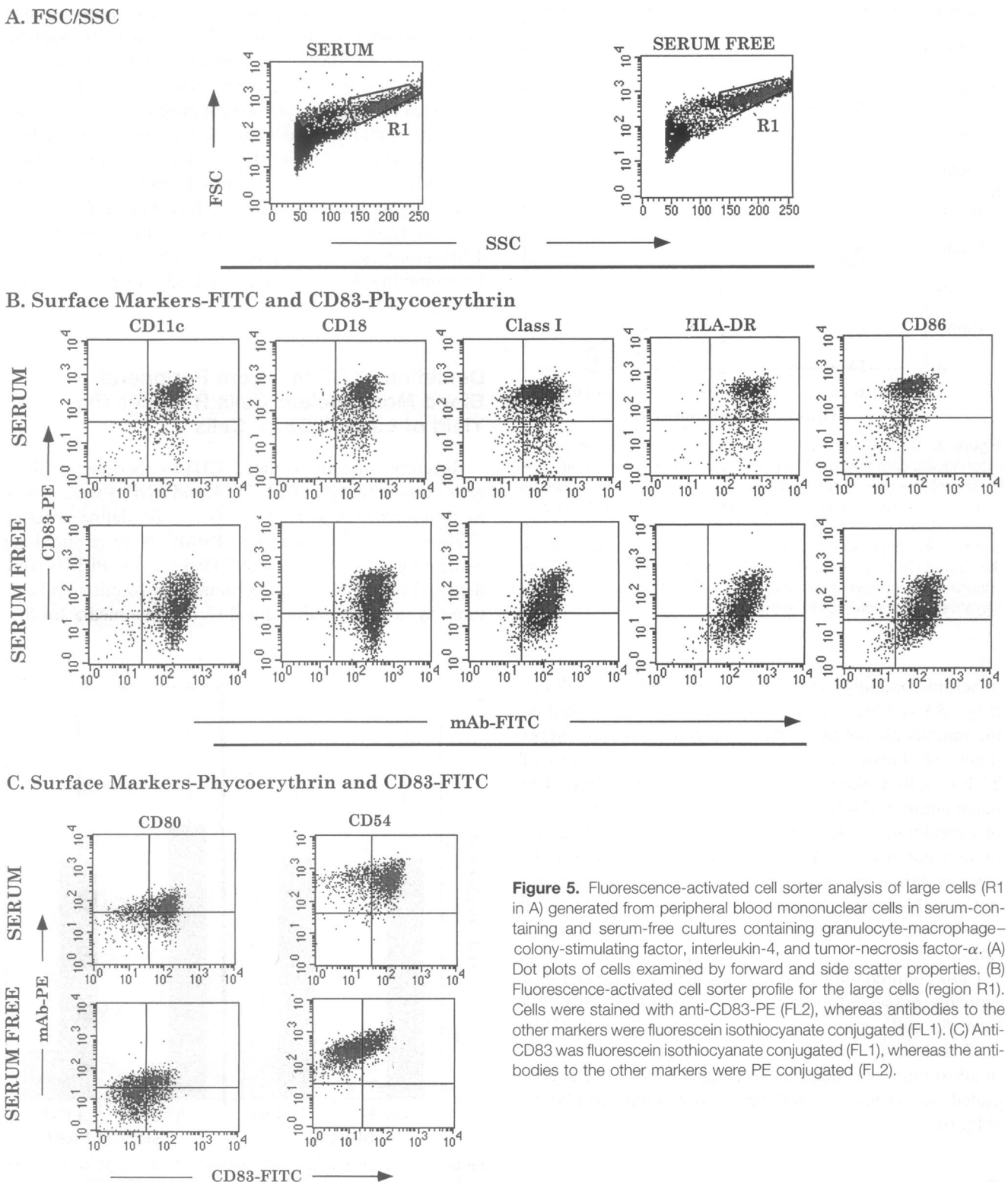

Figure 5. Fluorescence-activated cell sorter analysis of large cells (R1 in A) generated from peripheral blood mononuclear cells in serum-containing and serum-free cultures containing granulocyte-macrophage–colony-stimulating factor, interleukin-4, and tumor-necrosis factor-α. (A) Dot plots of cells examined by forward and side scatter properties. (B) Fluorescence-activated cell sorter profile for the large cells (region R1). Cells were stained with anti-CD83-PE (FL2), whereas antibodies to the other markers were fluorescein isothiocyanate conjugated (FL1). (C) Anti-CD83 was fluorescein isothiocyanate conjugated (FL1), whereas the antibodies to the other markers were PE conjugated (FL2).

7 days in the presence of GM–CSF, IL-4, and TNF-α. Greater than 98% of the CD14+ cells were eliminated from the PBMC before culture as determined by FACS analysis (data not shown). After culture, the number of large CD83+ cells was reduced 83% in serum-containing media and 73% in serum-free conditions, whereas the total cell number was not affected substantially in any condition (Fig. 8). The scatter properties and CD83 stain-

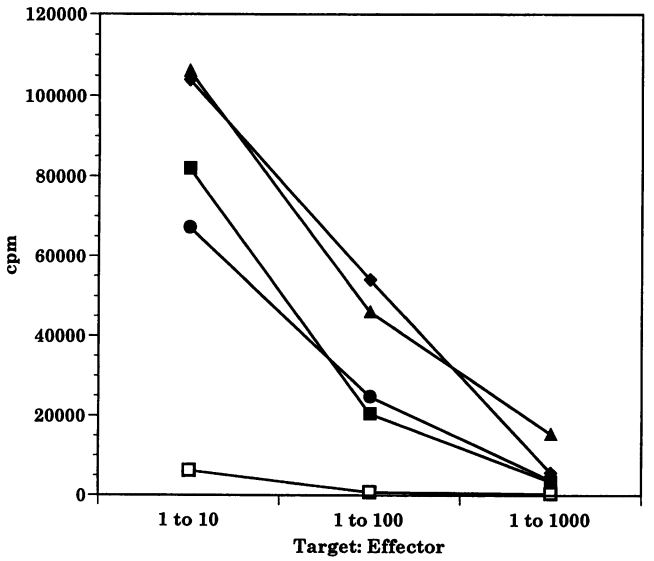

Figure 6. The proliferative response of allogeneic peripheral blood mononuclear cells to the heterogeneous population of cells isolated from serum-containing (●) and serum-free cultures (■) supplemented with granulocyte-macrophage–colony-stimulating factor and interleukin-4 compared to the heterogeneous population of cells isolated from serum-containing (♦) and serum-free cultures (▲) supplemented with granulocyte-macrophage–colony-stimulating factor, interleukin-4, and tumor necrosis factor, and peripheral blood mononuclear cells (□). The data are representative of eight individual experiments.

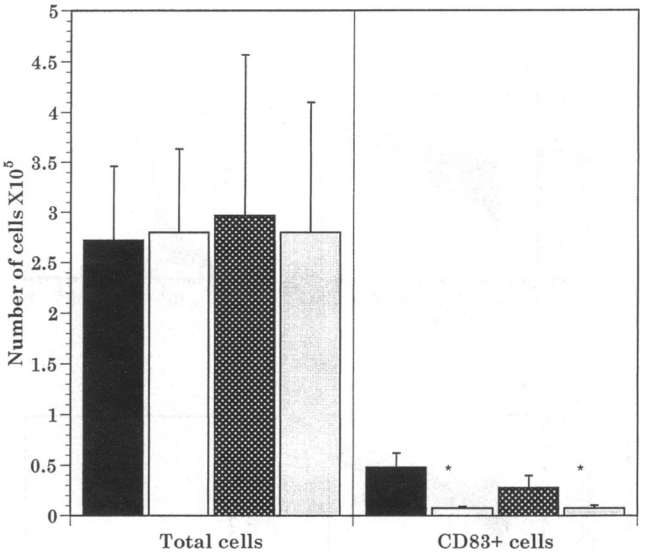

Figure 8. Reduction in the yield of large CD83+ cells after depletion of CD14+ cells from peripheral blood mononuclear cells (PBMC). The CD14-depleted PBMC were grown for 5 days in granulocyte-macrophage–colony-stimulating factor, interleukin-4, and tumor necrosis factor-α and compared to cultured PBMC containing CD14+ cells. The solid bars represent cells grown in serum-containing conditions, and the dotted bars represent cells grown in serum-free conditions. The light bars represent PBMC depleted of CD14+ cells. The data are the mean and standard error of three experiments. The * denotes a significance of $p < 0.05$.

ing of the few remaining large CD83+ cells in either culture condition were similar to those of the large CD83+ cells generated from PBMC that had not been depleted of CD14+ cells (Fig. 9).

DISCUSSION

Dendritic cells are considered among the most promising agents for inducing active immunity against tumors. Their use avoids difficulties encountered with other methods such as antivector immune responses, which limit viral vector gene therapy strategies,[39] relatively nonspecific responses seen with systemic cytokine therapies,[40] and the need to obtain and grow adequate numbers of autologous cancer cells for tumor cell vaccination techniques. Furthermore, animal studies now have shown both protection from tumor challenge[14,15] and regression of established metastases[14,41] after injections with DC pulsed with peptide[14,15] or tumor RNA.[41] To use DC in active immunotherapy strategies, define the conditions for obtaining large numbers under clinically acceptable conditions.

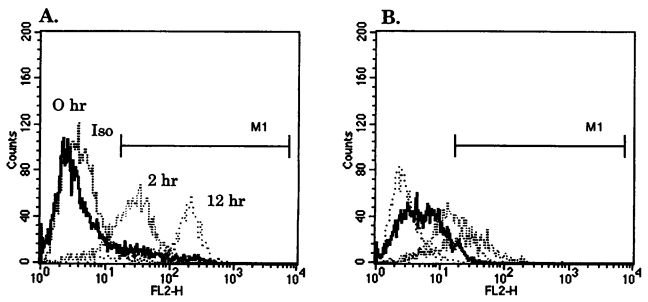

Figure 7. The time course of expression of CD83 after the addition of tumor necrosis factor-α to serum-containing cultures (A) and serum-free cultures (B) was measured by fluorescence-activated cell sorter analysis. Cells were stained with anti-CD83 and goat-antimouse-PE (FL2) or an isotypic control at baseline, 0 hour, 2 hours, and 12 hours as indicated (A).

In the current study, we sought to characterize the development of DC generated from PBMC in the presence of GM–CSF, IL-4, and TNF-α in clinically applicable serum-free conditions and to compare it with the development in the better characterized serum-containing conditions. Culturing PBMC with GM–CSF and IL-4 alone generated a heterogeneous population of cells comprised of large cells with the morphology and immunophenotype of DC and small cells with the morphology and immunophenotype of lymphocytes. The large cell population uniformly expressed high levels of class I, HLA-DR, CD86, CD18, and CD11c and failed to express CD14, T-cell markers, and B-cell markers. In the absence of TNF-α, only 5% to 15% of these large cells expressed the mature DC marker CD83. These large CD83+ cells were the

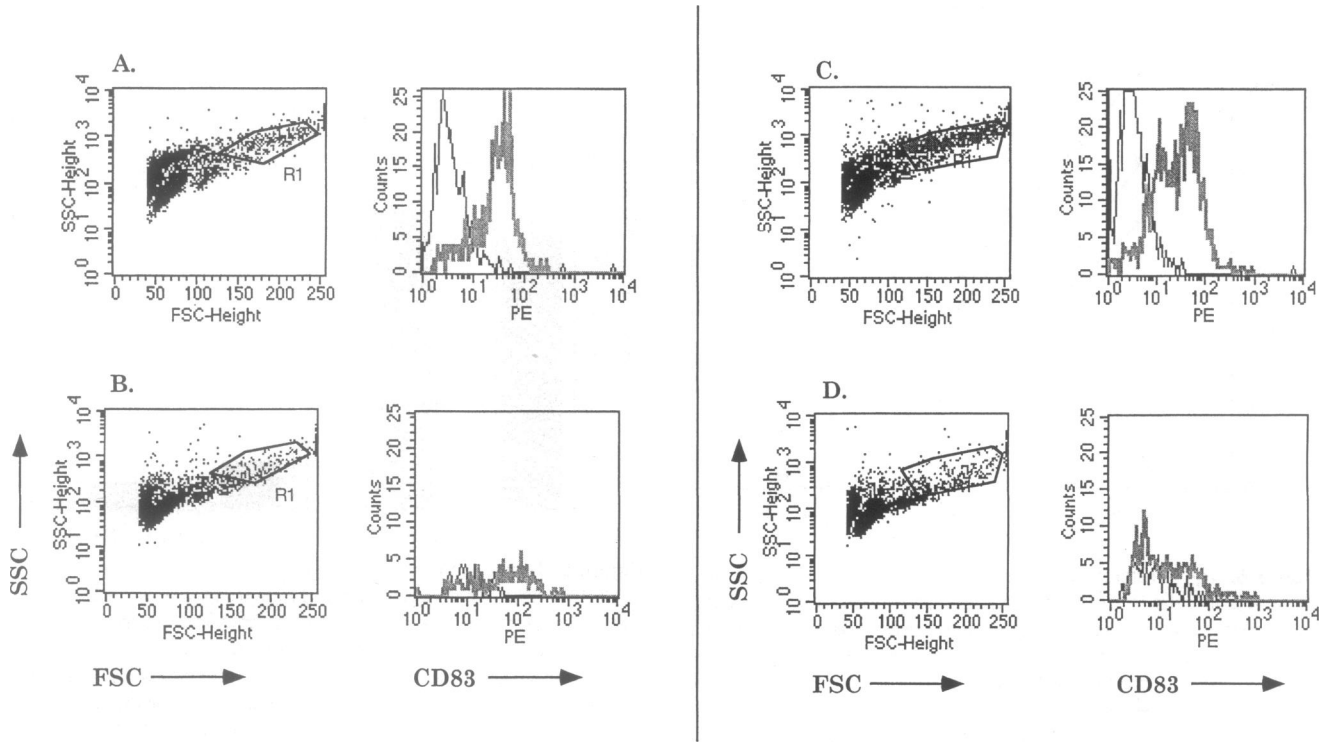

Figure 9. Fluorescence-activated cell sorter analysis of peripheral blood mononuclear cells grown in granulocyte-macrophage–colony-stimulating factor, interleukin-4, tumor necrosis factor-α with (B and D) and without (A and C) CD14+ depletion. (A and B) Serum-containing cultures and (C and D) serum-free cultures. The data are representative of three experiments.

most potent allostimulatory cells in mixed lymphocyte reactions, whereas large CD83− cells were less potent allostimulators. Both populations were significantly more effective than were small CD83+ cells, small CD83− cells, or PBMC as allostimulators. The addition of TNF-α to PBMC cultured in GM–CSF and IL-4 caused a significant increase in the number of large CD83+ cells predominantly via a rapid increase in CD83 expression in large CD83− cells. Depletion of CD14+ cells from the cultured PBMC significantly reduced the yield of large CD83+ cells.

Recently, Zhou and Tedder[34] have shown that purified peripheral blood CD14+ cells differentiate into mature DC via a two-step process. In the first step, CD14+ cells cultured with GM–CSF and IL-4 differentiate into CD1a+CD14+CD83− cells. In the second step, TNF-α induces the differentiation of these cells into CD1a+CD14−CD83+ cells that appeared to be mature DC based on their morphology, immunophenotype, and stimulating activity in MLR. These findings, along with the observations made in the current studies, support the following hypotheses. First, the large CD83+ cells generated from cultured PBMC are mature DC, whereas the large CD83− cells are immature DC. Second, the development of mature DC from PBMC depends largely on the presence of CD14+ cells in the starting cell population. Third, GM–CSF and IL-4 predominantly support the development of immature CD83− DC, whereas TNF-α promotes the differentiation of immature CD83− DC into mature CD83+ DC.

To determine whether factors in addition to GM–CSF, IL-4, and TNF-α may influence the development DC generated from PBMC, two sets of studies were performed. In the first set of studies, additional cytokines including rh-IL-1α, rh-IL-1β, rh-IL-2, rh-IL-3, rh-M–CSF, and c-kit ligand were added to the PBMC cultures. None of these cytokines influenced the yield of mature CD83+ DC, indicating that they do not play an obvious role in the development of mature DC from PBMC in these cultures. This is in contrast to the experience of some groups[32,42] that showed c-kit ligand increases the number of DC from CD34+ precursors. The more differentiated precursors in the PBMC population are likely not responsive to c-kit ligand. In the second set of studies, cultures of PBMC grown with GM–CSF and IL-4 and GM–CSF, IL-4, and TNF-α in serum-free conditions also were analyzed. Although the phenotypic and functional properties of DC generated in serum-free cultures were similar to DC generated in serum-containing cultures, several differences were noted. First, PBMC grown in serum-containing media grew in large nonadherent clumps, whereas the cells grown in serum-free conditions grew in smaller

clumps that were moderately to strongly adherent to the culture plate. Second, CD83 was expressed at higher levels in cells grown in serum-containing media supplemented with TNF-α compared with those of cells grown in serum-free media. Third, CD83 expression after treatment with TNF-α was delayed consistently in serum-free conditions. Despite these contrasts, no obvious differences were noted in the ability of DC grown in serum-free cultures to stimulate allogeneic T cells compared to DC grown in serum-containing cultures. These studies do indicate, however, that currently unknown factors contained in serum may affect the development of mature DC from PBMC.

The results of the experiments described in this report have several implications for the therapeutic applications of DC. First, the ability to generate cultures comprised largely of either immature or mature DC may be important for the way in which DC are used. Immature DC may be more effective at antigen processing and presentation than mature DC, whereas mature DC may be better at T-cell stimulation.[43] Consequently, one approach to using DC in a vaccination protocol would be to generate immature DC with GM–CSF and IL-4, pulse the DC with antigen, and then briefly treat the DC with TNF-α before inoculation. This approach may optimize the immunostimulatory properties of the DC vaccine. Second, there may be advantages to generating DC in serum-free conditions instead of serum-containing conditions. Vaccination of patients with cells grown in the presence of serum could result in allergic reactions to serum components or in the transfer of infectious agents to the vaccine recipients. In addition, growth of PBMC in serum could lead to the processing and presentation of serum proteins by the DC.[11] This potentially could diminish the potency of the DC vaccine as well as sensitize individuals to a variety of common serum proteins. Thus, because DC grown in serum-free conditions are functionally equivalent to those grown in serum containing media, they have been chosen for use in our current clinical active immunotherapy strategy. The other modest differences between the two conditions will provide an opportunity to define additional factors and cytokines that may affect DC development and function and be important for future generations of DC vaccines.

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