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In vitro characterization of rat bone marrow–derived dendritic cells and their precursors

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Abstract: Although the rat is commonly used for basic immunology and transplantation research, phenotypic and functional characterization of rat dendritic cells (DCs) lags behind similar studies in the human and mouse. Therefore, these features were examined using DCs propagated from cultures of rat bone marrow maintained in a medium supplemented with granulocyte-monocyte colony-stimulating factor. Analysis of cytospin preparations of cultured cells showed that DCs arise from OX7$^+$ myelomonocytic precursors. Typical mature rat DCs were morphologically similar to their mouse and human counterparts and expressed major histocompatibility complex (MHC) class II (common part determinant of Ia), OX62 (integrin molecule), OX7 (CD90), ICAM-1 (CD54), and CTLA4 counterreceptor, but were negative for OX8 (CD8), OX19 (CD5), W3/25 (CD4), and ED2, a rat macrophage marker. Functional analysis of OX62$^+$ sorted DCs showed that they could effectively present the soluble antigen ovalbumin to naive T cells in vitro. A combination of anti-MHC class II monoclonal antibody and CTLA4-immunoglobulin inhibited allostimulatory ability more effectively than either reagent alone. Implications for studying the role of DCs in immune responses in the rat are discussed. *J. Leukoc. Biol.* 59: 196–207; 1996.

Key Words: dendritic cells · rat · phenotype · major histocompatibility complex

INTRODUCTION

Dendritic cells (DCs) are well recognized as powerful stimulators of a mixed leukocyte reaction (MLR) and of syngeneic naive T cells in vivo [1, 2]. In fact, they are considered the primary initiators of immune responses and are present at the sites of early inflammation in rejection [3, 4] and autoimmune disorders [5]. However, donor-derived DCs have also been implicated in the induction of allogeneic tolerance [6–8]. According to this hypothesis, DCs have been propagated from precursors found within the liver [9] and, after solid organ transplantation, donor-derived DCs have been cultured from a variety of recipient tissues [10].

With the current availability of recombinant cytokines, including granulocyte-monocyte colony-stimulating factor (GM-CSF), interleukin-4 (IL-4), and tumor necrosis factor (TNF), bone marrow (BM)–derived DCs have been extensively investigated concerning their ontogeny, phenotypic profile, and biological functions in both mouse [11–13] and human [14] systems. Although the rat is extensively used for models investigating transplantation tolerance and other immunological phenomena, similar studies of DCs in this species have been less comprehensive. This is largely attributable to the difficulty of obtaining sufficiently large numbers of DCs for analysis and the paucity of well-defined immunologic reagents for this species.

Based on findings in previous publications [15–22], we have described a new and simple technique for the propagation of large numbers of rat BM-derived DCs using murine rGM-CSF with gelatin-coated tissue culture flasks [23]. The functional activity of the cultured cells was confirmed by studying their ability to stimulate an allogeneic MLR and their in vivo ability to home to T cell–dependent areas of the spleen [23]. DCs were quantitated by evaluating the coexpression of major histocompatibility complex (MHC) class II antigen (OX6) and OX62 [21], a molecule related to the integrin family and known to be present on rat DCs and γδ T cells.

In this study we define the developmental morphology, phenotype, and functional activity of cultured BM-derived DCs to serve as a baseline for investigations of DC functions in tolerance and autoimmunity in the rat. Particular attention was paid to maturational changes of precursor cells, the dynamics of surface marker expression, and allostimulating ability at various phases of maturation in

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Abbreviations: (m)Ab, (monoclonal) antibody; APC, antigen-presenting cells; BM, bone marrow; DC, dendritic cell; FcPA, Fc receptor–positive and plastic-adherent cells; FcR, Fc receptor; GM-CSF, granulocyte-monocyte colony-stimulating factor; Ig, immunoglobulin; IL, interleukin; LEW, Lewis; MLR, mixed leukocyte reaction; MHC, major histocompatibility complex; OVA, ovalbumin; TNF, tumor necrosis factor.

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culture. The functional importance of the surface molecules for rat DCs was determined using in vitro allostimulation blocking studies.

MATERIALS AND METHODS

Animals

Adult 7- to 9-week-old Lewis (LEW; $RT1^l$), ACI ($RT1^a$), and PVG ($RT1^c$) rats were purchased from Harlan Sprague Dawley (Indianapolis, IN) and maintained in specific pathogen–free facilities at the University of Pittsburgh.

Collection of rat bone marrow

BM cells were removed from the femurs, tibias, and humeri of LEW rats after sacrifice using methoxyflurane inhalational anesthesia (Pitman-Moore, Mundelein, IL). The cells were washed twice in complete medium [RPMI 1640 containing 5% fetal calf serum (FCS), 5 μg/ml gentamicin, 2 mM L-glutamine, and 10 mM HEPES buffer], purchased from Gibco (Life Technologies, Grand Island, NY).

Procedure for bone marrow culture

Before being placed in culture for the propagation of DCs [23], fresh BM cells were depleted of Fc^+ and plastic-adherent (FcPA) cells by panning on normal serum-coated petri dishes. Briefly, 1.2×10^8 FcPA-depleted BM cells were cultured at a concentration of 3×10^6/ml in gelatin-coated, 75-cm^2 Falcon tissue culture flasks. Complete medium, supplemented with 10% FCS, 5×10^{-5} M 2-mercaptoethanol (Gibco), 0.5×10^{-3} M N^G-monomethyl-L-arginine (Schweizerhall, Piscataway, NJ), and 0.4 ng/ml murine rGM-CSF (R&D Systems, Minneapolis, MN), was used for DC propagation. Cultures were fed every second day by exchanging half the medium for fresh rGM-CSF–containing medium, without discarding any cells (all medium being exchanged was centrifuged and any cells were restored to the culture flask). When the time in culture was completed, nonadherent cells were removed and redepleted of FcPA cells by panning on serum-coated dishes, before being used for functional assays and phenotypic analysis. Such FcPA-depleted, cultured BM cells will be referred to as DC-enriched BM cultures hereafter.

Selection of $OX7^+$ and $OX7^-$ BM cells for culture was achieved by labeling FcPA-depleted BM cells with OX7 monoclonal antibody (mAb) and panning on petri dishes coated with affinity column–purified goat anti-mouse immunoglobulin G (a mixture of 10 μg/ml affinity-purified mAb with 90 μg/ml normal goat IgG), according to a previously described technique [24]. $OX7^+$ and $OX7^-$ fractions and unfractionated control cells were then used for BM cultures.

Immunofluorescence labeling and flow cytometric analysis

DC-enriched BM cultures were stained using direct and/or indirect immunofluorescence techniques with a panel of monoclonal antibodies including OX1 (anti-rat CD45, leukocyte common antigen), OX6 (anti-rat nonpolymorphic determinant of MHC class II), OX7 (anti-rat CD90, Thy 1.1), OX8 (anti-rat CD8, suppressor/cytotoxic T cells, natural killer cells, some activated helper cells), OX19 (anti-rat CD5, a pan-T-cell marker), OX62 (an antibody that recognizes an integrin molecule [21] present on rat veiled DCs and γ/δ T cells), W3/25 (anti-rat CD4, T helper cells, and some macrophages), 1A29 [anti-rat CD54 (ICAM-1)], ED1 (anti-rat macrophages, monocytes, and dendritic cells), and ED2 (anti-rat tissue macrophages), all of mouse IgG1 isotype (Serotec, Oxford, England). Isotype control was purchased from Southern Biotechnology (Birmingham, AL). Rat anti-mouse B7/BB1 (B7-1, clone 1G10), B7-2 (clone GL-1), and normal rat IgG2a were purchased from Pharmingen (San Diego, CA). CTLA4-Ig, a soluble chimeric fusion protein combining CTLA4 (a structural homologue of CD28) and the human Ig-Cγ chain, which has high affinity for B7 (CTLA4 counterreceptor) on antigen-presenting cells (APCs) [25], was kindly provided by P.S. Linsley (Bristol Myers Squibb Pharmaceutical Research Institute, Seattle, WA). Normal human IgG was purchased from Sigma. Double immunofluorescent staining was performed as previously described [26] and 10,000 gated events were analyzed for each sample on an Epics Elite flow cytometer (Coulter Corporation, Hialeah, FL). The gate was chosen to include at least 90% of $OX62^+$ cells (a DC-specific surface marker) and to avoid counting autofluorescent cells, which can be problematic in cultured cells. For controls, the primary antibody was substituted with an immunoglobulin class–matched nonimmune antibody before completing the staining procedure.

Immunocytochemistry

Cytospin preparations of DC-enriched BM cultures were allowed to air dry overnight at room temperature and stored at -70°C until use. Before staining, cytospin slides were fixed with freshly prepared 2% paraformaldehyde, except those being labeled with CTLA4-Ig and normal human Ig control, which were not fixed. Staining was performed using a standard indirect avidin-biotin-peroxidase (ABC) staining technique (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA). The secondary Abs, F(ab')2 rat anti-mouse IgG (H+L) and goat anti-human Ig, were purchased from Jackson Immunoresearch (West Grove, PA). The slides were counterstained with hematoxylin and blued in Scott's bluing reagent. Control slides were processed in the same fashion as the controls described for immunofluorescence and flow cytometric studies.

Scanning electron microscopy

BM-derived DCs were enriched by bovine serum albumin gradient flotation using the previously described method of Klinkert et al. [15, 16]. Purified DCs were then placed on coverslips coated with poly-L-lysine (Sigma), critical point dried, sputter coated with gold, and viewed using a JEOL JSM-T300 microscope.

Cell sorting of rat DCs for functional analysis

Because there is some controversy regarding the ability of cultured mature DCs to present soluble proteins, pure populations of rat DCs were obtained by flow cytometric sorting, based on staining with the integrin molecule OX62. This marker was chosen on the basis of immunocytochemical analysis of DC-enriched BM cell cytospin preparations and prior publications [21, 23].

Mixed leukocyte reactions

Cervical lymph node cells from syngeneic LEW and allogeneic ACI and PVG rats were used as responders. Unsorted DC-enriched cultured BM cells, $OX62^+$ sorted DCs, and fresh LEW spleen cells (positive controls) were used as stimulators. One-way mixed leukocyte reactions (MLRs) were performed in U-bottom 96-well microtest plates (Falcon 3077). A constant number of responders (9×10^4/well) was used with various numbers of γ-irradiated (20 Gy) stimulator cells in each assay. Each dilution was performed in triplicate. Stimulator-to-responder (S/R) ratios ranged from 3:1 to 1:9 for spleen cell controls and 1:9 to 1:729 for unsorted DC-enriched cultured BM cells and $OX62^+$ sorted DCs. The medium used for MLRs was identical to that used for BM cultures, except that the 10% FCS was replaced with 10% LEW serum. Unless otherwise specified, each MLR was labeled with a final concentration of 0.2 μCi/ml [^{3}H]TdR (specific activity 2 Ci/mM; New England Nuclear, Beverly, MA) for a 7-h period, 65 h after starting the culture. Plates were automatically harvested (Skatron, Lier, Norway) and thymidine incorporation was determined using a liquid scintillation counter (1205 Betaplate, Wallac, Gaithersburg, MD).

Presentation of soluble antigen

After isolation using nylon wool columns, 5×10^4 syngeneic LEW lymph node T cells were used in each well as responders for this proliferation assay. DC-enriched BM cultures, $OX62^+$ sorted DCs, and

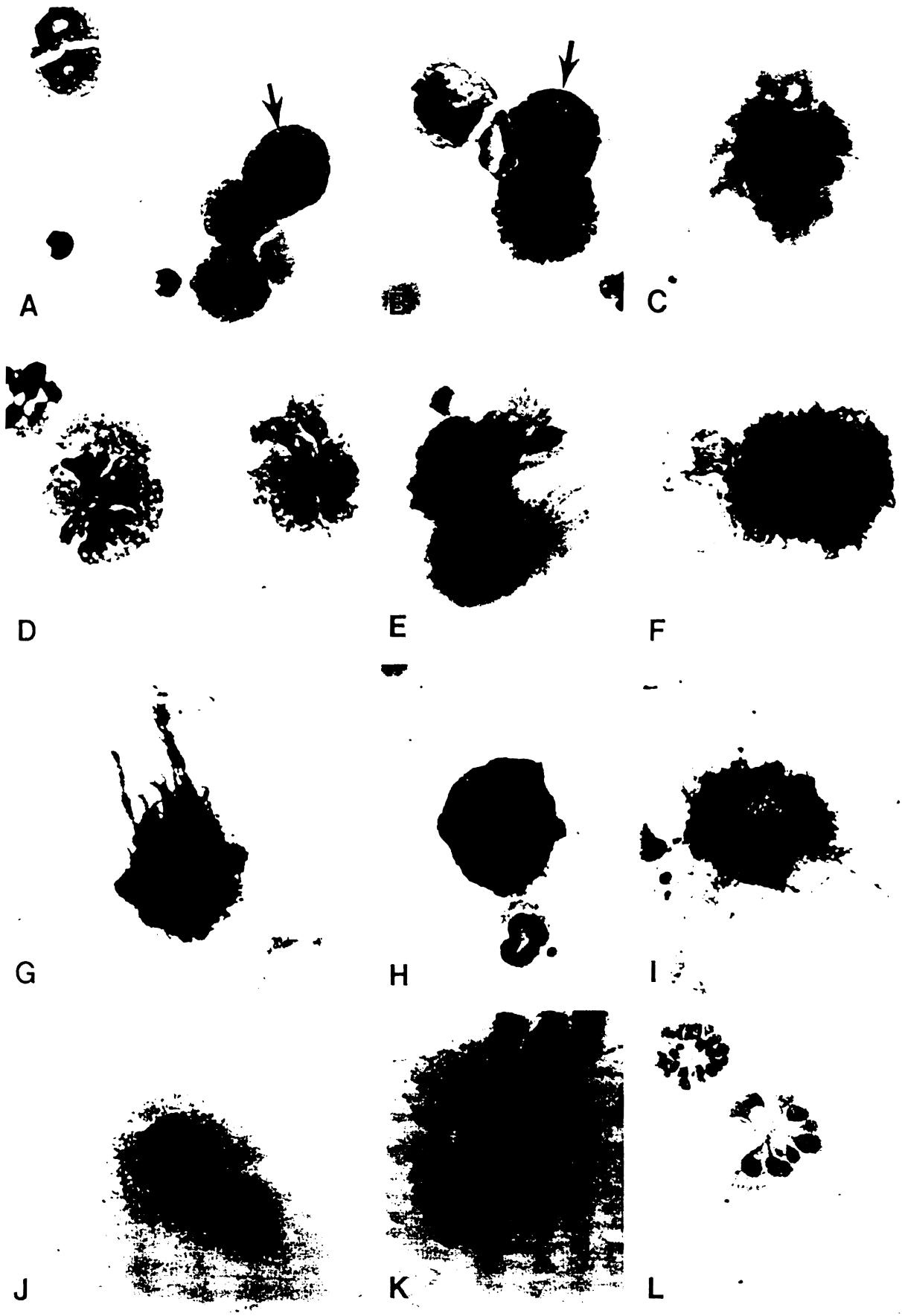
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Fig. 1. Giemsa and immunohistochemically stained cytospin preparations of DC-enriched BM cultures. (A) Blasts (arrow), immature myelomonocytic cells, and erythroid precursors with a small dense nucleus were present in day 1 cultures. (B) Slightly more mature precursors (arrow) could easily be identified on day 3. A small granulocyte is located in the center of the cluster and a monocyte/macrophage with a bubbly gray cytoplasm is present in the upper left corner. (C) DC-like cells (see text) appeared on day 4 with characteristic rectangular nuclei and smooth, blue-gray cytoplasm with short cytoplasmic processes. (D) These DC-like cells became more mature on day 5–6, showing early signs of the irregular nucleus typical of mature DCs. (E) Mature DC with typical cloverleaf-shaped nucleus and cytoplasmic dendrites. Immunohistochemical stain for: (F) class II MHC, OX6 (day 4); (G) OX6 (day 8); note the juxtanuclear clustering); (H) CTLA4-Ig (day 4); (I) CTLA4-Ig (day 8); (J) the integrin OX62 (day 4); (K) OX62 (day 8); (L) mouse IgG1 negative control.

fresh LEW splenocytes were used as stimulators and pulsed with 5 μg/ml ovalbumin (OVA) (Sigma, St. Louis, MO) for 2 h at 37°C before irradiation (20 Gy) and combination with the responders. The assay was labeled with 0.2 μCi/ml [^{3}H]TdR for 18 h (from 78 to 96 h of culture time), prior to harvesting and determination of [^{3}H]TdR incorporation.

Surface marker blocking experiments

The mAb OX6 and the fusion protein CTLA4-Ig were used in amounts of 2.5 and 1.25 μg/ml to determine whether the MLR response of allogeneic responders (in this case PVG lymph node cells) could be blocked. Mouse IgG1 (isotype control for OX6) and normal human Ig (control for CTLA4-Ig) were used as normal blocking controls. The irradiated DC-enriched BM cultures were incubated with either the blocking or control Ab for 1 h at 37°C, before the initiation of each assay. An S/R ratio of 1:27 was used for these experiments. Results are expressed as the percentage inhibition of isotype control values. Percent of inhibition was calculated as follows:

$$\% \text{ inhibition} = (1 - \frac{\text{counts in the presence of blocking Ab}}{\text{counts in the presence of control Ab}}) \times 100$$

Statistics

Student's *t*-test was used and $P < .05$ accepted as a significant result.

RESULTS

Phenotypic characterization of rat BM-derived DCs

Giemsa-stained cytospin preparations of DC-enriched BM cultures were examined daily for the first 8 days in culture. Representative photomicrographs of the observed sequence of changes are shown in **Figure 1.** Day 1 cytospins contained a large variety of cell types, very similar to those seen on smears of an unfractionated bone marrow aspirate. The predominant population consisted of cells belonging to the myelomonocytic lineage, although cells belonging to the normoblastic series were also present. Relatively mature, banded and segmented granulocytes outnumbered immature forms by a ratio of at least 4:1, but uncommitted blasts, myeloblasts, promonocytes, and pronormoblasts were also present. Mature monocytes or macrophages were uncommon and classic DCs were rare (less than 1 in 500 cells).

By day 3–4, cells in the normoblastic series had largely disappeared from the cultures, whereas the myelomonocytic elements increased proportionately and showed some features of maturation. These changes are probably related to the presence of GM-CSF and the lack of suitable erythropoietic growth factors in the medium. The immature myeloid cells formed small clusters composed of four to six cells, some of which had morphologic features of the myelomonocytic lineage. These included a streaked or raked chromatin pattern, one or two nucleoli, an irregular or reniform nucleus, and a moderate amount of blue or blue-gray cytoplasm. Occasional cells contained a small perinuclear hof of amphophilic cytoplasm. Overall, these immature cells accounted for about 25–30% of the population. There were also rectangular "DC-like" cells containing a large, twisted, oblong nucleus and smooth blue-gray irregularly shaped cytoplasm, distorted by short blunt processes. Besides the numerous mature and banded granulocytes and typical monocytes (described below), an occasional typical mature DC appeared.

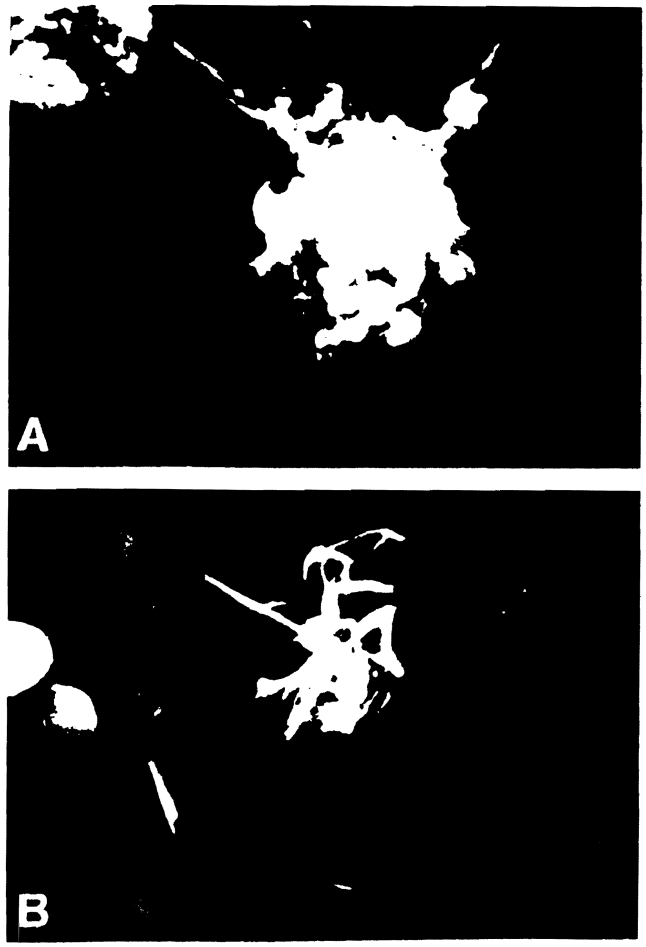

Fig. 2. Scanning electron micrograph of day 8 FcPA-depleted DC-enriched BM cultures shows the varied size and morphology of (A) cytoplasmic processes and (B) cytoplasmic veils of an individual DC.

TABLE 1. Expression of Phenotypic Markers of Cells Found in Rat BM Cultures Maintained in Medium Supplemented with GM-CSF[a]

	BM culture cell type			
Antibody	Day 3 myelomonocytic precursor	Immediate "DC-like" (d4) precursors (see text)	Day 8 mature DCs	Monocytes
OX1 (CD45)	+	+	+/-[c]	-/-[c]
OX6 (Ia)	+	++/+++[d]	+++[d]	+[c]
OX7 (CD90)	+	+	++/+++	-
OX8 (CD8)	0	0	0	0
OX19 (CD5)	0	0	0	0
OX62	+/-	+	+[c]	0
W3/25 (CD4)	0	+/-[c]	0	+[c]
ED1	+	+	+[c]	+
ED2	0	0[e]	0	-++
ICAM-1 (CD54)	0	+	+	-/-
CTLA4-Ig	0	+	+++[f]	-/- (weak)
Morphology (on Giemsa stain)	High N/C ratio Scant deep blue cytoplasm Nucleoli Rounded nucleus	Oblong twisted nucleus with "raked" chromatin Smooth blue-gray cytoplasm Irregular rectangular shape	Twisted, convoluted, or clover-leaf shaped nucleus Smooth blue-gray cytoplasm Distinct long cytoplasmic processes or dendrites	Small round nucleus Bubbly gray cytoplasm Low N/C ratio

[a] For the purpose of this table, cell type is distinguished by morphological criteria.
Scale: 0 = negative; +/- = very weak; + = weak; ++ = moderate; and +++ = strong staining, for the particular antigen listed.
[c] Variable staining.
[d] Distinct clustering in juxtanuclear area.
[e] Mostly negative, but variable staining.
[f] Generally strong but variable staining.

During days 5–8 the most noticeable shift in the population was a decreased number of immature myelomonocytic cells with a concomitant increase in mature monocytes/macrophages and typical dendritic cells. The monocyte/macrophage population consisted of cells that had a relatively low nuclear/cytoplasmic ratio, a small rounded or oval nucleus, and abundant bubbly, gray cytoplasm that formed distinct and round cell borders. Cells identified morphologically as typical DCs had a higher nuclear/cytoplasmic ratio, a larger, twisted, lobed, or cloverleaf-shaped nucleus, and moderate to abundant smooth blue-gray cytoplasm, teased out at the periphery into long delicate wavy processes or dendrites. Scanning electron microscopy showed the typical DC to have long cytoplasmic veils, although cells with a variety of cytoplasmic veil sizes were present in these cultures (**Fig. 2**). Immunocytochemical analysis of cytospin preparations of day 4 and day 8 DC-enriched BM cultures was carried out to correlate the expression of surface markers with the morphological observations (Fig. 1), described above. The results are presented in **Table 1.**

The expression of several of these markers was also studied by flow cytometry to quantify the number of stained cells and to examine simultaneous coexpression of two different surface markers. As seen in **Figure 3,** the percentage of $OX6^+/OX62^+$ cells and the intensity of OX6 and OX62 expression were reconfirmed to vary with time spent in culture [23]; double-positive cells increased from 4.6% (0.703 × 0.066) at day 4 to 16% (0.445 × 0.38) by day 8 (see rectangular gate in Fig. 3E and F). The percentage of cells expressing adhesion molecule ICAM-1 (CD54) and the costimulatory molecules labeled with CTLA4-Ig also increased with time in culture. The mean channel fluorescence intensity shifts from day 4 to day 8 were as follows: 8.6 to 16.2 for MHC class II antigen, 4.1 to 10.0 for ICAM-1, and 4.4 to 7.7 for CTLA4-Ig. Rat anti-mouse B7-1 also reacted with the $OX62^+$ subset in the DC-enriched BM cultures (data not shown). However, either the antibody recognizing mouse B7-2 did not react with rat B7-2 or B7-2 was not present on these cells (a range of concentrations of rat anti-mouse B7-2 was used, the maximal being 30 times higher than the amount routinely used for staining mouse splenic DCs in this laboratory).

Assessment and blocking of allostimulatory ability of DC-enriched BM cultures in MLR using the mAb OX6 and the fusion protein CTLA4-Ig

Because the intensity of surface marker expression varies between day 4 and day 8 cultures, it is pertinent to examine the contribution of these molecules to the in vitro allostimulatory function of cultured DCs. When the functional ability of these cells was assessed in the MLR, it was shown that DC-enriched BM cells obtained from day 8 cultures were between three and four times more potent than those from day 4 cultures at various S/R ratios (**Fig. 4**).

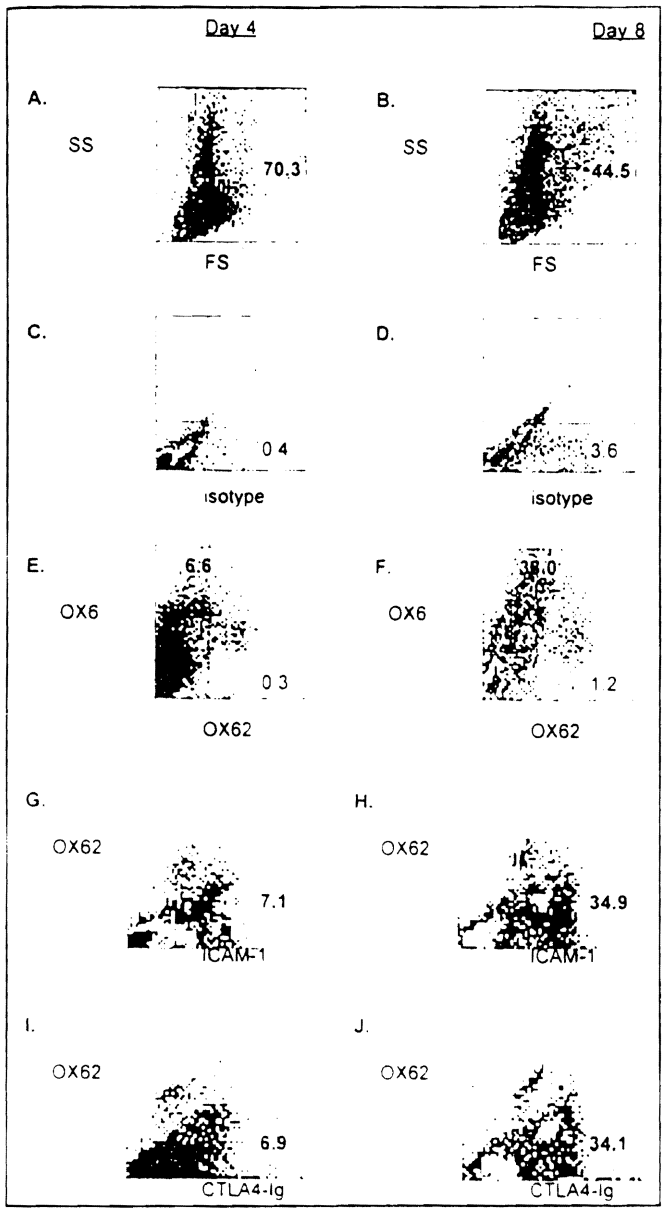

Fig. 3. Flow cytometric analysis of Fc^+ and plastic-adherent (FcPA) cell–depleted DC-enriched day 4 and day 8 BM cultures. (A and B) Forward and side scatter profiles of cultured cells and percentage of cells that fall within the DC gate, which was chosen to include at least 90% of the cells positive for OX62, a DC-specific surface marker. Many of the cells outside this gate are granulocytes, macrophage/monocytes. (C and D) Isotype controls in which a mixture of normal mouse IgG and normal human Ig was used to label the cells. Because OX62 was found to be the marker most restricted to DCs (see text), double labeling with OX62 and OX6 (E and F), ICAM-1 (G and H), or CTLA4-Ig (I and J) was determined on days 4 and 8 to detect the presence of these antigens on rat DCs. The bold number shows the number of doubly labeled cells within the rectangular gate

Due to the poor availability of reagents, only class II MHC (OX6) and CTLA4-Ig were used for blocking studies. The assays were performed using two concentrations of each reagent. Both the anti-MHC class II mAb OX6 and the fusion protein CTLA4-Ig had significant inhibitory effects when used separately, although OX6 was consistently more potent than CTLA4-Ig at blocking allostimulation in MLR (**Table 2**). Furthermore, the percent inhibition seen with OX6 was significantly greater in day 4 than day 8 cultures ($P < .05$), in contrast to that seen with CTLA4-Ig. When OX6 and CTLA4-Ig were combined, the inhibition was found to be greater than that seen with either mAb alone. This additive effect was especially noticeable when using day 8 cultures as stimulatory cells. As previously noted, the proliferation responses without Ig were greater for day 8 than day 4 DCs (14,919 ± 916 versus 10,035 ± 774; $P < .038$). However, the addition of normal mouse or human Ig as an isotype control augmented the level of proliferation and abrogated the statistically significant difference between the proliferative responses of day 4 and day 8 DC-enriched cultures. The mechanism of this increase is unclear; it is possible that some T cells have responded to the xenogeneic immunoglobulin.

Allostimulation and presentation of soluble antigen by $OX62^+$ sorted pure DCs

There is some controversy concerning the ability of mature DCs to process soluble antigen [27]. Experiments were designed, therefore, to examine and compare the ability of DC-enriched MB cultures to stimulate allogeneic T cells and to present the soluble antigen OVA to purified T cells. Although cells from these cultures could present OVA to purified T cells 100 times better than splenocytes (data not shown), the APC function could not be confidently attributed to DCs because of contaminating cell populations. To avoid these problems, the phenotypic characteristics identified in cytospin preparations were used to isolate pure DCs by flow cytometric sorting on $OX62^+$ cells. This resulted in a population of rat DCs that were 96.4% pure by FACS analysis. Morphologically, this purified population consisted mostly of cells that resembled the "immediate" DC precursors in culture cytospin preparations, with fewer typically mature DCs (**Fig. 5**). However, scarce relatively immature myelomonocytic forms were also present. The ability of $OX62^+$ sorted DCs to present OVA and to stimulate allogeneic T cells is shown in **Tables 3 and 4**, respectively. OX62 sorted cells were two to four times more efficient at presentation of OVA than unsorted DC-enriched BM cells (Table 3), which in turn were 100 times more potent than fresh splenocytes (data not shown). The same was true for stimulation of allogeneic lymphocytes—$OX62^+$ sorted DCs were 3 to 5 times more potent allostimulators than unsorted DC-enriched BM cells (Table 4), which in turn were at least 100 times more potent than fresh splenocytes.

The precursors of rat BM-derived DCs are contained in the $OX7^+$ subset of BM cells

Studies of human DC biology have been greatly facilitated by the availability of mAbs to specific progenitor cell types present in bone marrow and cord blood, such as CD33 and CD34 [28]. One of the factors that has limited similar studies in the rat has been the absence of a similar surface marker for progenitor cells. However, a report by Gieseler

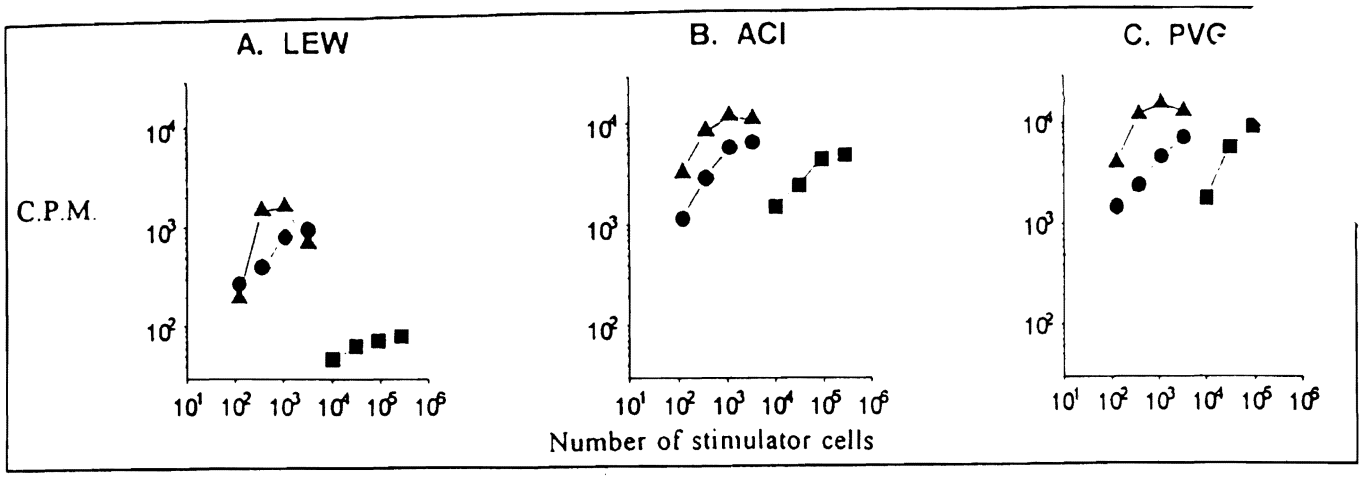

Fig. 4. Comparison of the allostimulatory ability of day 4 (●) and day 8 (▲) FcPA-depleted DC-enriched BM cultures. Fresh spleen cells (■) were used as a positive control.

et al. [29] has suggested that rat DC precursors belong to the myeloid cell lineage. Furthermore, others have shown that pluripotent hemopoietic stem cells in rat BM express OX7 as a surface marker [30]. Thus, it is likely that the BM precursors of rat DCs are contained in an $OX7^+$ subset, as suggested by Bowers et al. [31].

To determine whether the rat DC precursor is $OX7^+$, the following experiments were conducted. Fresh FcPA-depleted BM cells were separated by a panning technique using the mAb OX7 into $OX7^+$ and $OX7^-$ fractions. FACS analysis of four separate experiments showed that FcPA-depleted BM cells contained 67.4 ± 5.3% $OX7^+$ cells. After selection, means of 89.6 ± 2.6% and 7.0 ± 1.2% $OX7^+$ cells were present in $OX7^+$ and $OX7^-$ preparations, respectively. $OX7^+$ and $OX7^-$ fractions and unfractionated BM cells were then placed in culture with rGM-CSF, as described above. After periods of 2 and 6 days in culture, MLRs were used to examine the allostimulatory ability of $OX7^-$ and $OX7^+$ DC-enriched BM cultures. The number of cells recovered from cultures was similar among the three groups (data not shown). The results presented in **Figure 6A–C**, using day 2 BM cultures, show that the allostimulatory activity of cells from $OX7^+$ cultures was between six and nine times greater than that of cells derived from $OX7^-$ cultures. By day 6 (Fig. 6D–F), potent allostimulatory activity was also found in the $OX7^-$ fraction, and the difference in allostimulatory ability between the two groups had diminished to between two- and fourfold at various S/R ratios. These results are in agreement with the previous description of DC precursors as $OX7^+$ BM cells [31].

TABLE 2. Capability of the Monoclonal Antibody OX6 and Fusion Protein CTLA4-Ig at Blocking the Allostimulatory Ability of Cultured Rat BM-Derived DCs[a]

	Concentration	Day 4		Day 8	
Antibody	(μg/ml)	cpm	% inhibition	cpm	% inhibition
Normal human Ig (control)	2.5	17,600	—	16,819	—
	1.25	16,940	—	16,751	—
CTLA4-Ig	2.5	5,349	69.6	6,018	64.2[c, b]
	1.25	6,819	59.3	8,934	46.7[c, b]
Normal mouse Ig (control)	2.5	15,650	—	16,485	—
	1.25	16,859	—	15,361	—
OX6	2.5	910	94.2	3,422	79.2[*, b]
	1.25	1,334	91.5	6,381	61.3[c, b]
OX6 + CTLA4-Ig	1.25 + 1.25	423	97.3	547	96.7[c, b]

[a]DC-enriched BM cultures were used as stimulators in an MLR against allogeneic responders (PVG at an S/R ratio of 1:27. Results presented are means of two separate experiments. In the absence of Ig, the proliferative responses of day 8 and day 4 DC-enriched BM cultures were 14,919 ± 916 and 10,035 ± 774, respectively, $P < .038$.

[*]$P < .04$ vs. day 4.

[b, c]P not significant vs. day 4.

[c]$P < .007$ vs. day 4.

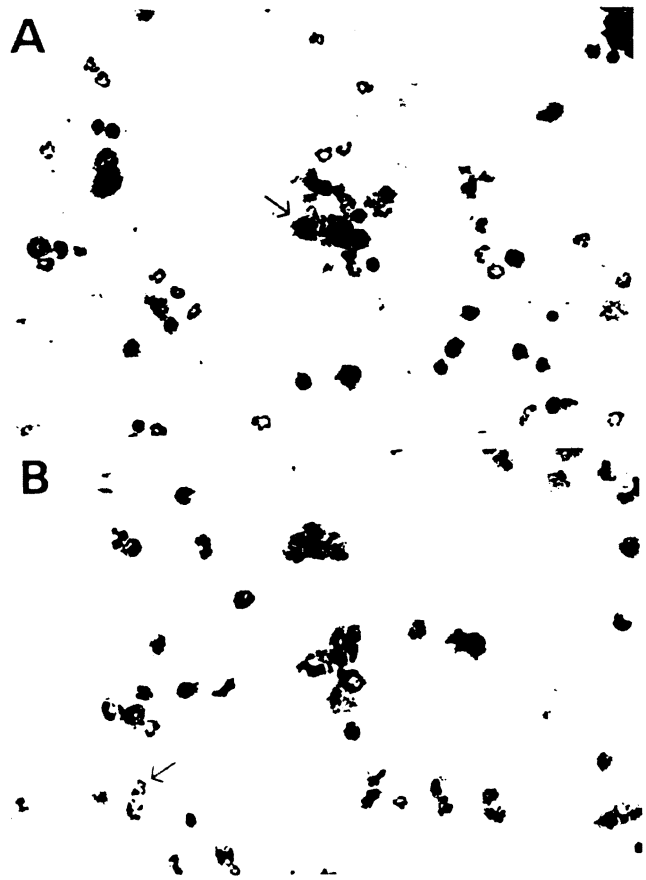

Fig. 5. Giemsa-stained cytospin preparations of day 6 unsorted DC-enriched BM cultures (A) and OX62$^+$ sorted DCs (B). Note the DC in the unsorted population (A, arrow) amid the contaminating granulocytes. In the OX62$^+$ sorted population (B), contaminating granulocytes are rare (arrow). This is an example of three separate experiments.

The increased allostimulatory activity seen using day 6 cultures of OX7$^+$ cells was an interesting observation; it may be explained by the 7% contaminating OX7$^+$ cells that differentiate or mature into functioning DCs. This is consistent with the observation that the proportion of OX7$^+$ cells (which is also the phenotype of cultured rat DC [31]) increased from 6.4% at day 0 to 16.7% by cultures.

Another point of interest is that the allo ity of unfractionated BM culture control exposed to OX7, was quite low, even were abundant in these cultures (69% on day 2). This suggested that binding of the a ligand to Thy 1.1 on DC precursors could speed differentiation or maturation into functional DCs. To test this hypothesis, the FcPA-depleted BM cells were treated with either OX7 or normal mouse IgG1 (isotype control) before being placed in culture. After 48 h, the OX7 and isotype control treated cells were used as stimulators in an MLR assay. The data in **Table 5** show that the OX7-pretreated culture outperformed the isotype controls, indicating that binding of OX7 to DC precursors promotes the differentiation and/or functional maturation of DCs.

DISCUSSION

In our initial report of this culture technique, double immunolabeling showed that DC-enriched BM cultures contained a population of dendritic-shaped cells that coexpressed MHC class II (OX6) and the integrin molecule OX62 [23]. These cells also showed potent allostimulatory ability in the MLR and an ability to migrate to T cell–dependent zones of the spleen in vivo. As OX62 has been previously shown to react with rat DCs and γδ T cells [21] and lymphocytes are rare in this system, it is reasonable to assume that the dendritic-shaped, OX6$^+$/OX62$^+$ cells in rat BM cultures are indeed DCs. However, few data were provided in our earlier report [23] concerning the DC precursor phenotype or the ontogeny, expression of costimulatory and adhesion molecules, or functional ability of DCs to present alloantigens or nominal protein antigens.

In the current study, the DC-enriched BM cultures were examined daily using Giemsa-stained cytospin preparations. Two separate time points (day 4 and day 8) were

TABLE 3 Comparison of the Ability of OX62$^+$ DCs and Unsorted DC-Enriched BM Cultures to Present Soluble Antigen OVA to Naive Syngeneic T Cells[a]

		Antigen	
Cells	APC	None	OVA
OX62$^+$ DCs	1×10^4	7,416 ± 850[*,b]	42,545 ± 989[*,b]
	3×10^3	5,507 ± 739[=,b]	31,642 ± 1642[*,b]
	1×10^3	2,355 ± 199[=,b]	19,464 ± 1977[*,b]
Unsorted control	1×10^4	4,393 ± 88	11,941 ± 1704
	3×10^3	3,458 ± 767	11,083 ± 2780
	1×10^3	2,117 ± 945	10,972 ± 741

[a]Day 6 DC-enriched BM culture was labeled with OX62 and sorted for OX62$^+$ DCs. The OX62$^+$ DCs and unsorted control were then used as APCs to present OVA (2.5 μg/ml) to 3×10^5/well of syngeneic T cells. The counts for responders alone and responders + OVA were 1621 ± 539 and 2284 ± 415, respectively. Results presented are means ± SD of one experiment.

[b]*$P < .02$ vs. unsorted control.

[b]=P not significant vs. unsorted control.

chosen to correlate cell morphology with various phenotypic markers, including costimulatory and adhesion molecule expression and antigen-presenting capabilities. These particular time points were chosen because by day 4 a distinct population of cells was present that were morphologically mature enough to be recognized as DC-like but immature enough to make an ontogenic phenotypic analysis meaningful. The phenotype of these immature cells was then examined during evolution of the population into typically mature DCs and monocytes/macrophages, both of which were present in day 8 cultures.

After combining this approach with the OX7 panning procedure, it became evident that typically mature DCs arise from an $OX7^+$ myelomonocytic precursor in rat BM cultures. The mature rat DC has a moderately high nuclear/cytoplasmic ratio, an oblong, twisted, or cloverleaf-shaped nucleus, and smooth blue-gray cytoplasm that is drawn out into long, thin, delicate cytoplasmic processes. They strongly express class II MHC antigens, which shows a distinct accentuation of positivity in the juxtanuclear region [32]. Mature rat DCs also express the integrin molecule OX62, OX7 (CD90), CTLA4-Ig, ICAM-1 (CD54), and B7-1 but are negative for OX8 (CD8), OX19 (CD5), W3/25 (CD4), and the rat macrophage marker ED2. In our hands, immunoperoxidase staining of cytospin preparations for OX1 was weak and equivocal. Flow cytometric analysis shows that the percentage of cells expressing the functionally important CTLA4-Ig, ICAM-1, and MHC class II antigens increases with time in culture, as does their level of expression of these antigens, shape, and allostimulatory ability.

Thus, we have extended the analysis of rat DCs to include a wider panel of monoclonal antibodies than previously used and correlated phenotype with morphology in culture to infer ontogeny. The results obtained for bone marrow–derived DCs were similar to those reported by Bowers et al. [31] and Gieseler and colleagues [33]. In contrast, mature rat thymic DCs [34] show ... ferences from bone marrow–derived DCs: $ED2^+$, $OX8^+$, and $OX19^+$, whereas the ... negative for these markers under the ... this study.

In our previous study, the percentage of O... started to decrease in the most mature cultures, an... they contained the most potent allostimulatory cells. Th... apparent paradox is now explained by examination of the cytospin preparations from day 8 that showed more than 30% typically mature DCs and strong expression of class II MHC and CTLA4-Ig. These typical mature DCs showed OX62 staining that was notably weaker by immunocytochemistry. The implication is that the use of $OX6^+$ $OX62^+$ cells as the "gold standard" phenotype may actually underestimate the number of DCs present, which otherwise are functionally and morphologically mature. This contention was confirmed by cytospin and functional analysis of $OX62^+$-sorted DCs, which showed a slightly less mature morphology than the typically mature DCs. However, these cells could effectively present OVA to naive T cells in vitro and stimulate an MLR much better than unsorted DC-enriched BM cultures. Therefore, similarly to the situation for other species, the broadest characterization of rat DCs should be based on a combination of distinct morphology and strong class II MHC antigen expression.

The relative importance of two surface antigens, MHC class II and CTLA4-Ig, in the allostimulatory ability of day 8 rat DCs was tested in functional assays using blocking antibodies. When used alone, CTLA4-Ig only partially blocked alloreactivity and even a fivefold increase in its concentration (to 12.5 μg/ml) allowed a maximum inhibition of no more than 72% (data not shown). These data indicate that either allostimulation in an MLR requires more than the CD28-B7 pathway or the homology between rat and mouse CTLA4 is incomplete. The former interpretation is, however, consistent with published findings that

TABLE 4. Comparison of the Ability of $OX62^+$ DCs and Unsorted DC-Enriched BM Cultures as Stimulators in MLR Assay[a]

	Responders		
Stimulators	LEW	ACI	PVG
$OX62^+$ DCs			
1×10^4	$7,416 \pm 850^{*,b}$	$39,341 \pm 2163^{*,b}$	$39,396 \pm 4470^{*,b}$
3×10^3	$5,507 \pm 739^{\ddagger,b}$	$29,145 \pm 168^{*,b}$	$32,362 \pm 1242^{*,b}$
1×10^3	$2,355 \pm 199^{\ddagger,b}$	$17,920 \pm 7132^{\ddagger,b}$	$18,025 \pm 5477^{*,b}$
Unsorted control			
1×10^4	$4,393 \pm 88$	$117,788 \pm 527$	$8,680 \pm 2061$
3×10^3	$3,458 \pm 767$	$11,676 \pm 3291$	$9,890 \pm 2471$
1×10^3	$2,117 \pm 945$	$8,308 \pm 1630$	$6,742 \pm 2687$

[a] Day 6 DC-enriched BM culture was sorted for $OX62^+$ DCs. The $OX62^+$ DCs and unsorted controls were then used as stimulators in an MLR assay against syngeneic (LEW) and allogeneic (ACI and PVG) nylon wool–purified T cells (3×10^5/well). The assay was labelled with [^{3}H]thymidine for 18 h before harvesting on day 4. Results presented are means ± SD of one experiment.

[b] $^*P < .05$ vs. unsorted control.

$^\ddagger P$ not significant vs. unsorted control.

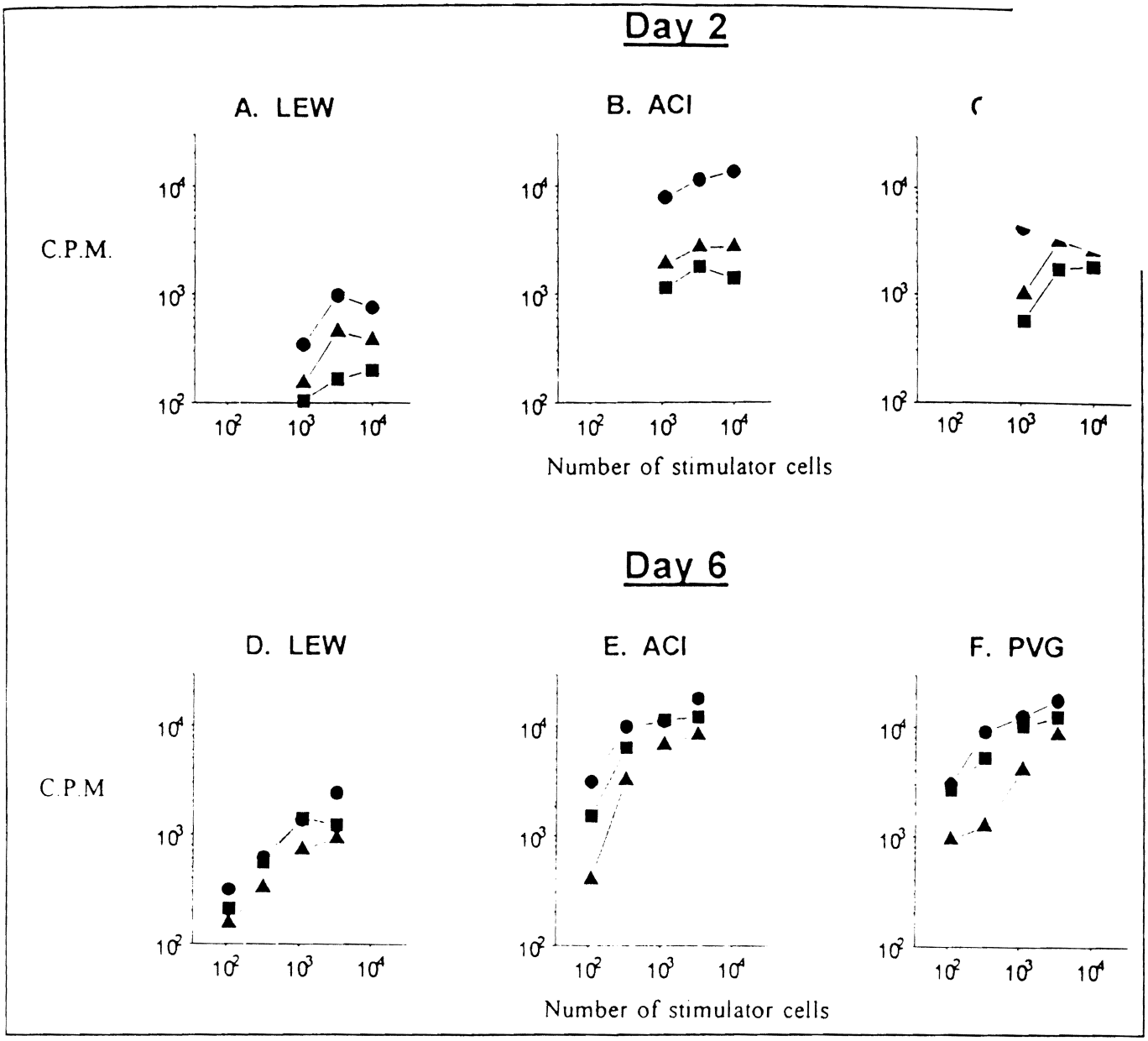

Fig. 6. DC-enriched BM cultures from unfractionated (■), OX7$^+$ (●), and OX7$^-$ (▲) BM cultures were used as stimulators in a one-way MLR against syngeneic (LEW) and allogeneic (ACI and PVG) responders. Cultures at 2 and 6 days were analyzed and the mean results of two separate experiments are shown.

serial administration of this reagent in vivo does not readily induce long-term acceptance of cardiac allografts [35] in rats. Although OX6 alone more effectively blocked allore-activity in an MLR, the almost complete prevention of proliferation seen with a combination of low-dose CTLA4-Ig and anti-MHC class II mAb (Table 2) reinforces the importance and complexity of costimulation pathways in the rat.

Considerable interest has arisen in using antigen-pulsed DCs as a vaccine adjuvant to induce protective immunity against infectious diseases or tumors, which otherwise do not incite protective immunity. For example, it has been shown that immature or freshly isolated DCs can process native proteins [36], and in vivo immature skin DCs acquire antigen, process it, and travel to draining lymph nodes [37]. In this study, pure OX62$^+$-sorted DC cells could effectively present the soluble antigen OVA to naive T cells and stimulate naive allogeneic T cells in vitro. Morphologically, this sorted population could be best classified as immediate DC precursors.

A simple panning procedure using OX7 to select Thy-1.1$^+$ cells enriches DC activity between six- and ninefold at various S/R ratios when assays are performed early in the culture period (day 2). These results are consistent with previous suggestions that the precursors of rat DCs are Thy-1.1$^+$ [31]. Although we have shown some usefulness for Thy 1.1 in identifying rat stem cells, its presence on 40–60% of fresh rat BM cells, including myelomonocytic

TABLE 5. Anti-CD90 mAb, OX7 Treatment Promotes the Maturation of BM-Derived DCs[a]

Treatment	Responder	Proliferative responses at various S/R ratios 1:9	1:27	1:81
Normal mouse IgG1	LEW	1,306 ± 276	642 ± 247	703 ± 262
	ACI	10,262 ± 2615	5,247 ± 1172	2,865 ± 52
OX7	LEW	7,901 ± 906*,[b]	8,176 ± 1703*,[b]	3,893 ± 10
	ACI	16,428 ± 5199‡,[b]	12,426 ± 1380*,[b]	8,083 ± 782

[a]Fresh Lew BM cells were treated with either normal mouse IgG1 (isotype control) or mAb OX7 before being put in culture. After 48 h in culture, the DC-enriched cultures were used as stimulators against syngeneic (Lew) or allogeneic (ACI) responders in an MLR. Results presented are means ± SD of one experiment.

[b]*$P <$ .03 vs. isotype control.

‡P not significant vs. isotype control.

precursors [38], makes attempts to enrich for Thy1.1$^+$ cells in the rat not that meaningful.

However, one interesting and unexpected finding was the high level of allostimulatory activity found in the OX7$^+$ fraction at day 2. In unfractionated cultures not exposed to OX7 binding, comparatively low levels of allostimulatory activity were found after 48 h of incubation. An additional experiment using unfractionated DC-enriched BM cells showed that binding of OX7 but not an isotype control antibody to cells in culture clearly enhanced the allostimulatory ability of this population. Although the functional role of Thy1 remains unknown, anti-Thy1.1 Ab has been shown to have a mitogenic effect on T cells and Thy1 transfected B cells [39]. Therefore, that anti-Thy1 mAb binding could promote DC maturation is not an unreasonable assumption.

In conclusion, mature DCs derived from rat BM cultures are potent stimulators of allogeneic T cells in an MLR and can efficiently present exogenous antigen to naive T cells. Rat DCs show a strong morphologic resemblance to their human and mouse counterparts and express a similar array of costimulatory and adhesion molecules, some of which are essential for their potent stimulatory ability in vitro. These studies provide a baseline for further analysis of the role of DCs in the many well-defined rat models of transplantation tolerance [6–8] and autoimmune disease.

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