Generation of DC from mouse spleen cell cultures in response to GM-CSF: immunophenotypic and functional analyses

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SUMMARY

In all tissues that have been studied to date, dendritic leucocytes constitute only a small proportion of total cells and are difficult both to isolate and purify. This study reports on a method for the propagation of large numbers of dendritic cells (DC) from mouse spleen using granulocyte–macrophage colony-stimulating factor (GM-CSF) and their characteristics. Within a few days of liquid culture in GM-CSF, B10 BR ($H-2^k$, $I-E^+$) mouse splenocytes formed loosely adherent myeloid cell clusters. Mononuclear progeny released from these clusters at and beyond 4 days exhibited distinct dendritic morphology and strongly expressed leucocyte common antigen (CD45), CD11b, heat-stable antigen, Pgp-1 (CD44) and intercellular adhesion molecule-1 (ICAM-1; CD54). The intensity of expression of the DC-restricted markers NLDC 145 and 33D1, the macrophage marker F4/80, and FcγRII (CDw32) was low to moderate, whereas the cells were negative for CD3, CD45RA and NK1.1. High and moderate levels, respectively, of cell surface staining for major histocompatibility complex (MHC) class II ($I-E^k$) and the B7 antigens (counter-receptors of CTLA4, a structural homologue of CD28) were associated with potent stimulation of unprimed, allogeneic T cells (B10; $H-2^b$, $I-E^-$). DC propagated in a similar fashion from DBA/2 mouse spleen proved to be strong antigen-presenting cells (APC) for MHC-restricted, syngeneic T-helper type 2 (Th2) cell clones specifically responsive to sperm whale myoglobin. Footpad or intravenous injection of GM-CSF-stimulated B10.BR spleen-derived DC into B10 ($H-2^b$, $I-E^-$) recipients resulted in homing of the allogeneic cells to T-cell-dependent areas of lymph nodes and spleen, where they strongly expressed donor MHC class II antigen 1–2 days later. These findings indicate that cells can be propagated from fresh splenocyte suspensions that exhibit distinctive features of DC, namely morphology, motility, cell-surface phenotype, potent allogeneic and syngeneic APC function and *in vivo* homing ability. Propagation of DC in this manner from progenitors present in lymphoid tissue provides an alternative and relatively convenient source of high numbers of these otherwise difficult to isolate but functionally important APC.

INTRODUCTION

Dendritic cells (DC) were first isolated from mouse lymphoid tissues during studies on the function of accessory cells in immune responses.[1] They constitute a minor population of bone marrow-derived leucocytes that is distributed ubiquitously throughout the body. In contrast to macrophages, from which they differ on a number of morphological, phenotypic,

enzymatic and behavioural criteria,[2–4] mature DC constitutively express high levels of cell-surface major histocompatibility complex (MHC) class II molecules and are the most potent initiators of T-lymphocyte responses, both *in vitro* and *in vivo*.[2–4] DC within secondary lymphoid tissues are present as interdigitating cells in T-cell areas (white pulp of spleen and paracortex of lymph nodes) and in the marginal zone of the spleen.[5] These cells represent the major MHC class II-positive stimulatory cell type within the spleen. The identity of critical costimulatory signals for resting T cells expressed by mature DC has, however, remained elusive. Recent evidence, based on studies of murine epidermal Langerhans' cells, suggests that up-regulation of cell-surface expression of CD80 (formerly termed B7 or BB1), the counter-receptor for the T-cell activation molecule CD28, may correlate with the functional maturation of DC.[6]

Studies on the properties and functional maturation of DC

have been restricted by the rarity of cells that meet criteria for classification as DC (< 1% of unfractionated spleen cells) and by the failure of such cells to survive beyond a few days or to grow in culture. Recently, however, DC progenitors have been propagated in response to granulocyte–macrophage colony-stimulating factor (GM-CSF) from precursors present in mouse bone marrow[7] and blood.[8] In addition, we have shown that DC lineage cells can be induced to proliferate in response to this cytokine from progenitors present in mouse liver.[9] In this study, we report on the propagation of DC from GM-CSF-stimulated progenitors present in unfractionated spleen cell suspensions. We have characterized these DC in terms of their phenotype and function, with particular regard to their role as inducers of T-cell activation.

MATERIALS AND METHODS

Animals

Adult 8–12-week-old male B10.BR (H-2^k, I-E$^+$), C57BL/10SnJ (B10, H-2^b, I-E$^-$) and DBA/2 (H-2^d) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). They were maintained in the specific pathogen-free facility of the University of Pittsburgh Medical Center.

Isolation and propagation of spleen cells

Fresh splenocytes were obtained by teasing the spleens under aseptic conditions; single-cell suspensions were prepared using standard procedures. Erythrocytes were removed by osmotic lysis. The nucleated cells were washed twice (5 min at 400 g) in RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with glutamine (2 mM/ml), non-essential amino acids (0·1 mM/ml), sodium pyruvate (1 mM/ml), 2-mercaptoethanol (20 μM), antibiotics (penicillin, 100 U/ml; streptomycin, 100 mg/ml) (complete medium) and 5% heat-activated fetal bovine serum (HIFBS; Gibco). To test the capacity of the spleen cells to proliferate in response to GM-CSF, 1–2 × 10^6 cells were placed in each well of a 24-well plate in 1 ml of complete medium, supplemented with 10% HIFBS and 0·4 ng/ml mouse recombinant (r) GM-CSF (R&D Systems, Minneapolis, MN). The cultures were 'fed' with GM-CSF every 48 hr by aspirating 50% of the supernatant, after gentle swirling, and replenishing with an equivalent volume of fresh, GM-CSF-supplemented medium. An objective of these washes was to remove non-adherent granulocytes without dislodging clusters of developing DC that had attached loosely to firmly adherent macrophages.[7] After 4 days, granulocytes were no longer significant contaminants of the cultures. These were maintained routinely for up to 10 days, at which time non-adherent cells released from growing clusters were harvested. In this manner, approximately 1·5 × 10^6 DC/spleen were obtained by day 10.

Flow cytometric analysis

GM-CSF-stimulated spleen cells were washed in complete medium (2 × 5 min each), then resuspended (5 × 10^5/tube) in Hanks' balanced salt solution (HBSS) with 1% w/v bovine serum albumin (BSA; Sigma, St Louis, MO) and 0·1% sodium azide (Sigma). They were stained either by direct or indirect immunofluorescence using a panel of monoclonal antibodies (mAb). T lymphocytes were identified using phycoerythrin (PE)-conjugated hamster anti-mouse CD3-ε (PharMingen, San Diego, CA). For the detection of B cells, rat anti-mouse B220

(CD45RA; TIB146; ATCC, Rockville, MD) was used, followed by FITC-conjugated AffiniPure goat anti-rat IgG (Jackson Immunoresearch Labs Inc., West Grove, PA). Anti-leucocyte common antigen (CD45; TIB122; ATCC), anti-macrophage antibody (F4/80; HB198; ATCC), anti-heat-stable antigen (J11D; TIB183; ATCC) and antibodies directed against DC-restricted markers (NLDC-145, derived from Kraal *et al.*,[10] and 33D1; TIB227; ATCC; kindly provided by Dr R. M. Steinman, Rockefeller University, New York, NY) were employed to characterize further the lineages of the isolated cells. The presence of natural killer (NK) cells was revealed using antibody against NK1.1 (Dr W. H. Chambers, Department of Pathology, University of Pittsburgh). The expression of accessory molecules was identified using antibodies against the interleukin-2 (IL-2) receptor (CD25; p55, PC61 5.3; TIB222; ATCC), intercellular adhesion molecule-1 (ICAM-1; CD54; Serotec, Indianapolis, IN), Pgp-1 glycoprotein (CD44; TIB235; ATCC), CD11b (MAC-1α unit, M1/70; TIB128; ATCC) and FcγRII (CDw32; PharMingen). Appropriate FITC-conjugated anti-mouse, anti-rat or anti-hamster secondary antibodies were used. Normal hamster serum or the appropriate rat immunoglobulin isotypes were used as negative controls. Biotin-conjugated mouse anti-mouse I-E^{k,d,p,r} (PharMingen) was used with FITC-steptavidin (Jackson Immunoresearch Labs) as the secondary reagent. Biotin-conjugated mouse IgG2a, together with FITC-streptavidin, was used as a negative control.

To identify counter-receptors of CTLA4 (a structural homologue of CD28), which include CD80 (B7-1) and CD86 (B7-2), the cells were first incubated with either human immunoglobulin (Sigma) as a negative control or with the CTLA4–immunoglobulin fusion protein (2·5 μg/ml; kindly provided by Dr P. S. Linsley, Bristol Myers Squibb Pharmaceutical Research Institute, Seattle, WA), which contains the extracellular portion of human CTLA4 and a human IgGγ1 chain.[11] They were washed and incubated with FITC-conjugated mouse IgG2a anti-human IgG (Fc; Sigma). Lipopolysaccharide (LPS)-activated splenic B-cell blasts, which express B7/BB1,[6] served as positive controls. After staining, the cells were fixed in 1% v/v paraformaldehyde before analysis in a FACSTAR® flow cytometer (Becton Dickinson, San Jose, CA). Five thousand events were acquired for each sample.

Immunocytochemistry

Cytocentrifuge preparations were stained either directly or indirectly using avidin–biotin–peroxidase complex (ABC)-staining procedures. Briefly, specimens were air-dried at room temperature, before fixing in acetone for 5 min. The slides were then washed in phosphate-buffered saline (PBS) and incubated for 1 hr at room temperature with biotinylated mouse IgG2a, anti-mouse I-E^{k,d,p,r} mAb. For indirect staining, primary rat anti-CD45, anti-NLDC145 or anti-2A1 (a gift from Dr R. M. Steinman) mAb were used (1 hr at room temperature) followed by biotinylated mouse IgG F(ab')$_2$ anti-rat IgG (Accurate Chemical and Scientific Corporation, Westbury, NY), for 1 hr at room temperature. After three washes of 5 min each in PBS, the slides were incubated with streptavidin–biotin–peroxidase complex (ABC-P; Boehringer Mannheim Corp., Indianapolis, IN) for 30 min at room temperature, and the colour reaction was developed for 6 min using a peroxidase chromogen kit
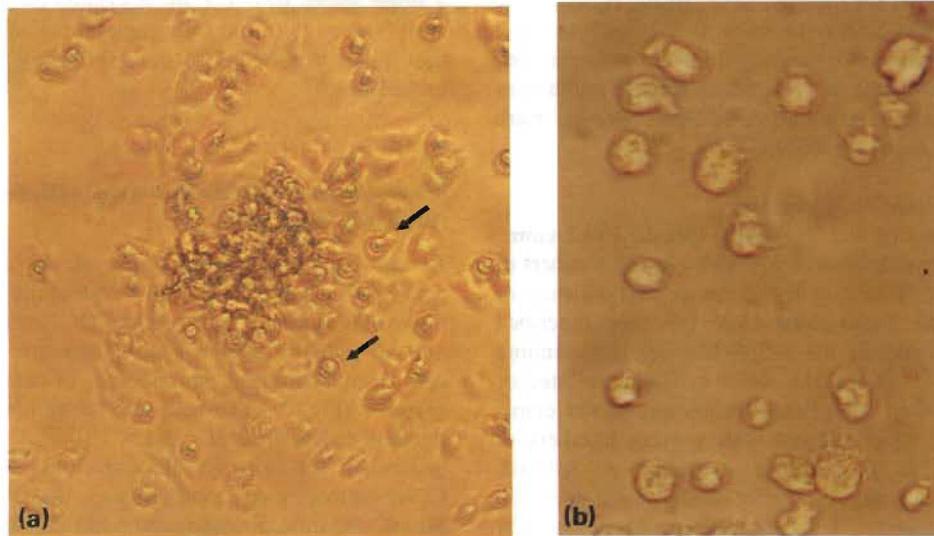
Figure 1. Phase–contrast micrographs illustrating the development and isolation of normal mouse spleen-derived DC in liquid cultures supplemented with GM-CSF. (a) An early aggregate of proliferating, putative DC progenitors (day 4) attached to strongly adherent macrophages and showing typical, loosely adherent cells (arrows) that were released from the aggregates. At 2 and 4 days, non-adherent granulocytes were removed by gentle aspiration of the supernatant and replaced with fresh, GM-CSF-supplemented medium. Magnification, × 100. (b) Higher power view of typical, non-adherent cells with cytoplasmic processes released from aggregates and harvested after 10 days of culture. Magnification, × 400.

(AEC; Biomedia Corp., Foster City, CA). Cells were counter-stained lightly with haematoxylin. Controls included the omission of antibody and the use of isotype-matched irrelevant mAb.

Mixed leucocyte cultures

To test the immunogenicity of freshly isolated or cultured spleen cells, one-way mixed leucocyte cultures (4×10^5 cells in 200μl/well) in 96-well, round-bottomed microculture plates (Falcon®; Becton Dickinson Labware, Lincoln Park, NJ) were performed with variable numbers of γ-irradiated (20 Gy) allogeneic (B10.BR) or syngeneic (B10) spleen-derived cells as stimulators. B10 spleen cells were used as responders, which were T-cell enriched by passage (1 hr at 37°) through a nylon wool column. In some experiments, designed to examine the functional expression of B7 antigens, CTLA4–immunoglobulin fusion protein ($2·5 \mu$g/ml) or human immunoglobulin was added at the start of the cultures. The cells were maintained in complete medium supplemented with 10% HIFBS for
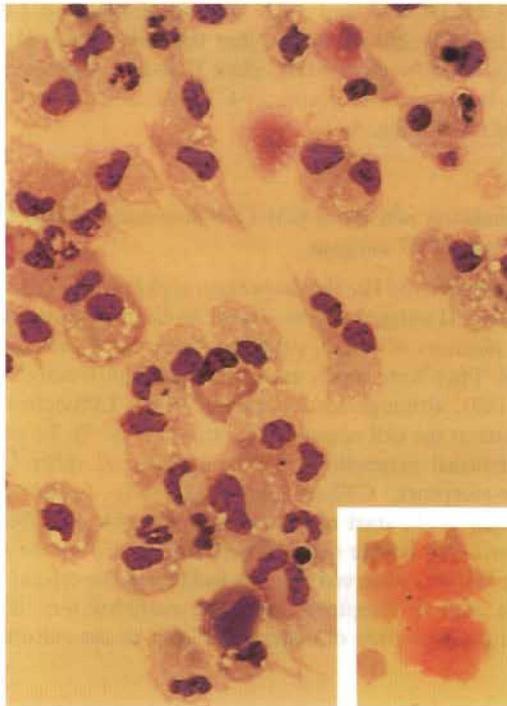

Figure 2. Giemsa-stained cytocentrifuge preparation showing cells released from GM-CSF-stimulated spleen cell aggregates (day 10), which exhibit irregular-shaped eccentric nuclei, variable degrees of cytoplasmic vacuolation, absence of prominent granules and distinct cytoplasmic processes. Magnification, × 600. Inset, immunoperoxidase-stained preparation, showing a high level of MHC class II (I-E^k) antigen expression on dendritic-shaped cells (also day 10). Magnification, × 600.
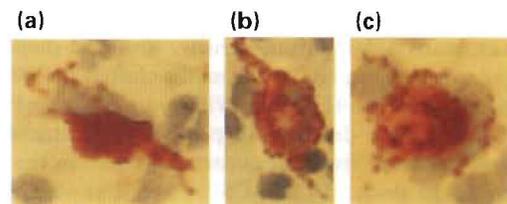

Figure 3. Immunoperoxidase labelling of cultured cells harvested after 10 days to show additional phenotypic features of released DC progeny. The primary mAb were (a) anti-CD45 (leucocyte common antigen; ATCC; TIB122), (b) NLDC-145 anti-interdigitating cell,[10] and (c) 2A1, which reacts primarily with granules within the cytoplasm of cultured DC *in vitro*.[8] Magnification, × 600.

72 hr in 5% CO_2 in air; for the final 18 hr, 10 μl [^{3}H]thymidine ([^{3}H]TdR; 1 μCi) was added to each well. Cultures were harvested onto glass fibre disks using a multiple cell harvester, and the degree of thymidine incorporation was determined in a liquid scintillation counter. Results were expressed as means (c.p.m.) $\pm$ 1 SD.

Stimulation of syngeneic Th2 cell clone

The sperm whale myoglobin (SpWMb)-specific T-cell clone, 13.26, was used in proliferation assays with varying numbers of spleen-derived DBA/2 cells in the presence and absence of specific antigen. The T-cell clone 13.26 has been described previously[12] and is specific for a SpWMb peptide spanning amino acids 132–147. To test the ability of freshly isolated or cultured spleen cells to stimulate proliferation of the clone, 5×10^4 T cells/well were cultured with varying numbers of spleen-derived cells in the presence or absence of SpWMb peptide 132–147 (20 μg/ml) in 200 μl of medium in 96-well flat-bottomed microtitre plates. The cells were incubated for 48 hr at 37°, pulsed with 0·5 μCi [^{3}H]TdR for an additional 24 hr, harvested and counted in a β-scintillation counter.

DC homing

GM-CSF-stimulated B10.BR spleen cells were washed in RPMI-1640 and injected subcutaneously (s.c.) (1 or $2 \cdot 5 \times 10^5$ cells in 50 μl) into one hind footpad, or intravenously (i.v.) (1×10^6 in 200 μl) via the lateral tail vein, of normal allogeneic B10 mice. One and 2 days later, the draining popliteal lymph nodes (where appropriate) and spleen were removed and embedded in Tissue-Tek® (OCT Compound; Miles Inc., Elkhart, IN) before freezing at $-70°$. Sections (10 μm) were cut using a cryostat microtome at $-30°$ and melted directly onto slides at room temperature, then air-dried. After brief acetone fixation, immunoperoxidase staining was performed, as described above. Controls included sections of normal recipient strain (B10) tissues.

RESULTS

Proliferation of spleen cells in response to GM-SCF

Since it has been reported recently that large numbers of DC can be induced to proliferate from mouse blood or bone marrow when cultured with GM-CSF,[7,8] we determined whether, using a similar approach, spleen-derived DC could be generated in liquid culture. After 4 days culture of freshly isolated splenocytes, during which time non-adherent granulocytes and mature DC were removed by gentle washes, growth of cell 'clusters' attached to a layer of adherent cells was evident (Fig. 1a); many low buoyant density, dendritic-shaped cells appeared to have been released from the clusters and exhibited cytoplasmic processes (Fig. 1b). With more prolonged culture in GM-CSF, these dendritic-shaped, loosely attached cells continued to detach from the clusters and float in the culture medium. In the absence of GM-CSF, no clusters were observed and cells did not proliferate. Macrophages and fibroblasts also expanded in the GM-CSF-stimulated cultures, but remained attached firmly to the plastic surface. Floating or loosely adherent, putative DC were harvested by gentle aspiration for various analyses. Approximately $1 \cdot 5 \times 10^6$ of these cells could be recovered consistently at 10 days from cultures derived from

a single spleen ($0 \cdot 7$–1×10^8 nucleated cells). This represented an increase of 10-fold over the number of mature DC/spleen that could be isolated in our laboratory using conventional procedures.[1,13]

Microscopic and immunophenotypic analysis of GM-CSF-stimulated spleen cells

At the microscopic level, most of the cells released from proliferating aggregates of GM-CSF-stimulated cells exhibited typical morphological features of DC, including (in many but not all cells) irregular shape, eccentric nuclei, numerous cytoplasmic dendrites, and absence of prominent cytoplasmic granules (Fig. 2). The cells with dendritic morphology were strongly MHC class II$^+$ (I-E^{k+}) (Fig. 2 inset), as described previously for cultured blood-derived DC,[8] and expressed CD45 (leucocyte common antigen), the DC-restricted marker NLDC-145 (interdigitating cells) and the DC-restricted granule antigen 2A1 (Fig. 3). In order to characterize further the surface phenotype of cells released from proliferating aggregates, flow cytometric analysis was performed after 7–10 days culture in GM-CSF. Staining for cells of lymphoid lineage, including NK cells, was absent. As shown in Fig. 4, the floating cells strongly expressed surface antigens that are known to be associated with mouse DC. These included CD45, heat-stable antigen (J11D), ICAM-1 (CD54), CD11b (MAC-1) and CD44 (non-polymeric determinant of Pgp-1 glycoprotein). In addition, staining of weak to moderate intensity was observed for the DC-restricted markers NLDC-145 and 33D1 and for F4/80 and FCγRII. FACS analysis also confirmed that the cells expressed a high intensity of cell-surface MHC class II (I-E^k). Moreover, they stained for counter-receptors of CTLA4, a structural homologue of CD28 (Fig. 5).

Allostimulatory activity of GM-CSF-propagated spleen DC and the role of B7 antigens

The spleen-derived DC that expressed high levels of cell-surface MHC class II antigen and moderate levels of B7 antigens were potent inducers of 3-day, primary allogeneic T-cell responses (Fig. 6). They were much more effective than freshly isolated spleen cells, although less stimulatory than LPS-activated B-cell blasts at the cell concentrations used (Fig. 7). To examine the functional expression of B7 antigens and other CTLA4 counter-receptors, CTLA4–immunoglobulin fusion protein was added at the start of the cultures. Complete inhibition of the mixed lymphocyte reaction (MLR) induced by the spleen-derived DC was observed (Fig. 7), indicating the crucial role of CTLA4 counter-receptors, including costimulatory B7 antigens, in the activation of unprimed T cells by the cultured DC.

Activation of syngeneic T-cell clones

The spleen-derived DC were also potent stimulators of antigen-specific T-cell activation. Significant proliferation of the SpWMb-specific Th2 clone 13.26 was observed with as few as 300 spleen-derived DC, with peak proliferation observed with 3000 stimulator cells/well (Fig. 8). In contrast, freshly isolated spleen cells were much less efficient.
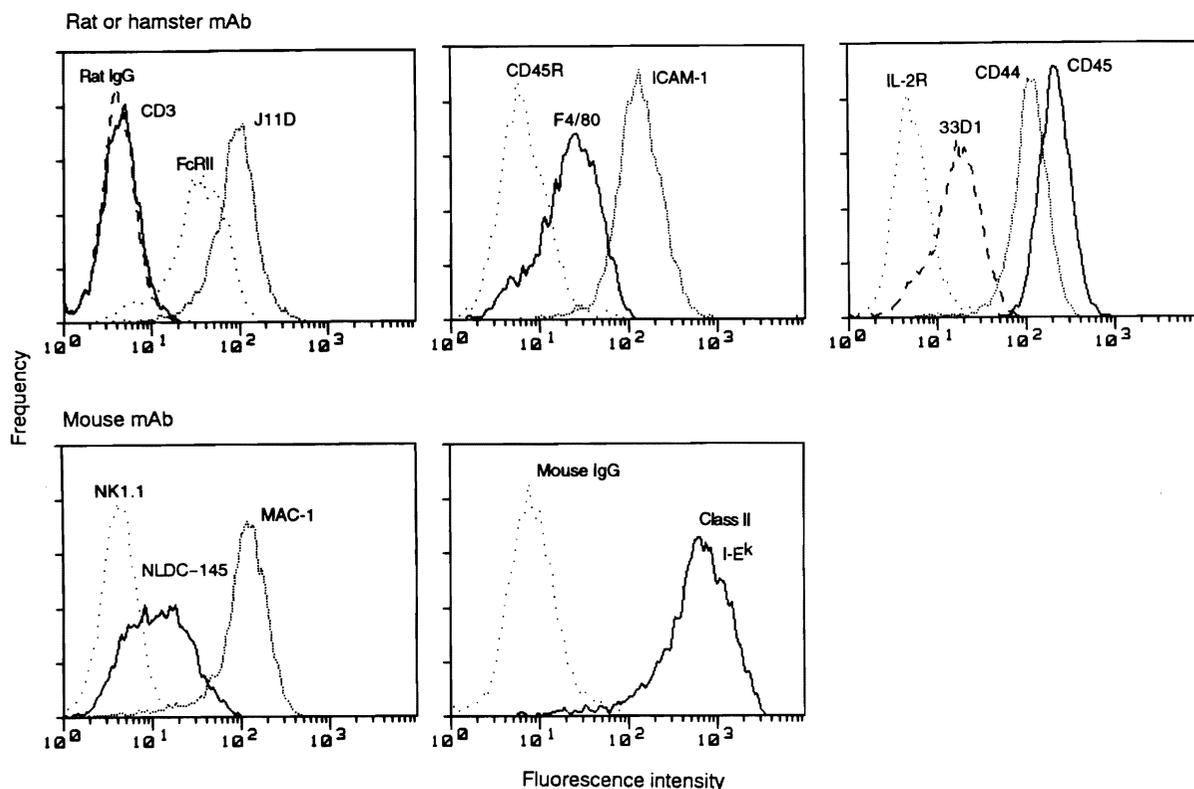
Rat or hamster mAb

Mouse mAb

Fluorescence intensity

Figure 4. Merged FACSCAN® immunophenotypic profiles of the GM-CSF-stimulated spleen-derived DC released from cell aggregates in liquid culture (day 10) and examined using rat, hamster or mouse mAb as detailed in the Materials and Methods. All data were obtained from the same cell preparation at the same time. The result is representative of three separate experiments performed using cells obtained from 6–10-day cultures.

In vivo homing of spleen-derived DC

A specialized property of DC is their capacity to 'home' to T-dependent areas of peripheral lymphoid tissues.[14,15] To determine the homing ability of the spleen DC propagated in culture, 10-day GM-CSF-stimulated cells were injected s.c. into one hind footpad or i.v. into allogeneic B10 (I-E$^-$) recipients. One and 2 days later, the mice were killed and cryostat sections of the draining lymph nodes (where appropriate) and spleens
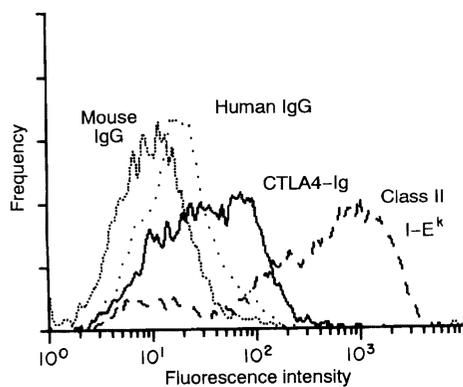

Figure 5. Flow cytometric analysis of MHC class II (I-E^k) and CTLA4– immunoglobulin counter-receptor expression on GM-CSF-stimulated mouse spleen-derived DC released from proliferating aggregates in 10-day cultures. The result is representative of three separate experiments.
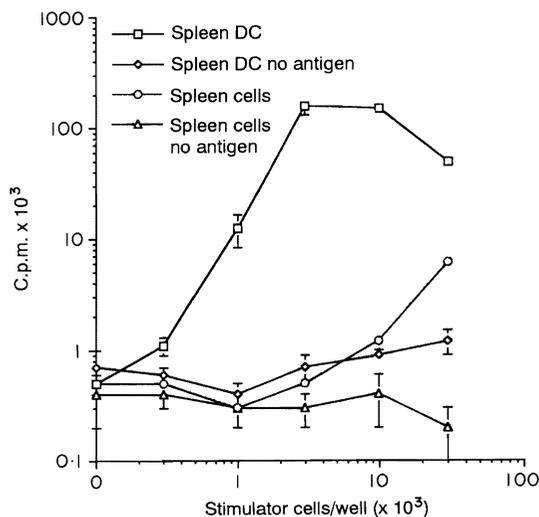

Figure 6. Allostimulatory activity of γ-irradiated, GM-CSF-stimulated B10.BR mouse spleen-derived DC using naive B10 (I-E$^-$) splenic T cells as responders. The non-adherent cells were harvested from 10-day GM-CSF-stimulated cultures and set up at various concentrations with 4×10^5 responder T cells. Cultures were maintained for 72 hr; [^{3}H]TdR was added 18 hr before harvesting. The MLR-stimulatory activity of freshly isolated allogeneic (B10.BR) and syngeneic (B10) spleen cells is also shown. The results are expressed as mean c.p.m. ± 1 SD and are representative of three separate experiments.

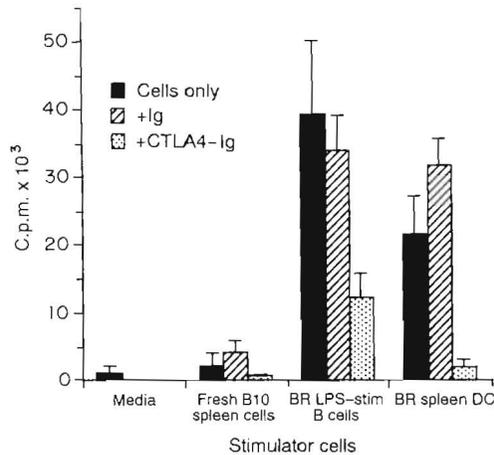**Figure 7.** Inhibition by CTLA4–immunoglobulin of spleen-derived DC-induced T-cell proliferation in an allogeneic MLR. Unprimed B10 nylon wool-passed T cells (4×10^5/well) were stimulated with 10^5 B10.BR spleen-derived DC or *Escherichia coli* LPS-activated ($10\,\mu g/ml$; 72 hr) B10.BR splenic B cells in the presence of $2.5\,\mu g/ml$ CTLA4–immunoglobulin or human immunoglobulin. Results are the means ± 1 SD of triplicate cultures. Similar results were obtained in three experiments.

were stained with donor-specific mAb to I-E^k. As shown in Fig. 9, spleen-derived cells propagated in GM-CSF-supplemented cultures homed almost exclusively to T-cell areas of the recipients' spleens in close proximity to arterioles. Similar observations were made in the draining lymph nodes of footpad-injected mice (data not shown). Moderate to intense I-E^k expression was detected on the spleen-derived cells, many of which also exhibited distinct dendritic morphology. As previously reported for non-lymphoid organ DC,[14-17] the

Figure 9. Homing ability of GM-CSF-stimulated B10.BR spleen-derived cells DC (I-E$^+$) released in culture from proliferating cell aggregates (harvested on day 10). The cells (2.5×10^5) were injected s.c. (day 0) into one hind footpad of B10 (I-E$^-$) recipients and detected by immunohistochemistry in cryostat sections of spleen 1 or 2 days later. The spleen sections were stained using the ABC peroxidase procedure with donor-specific mouse anti-I-E^k mAb with appropriate controls. (a) Strongly MHC class II-positive cells (one of which is arrowed) were detected in the periarteriolar lymphatic sheath. Magnification, $\times 45$. (b) At higher magnification, cells with dendritic morphology were readily identified in the T-dependent areas in close proximity to arterioles; day 2. Magnification, $\times 400$.

spleen DC propagated in culture also exhibited a key functional property of this cell lineage—the capacity to home to T-dependent areas of secondary lymphoid tissue and therein to express strong MHC class II cell-surface antigen.

DISCUSSION

Previous estimates of the frequency of the prototypic DC in mouse spleen have been about 0.1–0.3% of nucleated cells, with yields of approximately $2–5 \times 10^5$/spleen.[18,19] Similar results are obtained whether the DC are sorted[18] or isolated by more traditional adherence/rosetting methods,[19] which deplete contaminating macrophages. Once isolated, spleen DC are exquisitely dependent on GM-CSF for survival beyond 1 day of culture. To our knowledge, growth of splenic DC has not been reported in response to GM-CSF, nor has viability been maintained in culture for more than a few days.[18] Until recently, it has proved difficult to generate DC in cultures of
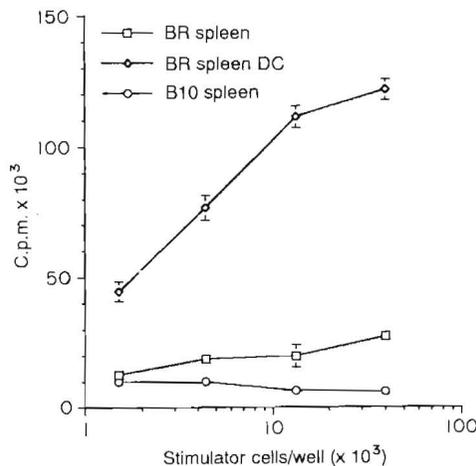

Figure 8. Spleen-derived DC efficiently present antigen to a SpWMb-specific Th2 clone, 13.26. The indicated numbers of stimulator cells were incubated with 5×10^4 13.26 cells in the presence ($20\,\mu g/ml$) or absence of SpWMb peptide 132–147. The cells were cultured for 72 hr and pulsed with $0.5\,\mu Ci$/well [^{3}H]TdR for the last 24 hr. The results are expressed as the mean ± 1 SD of triplicate wells. A representative of three similar experiments is shown.
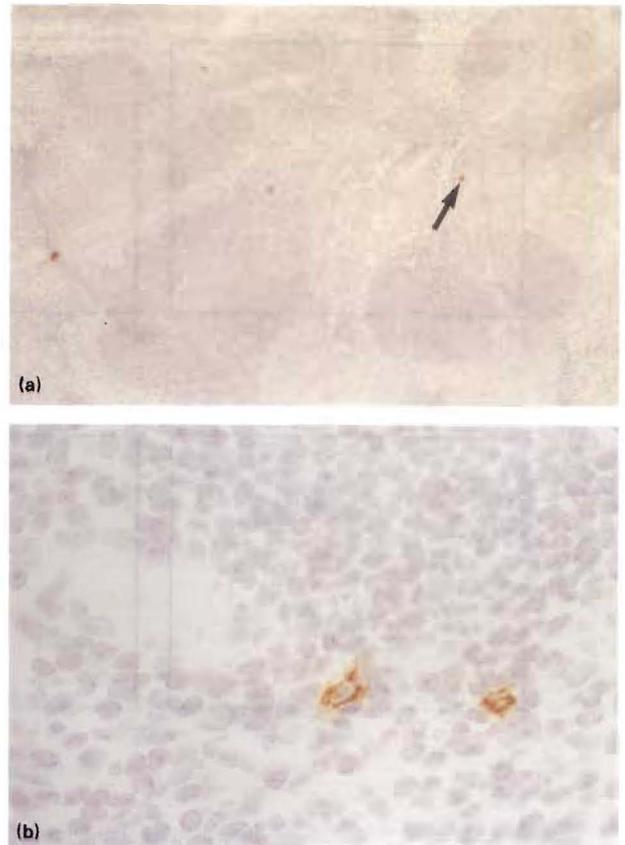
bone marrow,[20,21] from which these cells are ultimately derived, and little is known about the properties of progenitors cells of DC in spleen and other tissues.

Within the last 2 years, however, GM-CSF-dependent proliferating DC progenitors that give rise to large numbers of typical, mature DC have been identified in mouse blood,[8] bone marrow[7] and liver.[9] Initially, these studies centred on those murine tissues believed to be relatively deficient in mature DC (e.g. blood and bone marrow). More recently, propagation of DC progenitors for normal mouse liver[9] has shown that tissues from which Ia+ mature DC can be isolated using conventional procedures[22] also contain Ia⁻ progenitors of these cells. In the present study, the use of culture techniques which we have shown to facilitate the growth of DC progenitors from unfractionated, non-parenchymal cells in normal mouse liver,[9] also promoted the growth and differentiation of DC progenitors present in fresh spleen cell suspensions. The identity of the cells was confirmed by their morphology, immunophenotypic characteristics, antigen-presenting function and *in vivo* homing ability, all of which conformed to that of 'classical' murine splenic DC.[1-4]

Flow cytometric analysis of the GM-CSF-stimulated spleen-derived DC revealed many surface molecules characteristically associated with mouse DC; including leucocyte common antigen (CD45), heat-stable antigen (J11D), ICAM-1 (CD54), CD11b (MAC-1) and CD44 (non-polymorphic determinant of Pgp-1 glycoprotein). In addition, staining of moderate intensity was observed for the DC lineage-restricted markers NLDC-145 (interdigitating cells) and 33D1, for the DC-restricted granule antigen 2A1 and for F4/80 and FcγRII. Similar observations for each of these markers have recently been made on DC released from proliferating aggregates in cultures of GM-CSF-stimulated mouse blood or bone marrow.[7,8] In keeping with these latter findings on circulating or bone marrow DC progenitors, the spleen-derived, GM-CSF-stimulated cells expressed very high levels of the MHC class II surface antigen molecule. The cultured splenic DC also expressed moderate intensity of counter-receptors/ligands (B7 antigens) for CTLA4 (the structural homologue of CD28). Moreover, in blocking experiments, we found that CTLA4 counter-receptors on the cultured DC were required for proliferation of unprimed, allogeneic splenic T cells in primary mixed leucocyte reactions. These findings confirm recent observations of moderate cell-surface immunofluorescence staining and functional expression of B7/BB1 (CD80) on low density, transiently adherent DC-enriched populations isolated from mouse spleen cells suspensions[6] using conventional procedures.[23]

The intense cell-surface expression of MHC class II (I-E^k) and accompanying staining for B7 antigens was associated with potent antigen-presenting activity of the GM-CSF-stimulated spleen DC, both for allogeneic (I-E⁻) naive T cells and for antigen-driven proliferation of syngeneic (I-A^d) Th cell clones specifically responsive to sperm whale myoglobin. Recent findings suggest that up-regulation of the expression of B7 antigens on maturing mouse Langerhans' cells is of critical importance in the development of their capacity to activate naive T cells.[6]

A specialized property of DC is their ability to home to T-cell areas of peripheral lymphoid tissues.[8,14,15,17,24] Donor-derived DC expressing high levels of alloantigens have previously been identified within the spleens of unmodified murine allograft recipients, 1–4 days after heart transplantation[14] and up to 1 year after liver transplantation.[16] To test the homing capacity of the splenic DC (I-E⁺) produced in culture, cells were either injected into the footpad or i.v. into unmodified, allogeneic (I-E⁻) recipients. The precise homing of these MHC class II⁺ DC progeny to T-cell areas of the spleen confirmed the lineage of the cells and, together with our *in vitro* observations, indicated the potential of the GM-CSF-cultured cells to act as potent APC *in vivo*. *In vitro*, however, cytokines other than GM-CSF, such as tumour necrosis factor-α (TNF-α) and IL-4[25] may also affect their maturation and functional status.

Whilst mature or 'steady state' murine spleen DC (e.g. N418⁺ 'sorted' cells) fail to propagate *in vitro* in response to cytokines, it appears that GM-CSF-responsive progenitors present in bone marrow,[7,26] blood,[8] liver[9] and (as shown in this study) unfractionated suspensions of spleen cells, have the capacity for both macrophage and DC differentiation. Cytokine-driven propagation and differentiation of high numbers of viable DC progenitors from spleen-cell suspensions provides a convenient alternative to traditional labour-intensive methods of DC enrichment/sorting where low yields/viability and/or contamination can prove problematic. The method described also offers opportunities for further molecular analyses of the regulation of growth and differentiation of DC lineage cells, and of the mechanism of action of this cell lineage in the generation and regulation of adoptive immune responses.

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