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Generation of Tumor Immunity by Bone Marrow-Derived Dendritic Cells Correlates with Dendritic Cell Maturation Stage[1]

Marta S. Labeur, Berthold Roters, Birgit Pers, Annette Mehling, Thomas A. Luger, Thomas Schwarz, and Stephan Grabbe[2]

Bone marrow-derived dendritic cells (BmDC) are potent APC and can promote antitumor immunity in mice when pulsed with tumor Ag. This study aimed to define the culture conditions and maturation stages of BmDC that enable them to optimally function as APC in vivo. BmDC cultured under various conditions (granulocyte-macrophage CSF (GM-CSF) or GM-CSF plus IL-4 alone or in combination with Flt3 ligand, TNF-α, LPS, or CD40 ligand (CD40L)) were analyzed morphologically, phenotypically, and functionally and were tested for their ability to promote prophylactic and/or therapeutic antitumor immunity. Each of the culture conditions generated typical BmDC. Whereas cells cultured in GM-CSF alone were functionally immature, cells incubated with CD40L or LPS were mature BmDC, as evident by morphology, capacity to internalize Ag, migration into regional lymph nodes, IL-12 secretion, and alloantigen or peptide Ag presentation in vitro. The remaining cultures exhibited intermediate dendritic cell maturation. The in vivo Ag-presenting capacity of BmDC was compared with respect to induction of both protective tumor immunity and immunotherapy of established tumors, using the poorly immunogenic squamous cell carcinoma, KLN205. In correspondence to their maturation stage, BmDC cultured in the presence of CD40L exhibited the most potent immunostimulatory effects. In general, although not entirely, the capacity of BmDC to induce an antitumor immune response in vivo correlated to their degree of maturation. The present data support the clinical use of mature, rather than immature, tumor Ag-pulsed dendritic cells as cancer vaccines and identifies CD40L as a potent stimulus to enhance their in vivo Ag-presenting capacity. ***The Journal of Immunology*****, 1999, 162: 168–175.**

Dendritic cells (DC)[3] play a critical role in the activation of naive T lymphocytes and in the generation of primary T cell responses (reviewed in Refs. 1–3). These cells reside in a resting or immature state in nonlymphoid tissues, where they efficiently capture and process Ag. Upon activation they initiate a differentiation process that results in decreased Ag-processing capacities, enhanced expression of MHC and costimulatory molecules, and migration into secondary lymphoid organs, where they trigger naive T cells. This in vivo maturation process is efficiently regulated and controlled by a complex array of signals present in the DC microenvironment. A number of cytokines and other factors have been proposed to promote DC growth and differentiation from myeloid progenitor cells, including GM-CSF, TNF, Flt3L, IL-4, and possibly also stem cell factor, IFN-γ, TGF-β, PGE_2, ionomycin, and others (1–21). In vitro, and possibly in vivo as well, inflammatory stimuli (such as IL-1, IL-6, and TNF) (21–23) and contact with T cells (via CD40/CD40L interaction) (24, 25) further activate DC, resulting in mature DC with strong T cell stimulatory potential. Although several different protocols exist for generation of BmDC, resulting in cells with potentially different phenotypes and/or functions, the in vitro regulation of BmDC maturation by relevant molecules in a comparative way and the correlation with their function in vivo remain to be elucidated.

Due to their unique capacity to stimulate resting T cells, DC are the candidate cell type to use for immunization protocols, especially for induction of antiviral or antitumor immunity (26, 27). It has been demonstrated that murine BmDC are able to promote prophylactic and therapeutic antitumor immunity when pulsed with relevant tumor-associated T cell epitopes (28–32). The degree of DC differentiation (immature vs mature) may determine their subsequent function. Once pulsed with Ag in vitro, it may be argued that immature BmDC are the most appropriate DC to use for immunization protocols in vivo because of their capacity to internalize/process Ag and mature on their way to the regional LN. Alternatively, however, mature DC may be best suited for in vivo immunotherapy because of their capacity to efficiently present Ag to naive T cells, and because immature BmDC may induce tolerance rather than immunity. Since Ag processing (which is maximal in immature DC) and T cell sensitization (which is more effective in mature DC) are both essential for the development of antitumor immunity, it is crucial to understand the regulation of DC maturation to gain further insights into their in vivo function. Presently, no data are available that conclusively address whether terminally differentiated mature DC or incompletely differentiated immature DC are best to use for vaccinations in vivo.

Therefore, to understand the interplay of cytokines in the DC maturation process, BmDC generated under various culture conditions were directly compared with respect to morphology, phenotype, and in vitro function. These different BmDC populations were then analyzed in vivo by testing the ability of these cells to migrate into regional LN and to promote prophylactic and/or therapeutic antitumor

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[3] Abbreviations used in this paper: DC, dendritic cell(s); Flt3L, Flt3 ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; CD40L, CD40 ligand; BmDC, bone marrow-derived dendritic cells; LN, lymph node; CM, complete medium; TA, tumor antigen.

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immunity to define culture conditions that result in the generation of DC that are best suited for in vivo immunotherapy.

We report here that BmDC generated only in the presence of GM-CSF display an immature phenotype. CD40L and LPS are strong inducers of full DC maturation. DC cultured in the presence of GM-CSF and IL-4 with or without the addition of Flt3L or TNF-α exhibit an intermediate maturation stage with respect to phenotype and in vitro Ag-presenting capacity. The cytokine secretion profile of BmDC cultured in various ways differs significantly. The capacity to induce an antitumor immune response in vivo correlates to the degree of DC maturation. BmDC generated in vitro migrate rather inefficiently into regional LN after s.c. injection regardless of the culture conditions and maturation stage. Of all culture conditions tested, BmDC generated in the presence of CD40L mediate the most potent immune responses in vivo, including the generation of protective and therapeutic tumor immunity.

Materials and Methods

Mice

DBA2/N, BALB/c, and C57BL/6 mice (6–10 wk old) were obtained from Charles River (Sulzfeld, Germany) and housed according to government regulations.

Generation, culture, and flow cytometry of BmDC

BmDC were generated as described previously (6) with some modifications. Briefly, bone marrow was collected from tibias and femurs of female BALB/c mice, passed through a nylon mesh to remove small pieces of bone and debris, resuspended in complete medium (CM: RPMI 1640 containing 5% FCS, 50 μM 2-ME, 2 mM glutamine, 0.1 mM nonessential amino acids, and 20 μg/ml gentamicin; all from PAA, Linz, Austria), and cultured in tissue culture dishes (Becton Dickinson, Heidelberg, Germany) for 2 h. Nonadherent cells were collected, and aliquots of 1×10^6 cells were placed in 24-well plates (Becton Dickinson) containing 1 ml of CM with the cytokines listed below. Two-thirds of the medium was replaced on day 3. On days 5 and 7 of culture nonadherent cells were transferred into six-well plates in CM with cytokines (2.5×10^6 cells/2 ml/well) and maintained for 2 additional days in culture. On day 9 of culture, most of the nonadherent cells had acquired typical dendritic morphology, and these cells were used as the source of DC in subsequent experiments. At this time point supernatants from the different cultures were collected and stored at −20°C for cytokine determinations.

Six different combinations of cytokines were used for BmDC generation. 1) GM-CSF (150 U/ml; R&D Systems, Wiesbaden, Germany); 2) GM-CSF and IL-4 (75 U/ml; PharMingen, Hamburg, Germany); and 3) GM-CSF, IL-4, and Flt3L (250 ng/ml; Immunex, Seattle, WA) were used throughout the culture period. Alternatively, cells were maintained in GM-CSF and IL-4 for 9 days with the addition of 4) TNF-α (20 U/ml; Genzyme, Cambridge, MA), 5) CD40L (1 μg/ml; Immunex), or 6) *Escherichia coli*-LPS (0.1 μg/ml; Sigma, St. Louis, MO) for the final 2 days of cell culture.

Expression of surface molecules was quantitated by flow cytometry using the following Abs: I-$A^{b,d,q}$, I-$E^{d,k}$ (M5/114), B7-1 (1G10), B7-2 (GL-1), CD11b/Mac-1 (M1/70), and CD40 (all obtained from PharMingen, San Diego, CA); CD11c (N418, Endogen, Cambridge, MA); NLDC145 and ICAM-1 (YN1/1.7.4; from American Type Culture Collection, Manassas, VA; 10% culture supernatant); anti-rat or anti-hamster IgG-FITC (Boehringer Mannheim, Mannheim, Germany); and normal rat IgG2b (PharMingen) as isotype control (1 μg/ml, diluted in PBS/1% FCS(v/v)). For flow cytometry, aliquots of 1×10^5 BmDC were incubated with the mAbs for 60 min at 4°C. The cells were washed twice with PBS/0.1% FCS(v/v) and incubated with FITC-conjugated goat anti-rat or goat anti-hamster IgG (diluted 1/50 in PBS/1% FCS (v/v)) for 45 min on ice. At the end of this incubation, propidium iodide (100 nM; Sigma) was added to determine the percentage of dead cells, cells were washed twice and subsequently analyzed in a flow cytometer (EPICS XL, Coulter, Miami, FL). No gating was performed, except for elimination of dead cells.

Detection of cytokines

IL-1β, IL-10, IL-12, TNF-α, and IFN-γ production by BmDC was determined by ELISA (Endogen (Cambridge, MA) and Laboserv (Giessen, Germany)). Additionally, IL-2 bioactivity in supernatants was assessed in a bioassay based on the proliferation of the IL-2-dependent murine cell line CTLL-2 (33). Briefly, CTLL-2 cells were washed extensively, resuspended in CM, and seeded into 96-well, flat-bottom microtiter plates (5×10^3 cells/well) in the presence of serial dilutions of culture supernatants. In parallel, a standard curve was generated using recombinant murine IL-2 (R&D Systems). After 48 h of incubation, the proliferation of CTLL-2 cells was evaluated photometrically using the Alamar-Blue reagent (BioSource, Camarillo, CA).

Phagocytosis and endocytosis

Phagocytosis and endocytosis was assessed using FITC-conjugated *E. coli* particles and dextran, respectively (Molecular Probes, Leiden, The Netherlands). Briefly, 500 μl of *E. coli* particle suspension (0.625 mg/ml) or 20 μl of dextran (0.05 mM) was incubated with 5×10^5 BmDC, CTLL-2 (T cell line used as a negative control), and ML cells (murine macrophage cell line used as a positive control, provided by Dr. P. Ricciardi-Castagnoli) in RPMI 1640 medium at 37°C. After 2 h of incubation, cells were harvested and resuspended in medium with the addition of trypan blue, which quenches the fluorescence of extracellular particles. Next, BmDC were washed, resuspended in PBS, and analyzed in a flow cytometer.

Mixed lymphocyte reactions

BmDC were incubated in graded doses together with 2×10^5 allogeneic T cells in 96-well culture plates. The primary mixed lymphocyte reactions were performed in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.01 M HEPES, 50 μM 2-ME, 20 μg/ml gentamicin, and 5 μg/ml indomethacin. T cells were obtained from spleen cells of C57BL/6 mice by nylon wool nonadherence and subsequent elimination of residual contaminating cells with Ab-coated T cell isolation columns (Cellect mouse T cell immunocolumns, Biotex, Alberta, Canada). The resulting cell preparation contained $<0.1\%$ IA^+ cells. After 5 days, T cell proliferation was measured by adding [^{3}H]thymidine to the cultures and subsequent liquid scintillation counting after an overnight incubation period.

Presentation of peptide Ag to Ag-specific T-T hybridomas in vitro

To assess the ability of BmDC to present soluble protein Ag or peptide to primed T cells, the OVA-specific T-T hybridoma D011.1 was used. This hybridoma recognizes the $OVA_{323–339}$ peptide. For this assay, 1×10^4 BmDC/well or 1×10^5 A-20/well (B cell line, H-2^d, used as positive control) were incubated in the presence of 0.08–5 μg of $OVA_{323–339}$ peptide (recognized by D011.1 without requiring processing) (34). D011.1 cells (1×10^5) were added to each well, and the cultures were incubated in a total volume of 200 μl for 24 h at 37°C. One hundred microliters of culture supernatant was removed and assayed for IL-2 content using the IL-2-responsive cell line, CTLL-2, as described above.

In vivo migration

BmDC were labeled with the fluorescent dye, PKH2-2 (Sigma), according to the manufacturer's protocol. Briefly, the cells were washed three times with PBS to remove FCS. Cells were resuspended in PKH2-2 staining solution for 5 min. CM containing 10% FCS was added to the cells, followed by removal of unbound PKH2-2 by extensive washing with PBS. Thereafter, 5×10^5 labeled cells were s.c. injected in 40 μl of PBS into hind footpads of mice. Forty-eight hours after injection, mice were killed, and regional LN (inguinal and popliteal LN) were removed. Single cell suspensions of LN cells were subjected to a density gradient (Nycoprep 1.077, Nycomed Pharma, Oslo, Norway), and a low density cell fraction, enriched in DC, was harvested after centrifugation at $900 \times g$ for 30 min. Flow cytometric analysis was performed to detect fluorescent cells within the LN preparation.

Tumor cells and preparation of tumor Ag

The squamous cell carcinoma KLN205 (syngeneic to DBA2/N mice, H-2^d) was obtained from American Type Culture Collection (Manassas, VA) and maintained in tissue culture at 37°C in RPMI 1640 containing 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.01 M HEPES buffer, and 50 μM 2-ME. Upon injection of 2×10^5 tumor cells, the tumor grows progressively in syngeneic mice and can produce metastases at late tumor stages into regional LN. Previous experiments demonstrated that this tumor is very poorly immunogenic (K. Mahnke et al., manuscript in preparation). For preparation of TA, tumor fragments containing tumor-derived antigenic epitopes were prepared by disrupting 1×10^7 KLN205 cells/ml with

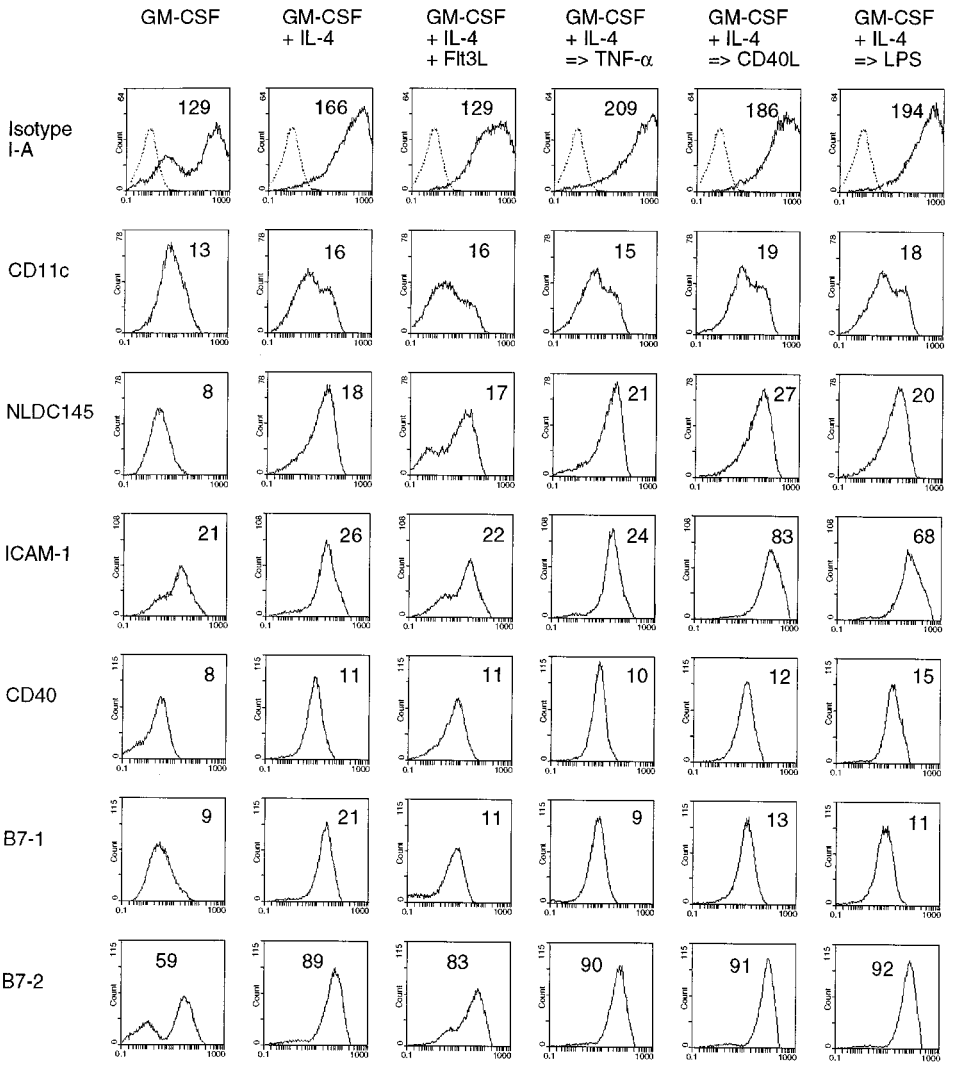

FIGURE 1. Surface phenotype of BmDC generated under different culture conditions. Cells differentiated under distinct culture conditions were analyzed by flow cytometry for the expression of I-A, CD11c, NLDC145, ICAM-1, CD40, B7-1, and B7-2 (thick lines). Dotted lines show the FACS profiles after staining with isotype control mAb; numbers indicate the mean fluorescence intensity. Results are representative of three different experiments. No gating was performed, except for elimination of dead cells.

four freeze-thaw cycles in liquid nitrogen. Lysates were centrifuged at 2000 × *g* for 10 min to remove insoluble cell fragments, and the supernatant was used as a source of tumor-associated Ag as previously described (35).

BmDC cultures were harvested and used for immunization experiments. To pulse BmDC with TA, 1 mg of TA from KLN205 cells was added to 1.5×10^6 BmDC in CM for 4 h at 37°C. Thereafter, cells were harvested and washed three times with PBS. Pulsed BmDC (2×10^4) were then injected s.c. into naive recipient mice on the lower abdomen. This immunization was repeated twice at weekly intervals. Two weeks before or 1 wk after the immunizations, mice were challenged with 2×10^5 live KLN205 cells s.c. on the lower lateral back, and tumor growth was assessed every 3 days by measurement with a spring-loaded caliper.

Statistical analysis

Student's *t* test was applied to reveal significant differences in cytokine production by BmDC generated under different culture conditions. $p < 0.05$ was accepted as the level of significance.

Results

Cell surface phenotype of BmDC generated under different culture conditions

The various culture conditions resulted in the generation of BmDC with typical morphology and fine membrane projections, although the number of dendritic processes was enhanced when CD40L or LPS were present in the culture medium and was decreased when cells were maintained in the presence of GM-CSF without addition of other factors. Homotypic BmDC aggregates were more abundant in cultures containing either CD40L or LPS, and cell yield was higher when GM-CSF or GM-CSF, IL-4, and Flt3L were present in the culture than with the remaining culture conditions.

Significant differences were observed in the morphology and expression of cell surface molecules involved in APC/T cell interactions. Compared with the other culture conditions, MHC class II, NLDC145, ICAM-1, CD11c, CD40, B7-1, and B7-2 expression was consistently lower in cells cultured in the presence of GM-CSF only or in cells cultured in GM-CSF, IL-4, and Flt3L, whereas the highest surface expression of these markers was observed in cells incubated with CD40L or LPS (Fig. 1). To assess the percentage of contaminating macrophages in the different culture conditions, the expression of the macrophage-related surface molecules CD11b, F4/80, ER-TR9, ER-HR3, and BM8 was determined. CD11b was broadly expressed on BmDC, and all BmDC also expressed high amounts of ER-TR9, indicating that these molecules are not macrophage specific. BmDC generated by culture in GM-CSF alone also broadly expressed BM8, ER-HR3, and F4/80 (F4/80, 60%; ER-HR3, 35%; BM8, 80% positive cells), but the expression of these markers could be down-regulated by subsequent culture in LPS or CD40L, which is consistent with the immature phenotype of these DC (data not shown). In all other BmDC culture systems, the percentages of cells expressing

Table I. *IL-12, TNF-α, and IL-1β production by BmDC generated under different culture conditions*[a]

Culture Conditions	IL-12 (pg/ml ± SEM)	TNF-α (pg/ml ± SEM)	IL-1β (pg/ml ± SEM)
GM-CSF	50 ± 12	320 ± 18	17 ± 3
GM-CSF + IL-4	164 ± 35	200 ± 27	21 ± 11
GM-CSF + IL-4 + Flt3L	183 ± 21	200 ± 30	22 ± 8
GM-CSF + IL-4 ⇒ TNF-α	267 ± 56	ND	27 ± 14
GM-CSF + IL-4 ⇒ CD40L	667 ± 23[b]	200 ± 18	27 ± 13
GM-CSF + IL-4 ⇒ LPS	1000 ± 40[b]	1800 ± 120[b]	1000 ± 294[b]

BmDC (1×10^6/ml) were cultured in medium containing different DC growth factors as described in *Materials and Methods*. Protein levels of cytokines secreted in supernatants during the last 24 h of the 9-day culture period were determined by ELISA. Data are shown as the mean ± SEM of three independent experiments.

[b] $p < 0.05$ with respect to the remaining cultures.

significant levels of ER-HR3, BM8, and F4/80 were <5%, indicating low numbers of contaminating macrophages in the BmDC cultures.

Cytokine production by BmDC generated under different culture conditions

To investigate the modulation of cytokine production and secretion profiles of BmDC emerging from the various culture conditions, supernatants from BmDC cultures were collected and analyzed for IL-1β, IL-10, IL-12, TNF-α, and IFN-γ contents using ELISA assays (Table I).

Interestingly, addition of LPS to BmDC stimulated the production of IL-12 as well as that of proinflammatory cytokines (IL-1β and TNF-α), whereas CD40L stimulation selectively induced the production of IL-12 but not that of IL-1β or TNF-α by BmDC. Lower amounts of IL-12, TNF-α, and IL-1β were measurable in supernatants of BmDC generated under GM-CSF and IL-4 with or without the addition of TNF-α or Flt3L, and very low levels of these cytokines were detected in supernatants from cells incubated with GM-CSF alone.

None of the tested stimuli induced endogenous production of IFN-γ by BmDC, and only minute amounts of IL-10 were detected in supernatants from LPS-cultured BmDC (mean ± SEM, 3.4 pg/ml ± 1.2), but not in any of the other culture conditions.

Phagocytosis and endocytosis

The endocytic capacity of BmDC that emerged from the distinct culture conditions is shown in Table II. Incubation of BmDC with FITC-labeled *E. coli* provided evidence of extensive phagocytosis in cells cultured in the presence of GM-CSF, as judged by the appearance of many FITC-positive cells with high mean fluorescence intensity. Incubation of cells with IL-4 resulted in a marked down-regulation of FITC-*E. coli* uptake. Further addition of Flt3L, TNF-α, CD40L, or LPS did not have additional effects on phagocytosis. Similar results were obtained using FITC-labeled latex beads instead of FITC-*E. coli* (data not shown).

Table II. *Phagocytosis and endocytosis by BmDC generated under different culture conditions*[a]

Culture Conditions	Mean Fluorescence Intensity	
	Phagocytosis	Endocytosis
GM-CSF	207	2.11
GM-CSF + IL-4	25	0.98
GM-CSF + IL-4 + Flt3L	18	0.88
GM-CSF + IL-4 ⇒ TNF-α	23	1.31
GM-CSF + IL-4 ⇒ CD40L	26	1.30
GM-CSF + IL-4 ⇒ LPS	17	0.76
Negative control (CTLL cells)	3	
Positive control (ML cells)	140	2.05

[a] BmDC were differentiated in the presence of distinct DC growth and differentiation stimuli as described in *Materials and Methods*. Phagocytosis and endocytosis were assessed using FITC-conjugated *E. coli* particles and FITC-dextran, respectively. CTLL-2 and ML cells were used as negative and positive controls. The data are representative of three independent experiments.

FITC-dextran was used as a marker for both the macropinosomal fluid phase and the clathrin-coated pit pathway of endocytosis (36). GM-CSF-treated cells most effectively incorporated this tracer, followed by cells differentiated in the presence of TNF-α and CD40L. Significantly less efficient endocytosis was detectable in the rest of the cultures. Thus, upon maturation, BmDC appear to shut off phagocytosis but maintain a reduced, but still detectable, capacity for endocytosis.

Allostimulatory activity and presentation of OVA peptide

To further characterize BmDC generated under distinct culture conditions, BmDC were compared for their capacities to stimulate alloreactive T cells. Graded numbers of BmDC from each culture condition were incubated with a fixed number of allogeneic T cells. Fig. 2 shows that approximately 150 BmDC could already trigger a substantial response in all the culture conditions tested. However, at low stimulator cell concentrations, DC cultured with either CD40L or LPS were at least twofold more effective than the remaining cultures in stimulating naive allogeneic T cells. In some experiments, as few as 30 BmDC/well from these cultures stimulated allogeneic T cell responses of >100,000 cpm (data not shown). BmDC generated from GM-CSF and IL-4 cultures with or without the addition of Flt3L or TNF-α exhibited intermediate ability to present alloantigen.

To evaluate the capacity of BmDC to present $OVA_{323–339}$ peptide to the OVA-specific T-T hybridoma DO11.1, BmDC were exposed to different concentrations of $OVA_{323–339}$ peptide and incubated in the presence of DO11.1 cells. As evident from Fig. 3, CD40L- and LPS-treated cells were the most efficient APC also in this assay system, since they were able to induce potent IL-2 production at low peptide concentrations. In contrast, cells cultured in the presence of GM-CSF were less efficient in OVA peptide presentation than BmDC grown under the other conditions.

In vivo migration

Syngeneic BmDC that had been labeled with a fluorescent dye were s.c. injected into the hind footpad of mice. After 2 days, regional LN were removed, and single cell suspensions were analyzed by flow cytometry. In all experiments, dye-labeled BmDC were clearly detectable in draining lymph nodes, but the vast majority of in vitro differentiated BmDC injected s.c. into syngeneic mice remained at the site of injection and failed to migrate into the draining LN regardless of the culture conditions and degree of BmDC maturation (Table III). In general, approximately 3–10% of

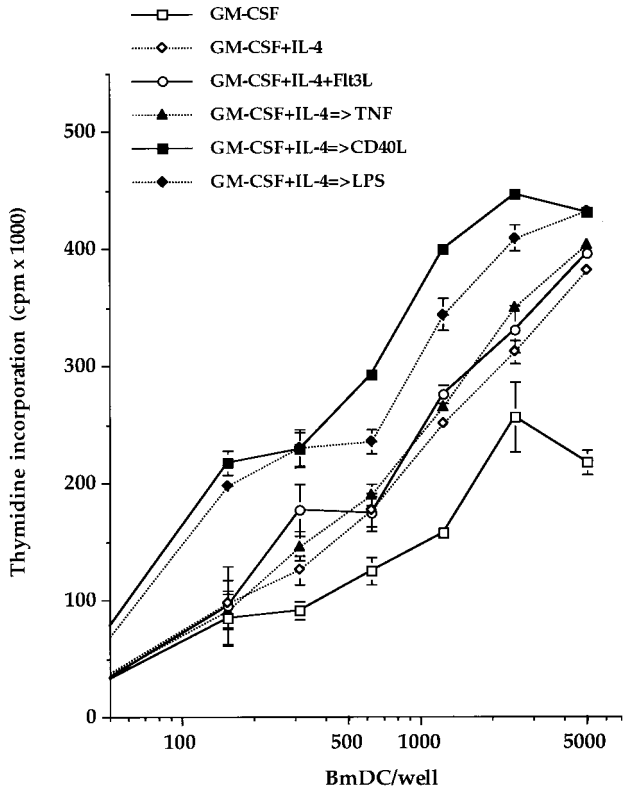

FIGURE 2. Allostimulatory capacity of BmDC. BmDC were differentiated in the presence of distinct DC growth and differentiation stimuli as described in *Materials and Methods*. Graded numbers of BmDC were coincubated with a fixed amount of allogeneic purified T cells for 4 days at 37°C, followed by overnight pulse with [^{3}H]thymidine. Background proliferation of BmDC or T cells alone was always <15,000 cpm. Results are the mean ± SEM of one representative experiment of three performed.

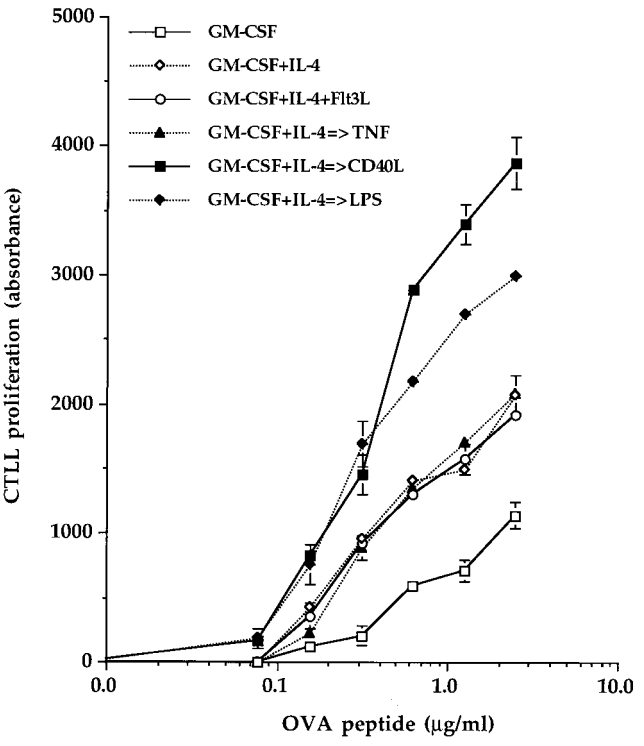

FIGURE 3. Presentation of OVA peptide by BmDC generated under different culture conditions. BmDC were differentiated as described in *Materials and Methods* and incubated in the presence of graded concentrations of $OVA_{323–339}$ peptide. The OVA-specific T-T hybridoma DO11.1 was used to assess the ability of BmDC to present soluble Ag. Results are the mean ± SEM of one representative experiment of three performed.

the injected BmDC were detectable in regional LN, with a maximum present 48 h after injection. The relatively poor migratory capacity of s.c. injected BmDC was not due to interference of the labeling procedure with BmDC viability or migration, since labeled and unlabeled BmDC 1) did not differ in vitro with regard to survival or allostimulatory capacity, 2) showed almost equal random migration activity when placed into three-dimensional collagen gels and subjected to time-lapse video microscopy, 3) were detectable in equally large numbers (>75% of the injected cells) as viable (trypan blue-excluding) cells in the skin at the injection site, and 4) were detectable in the regional LN in equally low numbers when investigated by direct fluorescence microscopy or after labeling by phagocytosis of fluorescent *E. coli* instead of using the membrane dye (data not shown). Thus, only a small percentage of BmDC migrates into the regional LN after s.c. injection in mice.

Induction of protective and therapeutic tumor immunity by TA-pulsed DC

To assess which type of DC culture and which DC differentiation stage are best suited for in vivo immunotherapy, we tested the ability of these BmDC to induce protective and therapeutic immunity against the poorly immunogenic squamous cell carcinoma, KLN205. For immunization against the tumor, mice were injected twice at weekly intervals with TA-pulsed BmDC as described in *Materials and Methods*. Control groups received TA only (data not shown) or BmDC that had not been pulsed with TA. One week after the last immunization, mice were challenged by s.c. injection of viable KLN205 cells. Control groups were not protected against tumor growth, suggesting that s.c. injected DC do not induce tumor immunity by Ag-independent mechanisms, and that immunization with TA alone does not induce a tumor-specific immune response. In contrast to data obtained by others using different tumor models, in the KLN205 tumor model immunization with TA-exposed BmDC that were generated by culturing cells in GM-CSF and IL-4 did not lead to consistent and profound protective tumor immunity, possibly due to the low immunogenicity of this tumor cell line. However, immunization of mice with TA-pulsed BmDC generated in vitro in the presence of CD40L dramatically reduced tumor growth and incidence, whereas mice immunized with TA-pulsed

Table III. *In vivo migration of s.c. injected BmDC*[a]

Culture Conditions	% Fluorescent Cells ± SEM
GM-CSF	1.17 ± 0.41
GM-CSF + IL-4	1.66 ± 0.58
GM-CSF + IL-4 + Flt3L	1.17 ± 0.43
GM-CSF + IL-4 ⇒ TNF-α	1.80 ± 0.23
GM-CSF + IL-4 ⇒ CD40L	2.35 ± 0.77
GM-CSF + IL-4 ⇒ LPS	1.45 ± 0.43
Dead BmDC	< 0.2

[a] BmDC were differentiated in the presence of distinct DC growth and differentiation stimuli as described in *Materials and Methods*. Migration of BmDC injected s.c. into footpads of mice toward the popliteal and inguinal lymph node was assessed by labeling the BmDC with the fluorescent tracer, PKH2-2, and subsequent flow cytometry of regional LN cells. Numbers are calculated as percent fluorescent cells in the DC-enriched LN cell suspension. Since the total cell number in popiteal and inguinal LN combined is approximately 2–3 × 10^6, this equals to a recovery rate of 3–10% of the injected BmDC. The data are representative of four independent experiments. No statistically significant differences in migration were present between the groups of BmDC injected (except for dead BmDC).

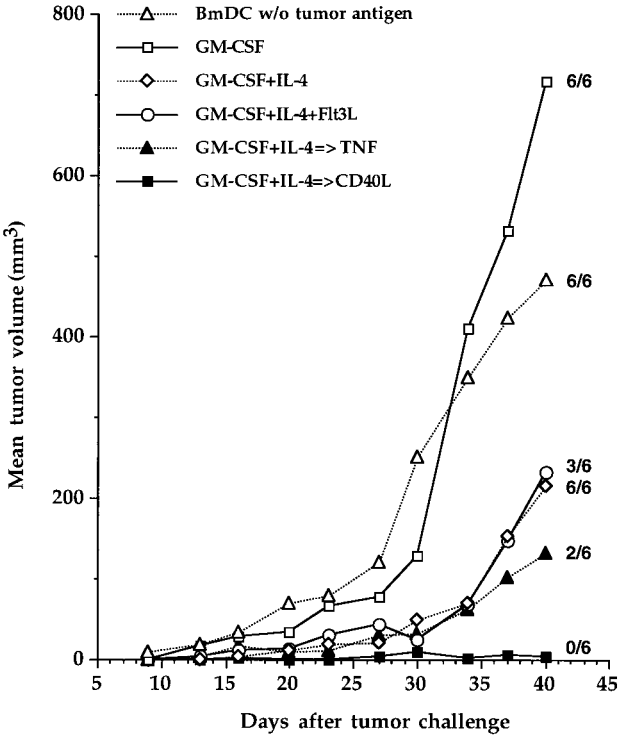

FIGURE 4. Induction of protective tumor immunity by BmDC. Groups of six mice were immunized with TA-pulsed BmDC as described in *Materials and Methods*. The immunizations were conducted twice at weekly intervals, and all mice were challenged with KLN205 cells 1 wk after the last immunization. Control animals received BmDC that had not been pulsed with TA. The graph shows the mean tumor volume over time. The number of tumor-bearing animals compared with the total number of mice in each group is indicated.

BmDC that were cultured with GM-CSF alone did not exhibit any protective tumor immunity (Fig. 4).

In a second set of experiments, cells emerging from the above-mentioned culture conditions were also tested for their ability to promote therapeutic tumor immunity. Tumors established for 14 days were treated by s.c. injection of TA-pulsed BmDC at a site distant from the tumor. In accordance with the other data obtained to date, tumor growth was markedly reduced when mice were injected with BmDC generated in the presence of CD40L. In addition, however, immunotherapy with TA-pulsed BmDC that had been cultured in GM-CSF, IL-4, and Flt3L also induced a marked reduction in tumor growth (Fig. 5*a*), whereas LPS-stimulated BmDC were not as potent as CD40L-treated cells when used in this immunotherapy model (Fig. 5*b*).

Discussion

One of the most promising approaches to establish vaccination protocols for the induction of T cell-mediated immune responses is to generate autologous DC, couple them ex vivo with Ag, and use these Ag-exposed APC for immunization purposes. The aim of this study was to directly compare and define culture conditions for BmDC that result in the generation of cells able to induce primary immune responses in vivo. In this respect our results emphasize the necessity of considering the stage of DC maturation when designing approaches for prophylaxis or therapy of tumors using DC-based immunization protocols. Our data indicate that, in general, the capacity to induce a tumor-specific immune response in vivo correlates to the degree of DC maturation. However, this correlation was not complete, since, for example, LPS-stimulated DC that were phenotypically and functionally mature generated a weaker in vivo immune response than those cells that were incubated in CD40L, which had largely the same degree of maturity but differed significantly in their cytokine secretion profile.

CD40L was found to be a strong inducer of full DC maturation, as evident by down-regulation of phagocytosis, low endocytic capacity, and up-regulation of MHC class II, the adhesion molecules CD11c and ICAM-1, and the costimulatory molecules B7-1 and B7-2. In addition, cells incubated with CD40L secreted high levels of IL-12 and were very efficient in presenting alloantigen or peptide Ag in vitro. In vivo, these mature DC were excellent in the promotion of antitumor immunity. Thus, the ability of the CD40L differentiated cells to promote antitumor immunity correlates to their high efficiency of stimulating resting T cells and to the high production of IL-12, which is in accordance with in vitro data obtained by others (24, 25). At present, we do not know yet whether these features are responsible for their potent in vivo immunostimulatory capacity. We speculate, however, that the IL-12 production by DC is critical for their in vivo function, since in other systems, IL-12 was shown to generate a polarization of the immune response toward the Th1 pathway in vivo. IL-12 is also a potent inducer of IFN-γ and TNF-α production by both NK cells and T cells (37), and these cytokines are critically involved in the development of cell-mediated immune responses (38), which is crucial for the induction of antitumor immunity. In this direction, Zitvogel et al. showed that the neutralization of both IFN-γ and TNF-α or the administration of anti-IL-12 Abs in mice totally abrogates the DC-induced anti-MCA205 tumor response (30). At the same time, however, LPS-stimulated DC were less capable of promoting tumor immunity than CD40L-treated BmDC, although they secreted similar amounts of IL-12 in vitro and had an in vitro maturation stage comparable to that of CD40L-cultured BmDC. Besides the functional characteristics previously described for the CD40L-generated cells, the cells cultured in the presence of LPS produced, in addition to IL-12, high levels of TNF-α and IL-1β. Although a role for these cytokines in tumor resistance has also been suggested (11, 39), the different cytokine secretion profiles of LPS- vs CD40L-stimulated BmDC may contribute to their different in vivo effectiveness.

In contrast to other laboratories (8), in our hands BmDC differentiated in the presence of only GM-CSF consistently generated immature DC as evident by morphological, phenotypical, and functional analyses in vitro as well as in vivo. Most likely, this difference is due to the use of different protocols for DC generation. Clearly, our data also show that IL-4 is a potent enhancer of mouse DC maturation, which is in agreement with data obtained by others (21, 29, 30, 32). Compared with DC differentiated in the presence of GM-CSF alone, supplementation of IL-4 significantly enhanced DC differentiation, leading to an intermediate degree of maturation. Expression of MHC class II Ags and the costimulatory molecules B7-1 and B7-2 was higher on DC cultured with GM-CSF and IL-4 than on those cultured with GM-CSF alone. Furthermore, DC grown in GM-CSF plus IL-4 were more potent stimulators of mixed lymphocyte reactions as well as more efficient in Ag presentation than cells grown in medium containing GM-CSF alone. Thus, and in agreement with other publications investigating human DC (9, 24, 25), this combination of cytokines provided good conditions for the generation of cells with the characteristic phenotype and functional properties of DC in the murine system. However, these in vitro generated BmDC retarded tumor growth only to some extent when DC were injected before tumor challenge and were poorly able to induce the rejection of preexisting tumors. These data contrast with the results of studies performed

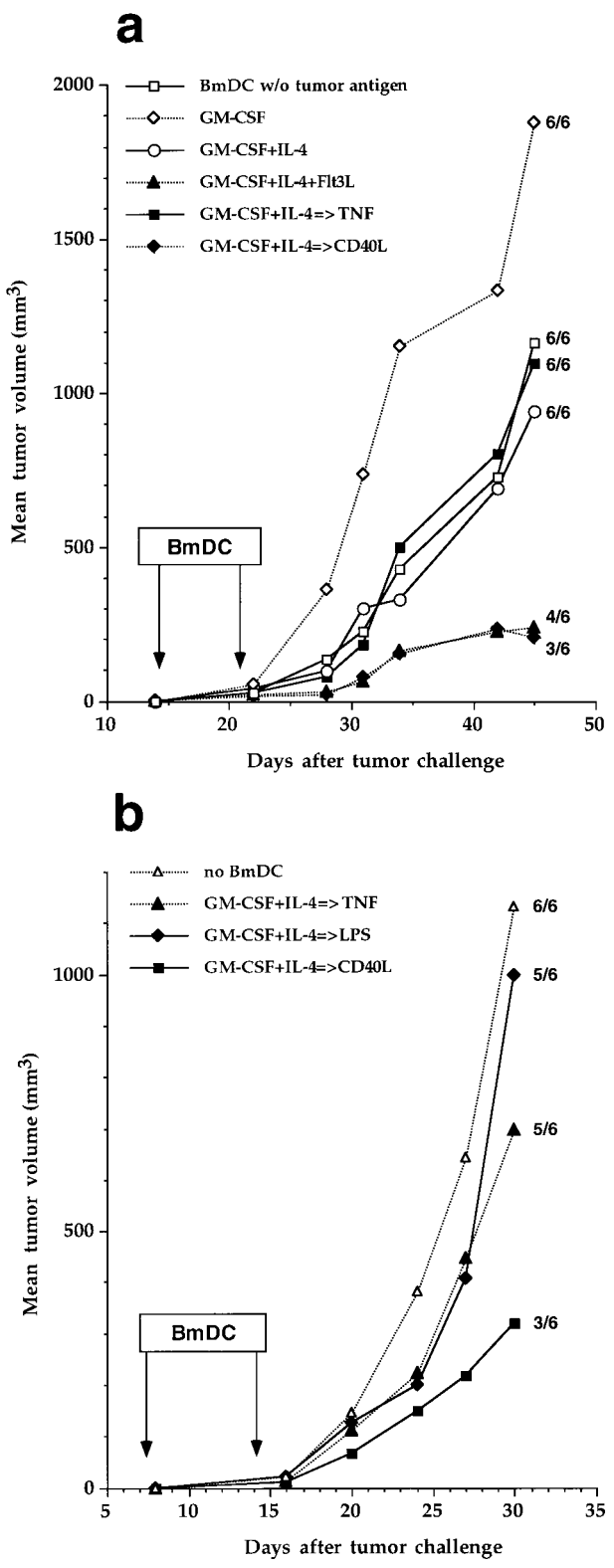

FIGURE 5. Induction of therapeutic tumor immunity by BmDC. Tumors established for 7 or 14 days (tumor volume, 10–100 mm^3) were treated with TA-pulsed BmDC or, as a control, with BmDC that had not been pulsed with TA. The immunizations were conducted twice at weekly intervals. The graphs (*a* and *b*) show the mean tumor volume in groups of six mice. Only a minority of the tumors regressed completely when treated with only two injections of TA-pulsed BmDC. In *b*, two different populations of mature BmDC (as judged by phenotype and in vitro function) were compared for the capacity to mediate regression of established KLN205 tumors, using the same experimental protocol as that described above. Figures indicate the number of tumor-bearing animals compared with the total number of mice in each group.

by Mayordomo et al. (32), which indicate that BmDC grown in medium containing GM-CSF and IL-4 are capable of generating a complete protective antitumor immune response. It is unlikely that this discrepancy is due to the inadequate quality of the BmDC generated in our study, given their immunophenotype and functional properties in vitro. Instead, it may be attributed to the use of different tumor models. The KLN205 is a very poorly immunogenic tumor, as determined by the fact that it generates no concomitant immunity and that vaccination with dead tumor cells alone or together with CFA, with irradiated cells, or with soluble TA did not affect tumor growth after a subsequent challenge with viable KLN205 cells (data not shown).

Surprisingly, the addition of TNF-α or Flt3L to the GM-CSF plus IL-4 cultures did not substantially affect the morphology or the in vitro functions of these cells. However, in vivo tumor experiments showed the ability of these cells to prevent tumor growth in some of the injected animals when tumor cells were injected after DC vaccination, whereas GM-CSF and IL-4 differentiated cells were able to retard tumor growth but not to prevent it. Interestingly, however, BmDC generated in the presence of Flt3L decreased tumor growth in a similar way as that observed by the CD40L differentiated BmDC, when tumor challenge preceded BmDC vaccination. Despite the functional similarities found in these cultures in vitro (GM-CSF and IL-4; GM-CSF, IL-4, and Flt3L; and GM-CSF, IL-4, and TNF-α), different antitumor responses were determined in vivo. The mechanisms underlying these differences remain to be elucidated.

The capacity of DC to migrate into T cell areas of LN is a key event in initiating immunity, and it may be critical for sensitization against tumor Ags. Thus, we were surprised by our consistent finding that BmDC appear to migrate very inefficiently into regional LN after s.c. injection, at least in the murine system. However, these data are in agreement with those of other groups (40–42), who also reported that the majority of DC that were s.c. injected remained at the site of injection and failed to migrate to the LN. In contrast, in vitro generated DC injected into the s.c. tissue of chimpanzees were reported to migrate rapidly and apparently completely to the regional LN (43). This different migratory behavior of BmDC may be due to different properties of chimpanzee and murine BmDC, respectively, or may reflect the fact that large numbers of DC (4×10^6) were injected directly adjacent to the inguinal LN in that study. We believe that the poor migratory capacity of s.c. injected BmDC was not a technical artifact, since labeled and unlabeled BmDC exhibited equal in vitro survival, random migration in collagen gels, and allostimulatory capacity, and identical results were obtained when using an entirely different labeling technique. Thus, s.c. injection may not be an optimal cell delivery system for in vitro generated BmDC, at least in the mouse. Nevertheless, although only a small number of labeled BmDC was detected in the LN, a potent in vivo immunostimulatory capacity was observed in most cases, resulting in the generation of protective and therapeutic tumor immunity. In our hands, incubation of BmDC with CD40L is currently the most potent stimulus to generate efficient in vivo immunostimulatory activity of murine BmDC. Thus, it might be crucial to optimize the maturation stage and migratory capacities of DC for designing future strategies using TA-pulsed DC for tumor immunotherapy.

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