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Transfection of Immature Murine Bone Marrow-Derived Dendritic Cells with the Granulocyte-Macrophage Colony-Stimulating Factor Gene Potently Enhances Their In Vivo Antigen-Presenting Capacity

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Ag presentation by dendritic cells (DC) is crucial for induction of primary T cell-mediated immune responses in vivo. Because DC culture from blood or bone marrow-derived progenitors is now clinically applicable, this study investigated the effectiveness of in vitro-generated murine bone marrow-derived DC (Bm-DC) for in vivo immunization protocols. Previous studies demonstrated that GM-CSF is an essential growth and differentiation factor for DC in culture and that in vivo administration of GM-CSF augments primary immune responses, which renders GM-CSF an attractive candidate to further enhance the effectiveness of DC-based immunotherapy protocols. Therefore, immature Bm-DC were transiently transfected with the GM-CSF gene and tested for differentiation, migration, and Ag-presenting capacity in vitro and in vivo. In vitro, GM-CSF gene-transfected Bm-DC were largely unaltered with regard to MHC and costimulatory molecule expression as well as alloantigen or peptide Ag-presenting capacity. When used for in vivo immunizations, however, the Ag-presenting capacity of GM-CSF gene-transfected Bm-DC was greatly enhanced compared with mock-transfected or untransfected cells, as determined by their effectiveness to induce primary immune reactions against hapten, protein Ag, and tumor Ag, respectively. Increased effectiveness in vivo correlated with the better migratory capacity of GM-CSF gene-transfected Bm-DC. These results show that GM-CSF gene transfection significantly enhances the capacity of DC to induce primary immune responses in vivo, which might also improve DC-based vaccines currently under clinical investigation. The Journal of Immunology, 1999, 163: 174–183.
cells within the local tissue environment, significantly affect DC function at various levels, including viability, morphology, migratory capacity, expression of surface molecules involved in Ag presentation, and binding and processing of antigenic peptides (reviewed in Refs. 1, 2, 5, 7, 13, and 14). In this respect, GM-CSF appears to have the most profound effects on DC function. There is a large body of literature suggesting that GM-CSF is essential for in vitro cultivation of DC and for differentiation of DC precursors into mature DC, both in human and in murine systems. Moreover, GM-CSF exposure of DC induces potent morphological and functional changes of DC (15). In addition, GM-CSF administration enhances primary immune responses in vivo, and it has been suggested that this effect might be due to stimulation of endogenous APC (16). Because GM-CSF is absolutely required for maintaining Bm-DC viability and differentiation in culture, it was hypothesized that the presence of GM-CSF might also be critical for viability and Ag-presenting capacity after injection of Bm-DC in vivo. Thus, we transfected immature murine Bm-DC with the GM-CSF gene, hypothesizing that this would enable the DC to generate a microenvironment that supports their own functional capacities in vivo. This study shows that GM-CSF gene transfection significantly enhances the in vivo functions of in vitro-expanded Bm-DC, which might lead to improved immunization and vaccination protocols in humans.

**Materials and Methods**

**Mice**

C3H/HeN, C57BL/6j, and BALB/c mice, 6–10 wk old, were obtained from Charles River (Sulzfeld, Germany) and housed according to government regulations.

**Generation and culture of DC**

DC were generated by culture of bone marrow cells in the presence of GM-CSF as described (17, 18). Briefly, bone marrow was collected from tibia of female BALB/c mice (Charles River). Erythrocytes were lysed, and the remaining cells were passed through a nylon mesh to remove small pieces of bone and debris. The cells were washed twice with cold PBS, resuspended, and cultured in petri dishes (Becton Dickinson, Heidelberg, Germany) at a density of 0.5 × 10⁸ cells/cm² for 2 h. Nonadherent cells were collected, and 1 × 10⁸ cells/ml were placed in 24-well plates (Becton Dickinson) in RPMI 1640, supplemented with 5% FCS, 50 μM 2-ME, 1% nonessential amino acids, 20 μg/ml gentamicin (all from PAA, Linz, Austria), and 100–200 U/ml GM-CSF (PharMingen, San Diego, CA) (BM medium). Two-thirds of the medium was replaced every 2 days, and nonadherent cells were harvested on day 7. DC aggregates were purified by sedimentation at 1 × g for 30 min and subcultured in 6-well plates in BM medium. After overnight cultivation, most of the nonadherent cells had acquired typical dendritic morphology. Expression of surface molecules characteristic for DC was determined by flow cytometry. For flow cytometry, aliquots of 1 × 10⁶ Bm-DC were incubated with mAbs against I-A<sup>d</sup>, I-E<sup>k</sup> (M5/114); Gr-1 (clone RB6-8C5), CD14 (rmc5-3), B7-1 (1G10), B7-2 (Gl-1) (all obtained from PharMingen); CD11c (N418, Endogen, Cambridge, MA), B220 (RA3-3A1), Thy-1.2 (30 H-12), LFA-1 (FD441.8), CD11b (M1/70), ICAM-1 (3E2) (all from American Type Culture Collection (ATCC), 10% culture supernatant), anti-rat IgG-FITC (Boehringer Mannheim, Mannheim, Germany), and normal rat IgG2b (PharMingen) as isotype control for 45 min on ice (1 μg/ml diluted in PBS, 1% BSA (v/v)). The cells were washed twice with PBS, 0.1% BSA (v/v) and incubated with FITC-conjugated goat anti-rat IgG (diluted 1:50 in PBS, 1% mouse serum (v/v)) for 45 min on ice. At the end of this incubation, propidium iodide (100 μM, Sigma, St. Louis, MO) 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). Bm-DC cultures of ≥70% I-A<sup>d</sup> cells were harvested and used as source of DC in subsequent experiments.

**Polymerase chain reaction**

Total RNA was isolated by lysing cells in RNAzol (Life Technologies, Grand Island, NY), followed by chloroform extraction and isopropanol precipitation. The RNA was treated with RNase-free DNase (Life Technologies) to eliminate genomic DNA contaminations and subsequently reverse transcribed by incubation of 2 μg RNA with 20 pmol oligo(dT) primers and 200 U Moloney murine leukemia virus reverse transcriptase at 42°C for 1 h in the presence of dNTPs and RNase inhibitor (Clontech Laboratories, Palo Alto, CA). The cDNA was amplified by 35 cycles of PCR (94°C for 40 s, 60°C for 40 s, and 72°C for 2 min) with pairs of oligonucleotides specific for mgGM-CSF or mgGPDH. The primer sequences were: GM-CSF: 5′-TGGTTGCTTACGCTCTTCAGACG-3′; CAAAGGGGATATCACTGAAAGGT. The expected length of the transcript is 368 bp. GPDH: 5′-TGAAGGTCCTGTTGACCGATTGGC-3′; CATGTAACGATCGTCACACAC. The expected length of the transcript is 983 bp.

**Transfection of Bm-DC**

The plasmid, pWSnmGM-CSF, in which the murine GM-CSF cDNA is expressed under the control of a CMV promoter, has been described elsewhere (19, 20). For control purposes, a CMV-driven LacZ construct (CMV<sup>β</sup>, kindly provided by G. MacGregor, Howard Hughes Institute for Molecular Genetics, Houston, TX), and a CMV-driven chloramphenicol acetyltransferase (CAT (PRV)<sup>γ</sup>460) construct, based on pBK-CMV (Stratagene, La Jolla, CA), was used. Mock transfections were performed with the plasmid psp65 (Boehringer Mannheim). All plasmids used were purified by two passages over CsCl<sub>2</sub> gradients and were completely free of LPS contaminations as determined by standard Limulus amebocyte lysate assay systems. Bm-DC were harvested on day 8 after onset of the Bm culture and transfected using the adenovirus-transferrin-polylysine transfection system as described before (19, 20). For transfection of Bm-DC, 3–10<sup>6</sup> particles of E4-defective, biotinylated, and porosarli/UV-inactivated adenovirus (human adenovirus type 5, strain d11014) in 200 μl HEPES-buffered saline (HBS) were mixed with 500 ng streptavidin-polylysine in 200 μl HBS. After 30 min of incubation at RT, 1.5–6 μg plasmid DNA in 300 μl HBS were added to the mixture and incubated for 30 min. Finally, 6 μg polylysine-modified transferrin (Sigma) in 300 μl HBS were admixed and incubated for additional 30 min at room temperature, resulting in generation of adenovirus-transferrin-polylysine complexes. Bm-DC (1 × 10⁶) in 4 ml culture medium were then transfected by adding adenovirus-transferrin-polylysine complexes equivalent to 3 μg plasmid DNA, resulting in ≥70% viability of Bm-DC after transfection. Four hours after transfection, BmDC were washed free of unbound transfection complexes and transferred into new medium without exogenous GM-CSF. After additional overnight cultivation, Bm-DC were washed, tested for viability by trypan blue staining, and used for additional experiments. Production of GM-CSF by the transfected Bm-DC was determined by assaying the supernatant every 24 h for GM-CSF bioactivity using the FDC-P<sub>1</sub>, bioassay as previously described (21). Bm-DC, 1 × 10⁶ cells/well were suspended in complete medium without FCS and cultured for 24 h in the presence of 100 μl culture supernatants in 96-well flat-bottom plates. In some wells, the DC culture supernatants were preincubated for 2 h at 37°C in the presence of neutralizing antisera against murine GM-CSF. Proliferation of FDC-P<sub>1</sub> cells was determined by [3H]thymidine uptake during the final 6 h of the culture period.

**Dye labeling and flow cytometry of Bm-DC**

Bm-DC or freshly prepared spleen cells, respectively, were labeled with the fluorescent dye PKH2-2 (Sigma, Deisenhofen, Germany) 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. Complete medium containing 10% FCS was added to the cells, followed by removal of unbound PKH2 by extensive washing with PBS. Thereafter, labeled cells were injected s.c. into mice on the lower abdomen. At various time points, mice were killed, and inguinal LN were removed. Single-cell suspensions of LN cells were subjected to flow cytometric analysis (EPICS; Coulter, Miami, FL) to detect fluorescent cells within the LN preparation.

**Mixed lymphocyte reaction**

Primary allogeneic mixed lymphocyte reactions (MLR) were performed as described (21). Briefly, nylon wool-purified C3H/HeN splenic T cells (H<sub>2</sub><sup>b</sup>) were cocultured with freshly prepared Bm-DC from BALB/c mice (H<sub>2</sub><sup>b</sup>) in RPMI 1640, supplemented with 1.5% mouse serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.1 mM essential and nonessential amino acids, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.01 M HEPES buffer, and 5 mg/ml indomethacin. Serial dilutions of triplicate samples of EC were mixed with a constant amount (2 × 10⁵) of allogeneic T cells in 96-well culture dishes. T cells were prepared by passing RBC-depleted spleen cells over a nylon wool column, followed by removal of remaining
contaminants using the mAbs M5/114, Mac-1, and B220 and immunomagnetic microbeads (MiniMACS, Miltenyi Biotech, Bergisch Gladbach, Germany). The resulting cell preparation contained <0.1% IA− cells. Cells were cultured in the MLR for 6 days and pulsed with 80 μg/well 5-bromo-2′-deoxyuridine (BrdU) for 18 h. Cell proliferation was measured using peroxidase-labeled anti-BrdU mAb and 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) as colorimetric indicator (BrdU labeling kit, Boehringer Mannheim).

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

To assess the ability of Bm-DC to present peptide Ag to primed T cells, the OVA-specific T-T hybridoma D011.1 was used (22). For this assay, Bm-DC were incubated in the presence of 0.1–2 μg OVA323–339 peptide, and 1 × 10⁴ D011.1 cells were added and incubated for 24 h at 37°C in a total volume of 200 μl. One hundred microliters of culture supernatant were removed and assayed for IL-2 content using the IL-2-responsive cell line, CTLL-2, as described (23).

Determination of Ag-specific activation of peripheral LN cells

Mice were sensitized by s.c. injection of Bm-DC that were either coupled to the hapten trinitrophenyl (TNP) by incubation in 1 mM trinitrobenzene-sulfonic acid in HBSS at pH 7.0 for 10 min at 37°C as described (23) or incubated with keyhole limpet hemocyanin (KLH) for 2 h in BM medium. Four days after sensitization, regional LN were obtained, and single-cell suspensions were prepared by pressing the cells through a metal sieve and subsequently through a nylon mesh. LN cells from immunized or control mice were either pooled and plated into 96-well round-bottom plates (serial dilutions, starting at 5 × 10⁵/well) in RPMI 1640, supplemented with 5% FCS, 5 × 10⁻³ M 2-ME, 1 mM l-glutamine, 1% sodium pyruvate, and 1% penicillin-streptomycin, or with without addition of exogenous Ag. After 48 or 72 h, 1 μCi/well [³H]thymidine was added to the cultures, and LN cell proliferation was measured by the amount of thymidine incorporation after additional overnight incubation.

Contact hypersensitivity (CHS)

CHS experiments were performed as described previously (23). Briefly, groups of 5–7 mice were sensitized by s.c. injection of TNCB-coupled Bm-DC as above, or (as controls) by painting 100 μl 0.15% TNCB in acetone-olive oil (4:1) on the abdominal skin. For elicitation of CHS, ears of mice were painted with 10 μl of 0.8% TNCB on one ear. CHS was determined by the degree of ear swelling of the hapten-exposed ear compared with the vehicle-treated contralateral ear and measured with a spring-loaded caliper (Oditest, Kroeplin, Schüchtern, Germany) 24 or 36 h after challenge. Mice that were ear challenged without prior sensitization served as negative controls.

Tumor cell culture, preparation of tumor Ag (TA), and determination of tumor-specific immunity in vivo

The tumor cell line KLN205 (spontaneously originated squamous cell carcinoma, syngeneic to DBA2/N mice, H-²) was obtained from ATCC and maintained in tissue culture at 37°C in RPMI 1640 containing 10% FCS, 1% nonessential amino acids, 1% HEPES, 1% glutamine, 50 μM 2-ME, and 1% penicillin-streptomycin (PAA). For preparation of TA, tumor fragments containing tumor-derived antigenic epitopes were prepared by disrupting KLN205 cells with four freeze-thaw cycles in liquid nitrogen. Ly-pectinase was cleaved by centrifugation (10,000 × g) to remove insoluble cell fragments, and the supernatant was used as a source of TA as described (24).

After injection of ≥10³ cells, tumors grew progressively in syngeneic mice and metastasized at late tumor stages into regional LN. In initial experiments, the immunogenicity of the KLN205 tumor was determined in several ways, including vaccination with X-irradiated tumor cells, vaccination with dead tumor cells with or without CFA, and secondary tumor challenge of mice in which a growing tumor had been surgically removed (concomitant immunity). In all of these instances, progressive tumor growth was observed after subsequent challenge with viable KLN205 cells (data not shown), indicating that this tumor is very poorly immunogenic in syngeneic mice. Moreover, mice immunized with soluble tumor lysates (used as source of TA for immunizations with DC) also developed tumors to the same degree as untreated control animals, indicating that injection of TA alone is not sufficient to induce tumor immunity.

To pulse Bm-DC with TA, TA from 1 × 10⁷ KLN cells was added to BM cultures for 16 h. Thereafter, cells were harvested and washed three times with PBS. Bm-DC (1 × 10⁴) were then injected s.c. into groups of five naive recipient mice on the lower abdomen. This immunization was repeated twice at weekly intervals. In all experiments, tumor challenge was performed by injection of 2 × 10⁵ live KLN205 cells s.c. on the lower lateral abdomen 1 wk after the last immunization, and tumor growth was assessed every 3 days with a spring-loaded caliper.

Statistical analysis

Differences between groups within experiments were tested for significance with Student’s t test. All experiments were performed at least twice.

Results

Transfection of Bm-DC with the murine GM-CSF gene

To ensure that the Bm-DC used for immunizations were optimally capable to endocytose and process exogenous protein Ag, we utilized Bm-DC that still displayed an immature phenotype and functional state, as assessed by surface expression of MHC and co-stimulatory molecules and by their capacity to present native protein Ag in vitro (data not shown). In general, Bm-DC were difficult to transfect without resorting to virus-mediated gene transfer, because standard transfection techniques including electroporation, calcium phosphate, or lipofectin-mediated transfection protocols resulted in poor cell viability and low expression of the transfected gene (data not shown). Therefore, we utilized the adenovirus-enhanced transfection protocol, which was reported to be a gentle and efficient transfection method (19, 20), resulting in high transient expression levels. Advantages of this system are that it does not require cloning of the gene of interest into a viral genome and that no viable virus particles are transmitted. Using this system, expression of the transfected gene in Bm-DC was readily detectable by RT-PCR (data not shown). By using β-galactosidase (LaCL) or chloramphenicol acetyltransferase reporter constructs, we estimate that ~5–10% of the Bm-DC were successfully transfected. Transfected Bm-DC produced low but clearly detectable amounts of bioactive GM-CSF (~300 pg/10⁶ cells/ml within the first 24 h after transfection), as determined by using the FDC-P1, bioassay for murine GM-CSF (Fig. 1). In the absence of exogenous cytokines, the majority of the transfected Bm-DC remained viable and continued to produce bioactive GM-CSF (although in decreasing amounts) until approximately day 6 after transfection. Because high amounts of transfection mixture resulted in reduced cell viability, the transfection mixture was adjusted to yield ~70% viability of Bm-DC 24 h after transfection (see Materials
In vitro Ag-presenting capacity of GM-CSF gene-transfected Bm-DC:

Presentation of protein Ag. The principal aim of this study was to investigate primary immune responses induced by sensitization with Ag-exposed Bm-DC in intact animals and to test whether GM-CSF gene transfection enhances the capacity of Bm-DC to present Ag in vivo. For this purpose, GM-CSF gene-transfected and control Bm-DC were pulsed with the protein Ag KLH, washed extensively, and injected s.c. into naïve syngeneic mice. Four days later, regional LN were removed, single-cell suspensions were prepared, and the proliferative response was determined. In animals immunized by injection of Ag-exposed Bm-DC, proliferation of regional LN cells was readily detectable, and injection of GM-CSF gene-transfected Bm-DC resulted in significantly enhanced proliferative responses compared with mock-transfected or untransfected cells (Fig. 4). Injection of naive mice with as little as $10^4$ Ag-pulsed, GM-CSF gene-transfected Bm-DC resulted in the generation of a detectable Ag-specific immune response in vivo, whereas mock-transfected Bm-DC elicited no proliferative response at this dose (Fig. 4a). In a second experiment, we wished to address the question of whether GM-CSF production by the Bm-DC themselves was necessary for enhanced Ag presentation in vivo or whether simply the presence of GM-CSF at the site and time of sensitization was responsible for the observed effect. For this purpose, mice were immunized with KLH-pulsed DC with or without additional s.c. injection of 10,000 U recombinant murine GM-CSF. Coinjection of GM-CSF indeed enhanced the KLH-specific primary proliferative response of LN cells (Fig. 4b). No significant proliferative response was induced by injection of GM-CSF alone (data not shown). To investigate further whether the presence of GM-CSF at the site of Ag presentation stimulated primary T cell responses in an autocrine or paracrine manner, we coinjected GM-CSF gene-transfected DC that were not KLH pulsed together with KLH-pulsed, untransfected DC, and we compared the immune response with that induced by coinjection of GM-CSF gene-transfected DC that were KLH pulsed and untransfected DC that were not pulsed with KLH. No significant differences in KLH-specific T cell proliferation were observed (Fig. 4c, hatched bars). These data suggest that the continuous presence of GM-CSF at the site of primary T cell activation and not the production of GM-CSF by those DC that actually present the Ag is responsible for the immunostimulatory effect of GM-CSF transfection.

Presentation of hapten. Similar results were obtained when Bm-DC were incubated in the presence of the hapten TNP instead of KLH (Fig. 5a). Again, GM-CSF gene-transfected Bm-DC were not different significantly from mock-transfected or untransfected Bm-DC with regard to their capacity to present an immunogenic peptide from chicken OVA that is recognized by the OVA-specific T cell hybridoma, D011.1 (Fig. 3a). Except for one data point, the capacity to present alloantigen in a primary mixed lymphocyte response did not differ in a statistically significant fashion between transfected cells and control cells that were incubated in exogenous GM-CSF, although we generally observed somewhat enhanced alloantigen presentation by GM-CSF gene-transfected Bm-DC, especially at low APC:T cell ratios (Fig. 3b). However, addition of exogenous GM-CSF to mixed lymphocyte cultures also enhanced the proliferative response of allogeneic T cells but required higher amounts of GM-CSF than those produced by the transfected DC (data not shown). As expected, mock-transfected BmDC incubated in the absence of exogenous GM-CSF lost most of their allostimulatory capacity (Fig. 3b).

*In vivo* Ag-presenting capacity of GM-CSF gene-transfected Bm-DC:

In vitro Ag-presenting capacity of GM-CSF gene-transfected Bm-DC

When used as APC in vitro, GM-CSF gene-transfected Bm-DC did not differ significantly from mock-transfected or untransfected
significant superior to untransfected or mock-transfected controls in inducing a hapten-specific proliferative response of regional LN cells four days after s.c. injection of hapten-coupled Bm-DC. Interestingly, low but statistically significant proliferative responses were also obtained by injection of hapten-coupled allogeneic Bm-DC, suggesting that immunization with large numbers of Bm-DC (>10^5 Bm-DC/immunization) might result in reprocessing of the Ag by host Ag-presenting cells.

Additionally, we compared the capacity of GM-CSF gene-transfected and mock-transfected Bm-DC to immunize mice for generation of a hapten-specific CHS response in vivo. Groups of mice were injected with 10^4 or 10^5 TNP-coupled GM-CSF gene-transfected Bm-DC and assayed for induction of TNP-specific immunity by topical application of TNCB to one ear and subsequent determination of CHS responses. As shown in Fig. 5b, sensitization with 10^5 TNP-coupled untransfected as well as GM-CSF gene-transfected Bm-DC were able to induce immunity. However, as in the in vitro readout systems, GM-CSF gene-transfected Bm-DC again induced the most profound sensitization. The superiority of GM-CSF gene-transfected cells became even more evident after sensitization with lower cell numbers, because immunization with 10^5 TNP-coupled GM-CSF gene-transfected Bm-DC resulted in profound CHS to TNCB, whereas sensitization with the same amount of mock-transfected Bm-DC did not lead to significant hapten-specific CHS (Fig. 5c), indicating that GM-CSF transfection of Bm-DC augments their sensitizing potential for in vivo immunizations.

**Presentation of tumor Ag for induction of protective tumor immunity.** To investigate the impact of GM-CSF gene transfection on the capacity of Bm-DC to induce protective tumor immunity in vivo, GM-CSF gene-transfected Bm-DC were pulsed with KLN205-derived TA and administered as a tumor vaccine as described in Materials and Methods. To determine induction of protective tumor immunity, mice were subsequently challenged by s.c. injection of 2 × 10^5 KLN205 cells, and tumor growth was measured as above. As shown in Fig. 6, immunization of mice with TA-pulsed, GM-CSF gene-transfected Bm-DC resulted in protective tumor immunity, whereas injection of mock-transfected or untransfected Bm-DC exhibited no effect on tumor growth. Neither injection of TA alone nor injection of GM-CSF gene-transfected but not TA-exposed Bm-DC affected tumor growth, indicating that induction of protective tumor immunity was caused by TA presentation by the injected TA-pulsed Bm-DC. Moreover, immunization with Bm-DC that were transfected with the empty plasmid vector, subsequently incubated in large amounts of exogenous GM-CSF for 24 h and exposed to TA, did not yield significant tumor immunity. Likewise, most tumors still grew progressively when untransfected Bm-DC were coinjected with 1000 U exogenous GM-CSF, although in some experiments concomitant administration of exogenous GM-CSF together with untransfected Bm-DC improved tumor Ag presentation somewhat (data not shown).

**Transfection of Bm-DC with the GM-CSF gene results in prolonged survival and enhanced migratory capacity in vitro as well as in vivo**

To determine why GM-CSF gene transfection of Bm-DC enhances their capacity to induce primary immune responses in vivo while leaving their in vitro functions largely unaltered, we investigated the impact of GM-CSF transfection on Bm-DC migration in vitro. Transfected and nontransfected cells, respectively, were stably labeled with a fluorescent dye and injected s.c. into mice at the lower abdomen. After 2 days, the skin at the injection site as well as the inguinal LN was removed, and single-cell suspensions were analyzed for labeled cells by flow cytometry. Indeed, fluorescent cells

**FIGURE 3.** Presentation of OVA peptide and alloantigen by GM-CSF gene-transfected Bm-DC. A, Presentation of OVA_{323-339} peptide by GM-CSF gene-transfected (Bm-DC/GM-CSF) and mock-transfected Bm-DC (Bm-DC). Bm-DC were incubated in BM medium without exogenous cytokines after transfection and harvested after 24 h. Mock-transfected cells were continuously incubated in BM medium with 100 U/ml exogenous GM-CSF. Bm-DC were incubated in the presence of 1 μg/ml OVA_{323-339} together with 1 × 10^5 OVA-responsive D011.1 hybridoma cells. Supernatants were obtained after 24 h and assayed for IL-2 content as a measure for Ag-specific T cell stimulation. B, Bm-DC were transfected with the GM-CSF gene-containing plasmid (Bm-DC/GM-CSF) or mock-transfected with the empty plasmid vector only (Bm-DC). GM-CSF-transfected Bm-DC were incubated in BM medium without additional cytokines for 24 h. Mock-transfected Bm-DC were either incubated in the presence of 100 U/ml exogenous GM-CSF (Bm-DC + exogenous GM-CSF) or in the absence of cytokines (Bm-DC). Twenty-four hours after transfection, graded numbers of BALB/c-derived (H-2^d) GM-CSF gene-transfected or mock-transfected Bm-DC were coincubated with 2 × 10^5 allogeneic purified T cells from C57BL/6 mice (H-2^b) in an allogeneic MLR and incubated for 4 days at 37°C, followed by overnight pulse with [3H]thymidine. Data are shown as mean ± SEM, *, Statistically significant differences between GM-CSF-transfected and GM-CSF-incubated DC (p < 0.05). Background proliferation of Bm-DC or T cells alone was always below 10,000 cpm.
could be readily detected in the skin 48 h after injection of labeled Bm-DC, regardless whether they were transfected or not (Fig. 7a and data not shown). However, hardly any fluorescent cells could be found in LN of mice injected with control or mock-transfected Bm-DC (Fig. 7, b and c). In contrast, a distinct population of dye-labeled cells was apparent in the LN of mice that had been injected with GM-CSF gene-transfected Bm-DC (Fig. 7c). To exclude the possibility that fluorescent cells within the LN preparation were host-derived phagocytic cells that had ingested dye-labeled Bm-DC, we injected dye-labeled Bm-DC that had been killed by treatment with paraformaldehyde before their injection. After examination of the regional LN, no fluorescence could be recorded by flow cytometry (Fig. 7c), showing that fluorescent label derived from dead cells is not accumulated in the regional LN. This rules out the possibility that phagocytosis and transportation of cellular debris are responsible for the fluorescent signal detected in the LN after injection of GM-CSF gene-transfected Bm-DC.

Discussion

DC are the most potent stimulators of primary immune responses known thus far, and DC have long been recognized as potential tools for immunotherapy and vaccination strategies, especially for the therapy of tumors (9, 10, 13, 24, 25). In the past, their use as immunotherapeutic reagents was inhibited by difficulties in obtaining sufficient numbers of DC because of the lack of in vitro culture systems. This problem, however, has mostly been overcome, because it is now routinely possible to generate large numbers of DC from murine and human bone marrow or peripheral blood (17, 26–28). Recently, several groups demonstrated that murine Bm-DC can be used to induce protective or therapeutic immunity against tumors in vivo (29–33). We and others are currently testing the applicability of DC-based vaccination protocols for tumor immunotherapy in humans (11, 12).

However, many details of this therapeutic strategy have yet to be clarified, mainly because DC exhibit extensive morphological and functional plasticity, and it is as yet unclear which DC subpopulation and which differentiation state of DC is best suited for in vivo immunotherapy protocols. When comparing the efficacy of freshly isolated Langerhans cells with that of Bm-DC, we recently noted that in vitro-generated immature Bm-DC (generated by incubation in either GM-CSF alone or GM-CSF + IL-4) were only weakly able to induce protective tumor immunity in the KLN205 system. In contrast, ex vivo-isolated epidermal Langerhans cells were readily capable of immunizing against this tumor. Moreover, Bm-DC that were terminally differentiated by culture in GM-CSF, IL-4, and CD40 ligand trimer were as potent APC as Langerhans cells for in vivo immunization against this poorly immunogenic tumor (34) (K. Mahnke, unpublished observation). Several other investigators conclusively demonstrated that DC can be used to induce protective immunity against a variety of, albeit mostly somewhat immunogenic, tumors as well as to induce immunological rejection of existing tumors (29–33), and it appears that the efficacy of DC-based immunizations increases with the degree of DC differentiation induced by in vitro culture before adoptive transfer (34). Likewise, we could also show that the capacity of
epidermal Langerhans cells to present tumor Ag in vivo increases with GM-CSF-induced Langerhans cell maturation (24). Those data suggest that one way to generate DC that are effective as APC in adoptive immunotherapy protocols is to use terminally differentiated, “mature” Bm-DC.

An alternative approach to enhance the efficacy of DC immunotherapy has been published recently. Here, several groups reported that adenovirus-mediated transfection of DC with genes encoding for tumor or viral Ags before adoptive transfer results in generation of profound immunity against the gene product of the transfected gene (35–41). A prominent adverse effect of adenovirus-mediated gene transfer, generation of immunity against the advenoviral vector itself, was not observed when using adenovirus-transfected DC (35). However, almost all of these studies used DC that displayed an immature phenotype for transfection, because also in our hands the efficacy of gene transfer into DC decreased significantly with maturation.

The data presented here demonstrate that yet another way to induce immunity with DC is to use immature Bm-DC that have been transfected with the GM-CSF gene. In contrast to other groups (35–41), our strategy was not to express in DC genes encoding for potential tumor Ags but to promote their survival and maturation in vivo, because we reasoned that DC already are extremely efficient in Ag uptake and processing per se. Thus, by transfecting DC with their principal growth and differentiation factor, we attempted to manipulate the microenvironment that DC require for optimal function. We demonstrate here that the efficacy of Bm-DC to induce primary immune responses in vivo can be enhanced by transfection with the GM-CSF gene. Morphologically, transfection of Bm-DC with the GM-CSF gene resulted in only subtle phenotypical alterations and almost unaffected surface expression of MHC class II and costimulatory molecules (Fig. 2). Likewise, no dramatic changes in their in vitro function were observed after GM-CSF gene transfection (Fig. 3). Despite minimal morphological and functional alterations, however, GM-CSF gene-transfected Bm-DC were significantly superior to untransfected Bm-DC when investigated for their ability to induce primary immune responses in vivo, which is demonstrated in three experimental systems (Figs. 4–6). These data suggest that GM-CSF gene delivery to Bm-DC might be a potential way to optimize their effectiveness in inducing prophylactic or therapeutic immune responses after adoptive transfer in vivo.

Which of these possible ways is superior is presently under investigation in our laboratory. In theory, immature DC bear the advantage of being more effective in Ag uptake and processing than mature DC. On the other hand, mature DC are more effective in Ag presentation to naive T cells. It has been demonstrated that mature DC are less dependent than immature DC on external growth factors such as GM-CSF (28), which might also explain why mature Bm-DC function well as APC in vivo. In addition, our data suggest that the capacity of DC to migrate to lymphatic organs might be of significant importance for their in vivo function after adoptive transfer into intact organisms. Indeed, our data suggest that in vitro-generated immature Bm-DC, in contrast to freshly

derived Bm-DC (H-2b) were GM-CSF gene transfected (GM-CSF) or mock transfected (plasmid), TNF coupled, and washed extensively to remove unbound hapten. Cells, 10^4 (b) or 10^5 (c) per mouse, were injected s.c. into naive BALB/c mice. Control mice were left untreated, injected with Bm-DC that were not coupled to TNF, or sensitized by epicutaneous application of 100 μl 0.15% TNCB. Six days later, all mice were challenged with TNCB on both sides of one ear to elicit TNP-specific contact hypersensitivity. Graphs show ear swelling responses after 30 h. n = 5/group. *, p < 0.01.
prepared spleen cells, fail to migrate to regional LN when injected into mice. This could be overcome by transfection of Bm-DC with the GM-CSF gene, because GM-CSF gene-transfected Bm-DC could readily be detected in LN after s.c. injection, indicating that GM-CSF stimulates the migratory capacity of Bm-DC in vivo (Fig. 7). It is equally possible that GM-CSF truly enhances the migration of Bm-DC in vivo or that it simply enhances viability of the injected DC upon GM-CSF transfection, resulting in better functional capacities. Indeed, GM-CSF is a well-known survival factor for DC in vitro, and GM-CSF transfection might have provided an in vivo survival factor for cells that would have died otherwise. However, when DC recovered from the injection sites were analyzed by flow cytometry, we found that a large portion of the injected untransfected Bm-DC stayed viable after injection but failed to migrate into regional LN. 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process that is associated with GM-CSF gene delivery might activate Bm-DC somewhat, although we have no evidence for a significant effect in this respect.

Because GM-CSF gene transfection of Bm-DC stimulated their Ag-presenting capacity, we propose that the presence of GM-CSF in the host at the site of Ag presentation augments the efficacy of Bm-DC in inducing primary immune responses. Our data suggest that the continuous presence of GM-CSF at the site of primary T cell activation but not the production of GM-CSF by the Ag-presenting DC themselves is necessary for the immunostimulatory effect of GM-CSF transfection (Fig. 4c). Thus, an alternative way to apply GM-CSF might be to simply inject it into the host together with the DC, which indeed augmented the subsequent immune response in some of our experimental systems (Fig. 4b). Likewise, administration of biodegradable GM-CSF-containing microspheres or of GM-CSF-transfected tumor cells enhanced antitumor immunity significantly (16, 46). Although in most of our own experiments tumors still grew progressively when untransfected Bm-DC were coinjected with exogenous GM-CSF, concomitant administration of exogenous GM-CSF and untransfected Bm-DC often improved the generation of tumor immunity to some extent, although inconsistently (K. Mahnke, unpublished observation). Possibly, repeated administration of GM-CSF or injection of larger doses might result in similarly augmented Ag-presenting capacity as seen with GM-CSF-transfected Bm-DC. Current studies are under way in our laboratory to test the adjuvant effect of GM-CSF administration in DC-based immunotherapy. However, targeting the GM-CSF delivery directly to DC has the advantage of requiring much lower cytokine concentrations. In addition, noncontrollable effects of this pluriplent growth factor on other hemopoietic cells, as well as a general activation of host APC rather than of the injected Ag-pulsed DC, are likely to be avoided by this application system.

In summary, our data suggest that transfection of Bm-DC with the GM-CSF gene results in only moderate alterations of their in vitro functions. In vivo, however, autocrine production of GM-CSF greatly enhanced the ability of Bm-DC to induce primary immune responses. These results may lead to more effective immunization strategies using Ag-exposed DC.

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References


