Granulocyte-Macrophage Colony-Stimulating Factor-Based Melanoma Cell Vaccines Immunize Syngeneic and Allogeneic Recipients via Host Dendritic Cells[1]

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Subcutaneous injection of GM-CSF-expressing cancer cells into experimental animals results in protective cancer immunity. To delineate the mode of action of such vaccines, we used trinitrophenyl, the antigenic moiety of the contact allergen trinitrochlorobenzene, as surrogate Ag. Trinitrophenyl-derivatized bone marrow-derived dendritic cells were found to elicit a contact hypersensitivity response in syngeneic, but not in allogeneic recipients, compatible with their expected mode of direct Ag presentation. When expressing GM-CSF, haptenized M3 melanoma cells were also able to induce a contact hypersensitivity response but, in contrast to bone marrow-derived dendritic cells, not only in syngeneic but also in allogeneic recipients. This argues for a critical role of host APC. To identify their nature, we introduced the β-galactosidase (βgal) gene into M3-GM cells. Their administration activated βgal-specific, L^d-restricted CTL in syngeneic BALB/c mice. Evaluation of lymph nodes draining M3-GM-βgal injection sites revealed the presence of cells presenting the respective L^d-binding βgal peptide epitope. Based on their capacity to activate βgal-specific CTL, they were identified as being $CD11c^+$ dendritic cells. These experiments provide a rational basis for the use of GM-CSF-based melanoma cell vaccines in an allogeneic setting. *The Journal of Immunology,* **2003, 171: 5180–5187.**

We and others have recently shown that genetically modified cancer cells can be used to induce a protective, T cell-mediated immune response in experimental animals (1–3). The most actively investigated approach was the introduction of cytokine genes into the tumor cells. Among the various cytokines tested, IL-2 and GM-CSF appeared to be the most potent ones (3–6). Modification of tumor cells with these cytokines abrogated their tumorigenicity and, more importantly, their administration protected animals against the growth of subsequently inoculated unmodified tumor cells in a T cell-dependent and tumor-specific manner (2, 7, 8). These encouraging results led to various clinical trials testing the safety and immunological efficacy of genetically modified cancer cells. Although most vaccines exerted some level of immunologic activity, only a minority of the vaccinated cancer patients experienced a clinical benefit (5, 9, 10).

Delineation of the mode of action of such vaccines provides a rational way to improve their clinical efficacy. Originally, it was thought that the modified cancer cells themselves activate the immune response by directly interacting with T lymphocytes (11, 12). This notion was challenged by the observation that the administration of genetically modified MHC class II-negative cancer cells results in the activation of tumor-specific, class II-restricted T lymphocytes and by the ability of allogeneic cancer cell vaccines to induce a tumor-specific T cell response which is restricted by the MHC type of host and capable of protecting against the growth of histogenetically identical syngeneic cancer cells (13–15). These and other data led to the conclusion that the specific T cells are activated indirectly, i.e., by host APC (16). Regarding the nature of the APC involved, Chiodoni et al. (17) observed that the s.c. administration of GM-CSF/CD40 ligand (CD40L)[3] L-transfected cancer cells results in the accumulation of dendritic cells (DC) at the injection site. These cells had engulfed apoptotic cancer cells and were capable of inducing a tumor-specific immune response when injected into the footpads of naive animals. In this study, we further explored the events occurring between the cutaneous administration of a GM-CSF-based cancer vaccine and the advent of protective cancer immunity, focusing particularly on the characterization of phenotype and migratory pattern of the cell type(s) inducing T cell sensitization. To this end, we used two independent systems: a contact hypersensitivity (CHS) model as well as the surrogate Ag β-galactosidase (βgal).

CHS denotes an eczematous skin reaction caused by a T cell-mediated immune response to an environmental allergen. Both sensitization and elicitation of the reaction involve contact of the allergen with the skin. Many of the offending allergens are organic chemicals or metals. They function as haptens and their binding to a carrier protein is an essential step in their immunogenicity. Trinitrochlorobenzene (TNCB) is one of the best studied model haptens (18). Various lines of evidence suggest that hapten-specific $CD8^+$ T cells are the main effector population in the CHS response to TNCB (6, 19–22). Specific T cells recognize trinitrophenyl (TNP), the reactive group of TNCB, as part of a complex consisting of a TNP-derivatized peptide present within the binding groove

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[3] Abbreviations used in this paper: CD40l, CD40 ligand; βgal, β-galactosidase; DC, dendritic cell; BMDC, bone marrow-derived DC; cLC, cultured Langerhans cell; CHS, contact hypersensitivity; TNCB, trinitrochlorobenzene; TNP, trinitrophenyl; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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of a MHC molecule (18, 23–28). There are two ways by which ex vivo TNP-exposed cells may induce a hapten-specific T cell response. First, upon transfer into the host, they themselves could directly interact with and thereby activate the T lymphocytes. This would require the MHC types of the cells and the host to be matched. Second, hapten-modified proteins of the TNP-exposed cells could be cross-presented by host APC. Because of the multitude of molecules expressed by cancer cells, their exposure to reactive TNP moieties will generate a large number of hapten-derivatized proteins. Processing of the latter for presentation in the context of MHC molecules can be expected to generate a huge pool of TNP-labeled peptides capable of associating with MHC class I and II molecules of various haplotypes. This being the case, the TNP system enables us to differentiate between direct and indirect Ag presentation simply by determining whether the vaccine of interest, upon TNP modification, is capable of immunizing allogeneic recipients.

To define the nature of the relevant APC, we evaluated draining lymph nodes for the presence of cells presenting tumor-derived Ags to T lymphocytes. In view of our only restricted knowledge of T cell epitopes displayed by M3 melanoma cells, we decided to express the model Ag βgal in M3 and M3-GM cells. This protein offers the possibility to readily visualize βgal-positive tumor cells and contains well-defined T (and B cell) epitopes (29). The availability of a CTL clone specifically recognizing its immunodominant, L^d-restricted T cell epitope allowed us to ask whether lymph nodes draining M3-GM-βgal injection sites contain cells capable of priming a βgal-specific immune response and to define their nature.

Materials and Methods

Animals

DBA/2, BALB/c (both H-2^d), C57BL/6 (H-2^b), and C3H/He (H-2^k) mice were obtained from Charles River Wiga (Sulzfeld, Germany) and used at the age of 6–8 wk. For injection of cells or the application of the contact allergen, animals were anesthetized by i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). All animal experiments were approved by the Austrian Ministry of Science and Transportation.

Cell lines

Cells were grown in DMEM, RPMI 1640, or αMEM supplemented with 10% FCS (PAA Laboratories, Linz, Austria), 25 mM (RPMI 1640) or 10 mM HEPES, respectively, 2 mM L-glutamine, 50 mM 2-ME, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids (hereafter referred to as standard RPMI, DMEM, or αMEM). If not indicated otherwise, all media and reagents were purchased from Life Technologies (Paisley, U.K.). M3 melanoma cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM standard medium as described previously (2). The Lipofectamine method (Life Technologies) was used to cotransfect M3 cells with the murine GM-CSF gene-containing plasmid pWSGM (30) and the pRSVneo resistance plasmid as described previously (31). M3-GM cells were grown in DMEM supplemented with 1 mg/ml geneticin sulfate (G418; Life Technologies). M3-GM cells produce 17.3 ng GM-CSF/10^5 cells per 24 h (as detected by ELISA, Endogen, Woburn, MA). The adenovirus-enhanced transferrinfection method was used to transfect M3 as well as M3-GM cells with the βgal gene as described elsewhere (2, 32). Transfection rates obtained with this technique ranged from 20 to 40% as assessed by 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) staining. Transferrinfection did not affect the phenotype of M3 or of the M3-GM cells. Both lines expressed low levels of MHC class I Ags and ICAM-1 but were negative for MHC class II, CD11c, CD40L, CD80, and CD86. The mastocytoma line P815 was cultured in RPMI standard medium and its βgal-expressing variant P13.1 in RPMI standard medium supplemented with 0.5 mg/ml G418. The βgal-specific CTL clone was maintained in αMEM standard medium containing 100 U IL-2/ml by weekly restimulations using 3×10^6 irradiated peptide (TPHPARIGL)-pulsed splenocytes (H2-L^d) (29) and used for experiments after a 7- to 10-day rest as described elsewhere (33). Langerhans cells were prepared and cultured as described previously (34).

Hapten modification of cells

Cells (M3, M3-GM, bone marrow-derived DC (BMDC), cultured Langerhans cells (cLC)) were washed twice in HBSS without Ca^{2+} and Mg^{2+} and then incubated (10^7 cells/ml) with 2,4,5-trinitrobenzenesulfonic acid (Sigma-Aldrich, St. Louis, MO) at a concentration of 5 mM (pH 7.2) for 10 min at 37°C.

Sensitization for and elicitation of CHS

Anesthesized mice were injected with TNP-modified or unmodified cells in 100 μl of PBS supplemented with 1% FCS s.c. into the back. Positive control mice were painted on dry-shaved abdominal skin with 50 μl of 2% TNCB (TCI, Tokyo Kasai, Tokyo, Japan) in a 4:1 acetone and olive oil carrier solution. The animals were challenged 5 days later by applying 20 μl of a 0.5% TNCB solution onto both sides of one ear. Ear thickness was measured using an engineer‘s micrometer (Hahn and Kolb, Stuttgart, Germany) and the ear swelling response was determined by subtracting the prechallenge value from that obtained 24 h thereafter.

Tumor challenge experiments

DBA/2 mice ($n = 6$/group) were injected on days 0 and 7 s.c. into the left side of the lower back with 3×10^5 irradiated M3 or M3-GM cells. Thus, treated and a group of naive animals were challenged on day 17 by the s.c. inoculation of 1×10^5 wild-type M3 melanoma cells. Animals were regularly assessed for the presence of tumors, and tumor volumes were determined at the indicated time points as described previously (2, 33). Tumor-bearing animals were sacrificed when the largest tumor diameter exceeded 1.5 cm or tumors became ulcerated.

CTL assay

Spleen cells (4×10^6/well) from immunized or naive mice were cultured in the presence of the L^d-restricted βgal peptide TPHPARIGL (50 ng/well) for 5 days in 24-well culture plates. The cytotoxic potential of these cells was assessed in an Europium (Eu^{3+}) release assay performed essentially as described previously (2, 33). Briefly, varying numbers of effector cells were mixed with 5×10^3 Eu^{3+}-labeled P13.1 and P815 cells used as targets. In all instances, spontaneous Eu^{3+} release was between 10 and 25% of the maximal release obtained by 1% Triton X-100.

ELISPOT assay

Enumeration of βgal-specific, IFN-γ-producing $CD8^+$ T cells was done essentially as described elsewhere (33). Briefly, whole spleen and lymph node cells of M3, M3-βgal, M3-GM, and M3-GM-βgal recipients were plated in duplicates at the indicated cell number into multiscreen 96-well assay plates (Millipore, Bedford, MA) that had been precoated with the anti-IFN-γ Ab R4-6A2 (5 μg/ml). Cells were stimulated with the H-2L^d-restricted βgal peptide TPHPARIGL (4 μg/well) for 18 h at 37°C in 5% CO_2. After extensive washing, the biotinylated anti-IFN-γ Ab AN18.17.24 (2 μg/ml) was added for 2 h. Detection was conducted with peroxidase conjugated to streptavidin (dilution 1/5000 for 1 h; Boehringer Mannheim, Mannheim, Germany) followed by the addition of 100 μl of substrate (0.8 mg/ml 3,3′-diaminobenzidine (Sigma-Aldrich)/0.4 mg/ml $NiCL_2$ (Sigma-Aldrich)/0.009% H_2O_2 in 0.1 M Tris, pH 7.5). Spots were counted using the Bioreader-2000 (Biosys, Karben; Germany). Preliminary experiments had shown that culture of splenocytes in medium alone or with an irrelevant H-2K^d-restricted influenza hemagglutinin peptide (LFEAIEGFI) consistently led to low spot numbers. Therefore, the number of peptide-specific IFN-γ-positive spots was obtained by subtracting the number of spots in the medium-alone group from that in the peptide group.

Generation of BMDC

BMDC were prepared according to the protocol by Inaba et al. (35) with slight modifications. Briefly, bone marrow cells were depleted of lymphocytes, granulocytes, and MHC class II-positive cells by treating them with a mixture of mAbs (M5/114, GK1.5, 2B6.2D8, 3.168, 53.6-72, RB6.8C5) for 30 min at 4°C followed by incubation with mouse anti-rat κ-chain mAb (1 mg/10^7 cells) and Low Tox-M rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) at a final dilution of 1/10 for 45 min at 37°C (2). The resulting cells were cultured in RPMI 1640 supplemented with 5% FCS, GM-CSF (250 U/ml), IL-4 (1000 U/ml), HEPES, L-glutamine, 2-ME, and nonessential amino acids (at the concentrations indicated above) in 24-well plates (1.5×10^6 cells/well). At day 3, nonadherent cells were removed by gentle washings. Day 6 nonadherent cells were collected and subcultured for another 24 h in fresh cytokine-supplemented medium. By day 7, three different populations could be identified based on their MHC class II expression: negative (5–10%), medium (10–20%), and bright (70–85%), with the latter representing mature DC.

Peptide synthesis

Peptides were manufactured on a peptide synthesizer (model 433 A with feedback monitoring; Applied Biosystems, Foster City, CA) using an aminomethylated polystyrene resin with *p*-carboxytrityl alcohol linker (PepChem, Tübingen, Germany) as solid phase with the Nα-9-flourenyammonium strategy as described previously (36). Peptides were dissolved in 1 M triethylammonium acetate (pH 7.3), purified by reversed-phase chromatography, and their identity was confirmed by time of flight mass spectrometry (36).

Excision of the injection site

Panels of DBA/2 mice ($n = 20$) were injected s.c. in the middle of the back on day 0 with TNP-derivatized M3-GM, BMDC, and cLC. To later identify the injection sites, the cells were admixed with carbon particles. One, 2, and 3 days later, thus labeled injection sites (5 mice/panel and time point) were excised. Five mice of each panel were left untreated and served as reference. On day 5, all groups, including one of naive mice, were challenged with the hapten and their ear swelling responses were determined as described. The CHS response of a group at a given time point is expressed relative to the respective positive control group as calculated by the formula: (mean ear swelling of *panel A* injection site excised at time point X − mean ear swelling of naive group)/(mean ear swelling of nonexcised *panel A* group − mean ear swelling of naive group) = relative ear swelling response at time point X (day 1, 2, or 3) of a given *panel A* (TNP-derivatized M3-GM, BMDC, or cLC).

Isolation of draining lymph node cells and coculture with βgal-specific CTL

To test whether lymph nodes draining vaccination sites contain cells presenting tumor Ags, whole lymph node cells or subpopulations thereof were incubated for 48 h with 4×10^4 βgal-specific CTL in flat-bottom 96-well plates. The supernatants of these cultures were assayed for IFN-γ by ELISA. MACS and FACS sorting techniques were used to prepare various subpopulations of lymph node cells. To prepare B cells, lymph node cells were reacted with anti-CD11c-conjugated magnetic beads for 15 min at 4°C according to the manufacturer′s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Thereafter, they were loaded onto prewashed MACS columns. Nonbound cells were then washed through while the column was still attached to the magnet. These were incubated with a FITC-labeled mAb to CD11c (clone HL3; BD PharMingen, San Diego, CA) and a PE-labeled B220-specific mAb (CD45/B220; BD PharMingen). Using a FACSVantage (BD Biosciences, Mountain View, CA), we isolated a $CD11c^-B220^+$ (purity >96%) and a $CD11c^-B220^-$ population. The latter contained mainly $CD3^+$ T cells. To isolate DC, the retained population was eluted by removing the column from the magnet and adding MACS buffer. Approximately 30% of the resulting cells were $CD11c^+$; these were further enriched by FACS sorting using a FITC-labeled mAb to CD11c (clone HL3). This resulted in a >98% pure DC population as defined by CD11c expression.

Cell transfer experiments

Cells of lymph nodes draining M3-GM or M3-GM-βgal injection sites were harvested on day 3 after tumor cell inoculation, gamma-irradiated (15 Gy, Philips RT 305; Philips, Hamburg, Germany; 1.5 Gy/min) and injected (5×10^6 cells/mouse) i.v. into syngeneic naive recipients. Ten days later, spleens of these as well as positive (animals injected with irradiated βgal-expressing P13.1 cells) and negative (untreated) control mice were assayed for βgal-specific CTL as described above.

X-Gal staining of draining lymph node cells

Lymph nodes draining M3-GM or M3-GM-βgal injection sites were harvested 1, 2, 3, 4, 5, 6, and 7 days after application of the tumor cells. Cytospin preparations of the draining lymph node cells (1×10^5 cells/slide) were fixed in 0.5% glutaraldehyde for 5 min, washed, and then exposed to X-Gal (Sigma-Aldrich) for 10 min at 37°C. Controls included M3-GM-βgal cells alone (2×10^4 cells/slide) or admixed with mesenteric lymph node cells of untreated animals (1:1 mixture, 4×10^4 cells/slide).

Statistical analysis

The Sigma Stat software (SPSS, Chicago, IL) was used to evaluate statistical differences in the CHS tumor protection experiments. Based on the raw data, this program uses either Student's *t* test (normal distribution applies) or the Kruskal-Wallis one-way analysis (normal distribution does not apply). A value of $p < 0.05$ was considered to be significant. All series of experiments were repeated at least once with comparable results.

Results

TNP can be used as a surrogate Ag to test the mode of action of a GM-CSF-based M3 melanoma vaccine

The molecular basis of TNP recognition by T cells suggested that it can serve as a surrogate Ag to evaluate the mode of action of GM-CSF-transfected cancer cells. To test this, we injected TNP-derivatized parental as well as GM-CSF-transfected M3 melanoma cells s.c. into syngeneic animals and challenged them 5 days later by epicutaneous TNCB application. Administration of M3-GM-TNP was found to trigger a CHS response comparable in magnitude to the one observed in positive control mice that had been immunized by epicutaneous TNCB application (Fig. 1). No CHS response was induced by the s.c. injection of untreated parental and GM-CSF-transfected M3 cells as well as TNP-derivatized M3 cells, suggesting that the elicitation of a CHS response requires both Ag expression and GM-CSF production by the vaccine (Fig. 1). This pattern of response was compared with the one obtained in prophylactic tumor challenge experiments. To this end, DBA/2 mice were injected twice at a 1-wk interval with irradiated parental or GM-CSF-transfected M3 cells. Thus, treated and control animals were challenged 10 days later by the s.c. inoculation of parental M3 melanoma cells. As shown in Fig. 2, all naive control mice and five of six M3 recipients developed rapidly growing tumors. By contrast, s.c. administration of irradiated M3-GM cells was found to prevent melanoma formation in six of six mice in a tumor-specific manner. Based on these results, the ability of TNP-derivatized parental and GM-CSF-transfected M3 cells to induce a specific CHS response was found to correlate with their capacity to induce a protective antitumor immune response.

Administration of TNP-derivatized, GM-CSF-transfected melanoma cells induces a CHS response in syngeneic and allogeneic animals

We next asked whether TNP-derivatized, GM-CSF-secreting M3 cells are able to immunize allogeneic recipients for CHS. This experiment allowed us to discriminate between the two possible ways of Ag presentation. In case of direct Ag presentation, one would expect a CHS response to occur only in the syngeneic setting. By contrast, if the T cells are activated indirectly, i.e., by host

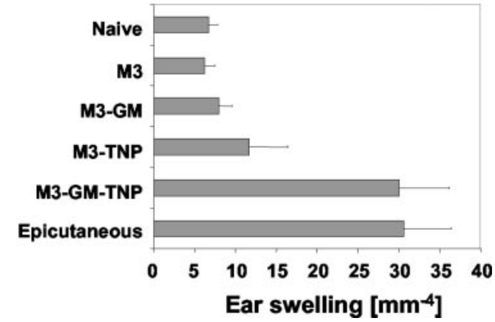


FIGURE 1. Subcutaneous administration of TNP-derivatized M3-GM cells immunizes syngeneic recipients for CHS. BALB/c mice (n = 5/group) were injected with 3×10^5 untreated or TNP-modified M3 and M3-GM cells. One group of mice was left untreated. Another group was sensitized by epicutaneous TNCB application (50 μl of a 2% solution). On day 5 after immunization, animals of all groups were challenged by applying 20 μl of a 0.5% TNCB solution onto both sides of one ear. The ear swelling response was measured 24 h later. The differences between the following groups were statistically significant: M3-GM-TNP recipients vs naïve, $p < 0.001$; M3-GM-TNP recipients vs M3-TNP-injected animals, $p < 0.001$; skin painted mice vs naive mice, $p < 0.001$; skin painted mice vs M3-TNP-injected animals, $p < 0.001$.

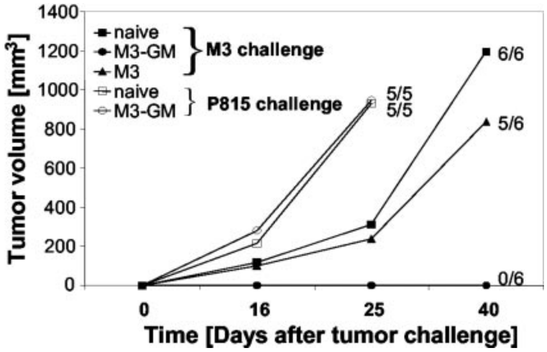


FIGURE 2. Subcutaneous administration of irradiated M3-GM, but not of M3 cells protects DBA/2 mice against a subsequent challenge with a lethal dose of wild-type M3 cells. Groups of DBA/2 mice ($n = 6$) were injected twice (days -17 and -10, lower back, left side) with irradiated M3 or M3-GM cells and challenged on day 0 by the s.c. inoculation (lower back, right side) of wild-type M3 melanoma cells. Control animals were only challenged with parental M3 cells ($n = 6$) or with histogenetically unrelated P815 mastocytoma cells or were immunized by the application of M3-GM cells and challenged by the inoculation of P815 cells ($n = 5$). Tumor incidence and volume were regularly assessed and documented. The differences between the tumor volumes of the following groups were statistically significant: M3-GM vs naïve, $p < 0.05$, M3-GM vs M3, $p < 0.05$.

APC, the haplotype of the injected Ag-bearing cell is irrelevant. BMDC were used to set up the experimental system. Compatible with their known mode of direct Ag presentation, DBA/2-derived and haptenized BMDC (H-2^d) induced a CHS response only when injected into syngeneic (BALB/c; H-2^d) but not when applied to allogeneic animals (C3H/He, H-2^k; C57BL/6, H-2^b) (Fig. 3). The failure of the allogeneic recipients to respond to an epicutaneous TNCB challenge (20 μl of a 0.5% solution) was immunologically spoken a null event since the epicutaneous application of TNCB at a dose (50 μl of a 2% solution) that usually immunizes mice of these strains resulted in a primary hapten-specific immune response in these animals (data not shown). By contrast, TNP-derivatized M3-GM cells (H-2^d) induced a vigorous ear swelling response both in syngeneic (BALB/c; H-2^d) and allogeneic (C3H/He, H-2^d; C57BL/6, H-2^b) recipients (Fig. 3).

Role of the vaccination site for the induction of the CHS response

The above findings were compatible with the view that host-derived APC mediate the immunologic effects of M3-GM-TNP administration. To test this more directly, we removed the immunization sites of M3-GM-TNP- and DC-TNP recipients at defined time points after vaccination and analyzed thus treated mice for their ability to mount a hapten-specific immune response. We found that for a significant CHS response to occur, M3-GM-TNP injection sites had to stay intact for 3 days (Fig. 4). For injection sites treated with TNP-derivatized BMDC or 3-day cLC, an in situ presence of <2 days only was needed for the induction of a CHS response (Fig. 4).

Subcutaneous injection of M3-GM-βgal cells induces a specific MHC class I-restricted CD8 T cell response

The above results suggested that host APC mediate the immunologic effects of GM-CSF-expressing M3 cells administered to the skin. To further test the validity of this concept, we cotransfected GM-CSF-expressing M3 cells with a gene encoding βgal (M3-GM-βgal). These as well as control cells (M3, M3-βgal, M3-GM) were s.c. administered to BALB/c mice. Using an ELISPOT system, their draining lymph nodes and spleens were assessed at defined time points (3, 6, and 8 days after tumor cell administration) for the presence of βgal-specific, MHC class I-restricted T cells. M3-GM-βgal were found to be the only cell type capable of inducing MHC class I-restricted, βgal peptide-specific T lymphocytes (Fig. 5, *A* and *B*). Regional lymph nodes of M3-GM-βgal recipients contained few if any βgal-specific T cells 3 days after

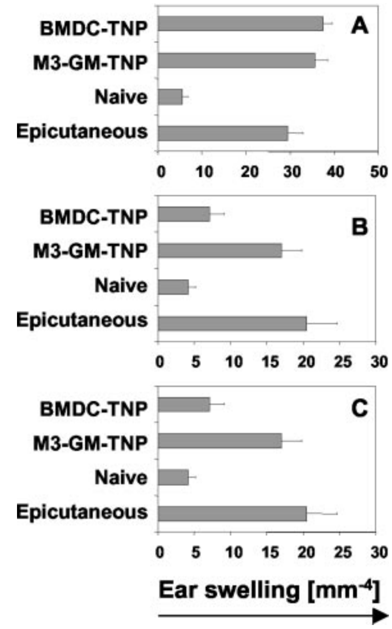


FIGURE 3. TNP-derivatized M3-GM cells immunize syngeneic as well as allogeneic recipients for CHS. DBA/2-derived BMDC (H-2^d) and M3-GM cells (H-2^d) were haptenized and s.c. injected (3×10^5/mouse) into (*A*) BALB/c (H-2^d), (*B*) C3H/He (H-2^k), and (*C*) C57BL/6 (H-2^b) mice ($n = 5$/group). Negative control mice ($n = 5$/group and strain) were left untreated. Positive control mice ($n = 5$/group and strain) were sensitized by epicutaneous hapten application (50 μl of a 2% TNCB solution). On day 5 after immunization, animals of all groups were challenged by applying 20 μl of a 0.5% TNCB solution onto both sides of one ear. The ear swelling response was measured 24 h later. The differences between naive and M3-GM-TNP-injected mice were statistically significant in all three strains analyzed: BALB/c, $p < 0.001$; C3H/He, $p < 0.001$; C56BL/6, $p < 0.001$. The CHS response of BMDC-TNP-injected and naive animals differed significantly only in BALB/c mice ($p < 0.001$).

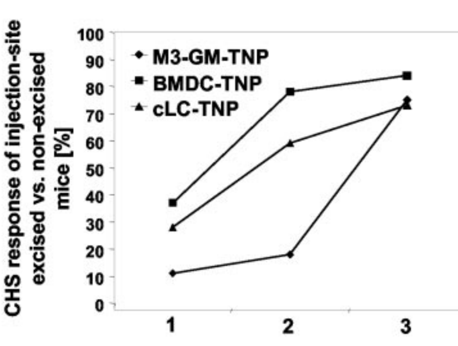


FIGURE 4. Role of the injection sites for the induction of a CHS response. Injection sites were removed 1, 2, and 3 days after the administration of TNP-derivatized M3-GM (1×10^5), BMDC (1×10^5), and cLC (2×10^4). CHS responses were elicited on day 5 and are expressed relative to the respective nonexcised control groups as detailed in *Materials and Methods*.

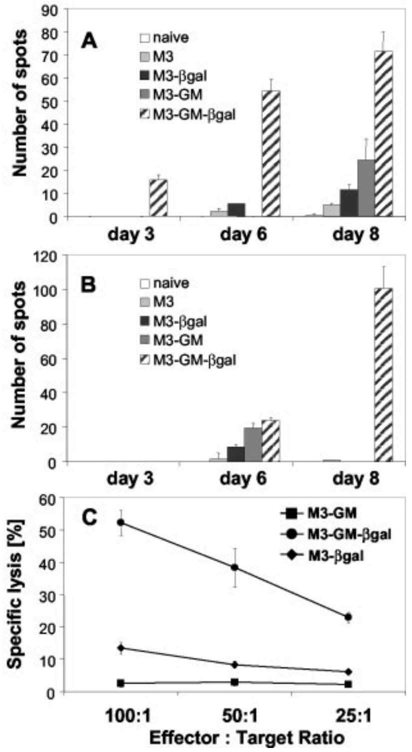


FIGURE 5. The s.c. administration of M3-GM-βgal cells induces βgal-specific CTL. Groups of BALB/c mice ($n = 2$) were injected with M3, M3-βgal, M3-GM, and M3-GM-βgal (3×10^5 cells/mouse) cells s.c. into the middle of the back. At days 3, 6, and 8, draining lymph node cells (1×10^6 cells/well; *A*) and splenocytes (0.5×10^6 cells/well; *B*) of thus treated animals as well as untreated control mice were analyzed for the presence of βgal-specific MHC class I-restricted T cells by ELISPOT analysis. *C*, Splenocytes of M3-GM, M3-GM-βgal, ands M3-βgal recipients were harvested on day 8 after s.c. administration of the tumor cells and stimulated for 5 days with the L^d-restricted βgal epitope TPHPARIGL. Thereafter, they were assayed for the presence of βgal-specific CTL using a Eu^{3+} release assay. Ag-specific lysis (as determined by subtracting lysis of P815 from that of P13.1 (βgal-transfected P815) cells) is shown.

vaccine administration, but were found to harbor substantial numbers of these cells on days 6 and 8 (Fig. 5*A*). This contrasted with the kinetics of the T cell response observed in the spleen. There,

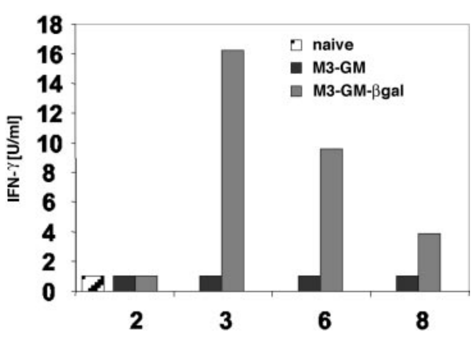
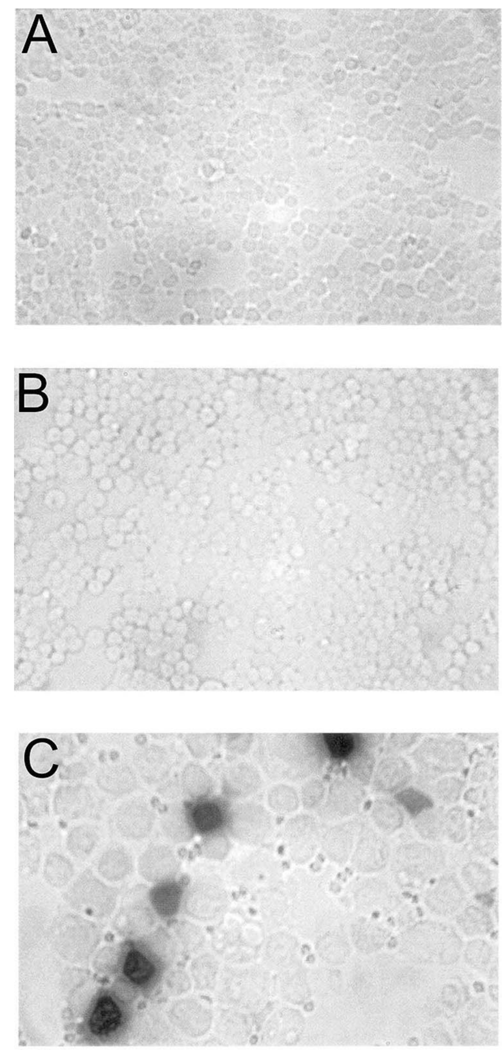


FIGURE 6. Lymph nodes draining M3-GM-βgal injection sites contain cells capable of stimulating βgal-specific CTL. BALB/c mice were s.c. injected into the lower back with either M3-GM or M3-GM-βgal cells (3×10^5 each). Draining inguinal lymph node cells were prepared 2, 3, 6, and 8 days later and cocultured at a dose of 4×10^5 cells with 4×10^4 βgal-specific CTL. Supernatants were harvested 48 h later and assayed for their IFN-γ content. Coculture of CTL with lymph node cells of untreated animals served as negative control.

specific T cells were first detectable by day 8 (Fig. 5*B*), suggesting that the T cells are primed within the regional nodes from where they spread and then circulate through the whole organism.

To determine the cytotoxic activity of the M3-GM-βgal-induced specific T cells, splenocytes of thus treated and control mice were stimulated for 5 days with the immunodominant MHC class I-restricted βgal epitope and then tested for their capacity to lyse Europium-labeled target cells. Results showed that spleen cells from M3-GM-βgal-injected mice were able to lyse syngeneic βgal-expressing P13.1 cells in an Ag-specific manner (Fig. 5*C*). Little if any lytic activity was detected when spleen cells from M3-βgal and M3-GM recipients were used (Fig. 5*C*).



D

	d1	d2	d3	d4	d5	d6	d7
M3-GM	0	0	0	0	0	0	0
M3-GM-βgal	0	0	0	0	0	0	0

FIGURE 7. Lymph nodes draining M3-GM-βgal injection sites do not contain X-Gal-reactive cells. Lymph nodes draining M3-GM (*A*) and M3-GM-βgal (*B*) injection sites were isolated 4 days after tumor cell administration. Cells prepared thereof were subjected to X-Gal staining. Mixtures of mesenteric lymph node cells derived from untreated mice and M3-GM-βgal cells served as assay control (*C*). At the time points indicated, no X-Gal-reactive cell was found within lymph nodes draining M3-GM or M3-GM-βgal injection sites (*D*).

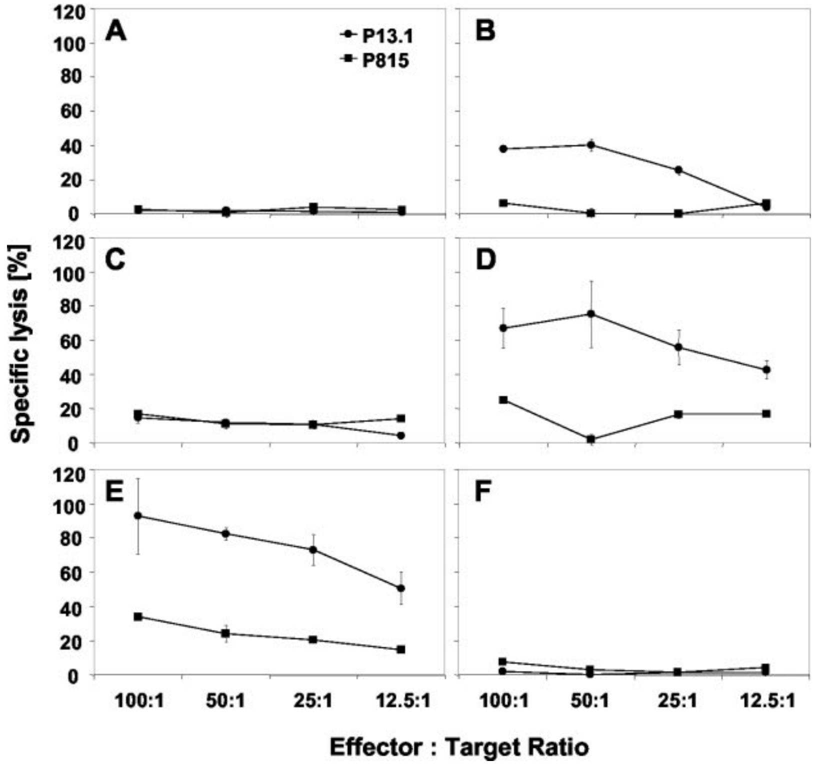


FIGURE 8. Intravenous injection of cells isolated from lymph nodes draining M3-GM-βgal immunization sites induces a βgal-specific CTL response in naive recipients. Cells of lymph nodes draining M3-GM (*A*) and M3-GM-βgal (*B*) injection sites were isolated on day 3 after vaccination. After being irradiated, 5×10^6 cells of each group were i.v. injected into naive BALB/c mice. Thus treated animals were analyzed 10 days later for the presence of βgal-specific CTL. Controls included BALB/c mice that had been injected with M3-GM (*C*), M3-GM-βgal (*D*), and P13.1 cells (*E*) as well as untreated ones (*F*). The experiment was repeated once with similar results.

DC activate the protective T cell response induced by the administration of cytokine-based cancer vaccines

Assuming that the specific T cells have been activated within the regional lymph node by APC that had captured the Ag at the immunization site, we evaluated the draining lymph node for the presence of βgal-displaying APC. Therefore, regional lymph node cells isolated at defined time points after M3-GM-βgal inoculation were cocultured with an L^d-restricted, βgal-specific CTL clone. Results obtained showed that the regional lymph node did indeed contain cells capable of activating the CTL clone to produce IFN-γ (Fig. 6). These cells appeared by day 3 and were detectable until day 6 after administration of the M3-GM-βgal cells (Fig. 6). This was in sharp contrast to the situation in M3-GM-injected control mice (Fig. 6). Their regional lymph node cells failed to activate the CTL clone. We next sought to determine the nature of the APC involved. To test whether M3-GM-βgal cancer cells are the critical APC, lymph nodes draining M3-GM or M3-GM-βgal injection sites were evaluated at defined time points after tumor cell application (days 1–7) for the presence of βgal-positive cells. At no time point did we find X-Gal-reactive cells within regional lymph nodes of M3-GM and M3-GM-βgal recipients (Fig. 7 and data not shown). We then checked whether cells isolated from lymph nodes draining M3-GM or M3-GM-βgal injection sites would be able to elicit a βgal-specific T cell response upon i.v. injection into naive recipients. Therefore, respective lymph nodes were harvested 3 days after administration of the tumor cells. Cells prepared therefrom were first irradiated and then i.v. injected into naive BALB/c mice. These were tested 10 days later for the presence of βgal-specific CTL. Results obtained showed that spleens of M3-GM-βgal recipients did indeed contain βgal-specific T lymphocytes while those of M3-GM-injected animals were essentially devoid of such cells (Fig. 8). To identify the APC, we prepared various cell populations of draining lymph nodes derived from animals that had been injected 3 days earlier with M3-GM-βgal and tested them for their ability to stimulate a βgal-specific CTL clone. As shown in Fig. 8, $CD11c^-B220^+$ B cells and a preparation of cells that neither expressed CD11c nor B220 (i.e., it was devoid of both B lymphocytes as well as DC) failed to do so. Only the $CD11c^+$ DC fraction was capable of stimulating the βgal-specific CTL clone to produce IFN-γ (Fig. 9). It remains to be seen though whether the APC belongs to the classical type of DC ($CD11c^+B220^-$) and/or to the subgroup of so-called plasmacytoid DC ($CD11c^+B220^+$) (37–39).

Discussion

The results of our study imply that the protective effect of a GM-CSF-based melanoma cell vaccine is due to the presentation of tumor-associated Ags by host APC rather than by the transfected melanoma cells themselves.

Several pieces of evidence support this conclusion. The first argument was provided by the finding that TNP-derivatized

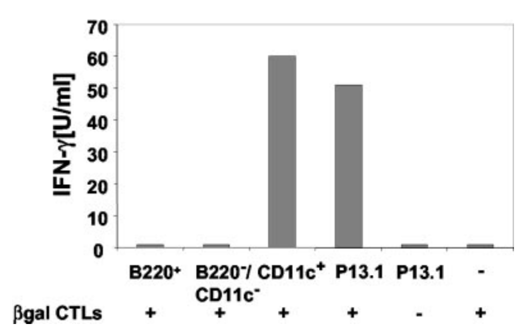


FIGURE 9. The capacity to activate βgal-specific T cells resides exclusively within the $CD11c^+$ DC population. Draining lymph nodes were harvested 3 days after the s.c. administration of M3-GM-βgal cells. Various subpopulations thereof ($CD11c^+$ DC (2×10^4), B lymphocytes (2×10^5) and a population devoid of $CD11c^+$ and $B220^+$ cells (2×10^5)) were cocultured for 48 h with 4×10^4 βgal-specific CTL in flat-bottom 96-well plates. The supernatants of these cocultures were assayed for IFN-γ by ELISA. The experiment was repeated twice with similar results.

BMDC and cLC induce a CHS response in syngeneic mice only, whereas hapten challenge of M3-GM-TNP-injected animals led to an ear swelling response in both syngeneic and allogeneic recipients. We do not know whether BMDC/cLC also caused allosensitization, but it is clear that the elicitation of a CHS response by epicutaneous application of hapten can only take place when APC and T cells are MHC matched in the respective animal.

We further observed that for the tumor-specific immune response to occur, the vaccination site had to stay intact for 3 days in the case of M3-GM-TNP recipients as compared with only 1.5 days in animals injected with DC-TNP. This observation can be explained in two ways. One is that both types of cells can migrate to the draining lymph node, but that DC do this more rapidly. An alternative explanation stems from observations with IL-2-transfected M3 cells (31, 40). When injected s.c., they attract and activate leukocytes that lead to their own demise (31). Ags released are then taken up and processed by professional APC of the host (16), which, following migration to the draining lymph nodes, present them to T cells. These complex processes require more time than the journey of injected DC (41, 42).

Another argument against direct presentation of tumor-associated Ags by the genetically modified melanoma cells is the absence of immunohistochemically detectable M3-GM or M3-GM-βgal cells within draining nodes, although this method might have been too insensitive for this particular purpose.

The involvement of host APC gained further support from experiments with M3-GM cells expressing the model Ag βgal. Similar to the results obtained in the tumor protection and CHS assays, only M3 cells that coexpressed βgal and GM-CSF but neither M3-βgal nor M3-GM were found to prime βgal-specific, class I-restricted T lymphocytes. Based on this information, we analyzed regional and nonregional lymph nodes of M3-GM-βgal recipients for the presence of cells presenting the immunodominant βgal epitope on their MHC class I moieties. Using a functional approach, such cells were indeed found in draining, but not in nondraining lymph nodes or the spleen (data not shown) and were confined to the CD11c$^+$ subpopulation. This finding did not only identify them as being DC but excluded a possible contribution of M3-GM-βgal cells since these are CD11c$^-$. The view that DC represent the critical APC in our system was further strengthened by the demonstration that irradiated draining lymph node cells of M3-GM-βgal recipients induced a βgal-specific CTL response upon i.v. injection into naive syngeneic animals since DC are the only cells capable of initiating a productive immune response by this route (43). It was also in line with results of other investigators demonstrating that the transport of Ag from the endosome to the cytosol, a prerequisite for the introduction of exogenous Ags into the MHC class I presentation pathway, is restricted to DC (44, 45).

The finding that βgal-presenting DC were confined to the regional lymph node suggested that this lymphoid organ represents the site of T (and B) cell sensitization. This view was further strengthened by the kinetics of the Ag-specific CD8 T cell response. Such cells were first found within the draining lymph node by day 6 after vaccination. In other organs, including the spleen and distant lymph nodes (data not shown), βgal-specific CD8 cells were first detected by day 8 after vaccine administration. These findings imply that T cell priming occurs within the regional nodes from where the specific T cells spread and then circulate through the entire organism.

Concerning the question as to how the Ag gains access to the draining node, two mutually nonexclusive possibilities can be entertained. Similar to components of interstitial fluids, Ags released from dying cells could be drained by afferent lymphatics to the regional nodes. There, resident APC could capture them and present their antigenic epitopes to T and B lymphocytes (33, 46, 47). The observations that the injection site is required for ~3 days for an immune response to occur and that DC presenting tumor-derived Ags were first detected by day 3 after immunization indicate that APC pick up the Ag at the injection site and carry it to the regional node where they activate Ag-specific T lymphocytes. Evidence supporting this view has been recently provided by Chiodoni et al. (17). Investigating the injection sites of colon carcinoma cells that coexpressed GM-CSF and CD40L, they found DC that contained tumor-derived apoptotic bodies (17).

In conclusion, we propose that the s.c. injection of GM-CSF gene-transfected melanoma cells results in the formation of a cutaneous sensitizing depot. Infiltrating DC take up tumor material and process it on their way to the regional lymph node where they activate the tumor Ag-specific and protective T cell response. Once activated, these cells circulate through the whole organism to detect and attack Ag-displaying tumor cells.

Together with the existence of tumor Ags that are shared between the cancer cells of various patients (e.g., differentiation Ags, cancer testis Ags), our results provide a rational basis for the use of cancer vaccines in an allogeneic setting. Measures to increase the numbers of DC recruited to the vaccination site (e.g., coexpression of chemokines) and/or their activation status (e.g., coexpression of molecules such as CD40L) may prove useful to augment the immunological and, perhaps, clinical efficacy of GM-CSF-based cancer vaccines.

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