Brief Definitive Report

Murine Dendritic Cells Loaded In Vitro with Soluble Protein Prime Cytotoxic T Lymphocytes against Tumor Antigen In Vivo

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Summary

The priming of an immune response against a major histocompatibility complex class I–restricted antigen expressed by nonhematopoietic cells involves the transfer of that antigen to a host bone marrow–derived antigen presenting cell (APC) for presentation to $CD8^+$ T lymphocytes. Dendritic cells (DC), as bone marrow–derived APC, are first candidates for presentation of tumor-associated antigens (TAA). The aim of this study was to see whether DC are able to prime in vivo antigen-specific cytotoxic T lymphocytes after exposure to a soluble protein antigen in vitro. Lacking a well-defined murine TAA, we took advantage of β-galactosidase (β-gal)–transduced tumor cell lines as a model in which β-gal operationally functions as TAA. For in vivo priming both a DC line, transduced or not transduced with the gene coding for murine GM-CSF, and fresh bone marrow–derived DC (bm-DC), loaded in vitro with soluble β-gal, were used. Priming with either granulocyte macrophage colony-stimulating factor–transduced DC line or fresh bm-DC but not with untransduced DC line generated CTL able to lyse β-gal–transfected target cells. Furthermore, GM-CSF was necessary for the DC line to efficiently present soluble β-gal as an H-$2L^d$–restricted peptide to a β-gal–specific CTL clone. Data also show that a long-lasting immunity against tumor challenge can be induced using β-gal–pulsed bm-DC as vaccine. These results indicate that effector cells can be recruited and activated in vivo by antigen-pulsed DC, providing an efficient immune reaction against tumors.

The cloning of genes encoding tumor-associated antigens (TAA) recognized by T cells in both humans and rodents (1, 2) have reopened the never-ending hope of curing cancer through immunological interventions. Active immunization with such antigens is expected to activate tumor-specific CTL, a process requiring T cell priming in the context of MHC class I pathway (3). CTL induction usually follows antigen processing via endogenous pathway, although exceptions have been reported (4, 5). Host bone marrow–derived APC induced in vivo a CTL-mediated protective immunity against an MHC class I–negative tumor by uptaking and processing for presentation within their own MHC-I (6). Among bone marrow–derived APC, dendritic cells (DC) are the most attractive candidates for this purpose since several studies suggest that they are particularly effective in stimulating both $CD4^+$ and $CD8^+$ naive T lymphocytes (7–12). In fact DC express the costimulatory molecules B7.1 and B7.2, adhesion molecules, and may release soluble factors, all needed for an efficient triggering of the immune response (13). Furthermore, DC were shown to be necessary to generate T cell–mediated tumor-specific immunity in some murine models (14–16). This response was dominated by $CD4^+$ T cells and was generally unable to completely inhibit tumor take unless, as in the case of BCL1 lymphoma, an antiidiotypic antibody response can be generated (16). This limited effect on tumors could be due to the incomplete activation of $CD8^+$ T cells, and particularly of CTL, which are considered as the main effectors able to destroy tumor cells in vivo (3). DC, exposed to soluble antigen in vitro, are able to sensitize antigen-specific T lymphocytes in vivo, in an MHC-II–restricted fashion (7). Since CTLs appear to be necessary to induce tumor rejection in several animal models, we addressed the question of whether DC pulsed in vitro with a soluble antigen were able to induce cytolytic MHC-I–restricted $CD8^+$ T cells as well as a protective antitumor immunity in vivo.

A preliminary account of this work was presented to the Keystone Symposia Conference on Dendritic Cells: Antigen-presenting Cells of T and B Lymphocytes, in Taos, NM, 10–16 March 1995.

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Materials and Methods

Animals. Female BALB/c (H-2^d) of 8 and 12 wk of age were purchased from Charles River Laboratories (Calco, Italy). This study was approved by the Institutional Ethic Committee for the use of animals in experimental research.

Cell Lines. D2SC/1 (H-2^d) is a DC line obtained by retroviral immortalization of BALB/c spleen-derived DC, through a previously described procedure (17), and generously provided by Dr. P. Ricciardi-Castagnoli (Consiglio Nazionale delle Ricerche, Milano, Italy). D2SC/1-GM was obtained by transduction of D2SC/1 cell line with the retroviral vector LmGMSN. GM-CSF cDNA was obtained from a murine CTL line by reverse transcription PCR using GM-CSF–specific primer ends modified to include EcoRI and BamHI sites at 5′ and 3′, respectively. The resulting 517-bp GM-CSF insert was ligated into EcoRI and BamHI of the LXSN retroviral vector (18), to obtain the LmGMSN vector. Retroviral particles were obtained by *trans*-infection technique, as described (19, 20). D2SC/1-GM cells released ~40 ng/ml per 10^6 cells of GM-CSF as measured by capture ELISA. The clone F1.A11 (H-2^d) expressing β-gal was obtained by transduction of spontaneously transformed BALB/c fibroblast cell line F1 (Colombo, M.P., unpublished results) with the LBSN retroviral vector (19). P13.1 (H-2^d), a P815 mastocytoma line derivative expressing β-gal (5), and the CTL clone 0805.B, (21) recognizing the β-gal naturally processed, H-$2L^d$–restricted, epitope p876-884 (22) were kindly provided by Professor H. G. Rammensee (Deutsches Krebsforschungzentrum, Heidelberg, Germany). P815 (H-2^d) and EL4 (H-2^b) tumor lines were used as negative control targets in ^{51}Cr release assay. All cells were maintained in RPMI 1640 supplemented with 10% FCS except for DC lines, which were grown in 5% FCS-supplemented IMDM. The synthetic peptide βGP1 (β-gal, p876-884, TPHPARIGL) was a generous gift of Dr. N. Restifo (National Cancer Institute, Bethesda, MD).

Bone Marrow DC Preparation. Fresh DC were obtained from mouse bone marrow precursors as previously described (23). Bone marrow–derived (bm) DC were used for in vivo priming after 9–11 d of in vitro maturation driven by recombinant mouse GM-CSF at 500 U/ml. The percentage of mature DC was determined by cell surface and intracellular antigens staining and MLR assay as previously described (17). Preparations always resulted in >90% potent MLR stimulator DC (not shown).

Immunization and CTL Assay. Before injection in vivo, DC lines as well as fresh bm-DC were exposed in vitro to 100 μg/ml β-gal grade X (Sigma Chemical Co., Milano, Italy) for 18–20 h to allow protein processing. 2–5 × 10^6 D2SC/1 and D2SC/1-GM cells or 5 × 10^5 fresh DC were injected intraperitoneally in 200 μl PBS after extensive washing in FCS-free medium. After 10–12 d, spleens were removed from three to four immunized mice, pooled, and a single cell suspension prepared by mechanical dissociation. Splenocytes were restimulated at 5 × 10^6 cells/ml with the synthetic peptide βGP1 (1 μM) or 5 × 10^5 irradiated (150 Gy) F1.A11 cells/ml in the presence of 20 U/ml recombinant human IL-2 (EUROCETUS, Milano, Italy). After 5-7 d, viable cells were harvested and tested in a ^{51}Cr release assay (24) for their ability to lyse the β-gal–expressing tumor cell line P13.1.

Flow Cytometry. The CD4/CD8 profile of in vivo primed T lymphocytes were analyzed by flow cytometry after conventional mAb staining (17). The following mAbs were used: FITC-conjugated anti-CD4, clone RM4-5, and anti-Thy1.2, clone 53-2.1, and PE-conjugated anti-CD8, clone 53-6.7 (Pharmingen, San Diego, CA). Analysis was performed on a FACScan® (Becton-Dickinson, Milano, Italy); data were collected on 5,000–10,000 viable cells and analyzed using Lysis II® software. To detect cell surface expression of leukocyte markers on DC cell lines, immunostaining was performed as previously described (17) using the following mAbs: M1/42, anti-MHC-I; B21.2, anti-MHC-II (I-$A^{b,d}$); 2D2C, anti-CD44; M1/69, anti–heat-stable antigen; 3E2, anti-ICAM-1; 1G10, anti-B7-1; GL1, anti-B7-2; and unrelated isotype-matched mAbs as controls.

In Vivo Protection Studies. Mice were immunized following the schedule of Table 1, and control naive animals received in the left rear flank a subcutaneous challenge of F1.A11 living cells (10^4) 10–12 d after vaccination. Mice given the tumor subcutaneously were inspected for tumor growth and size twice a week. The differences in tumor take between control and immunized mice were statistically evaluated by chi-square test, considering a χ^2 value >3.8 as indicative of significant difference at <0.05 level.

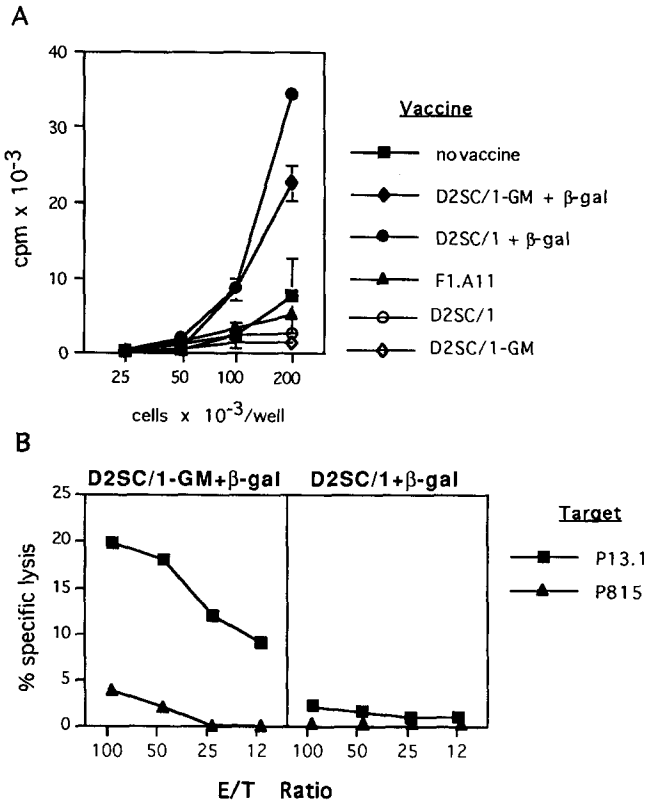

Figure 1. (*A*) Secondary response in mice that have been primed with the antigen-pulsed DC lines. CD4+ T lymphocytes, purified from spleen of naive or from mice immunized with DC lines pulsed in vitro with soluble β-gal or with irradiated F1.A11 tumor cells, were cultured at the indicated cell numbers in presence of β-gal (10 μg/ml) and syngeneic irradiated spleen APC. After 72 h the cultures were pulsed overnight with 1 μCi/well [^{3}H]TdR and harvested on glass fiber filters 18 h later. Incorporated radioactivity was measured by liquid scintillation counting. CD4+ T cells were immunoselected from total spleen cells population by nylon wool purification followed by complement-mediated lysis of CD8+ T cells in presence of the rat anti-mouse mAb 53-6.7 (TIB105), resulting in >85% pure CD4+ T cells as confirmed by immunostaining and flow cytometry (not shown). (*B*) Recognition of MHC-I–restricted β-gal epitope by lymphocytes primed in vivo with DC lines. After immunization with the indicated vaccine (*top*), lymphocytes were restimulated in vitro (5 d) in the presence of βGP1 peptide (1 μM); at the end of the culture lymphocytes were tested in a ^{51}Cr-release assay using P815 (▲) and P13.1 (■) as targets.

Table 1. *Schedule for In Vivo Priming Using bm-DC Pulsed In Vitro with Soluble β-gal*

Group*	Day 0 Vaccine (dose/mouse)	Day 5 Boost (dose/mouse)
1	bm-DC (5×10^5 cells)	—
2	bm-DC (5×10^5 cells)	β-gal (50 μg)
3	bm-DC + β-gal‡ (5×10^5 cells)	—
4	bm-DC + β-gal‡ (5×10^5 cells)	β-gal (50 μg)
5	β-gal (50 μg)	—
6	β-gal (50 μg)	β-gal (50 μg)
7	β-gal (50 μg) in CFA (1:1)	—
8	β-gal (50 μg) in C. parvum (100 μg)	—
9	—	—

*Vaccines and soluble protein boost were administered intraperitoneally. All groups received a challenge of live F1.A11 (10^4 cells/mouse; subcutaneously) on day 12 after immunization.
‡Before injection, bm-DC were pulsed in vitro with 100 μg/ml β-gal, for 18–20 h.

Results

The DC Line D2SC/1 Exposed to Soluble β-gal In Vitro Primes an Antigen-specific Cytolytic T Cell Response after a Single Immunization In Vivo. To assess the ability of DC to prime a CTL response against a soluble protein in vivo, mice were injected intraperitoneally with 2–5 × 10^6 D2SC/1 or D2SC/1-GM cells previously exposed in vitro to soluble β-gal. 10–12 d after immunization, mice were killed and their spleen cells harvested and placed in secondary culture in vitro. As expected, a CD4-mediated β-gal–specific proliferative response was elicited (Fig. 1 *A*). To assess cytolytic activity, splenocytes from vaccinated mice were placed in a secondary in vitro culture with the β-gal synthetic peptide βGP1, or β-gal gene-transduced tumor cells (not shown), and tested, 5 d later, for lytic activity on β-gal–transfected P13.1 and parental P815 cells (Fig. 1 *B*). Splenocytes from mice primed in vivo with D2SC/1 cells + β-gal were unable to specifically lyse P13.1 whereas splenocytes from mice primed with D2SC/1-GM + β-gal lysed P13.1 but not P815. Thus only the priming with the GM-CSF–transduced cell line elicited antigen-specific CTL. Vaccination with DC lines not pulsed with the soluble protein did not induce β-gal–specific CTL, while a proliferative response against FCS components could not be avoided.

GM-CSF Allows the In Vitro MHC-I–restricted Presentation of Soluble Antigen by D2SC/1 Cell Line. Further investigation of the effect of GM-CSF transduction on the DC line D2SC/1 did not reveal modifications in the pattern of cell surface markers but, as already observed after addition of recombinant mouse GM-CSF to another immortalized cell line (17), GM-CSF–transduced DC became free in suspension and acquired a more dendritic morphology (not shown).

Moreover, that GM-CSF modifies the ability of D2SC/1 to present β-gal was clearly shown by experiments in which D2SC/1 and D2SC/1-GM pulsed with either βGP1 peptide or soluble β-gal were used as targets of the β-gal–specific, CTL clone 0805.B. In fact, while both D2SC/1 and D2SC/1-GM were lysed if pulsed with the peptide βGP1 (Fig. 2 *A*), only D2SC/1-GM was lysed after pulsing with soluble β-gal (Fig. 2 *B*). This result clearly indicates that the peptide was correctly displayed within the

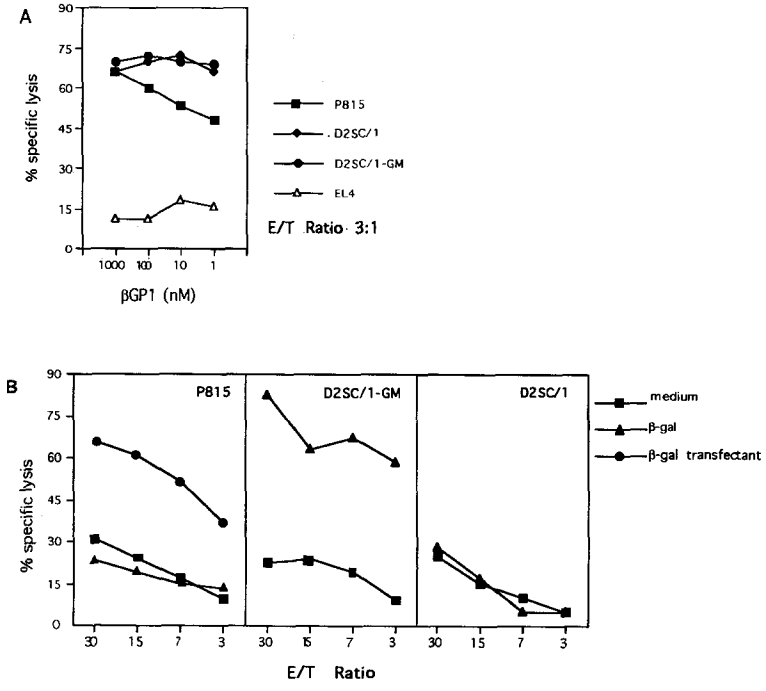

Figure 2. Recognition of β-gal naturally processed epitope by β-gal–specific 0805.B CTL clone. (*A*) Recognition of βGP1-pulsed targets; (*B*) recognition of soluble β-gal–pulsed targets. Lytic activity was assayed in a ^{51}Cr release after incubation of target cells with soluble β-gal (100 μg/ml, 18–20 h) or βGP1 peptide (1–0.001 μM; 2 h).

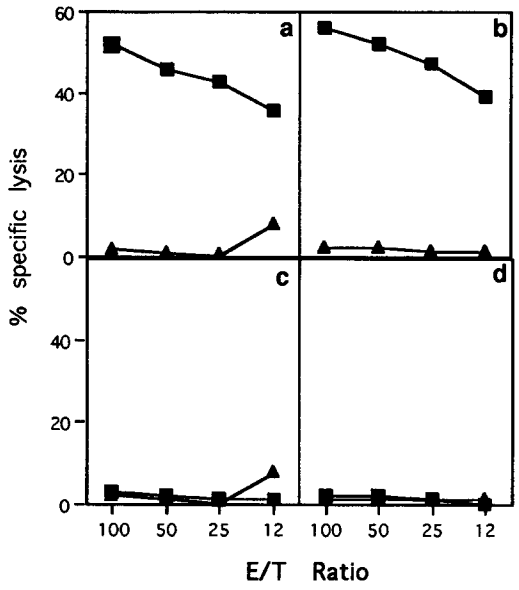

Figure 3. Recognition of MHC-I–restricted β-gal epitope by lymphocytes primed in vivo with bm-DC. After immunization with the indicated vaccine, lymphocytes were restimulated in vitro (5 d) in the presence of βGP1 peptide. At the end of the culture live lymphocytes were tested in a ^{51}Cr release assay using P815 (▲) and P13.1 (■) as targets. (*a*) bm-DC pulsed with β-gal; (*b*) bm-DC pulsed with β-gal plus β-gal boost; (*c*) β-gal; (*d*) β-gal plus β-gal boost.

MHC-I of both DC lines but that only DC transduced with GM-CSF can process the soluble protein for MHC-I presentation.

Fresh bm-DC Process Soluble β-gal In Vitro and Prime Antigen-specific CTL In Vivo. To investigate whether the in vivo priming of CTL is a property restricted to immortalized DC lines, experiments were run in parallel by using bm-DC. Fresh DC were exposed to soluble antigen in vitro with the same procedure and concentration used for D2SC/1 cells and injected intraperitoneally in BALB/c mice (Table 1). 500,000 bm-DC, pulsed in vitro with soluble β-gal, were sufficient to prime antigen specific CTL in vivo, whereas no evidence of CTL activation was seen in mice immunized once or twice with soluble β-gal or with unloaded bm-DC (Fig. 3).

Lymphocytes primed in vivo with DC and expanded by secondary in vitro cultures, were enriched in T cells. Particularly, we found that $CD8^+$ cells were 55–70% and 15–35% in DC versus tumor cell–primed lymphocytes, respectively. Furthermore, lymphocytes from mice primed with DC showed a blastlike morphology, indicating a probable entry in G1 phase, since their forward scatter/side scatter parameters were 500:250 while parameters of lymphocytes from mice primed with tumor cells were 200:50, the latter without a significant difference from naive lymphocyte size (Table 2).

Vaccination with DC Pulsed with β-gal Protects Mice Against a Challenge with β-gal–transduced Tumor Cells. To test whether the immune response induced by bm-DC can induce resistance against a challenge with live tumor cells, 10–12 d after immunization mice were injected subcutaneously with 10^4 viable F1.A11 cells. Immunization with bm-DC pulsed with soluble β-gal protected 60% of challenged mice while mice receiving also a soluble β-gal boost 5 d after the priming with pulsed bm-DC were completely protected against tumor challenge. Mice immunized with β-gal, boosted or not with the soluble protein, did not show statistically significant difference in tumor take as compared to naive mice which received the same challenging dose. No protection was seen also in mice receiving unloaded bm-DC accompanied or not by a boost of soluble protein (Fig. 4 *A*). Vaccination with soluble β-gal admixed with CFA or *Corynebacterium parvum* adjuvants did not result in tumor protection (Fig. 4 *B*).

Discussion

To directly investigate the in vivo priming of a CD8-mediated T cell response after prophylactic vaccination with soluble protein loaded DC, we took advantage of a previously described immortalized DC line (17) to set up the model, and from the complete set of reagents that an antigen like β-gal may offer. In fact, β-gal–soluble protein, its immunogenic peptide as well as a retroviral vector able to transduce the gene into tumor cells, were all available. In addition, β-gal has been chosen because, as soluble protein,

Table 2. *Activation of In Vivo Primed Lymphocytes after In Vitro Secondary Culture with β-gal–derived Synthetic Peptide*

Vaccine	Mean forward scatter	Percent CD4	Percent CD8	CD4/CD8
bm-DC	200	24	11	2.18
bm-DC + β-gal boost	200	32	9	3.5
bm-DC + β-gal*	500	6	70	0.08
bm-DC + β-gal + β-gal boost	400	20	60	0.33
β-gal	200	65	22	3.03
β-gal + β-gal boost	200	70	24	2.93
F1.A11	200	48	38	1.26

Lymphocytes were restimulated in presence of βGP1 (1 μM) for 5 d in vitro.
*Before injection, bm-DC were pulsed in vitro with 100 μg/ml β-gal for 18–20 h.

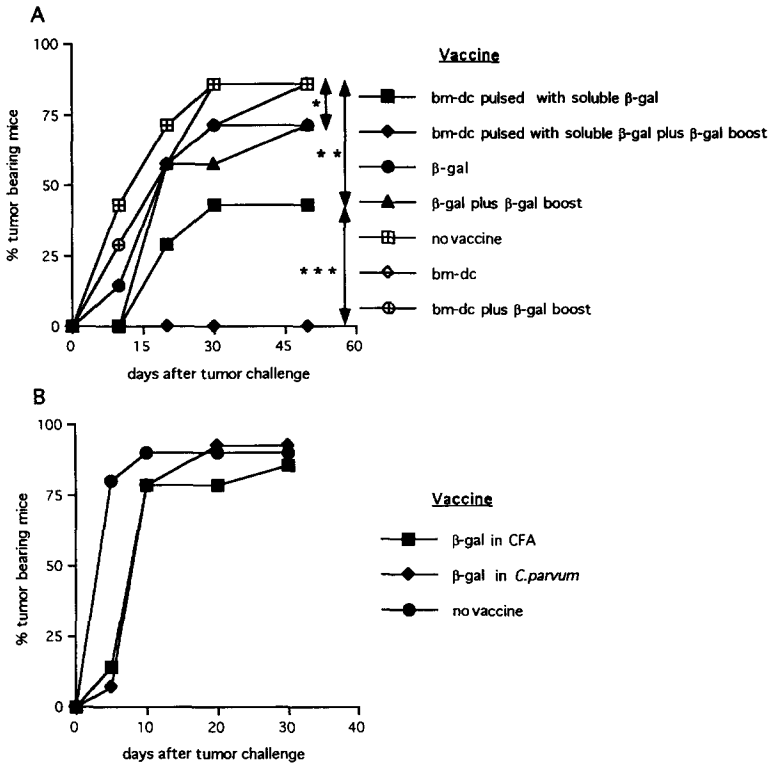

Figure 4. Ability of immunized mice to reject a β-gal–expressing tumor challenge. After immunization with the indicated vaccine, BALB/c mice were injected subcutaneously with 10^4 F1.A11 tumor cells. Tumor growth was inspected twice a week by palpation. Seven mice were included in each group. *Statistically not different; **$P < 0.05$; ***$P < 0.01$.

it lacks intrinsic properties that allow its entry into the cell outside the endosomal compartment and because it is unable to stimulate CTL in vivo (5).

We observed that DC lines loaded in vitro with soluble antigen were able to activate antigen-specific CTL if transduced with GM-CSF gene. This confirms previous observations which indicate GM-CSF as a "pro-presentation" cytokine (25). In fact, GM-CSF is able to enhance the immune response initiated by DC, including immunity against tumors, by directly stimulating maturation of their accessory properties (15, 26). Availability of DC lines either transduced or not with the GM-CSF gene was instrumental to define clearly the role of GM-CSF, since bm-DC could be obtained only in the presence of GM-CSF, thus lacking the control counterpart. Our results demonstrate that GM-CSF can activate a pathway of antigen processing that allows exogenous soluble protein to be presented by MHC-I molecules as shown by the recognition and killing of GM-CSF–transduced DC loaded with soluble β-gal by a CTL clone specific for the β-gal, H-2L^d–restricted epitope. Furthermore, CTL activation in vivo was obtained by vaccination with fresh bm-DC loaded with soluble β-gal in vitro, and vaccinated mice were completely protected against live tumor challenge if boosted with the soluble antigen. Here, GM-CSF was necessary to drive maturation of DC in vitro, while antigen boost shows that concomitant recall of DC-primed T helper cells is likely to be beneficial for sustaining a protective immune response against tumors as it was shown to occur for the induction of reaction against influenza virus in vitro (27). Along with CTL, Th were certainly activated by immunization with β-gal–loaded DC since both antigen-specific $CD4^+$ T cell proliferation and anti–β-gal antibody production were observed (not shown).

That β-gal could be considered a model to overcome the limited availability of well-characterized tumor antigen in the mouse is supported by our preliminary results showing that bm-DC pulsed with a 20-mer length peptide encompassing the Arg^{12} mutation of the K-*ras* oncogene which, by itself, can not be accommodated within the MHC-I groove, immunize naive mice against a fibrosarcoma carrying this mutation (Carbone, G., et al., manuscript in preparation). Identification and cloning of tumor antigens expressed by melanomas (2) and identification of immunogenic peptides within mutated or overexpressed oncogenes, i.e., RAS and HER-2/neu (3), prompted the possibility to immunize cancer patients with well-defined T cell epitopes. Since immunogenicity of antigenic peptides may depend on the type of adjuvant, pulsed DC may represent the ideal cell-based vaccine even for human tumors, especially now that obtaining a consistent number of DC from human $CD34^+$ precursor by CSF administration is possible (28).

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