Retrovirally Transduced Bone Marrow-Derived Dendritic Cells Require CD4$^+$ T Cell Help to Elicit Protective and Therapeutic Antitumor Immunity

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It has been extensively documented that murine dendritic cells loaded with tumor-associated Ag (TAA)-derived peptides or protein can prime Ag-specific CD8$^+$ cytotoxic T cells in vivo and can elicit Ag-specific immunity. Optimal presentation of TAA might be achieved by retroviral transduction of DCs allowing long term and stable expression of the TAA-peptides as well as the presentation of multiple epitopes in the context of MHC class I and/or class II molecules. Here we show that retroviral transduction of bone marrow-derived dendritic cells (DCs) with chicken OVA cDNA or the reporter gene green fluorescent protein retained their potent stimulatory capacity and that the transduced DCs could process and present the endogenously expressed OVA protein. The DCs transduced with cDNA encoding native OVA protein presented OVA-derived peptides in the context of MHC class I as well as MHC class II and induced a strong Ag-specific CTL response. DCs expressing a cytosolic form of OVA presented OVA peptides only in the context of MHC class I and failed to induce an OVA-specific CTL response in vivo when they had been cultured in the absence of exogenous protein. Immunization with retrovirally transduced DCs resulted in an Ag-specific immunity and rejection of a tumor cell challenge and a significant survival advantage in tumor-bearing mice. These results obtained in this rapidly lethal tumor model suggest that DCs transduced with TAA may be useful for tumor immunotherapy and underscore the importance of the simultaneous delivery of T cell help in the development of Ag-specific cytotoxic T-cells. ***The Journal of Immunology,*** **1999, 162: 144–151.**

During the last decade and due to advances in cellular and molecular techniques and the availability of crucial growth factors, dendritic cells (DCs)[3] have been actively studied. These cells are highly specialized APCs equipped with unique immunostimulatory properties. DC are the principal activators of resting naive T cells. As immature motile cells they have cytoplasmic processes and a veiled morphology, are specialized for Ag uptake by pinocytosis and receptor-mediated endocytosis, and transport their cargo to the T cell areas of lymphoid organs. T cell activation is mediated by the expression of important cell surface molecules, such as high levels of MHC class I and II molecules, adhesion molecules, and costimulatory molecules (reviewed in Refs. 1 and 2). These properties have attracted the attention of many investigators, and a large number of papers have reported on the use of DC for antitumor therapy and immunotherapy of infectious diseases. Many different methods have been used to load DC with Ags before in vivo injection. Protein- or peptide-pulsed DCs have been shown to be potent inducers of Ag-specific antitumor responses both in a variety of experimental animal models as well as in patients suffering of non-Hodgkin lymphoma (3–12). Except for the Id protein serving as tumor-associated Ag (TAA) in lymphoma and myeloma, the proteins corresponding to most TAA expressed by nonhematological tumors are seldom available (13). Synthetic peptides corresponding to the TAA epitopes recognized in the context of particular MHC class I molecules or autologous acid-eluted tumor peptides can be used to pulse onto DCs. However, these peptide-based strategies may have some drawbacks, such as the transient presentation of the antigenic epitopes to the T cells due to the rapid turnover of the preformed peptide/MHC complexes and the variability of the binding affinities of the synthetic peptides. The use of peptides also relies on the knowledge of the MHC haplotype of each patient and the corresponding class I binding motifs of the TAA. Moreover, while the peptide motifs for MHC class I presentation are relatively well known, the sequences necessary to load MHC class II molecules are not as well defined. The lack of Ag-specific help for the induction of cytotoxic T cell responses might result in a less efficient or suboptimal immune response (14–18).

In an attempt to optimize the use of DCs to induce an antitumor response we have focused our efforts on the expression of the entire tumor Ag polypeptide in DCs. Genetic modification of DCs using liposome-mediated (19) or biolistic transfection strategies or adeno- and retroviral transduction (20–23) has a number of potential advantages to peptide- or protein-based methods. Genetic modification does not require knowledge of the HLA haplotype of

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[3] Abbreviations used in this paper: DC, dendritic cell; TAA, tumor-associated Ag; GFP, green fluorescent protein; tOVA, truncated OVA; rmGM-CSF, recombinant murine granulocyte-macrophage CSF; rmIL, murine rIL; rhFLT3-L, recombinant human FLT3 ligand; PE, phycoerythrin; NMS, normal mouse serum.

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the patients or of the particular TAA peptides binding to the restriction elements. In addition, multiple and not yet defined peptide epitopes encoded by the whole tumor Ag polypeptide might contribute to T cell activation. This approach also may lead to long term presentation of the antigenic epitopes to the immune system.

We have chosen to develop this strategy by retroviral transduction of DCs with cDNA encoding a model Ag, chicken OVA. This Ag was chosen for several reasons. The E.G7-OVA cell line has been widely used for the development of immunotherapeutic strategies directed toward the surrogate TAA (i.e., OVA), and multiple reagents and tools are available (6, 10, 24–27). OVA in its native form is a secreted protein, and well-defined OVA peptides are presented in the context of MHC class I and class II molecules. By deletion of part of the OVA-encoding cDNA we could limit the expression of the tumor Ag polypeptide to the cytosol, thus mimicking the expression of most TAA identified to date, and demonstrate the effect of the lack of MHC class II presentation.

We demonstrate the efficient transduction of bone marrow-derived DCs with retroviral vectors encoding GFP, the entire OVA cDNA, and a truncated OVA (tOVA) cDNA. These gene-modified DCs differentiate in vitro and express, process, and present the gene products in the context of MHC molecules. Injection of OVA cDNA-modified DCs results in potent CTL response compared with the injection of tOVA cDNA-transduced DCs. Moreover, when the transduced DCs are cultured in autologous serum and in the absence of FCS, only the DCs presenting OVA peptides in the context of both MHC class I and class II are able to induce an OVA-specific CTL response. Genetically modified DCs are potent inducers of an effective antitumor immunity against a challenge of tumor cells and against an established tumor.

Materials and Methods

Mice, cell lines, and reagents

C57BL/6 (H-2^b), BALB/c (H-2^d), and (C57BL/6 × BALB/c)F1 (H-2^b × H-2^d) female mice, 6–8 wk old, were obtained from Charles River Wiga (Sulzfeld, Germany). Animals were maintained and treated according to the institutional guidelines. The care and use of mice were in accordance with the guidelines of the Vrije Universiteit Brussel.

The tumor cell lines used were EL-4 (C57BL/6, H-2^b, thymoma) and E.G7-OVA (EL-4 cells transfected with chicken albumin cDNA; American Type Culture Collection, Manassas, VA). Cells were maintained in DMEM (Life Technologies, Ghent, Belgium) supplemented with 5% heat-inactivated FCS, 2 mM glutamine, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. E.G7-OVA cells were grown in the same medium containing 400 μg/ml G418 (Calbiochem, Bierges, Belgium). When used as targets in CTL assays, these cells were grown in the same medium containing 1% autologous serum during the last 48–72 h before the assay. The T cell hybridoma RF33.70 recognizes an H-2-K^b OVA peptide (amino acids 257–264) and was provided by Dr. K. L. Rock (Boston, MA). The H-2-I-A^d-restricted OVA-specific T cell hybridoma DO11.10 recognizes an I-A^d-binding epitope located within amino acids 323–339 of the OVA protein sequence. This cell line was provided by Dr. P. Marrack. Upon recognition of the OVA epitopes in the context of the corresponding restriction element, these T cell hybridomas secrete IL-2. The amount of IL-2 released in the supernatant of the DC-T cell hybridoma cocultures was determined using the indicator cell line CTLL-2. All cell lines were free of mycoplasma as tested by PCR analysis (*Mycoplasma* PCR primer set, Stratagene, Westburg, Leusden, The Netherlands).

The rmGM-CSF was produced in-house as previously described (28). The rmIL-4 was 11B11 mAb affinity purified from supernatant of sF9 cells infected with a recombinant baculovirus provided by Dr. J. Van Snick (UCL, Brussels, Belgium). The biological activity of rmIL-4 was determined using IL-4-dependent TS1 cells. As a source of rhFLT3-L we used a 200-fold dilution of a supernatant of CHO cells transfected with pCDNA3-FL (containing 2 μg of rhFLT3-L/ml). This vector was generated by PCR using HS86 DNA as template. The HS86 plasmid was given to us by O. Rosnet (Laboratoire d'Oncologie Moléculaire, Marseille, France). The biological activity of rhFLT3-L was determined with OCI-AML-5 cells. All cytokines produced by ourselves were endotoxin-free (<15 pg/ml) as analyzed by a colorimetric assay (Chromogenic, Molndal, Sweden).

Generation of bone marrow-derived DCs and cell phenotype

The procedure used to generate DCs from bone marrow cultures was that described by Mayordomo et al. (6) with minor modifications. Briefly, bone marrow was flushed from the long bones of the limbs, filtered through a nylon mesh, and depleted of red cells with ammonium chloride. Bone marrow cells were incubated for 30 min with a panel of mAbs for the depletion of lymphocytes, granulocytes, and MHC class II-positive cells. All the Abs used were purchased from PharMingen (San Diego, CA). Ab-labeled cells were incubated with sheep anti-rat Ab-coated magnetic beads (Dynal, Oslo, Norway) at a cell-to-bead ratio of 1:5. Lineage marker-negative cells were plated in 24-well culture plates (10^6 cells/ml; 1 ml/well) in DMEM supplemented with 5% heat-inactivated FCS or 1% heat-inactivated autologous serum, 50 μM 2-ME, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml rhFLT3-L. After overnight incubation the cells were replated (2.5×10^5 cells/well in 1 ml) in the same medium containing cytokines (rmGM-CSF, 200 ng/ml; rmIL-4, 1000 U/ml; rhFLT3-L, 10 ng/ml). From day 6 on the DC were cultured in the absence of serum. The nonadherent cells were harvested on day 6, extensively washed with Opti-MEM (Life Technologies), and further cultured in the same medium in the presence of rmGM-CSF and rmIL-4. On days 8–10 DC were harvested by gentle pipetting.

For phenotypic analysis, DCs were incubated with the biotinylated mAbs directed against the surface molecules B7-1, B7-2, CD11c, MHC classes I and II, or appropriate isotype-matched controls in the presence of 2.4G2 supernatant, a rat Ab directed against the mouse FcRIIγ (CD32) receptor. Binding of the mAbs was revealed by a second incubation with PE-labeled streptavidin (PharMingen).

Cloning of retroviral vector constructs

For retrovirus production the retroviral vector MFG, derived from Moloney murine leukemia virus, was used. This vector does not contain a drug resistance marker, nor does it express any potential antigenic protein other than the inserted cDNA (29). All the cDNAs were obtained by PCR. The amplification products were sequenced before insertion into the MFG vector. For amplification of OVA cDNA we used the pAc-neo-OVA plasmid (provided by Dr. Bevan, La Jolla, CA) as template. Two OVA-MFG vectors were generated: MFG-OVA containing the whole OVA cDNA (amino acids 1–386) and MFG-tOVA containing the cDNA encoding a truncated form of the OVA protein sequence (amino acids 40–386). The cDNA encoding the reporter gene GFP was amplified from plasmid pRS-GFP-C1 (Clonetech, Westburg, Leusden, The Netherlands).

Ten million PhoenixECO producer cells (provided by Dr. G. P. Nolan, Stanford, CA) were transfected with 40 μg of retroviral vector DNA by the calcium phosphate precipitation method (following the instructions given by Dr. Nolan) (30). Cells were incubated in chloroquine (Sigma, St. Louis, MO) containing medium at 37°C for 10 h. The medium was again changed (Opti-MEM) after 14 h, and the retrovirus-containing medium was harvested 48 h after transfection. The retroviral supernatants were filtered (0.45 μm pore size), snap-frozen, and stored at −80°C.

Transduction of DCs

On days 2, 3, and 4 after the start of the bone marrow cell culture, the medium was removed and replaced with 1 ml of virus supernatant containing 8 μg/ml polybrene (Sigma). The cells were transduced during centrifugation of the 24-well plates during 2 h at 2500 rpm and at 32°C. The retroviral supernatant was then removed, and the cells were resuspended in cytokine-containing medium. This transduction procedure was repeated two more times.

Mixed lymphocyte reaction

The ability of transduced and nontransduced DCs to stimulate resting T cells was assessed by a mixed lymphocyte reaction. Mitomycin C-treated C57BL/6 bone marrow-derived DCs harvested on day 8 or 10 or mitomycin C-treated splenocytes were plated at graded concentrations in the presence of 3×10^5 BALB/c nylon wool-purified T cells in 200 μl of DMEM with supplements. After 3-day incubation at 37°C, 5% CO_2 the cells were pulsed overnight with 1.0 μCi/well [^{3}H]thymidine. [^{3}H]thymidine incorporation was measured using a beta scintillation counter (Microbeta, Wallac, Turku, Finland).

Ag presentation assay

To determine whether the transduced DCs presented OVA-derived peptides in the context of MHC class I and class II molecules, OVA-specific

T cell hybridoma cells (5×10^4 cells/well in a final volume of 200 μl) were cocultured with graded numbers of transduced DCs harvested on days 6–8 in round-bottomed 96-well tissue culture plates and incubated at 37°C in a 5% CO_2 humidified incubator. After 20–22 h, the supernatant was harvested, and IL-2 release was measured by the proliferation assay with CTLL-2 cells.

Induction of Ag-specific CTLs in vivo and cytotoxicity assay

Naive mice were immunized with transduced DCs (10^5 cells in 200 μl of PBS) by a single i.v. injection. To demonstrate the dependence on $CD4^+$ T lymphocytes during the induction phase, $CD4^+$ T cells were depleted before immunization, by i.p. injection of 1 mg of anti-CD4 Ab GK1.5 (5 days before the immunization) followed by injections of 250 μg 7 and 14 days later.

Fourteen days after the injection of the DCs, the splenocytes from the immunized mice were depleted of APCs by plastic adherence and were restimulated in vitro with mitomycin C-treated E.G7-OVA cells at a responder to stimulator ratio of 10:1 for 5 days in culture medium supplemented with 1% autologous mouse serum and 10 U of IL-2/ml. On day 6 restimulated splenocytes were tested for Ag-specific cytolytic activity using E.G7-OVA and EL-4 cells as targets. These cells were labeled with 200 μCi of $Na_2{}^{51}CrO_4$ (Amersham, Arlington Heights, IL). Restimulated splenocytes were mixed in graded doses with 10^4 labeled target cells in 200 μl of tissue culture medium in V-bottom 96-well tissue culture plates (Costar, Cambridge, MA). Cells were incubated for 4 h at 37°C in a 5% CO_2 humidified incubator. The release of ^{51}Cr was measured with a gamma counter (Kontron, Van Hopplynus, Brussels, Belgium), and the percent specific lysis was calculated as follows: $100 \times$ [(experimental release − spontaneous release)]/[(maximal release − spontaneous release)]. Spontaneous and maximal release were determined in the presence of either medium or 10% SDS. Spontaneous release was <10%. The SD of triplicate wells was <10%.

Protection against tumor challenge by transduced DCs

C57BL/6 mice were immunized by i.v. injection with 2×10^5 transduced DCs on days 0 and 7. One week after the last immunization (day 14), 10^7 E.G7-OVA or EL-4 cells were injected s.c. in the interscapular region. Mice were monitored on a regular basis for tumor growth and tumor volume (smallest diameter^2 × largest diameter) (31). This method of tumor volume assessment correlated very well with the tumor weight determined after resection of tumors. Statistical significance was calculated using the one-sided *t* test. Mice with a tumor diameter >3 cm were killed.

Suppression of tumor growth by transduced DCs

Groups of five to eight C57BL/6 mice were inoculated with 5×10^6 E.G7-OVA cells on day 0. These mice were treated with four i.v. injections of 2×10^5 transduced DCs (days 4, 8, 12, and 16). Mice were monitored as described above. The Ag expression by the tumors escaping the immunotherapy was analyzed by RT-PCR. RNA was extracted from the resected tumor tissue by TRIzol extraction (Life Technologies). First-strand cDNA was synthesized using a preamplification cDNA synthesis kit (Life Technologies). RT-PCR was performed with the following primers: sense primer, 5′-gggggatccaggggaaacacatctgccaa; and antisense primer, 5′-gggg gatccatttgccagtgggacaat. An RT-PCR using actin-specific primers was used to assess the quantity and the quality of the cDNA.

Results

Retroviral transduction of bone marrow-derived DC

Lineage marker-negative bone marrow cells were cultured in the presence of several cytokines and growth factors to enhance the expression of retroviral receptors and cellular division. After a 24-h culture period in the presence of rhFLT3-L, the cells were transduced during a 120-min centrifugation step at 32°C in the presence of supernatant of the ecotropic packaging cell line PhoenixECO and 8 μg/ml polybrene. The transduction was repeated three times. This method of gene transduction has been used for the successful transduction of human DCs (20), has been reported to enhance the transduction efficiency compared with other methods (32), and avoids the potential contamination of the differentiating cells with packaging cells. The transduced cells were allowed to differentiate into DCs during another 7–8 days in the presence of rmGM-CSF and rmIL-4. Transduction efficiency and the phenotype of the DCs were monitored cytofluorographically. Using GFP as reporter system, we consistently obtained a cell population of which the majority of the cells displayed a green fluorescence (mean, 74; range, 52–86%; in 27 independent experiments). A representative FACS analysis of mock-, GFP-, OVA-, or tOVA-transduced cells, cultured in 1% NMS, and stained with DC surface markers (CD80, CD86, MHC class II, and CD11c) is shown in Fig. 1. The cells displayed the characteristic DC morphology, with large extensions (not shown). DC cultured in 5% FCS displayed the same characteristics (not shown). It is important to mention here that there was no difference in the expression of surface markers or viability between the DCs transduced with the different constructs.

Allogeneic MLR using retrovirally transduced DCs

The effect of retroviral transduction of the DCs on their stimulatory capacity was assessed in an allogeneic MLR (Fig. 2). Both the mock-transduced as well as the GFP- or OVA-transduced DC populations induced a strong proliferation of allogeneic T cells. No significant differences were noted among the different retroviral constructs used or the culture conditions during the expansion and the differentiation of DCs. The stimulatory capacity of the DCs was consistently at least 1000-fold stronger than the stimulatory effect of bulk splenocytes.

Retrovirally transduced DCs process and present OVA-derived peptides in the context of MHC class I and class II molecules

The capacity of the retrovirally transduced DCs to process and present OVA-derived peptides in the context of MHC complexes was determined by their coculture with the OVA-specific T-T hybridoma cell lines DO11.10 ($OVA_{323–339}$-A^d specific) and RF33.70 ($OVA_{257–264}$-K^b specific). After a 22-h coculture of graded numbers of transduced CB6F1 (BALB/c × C57BL/6) DCs with the Ag-specific cell lines, the IL-2 released in the supernatant was measured. DCs transduced with the entire OVA cDNA presented OVA peptides in the context of both MHC class I and class II (Fig. 3). DCs expressing the truncated OVA cDNA (amino acids 40–386) presented OVA peptides only in an MHC class I-restricted fashion. OVA peptide-pulsed DCs were used as positive controls (not shown). Mock-transduced or GFP-transduced DCs did not activate the OVA-specific cell lines, demonstrating the specificity of the T-T hybridoma response. These results indicate that the endogenously expressed cDNAs were processed and presented on the surface of the DCs. However, the cytosolic expressed tOVA retaining the $OVA_{323–339}$ sequence but lacking a signal sequence for translocation into the ER was not presented in association with MHC class II molecules, while the $OVA_{257–264}$ presentation in the context of H2-K^b was indistinguishable between the two DC populations transduced with either OVA or tOVA cDNA.

Induction of OVA-specific CTLs by transduced DCs and $CD4^+$ T cell help requirement

To determine whether the transduced DCs could induce an OVA-specific CTL response in vivo, C57BL/6 mice were immunized with a single i.v. injection of 10^5 transduced DCs. Splenocytes from immunized mice were harvested 2 wk later, restimulated in vitro with E.G7-OVA cells, and then assayed for specific lysis of target cells in a standard ^{51}Cr release assay. When the DCs had been cultured in the presence of FCS until the day of immunization, OVA-transduced as well as tOVA-transduced DCs induced an Ag-specific CTL response. The CTL response in tOVA-transduced DC-immunized mice was consistently weaker than that in the mice immunized with OVA-transduced DCs, a finding repeatedly observed in several independent experiments. Immunization with GFP-transduced DCs failed to generate specific cytolytic

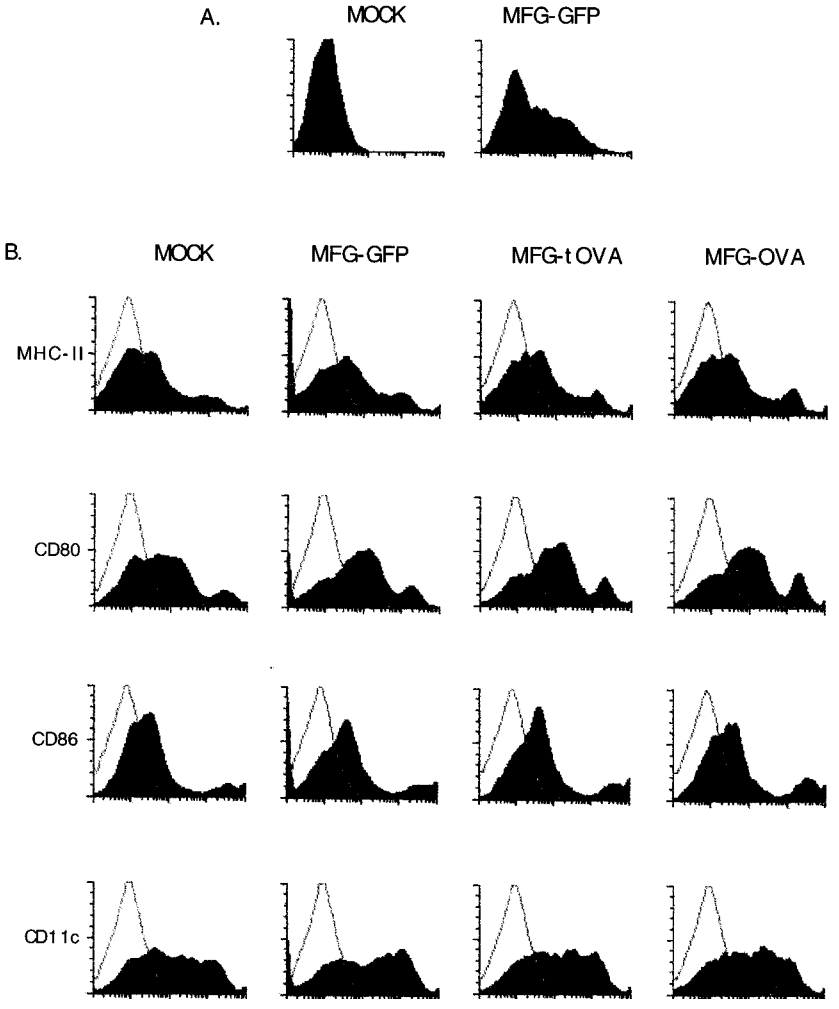

FIGURE 1. *A*, GFP expression in mock- or pMGF-GFP-transduced DCs. *B*, Cell surface phenotype of transduced bone marrow-derived dendritic cells cultured in 1% NMS. DCs were harvested on day 10 and incubated with biotinylated Abs with the indicated specificity.

CTLs. Negligible lysis of non-OVA-expressing EL4 target cells was detected when the in vivo primed pCTLs were restimulated in vitro in the presence of autologous normal mouse serum (data not shown). When the transduced DCs were cultured in the absence of FCS for 72 h before immunization, the difference in CTL response after OVA- and tOVA-transduced DC immunization became even more pronounced. Fig. 4 shows the data from five independent experiments. Similar results were obtained when F1 DCs pulsed in vitro with class I-restricted peptides alone or with class I plus class II peptides were used for immunization (data not shown). When the DCs had been cultured in the presence of autologous serum from day 1 on, no OVA-specific CTLs could be induced by the tOVA-transduced DCs, while the OVA-transduced DCs were still potent inducers of a strong CTL response (Fig. 4).

To determine whether $CD4^+$ T cell help was required for the generation of OVA-specific CTL by OVA-transduced DCs, the response was examined in $CD4^+$ T cell-depleted mice (Fig. 4). The lack of any response in these mice indicated that $CD4^+$ T cells are essential for CTL priming in vivo.

Induction of protective immunity against E.G7-OVA tumor cells by immunization with transduced DCs

To determine whether transduced DCs would induce protective immunity against a tumor cell challenge, mice were immunized i.v. with 2×10^5 irradiated OVA- or GFP-transduced or nontransduced DCs on days 14 and 7 before tumor challenge (on day 0). The mice were challenged with 10 million tumor cells and were monitored for tumor growth. Compared with untreated mice, the tumor growth was slightly retarded in mice immunized with GFP-transduced DCs (data not shown). In mice immunized with OVA-transduced DCs, tumor growth was significantly slowed (Fig. 5*A*).

Inhibition of tumor development by immunotherapy with transduced DCs

The therapeutic efficacy of retrovirally transduced DCs was also assessed (Fig. 5*B*). Five million tumor cells were injected on day 0, and these mice (groups of five to eight mice) were treated with four injections of 2×10^5 irradiated OVA- or GFP-transduced DCs (days 4, 8, 12, and 16). Mice treated with DCs transduced with OVA showed a significant ($p < 0.05$) retardation of tumor growth compared with mice treated with GFP-transduced DCs, indicating the Ag specificity of the antitumor immunity. The results shown are representative of data obtained from two independent experiments. At the highest tumor load the effect of the irradiation of the DCs was most apparent, while in the mice with a smaller tumor burden no difference between irradiated and nonirradiated DCs was noted (data not shown). However, after 3 wk tumors started to grow progressively in the mice immunized with OVA-transduced DCs. Analysis of the resected tumor tissues indicated that Ag-negative variants had been selected, since none of

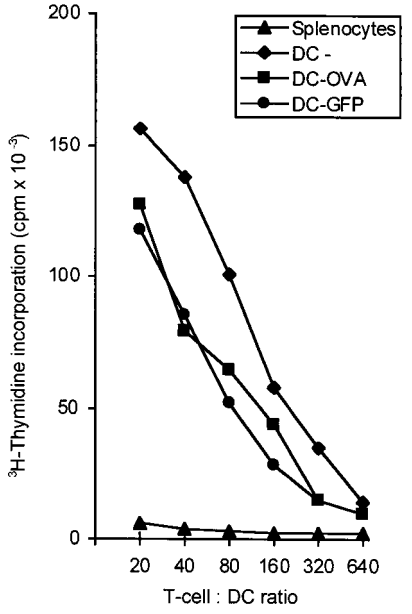

FIGURE 2. Allogeneic mixed lymphocyte reaction using nontransduced (DC−) or DCs transduced with OVA cDNA (DC-OVA) or with GFP cDNA (DC-GFP) and splenocytes. C57BL/6 bone marrow cells were transduced with GFP cDNA or OVA cDNA and differentiated into DCs in the presence of cytokines. Mitomycin C-treated DCs harvested on day 8 or bulk C57BL/6 splenocytes were cocultured with nylon wool-purified BALB/c T cells. After 3 days, the cells were pulsed with [^{3}H]thymidine, and the incorporation was measured with a scintillation counter.

the biopsies taken from OVA-transduced DC-treated mice generated an OVA-specific PCR amplification product, while tumors resected from mice treated with GFP-transduced DC all showed a strong PCR signal (data not shown). This further indicates that the transduced DCs induced a strong immune response eradicating all Ag-expressing tumor cells.

Discussion

The identification of immunogenic tumor Ags that can serve as targets for effector cells has led to different vaccination strategies aimed at the induction of a potent CTL response against such Ags. Since the proteins corresponding to most TAA are seldom available, most immunization strategies have used DCs charged in vitro with defined tumor antigenic peptides, acid-eluted peptides, whole tumor cell lysates, total RNA, or in vitro transcribed RNA. These approaches have been shown to be very effective for the induction of Ag-specific immunity and antitumor responses. The use of genetically modified DCs offers great potential for the generation of antitumor immunity. This approach circumvents the drawbacks of the strategies mentioned above, such as, for example, the need to synthesize tumor-specific peptides that have stringent MHC restrictions. Cytoplasmic expression of the tumor Ag potentially containing multiple immunogenic CTL epitopes allows the APC to tailor those peptides fitting their own MHC molecules. Also, the constitutive expression of the Ag by the APC will result in a prolonged presentation in vivo compared with peptide-loaded DCs, which display a rapid turnover of their MHC class I molecules.

In this report we have presented a strategy allowing optimal presentation of TAA by DCs independent of the limitations described above. We have chosen to develop this strategy by retroviral transduction of DCs with a model Ag gene encoding chicken OVA. Several groups have reported the successful transduction of human and murine DCs in vitro by retrovirus vectors encoding a model Ag (β-galactosidase) and tumor Ags including the melanoma MART-A Ag and the human epithelial tumor Ag mucin (20, 23, 33, 34). We show that bone marrow progenitor cell-derived DCs can be efficiently retrovirally transduced with MFG-OVA encoding OVA in its native form or MFG-tOVA, a truncated form of OVA lacking a signal sequence for translocation in the ER. The data provide evidence that the transduced DCs are able to process and present endogenously expressed OVA. The native form of OVA is presented in the context of both MHC class I and MHC class II molecules, as evidenced by recognition and specific IL-2 release by OVA-specific T cells. The truncated form of OVA, tOVA, is presented in the context of MHC class I only. Efficient presentation of Ag in class II requires endocytosis of the protein and processing in the early endosomes, where peptides can be loaded in the class II dimers. The complete OVA protein contains a targeting sequence that allows translocation into the ER and the secretory pathway of the Golgi apparatus. After secretion, the protein can be endocytosed by the cells and enter the exogenous class II presentation pathway. The fact that there is indeed a secretion of the OVA protein when DCs were transduced with the native protein is proven by the detection of OVA protein in the supernatant of cultured transduced DCs by Western blot (data not shown). The truncated form of OVA however lacks the ER translocation sequence and is present in the cells as a cytoplasmic protein. The

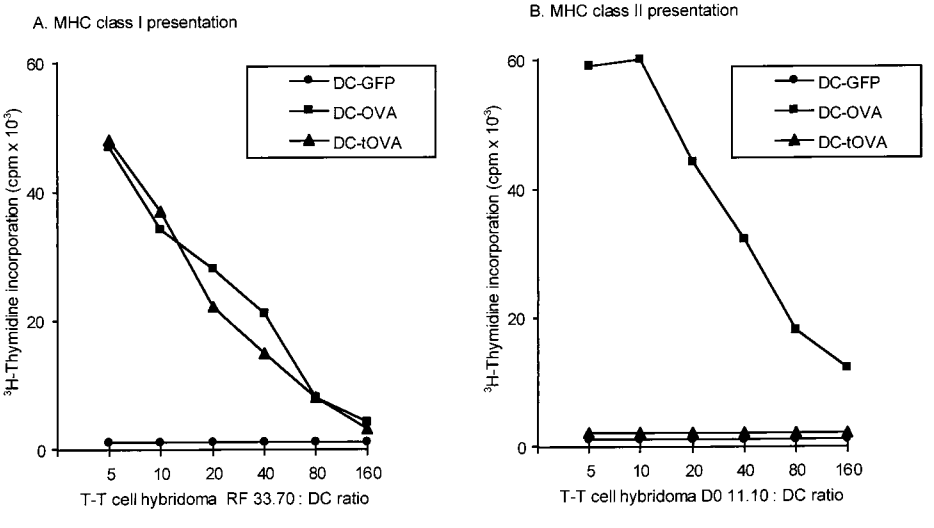

FIGURE 3. OVA peptide presentation by transduced CB6F1 (BALB/c × C57BL/6) DCs. Graded numbers of GFP-, OVA-, or tOVA-transduced DCs were cocultured for 22 h with 5×10^4 OVA-specific T-T hybridoma cells (*A*, RF33.70 recognizing OVA in the context of K^b; *B*, DO11.10 recognizing OVA in the context of I-A^d). The supernatant was analyzed for IL-2 content by the induction of [^{3}H]thymidine incorporation by CTLL-2 cells.

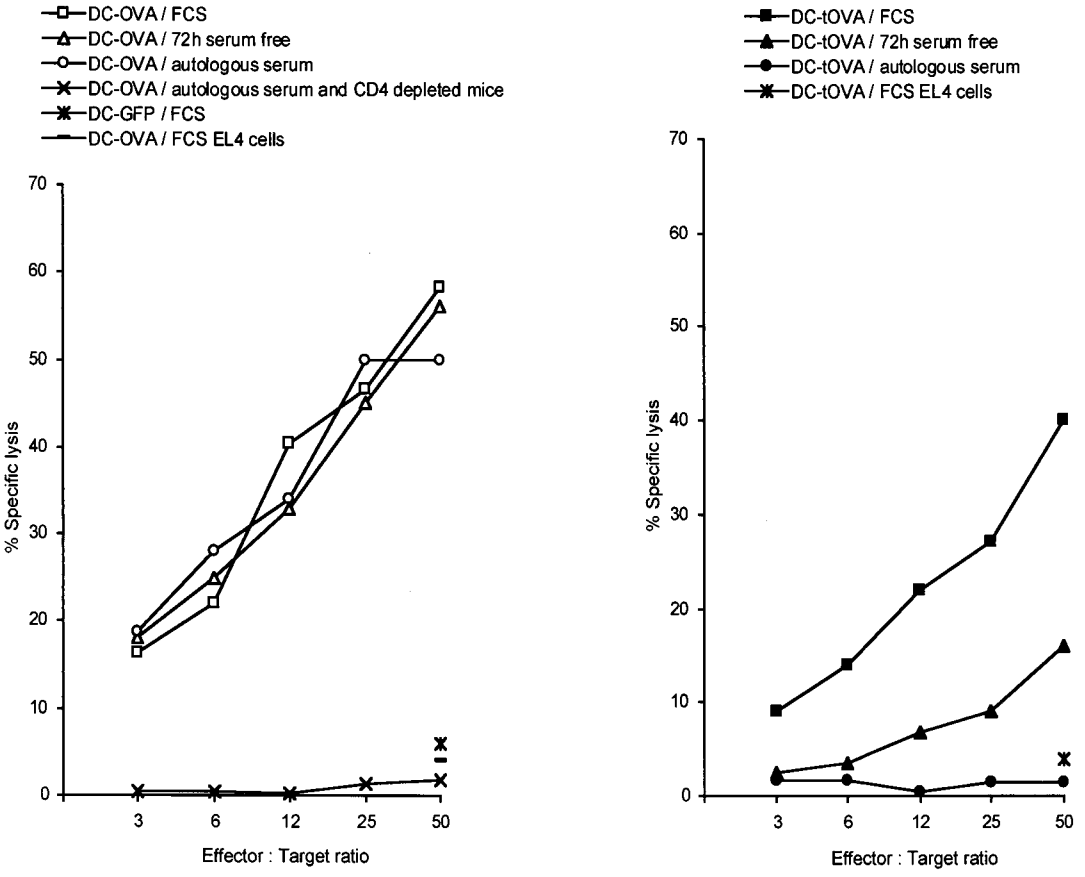

FIGURE 4. Lysis of E.G7-OVA cells by CTLs induced by immunization with transduced DCs. Retrovirally transduced DCs (OVA, open symbols, *left*; truncated OVA, closed symbols, *right*) were cultured in the presence of FCS until the day of injection (squares), cultured in Opti-MEM medium for 72 h before injection (triangles), or cultured in autologous serum during the in vitro expansion and differentiation (circles). The cells were extensively washed in PBS before i.v. injection. Splenocytes from immunized mice were harvested, and the APCs were depleted by plastic adherence and restimulated with irradiated E.G7-OVA cells in the presence of 1% autologous mouse serum for 6 days. CTLs were incubated in graded numbers with ^{51}Cr-labeled target cells for 4 h at 37°C in a 5% CO_2 humidified incubator. The data shown are the mean of five independent experiments. No CTLs could be induced by immunization with OVA-transduced DCs in $CD4^+$ T cell-depleted mice (x, mean of two experiments). No CTL were induced in mice immunized with GFP-transduced DC (j). The lysis was specifically directed against OVA epitopes, since the EL4 cell line was not lysed by splenocytes from mice immunized with OVA-transduced DCs (−).

truncated OVA will therefore enter the proteasome degradation pathway, thus generating peptides that will be loaded onto the MHC class I proteins. The fact that indeed OVA was no longer secreted after deletion of the signal sequence was proven by the fact that we were unable to detect OVA protein in the supernatant of tOVA-transduced DCs.

We show that retroviral transduction of DCs does not influence their capacity to be potent stimulators in an allo-MLR compared with non- or mock-transduced DCs, nor did it influence the expression of DC-specific markers. We enhanced the expression of retroviral receptors and cellular division of lineage-bone marrow-derived progenitor cells by incubating the cells with FLT3-L, granulocyte-macrophage CSF, and IL-4. Kotani et al. (32) have reported that centrifugation of retroviral vector supernatant onto the target cells increased the gene transfer efficiency 4- to 18-fold in NIH-3T3 fibroblasts and 3-fold in HUT78 cells. This in combination with the enhanced proliferation of the cells by using FLT3-L resulted in a transduction efficiency of a mean of 74%, ranging from 52–86%, as shown with the reporter gene GFP when DCs were cultured in 5% FCS. Transduction of DCs cultured in NMS resulted in lower transduction efficiencies, ranging from 40–75%. This approach offers an advantage to the method described by Specht et al. (23), where bone marrow cells are transduced in co-culture with the producer line, thus avoiding any potential contamination with the packaging cells.

The two versions of the same Ag, whole OVA and a truncated form of OVA, allowed us to study the requirement of $CD4^+$ T cell help for the in vivo priming of CTLs. The CTL response elicited by MFG-tOVA-transduced DCs was enhanced when the DCs were cultured in the presence of FCS, indicating that significant Th cell responses are generated in vivo because of the exposure of DCs to FCS during the in vitro expansion and differentiation. This finding has major implications for the design of human trials using DCs, in which the use of FCS should be avoided. The nonspecific help derived from FCS components was only partially reduced by culturing the DCs for 72 h before injection in serum-free medium. When the DCs had never been exposed to FCS but instead were generated in the presence of autologous mouse serum, no CTLs were primed in vivo. In contrast, presentation of the antigenic peptides in the context of both MHC class I and class II by the MFG-OVA-transduced DCs cultured in the absence of FCS resulted in a strong CTL response. This response was absent in $CD4^+$ T cell-depleted B6 mice. This indicates that a washout period of 72 h is not sufficient to empty all the preformed MHC class II-peptide complexes and that the OVA-specific CTL response is $CD4^+$ T cell dependent. These data are in agreement with the findings that

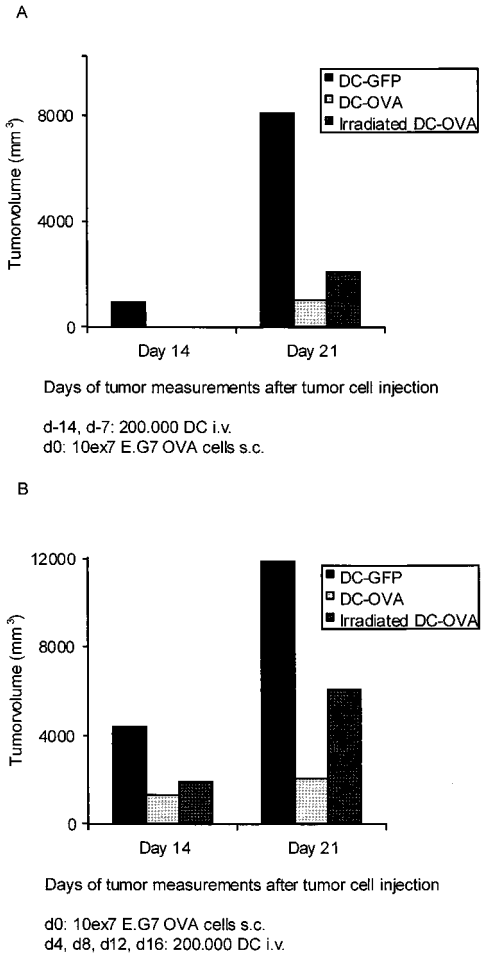

FIGURE 5. Induction of antitumor immunity (*A*) and therapeutic effect (*B*) in mice immunized with bone marrow-derived DCs transduced with either GFP or OVA. *A*, Mice were immunized with 2×10^5 transduced DCs by tail vein injection 14 and 7 days before s.c. injection of 10^7 E.G7-OVA tumor cells. Tumor volumes on days 14 and 21 post-tumor injection are indicated. *B*, Mice were injected with 5×10^6 tumor cells on day 0 and were treated with transduced DCs on days 4, 8, 12, and 16. Tumor volumes on days 14 and 21 are indicated.

the (serum) proteins endocytosed by the immature DCs may persist for a long time and are presented when the cells mature. These mature DCs have long-lived MHC class II/peptide complexes on their surface (35, 36). We want to underline the importance of our methodology, i.e., using DCs generated and transduced in the absence of any foreign Ag, in particular fetal bovine protein. Indeed, a prerequisite for translating the promising results obtained in experimental animal models to human studies is the ability to induce T cell responses with DCs that have not been exposed to bovine Ag.

These results confirm the idea that in vivo priming of CTLs requires the interaction of APCs, $CD4^+$, and $CD8^+$ T cells. There are many examples of $CD4^+$ T cell-dependent CTL responses (16, 17). Animal models of tumor and viral immunity have shown that the generation and persistence of a CTL response are dependent upon the presence of a $CD4^+$ Th cell response. Recently, Bennett et al. (14) clearly demonstrated that the induction of a CTL response by cross-priming requires cognate $CD4^+$ T cell help. Moreover, their experiments indicated that the $CD4^+$ and $CD8^+$ T cells need to recognize Ag on the same APC. They further suggested that the APC-$CD4^+$ T cell interaction in some way alters the APC function so that they become superactivated and stimulatory for $CD8^+$ T cells. This interaction, as first suggested by Grueder and Matzinger (15), involves the CD40-CD40 ligand molecules expressed on DCs and T cells (37–39). In line with this study, Ossendorp et al. (18) showed that specific Th cells are required for optimal induction of CTL against MHC class II-negative tumors.

A single immunization of no more than 10^5 genetically modified DCs resulted in high and transgene-specific tumor cell lysis. We successfully used these transduced DCs in the vaccination of naive animals that were protected against a subsequent tumor challenge after receiving two i.v. injections of no more than 2×10^5 DCs. We also significantly suppressed the tumor growth in tumor-bearing animals by treating them with DCs transduced with the native form of OVA. Radiation of the DCs had no influence when we used a tumor dose of $<10^7$ E.G7 OVA cell/mouse. Radiation of DCs was performed in the context of using this approach in the treatment of human malignancies. However, it is known that radiation of cells renders them apoptotic, thus reducing the time of Ag presentation in vivo. This was clearly only important when we injected the mice with high tumor doses (10^7 E.G7 OVA cell/mouse). However, when we transduced the DCs with the native OVA and tested the presence of OVA peptide in the context of MHC class I or class II molecules, we observed no difference between irradiated and nonirradiated DCs (data not shown). Thus, it is clearly not a matter of a reduction in presentation as a consequence of the irradiation but, rather, of a decrease in DC viability and duration of presentation in vivo.

Cells that eventually grew out of the tumor were tumor Ag-negative variants, as shown by an OVA-specific RT-PCR. This finding could be interpreted as either an indication of the effectiveness of the immunotherapy or as an important mechanism of tumor escape. Further studies are needed to analyze the underlying escape mechanism.

Our current and future efforts are focused on the presentation of the tumor-derived antigenic peptides in the context of both MHC class I and class II by specifically targeting the Ag to the endosomal/lysosomal pathway and/or to enhance the cytosolic degradation. The efficient presentation of tumor Ags in the context of both MHC class I and class II molecules should lead to more powerful immune responses. Strong $CD4^+$ Th cell function is desirable to induce tumor-specific $CD8^+$ effector T cells and long term immune memory.

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