

# Dendritic Cells Pulsed with RNA are Potent Antigen-presenting Cells In Vitro and In Vivo

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## Summary

Immunization with defined tumor antigens is currently limited to a small number of cancers where candidates for tumor rejection antigens have been identified. In this study we investigated whether pulsing dendritic cells (DC) with tumor-derived RNA is an effective way to induce CTL and tumor immunity. DC pulsed with *in vitro* synthesized chicken ovalbumin (OVA) RNA were more effective than OVA peptide-pulsed DC in stimulating primary, OVA-specific CTL responses *in vitro*. DC pulsed with unfractionated RNA (total or polyA<sup>+</sup>) from OVA-expressing tumor cells were as effective as DC pulsed with OVA peptide at stimulating CTL responses. Induction of OVA-specific CTL was abrogated when polyA<sup>+</sup> RNA from OVA-expressing cells was treated with an OVA-specific antisense oligodeoxynucleotide and RNase H, showing that sensitization of DC was indeed mediated by OVA RNA. Mice vaccinated with DC pulsed with RNA from OVA-expressing tumor cells were protected against a challenge with OVA-expressing tumor cells. In the poorly immunogenic, highly metastatic, B16/F10.9 tumor model a dramatic reduction in lung metastases was observed in mice vaccinated with DC pulsed with tumor-derived RNA (total or polyA<sup>+</sup>, but not polyA<sup>-</sup> RNA). The finding that RNA transcribed *in vitro* from cDNA cloned in a bacterial plasmid was highly effective in sensitizing DC shows that amplification of the antigenic content from a small number of tumor cells is feasible, thus expanding the potential use of RNA-pulsed DC-based vaccines for patients bearing very small, possibly microscopic, tumors.

The dendritic cell (DC)<sup>1</sup> network is a specialized system for presenting Ag to naive or quiescent T cells, and consequently plays a central role in the induction of T cell and B cell immunity *in vivo* (1). Consistent with this notion, several studies have documented the exceptional ability of DC to stimulate naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells *in vitro* and *in vivo* (1). Immunization using DC loaded with tumor antigens may therefore represent a potentially powerful method of inducing antitumor immunity. Indeed, recent studies have shown that vaccination with dendritic cells pulsed with specific antigens in the form of protein (2–5) or peptide (5–9) were capable of priming CTL in mice and engendering tumor immunity.

Immunization with defined tumor antigens is currently limited to a small number of cancers in which candidates for tumor rejection antigens have been identified (10, 11). Furthermore, it is unclear whether or which of the recently identified human tumor-specific or tumor-associated anti-

gens are the best choice to mount an effective anti-tumor immune response *in vivo*, an issue whose resolution must await clinical studies. This potential concern was underscored in a report by Anichini et al. who have shown that the majority of CTL present in HLA-A2.1 melanoma patients were not directed to the recently identified tumor antigens, Melan-A/Mart-1, tyrosinase, gp100, or MAGE-3 (12). This study suggested that immunization with other, yet unidentified, antigens would be more effective in eliciting tumor immunity in these patients. In addition, a recent study by Johnston et al. has demonstrated that improved immunogenicity of tumor cells engineered to express the B7-1 gene was caused by expansion of the antigenic repertoire of the tumor thereby implying that vaccination with multiple tumor antigens may be superior to using a single dominant epitope (13).

An alternative approach, not encumbered by these limitations, is to use unfractionated tumor peptides or tumor proteins as a source of tumor antigen. Two studies have shown that vaccination of mice with splenic antigen-presenting cells (APC) or with epidermal Langerhans cells pulsed with tumor fragments were capable of inducing protective immunity against a tumor challenge (14, 15). More recently, Zitvogel et al. have shown that vaccination of mice with bone marrow-derived DC pulsed with un-

<sup>1</sup>Abbreviations used in this paper: CEA, carcinoembryonic antigen; DC, dendritic cells; GT, guanidinium isothiocyanate; HAAT, human alpha-1-antitrypsin; IVT, *in vitro* transcribed; RT, room temperature.

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fractionated tumor peptides was capable of reducing the growth of subcutaneously established, weakly immunogenic tumors (16).

There are, however, three potential drawbacks in vaccinating cancer patients with unfractionated tumor derived peptides or proteins, as compared to using purified antigens. First, immunization with unfractionated tumor material may be less effective due to the low concentration of effective tumor antigens in the mixture, a possibility which has not yet been evaluated. Second, use of unfractionated tumor material as a source of tumor antigen will depend on the availability of substantial amounts of tumor tissue from the patient. This is a serious limitation of a practical nature because a large segment of cancer patients have only small, often microscopic tumor burdens. A third potential drawback is that vaccinating with unfractionated tumor derived antigens could induce autoimmune responses directed against "self" antigens (17, 18).

One approach to overcome the drawbacks stemming from the use of unfractionated tumor antigens is to use messenger RNA (mRNA) from tumor cells as a source of antigen. mRNA can be amplified from a very small number of cells, permitting the generation of sufficient amounts of antigen from microscopic amounts of tumor tissue. Moreover, tumor-specific RNA can be enriched by subtractive hybridization with RNA from normal tissue. This will increase the concentration of the relevant tumor-specific antigen(s) and hence the potency of the vaccine. More importantly, this will reduce the concentration of non-tumor specific antigens or possibly self-antigens and lessen the potential for autoimmunity.

Recent studies by Martinon et al. have demonstrated priming of virus-specific CTL in mice immunized with liposome encapsulated *in vitro* synthesized RNA corresponding to the influenza nucleoprotein (19). Conry et al. were able to elicit antibodies in mice after intramuscular injection of *in vitro* synthesized RNA corresponding to the human carcinoembryonic antigen (CEA) (20). Most recently, Qui et al. have used a gene gun to deliver human alpha-1-antitrypsin (HAAT) mRNA into the epidermis of mice to elicit an anti-HAAT antibody response (21).

In the current study we tested whether pulsing dendritic cells with RNA is an effective way to induce CTL responses and tumor immunity. We used two very stringent experimental systems to assess the antigen presenting function of DC pulsed with RNA; induction of a primary CTL response *in vitro* and regression of spontaneous lung metastases *in vivo*.

There have been few reports of primary CTL induction *in vitro* and these were achieved only by using professional APC such as dendritic cells (22, 23), or non-professional APC engineered to display high densities of specific peptide epitopes on the cell surface (24, 25). Induction of tumor immunity was investigated in the B16/F10.9 melanoma tumor system which was developed by Porgador and his colleagues and constitutes a highly relevant model to evaluate tumor vaccines (26). In this particular model one measures the efficacy of various vaccine strategies to elimi-

nate preexisting lung metastases in animals whose primary tumor was surgically removed. The only treatment that has shown significant therapeutic benefit in this model was vaccination with B16/F10.9 cells transduced with both the IL-2 and H-2K<sup>b</sup> genes (27). As is the case for most human tumors, no tumor-specific antigens have been identified in the B16/F10.9 system.

In this study we show that DC pulsed with RNA are highly potent APC, capable of stimulating primary CTL responses *in vitro* which were equal to or more efficient than those elicited by peptide pulsed DC. Moreover, treatment of tumor bearing mice with DC pulsed with tumor-derived RNA led to a dramatic reduction in the extent of lung metastasis.

## Materials and Methods

### Mice

7–8-wk-old C57BL/6 mice (H-2<sup>b</sup>) were obtained from the Jackson Laboratory (Bar Harbor, ME). In conducting the research described in this paper, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as proposed by the committee on care of Laboratory Animal Resources Commission on Life Sciences, National Research Council. The facilities at the Duke vivarium are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

### Cell Lines

The F10.9 clone of the B16 melanoma of C57BL/6 origin is a highly metastatic, poorly immunogenic and a low class I expressing cell line. F10.9/K1 is a poorly metastatic and highly immunogenic cell line derived by transfecting F10.9 cells with H-2K<sup>b</sup> cDNA (26). RMA and RMA-S cells are derived from the Rauscher leukemia virus-induced T cell lymphoma RBL-5 of C57BL/6 (H-2<sup>b</sup>) origin (28). Other cell lines used were EL4 (C57BL/6, H-2<sup>b</sup>, thymoma), E.G7-OVA (EL4 cells transfected with the cDNA of chicken ovalbumin (OVA) (29)). Cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 25 mM Hepes, 2 mM L-glutamine, and 1 mM sodium pyruvate. E.G7-OVA cells were maintained in medium supplemented with 400 µg/ml G418 (GIBCO BRL, Gaithersburg, MD) and F10.9/K1 cells were maintained in medium containing 800 µg/ml G418.

### Antigen-presenting Cells (APC) and Responder T Cells

Splenocytes obtained from naive C57BL/6 females were treated with ammonium chloride Tris buffer for 3 min at 37°C to deplete red blood cells. Splenocytes (3 ml) at  $2 \times 10^7$  cells/ml were layered over 2 ml metrizamide gradient column (Nycomed Pharma AS, Oslo, Norway; analytical grade, 14.5 g added to 100 ml PBS, pH 7.0) and centrifuged at 600 g for 10 min. The DC-enriched fraction from the interface was further enriched by adherence for 90 min. Adherent cells (mostly DC and a few contaminating Mø) were retrieved by gentle scraping and subjected to a second round of adherence at 37°C for 90 min to deplete the contaminating Mø. Non-adherent cells were pooled as splenic DC and FACS<sup>®</sup> analysis showed ~80–85% DC by staining with mAb 33D1, 1–2% Mø by staining with mAb F4/80, 10% T cells, and <5% B Cells (data not shown). The pellet was resuspended and enriched for Mø by two rounds of adherence at 37°C for 90 min each. More than 80% of the adherent population was identified as Mø by FACS<sup>®</sup> analysis with 5% lymphocytes and <5%

DC. B cells were separated from the nonadherent population (B and T cells) by panning on anti-Ig-coated plates. The separated cell population which was comprised of >80% T lymphocytes by FACS<sup>®</sup> analysis was used as responder T cells.

#### Isolation of Total and PolyA<sup>+</sup> Cellular RNA

Total RNA was isolated from actively growing tissue culture cells as previously described (30). Briefly, 10<sup>7</sup> cells were lysed in 1 ml of guanidinium isothiocyanate (GT) buffer (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7.0; 0.5% sarcosyl, 20 mM EDTA, 0.1 M 2-mercaptoethanol). Samples were vortexed followed by sequential addition of 100  $\mu$ l 3M sodium acetate, 1 ml water saturated phenol and 200  $\mu$ l chloroform/isoamyl alcohol (49:1). Suspensions were vortexed and placed on ice for 15 min. The tubes were centrifuged at 10,000 g, 4°C for 20 min and the supernatant was carefully transferred to a fresh tube. An equal volume of isopropanol was added and the samples were placed at -20°C for at least 1 h. RNA was pelleted by centrifugation as above. The pellet was resuspended in 300  $\mu$ l GT buffer which was then transferred to a microcentrifuge tube. RNA was reprecipitated by adding an equal volume of isopropanol and placing the tube at -20°C for at least 1 h. Tubes were microcentrifuged at high speed at 4°C for 20 min. Supernatants were decanted and pellets were washed once with 70% ethanol. Pellets were allowed to dry at RT and then resuspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). Possible contaminating DNA was removed by incubating RNA in 10 mM MgCl<sub>2</sub>, 1 mM DTT and 50 U/ml RNase free DNase (Boehringer-Mannheim, Indianapolis, IN) for 15 min at 37°C. The solution was adjusted to 10 mM Tris, 10 mM EDTA, 0.5% SDS and 1 mg/ml Pronase (Boehringer-Mannheim) followed by incubation at 37°C for 30 min. Samples were extracted once with phenol-chloroform and once with chloroform, and RNA was then reprecipitated in isopropanol at -20°C. After centrifugation the pellets were washed with 70% ethanol, air dried, and resuspended in sterile water. Total RNA was quantitated by measuring OD at 260 and 280 nm. OD 260/280 ratios were typically 1.65–2.0. RNA was stored at -70°C. PolyA<sup>+</sup> RNA was either isolated from total RNA using Oligotex (Qiagen, Chatsworth, CA) or directly from tissue culture cells using the Messenger RNA Isolation kit (Stratagene, La Jolla, CA) as per manufacturer's protocols.

#### Production of In Vitro Transcribed (IVT) RNA

Chicken ovalbumin cDNA in pUC18 was kindly provided by Dr. Barry T. Rouse (University of Tennessee, Knoxville). The 1.9-kb EcoR1 fragment containing the coding region and 3' untranslated region was cloned into the EcoR1 site of pGEM4Z (Promega, Madison, WI). Clones containing the insert in both the sense and anti-sense orientations were isolated and large scale plasmid preparations were made using Maxi Prep Kits (Qiagen). Plasmids were linearized with BamH1 for use as templates for in vitro transcription. Transcription was carried out at 37°C for 3–4 h using the SP6 MEGAscript In vitro Transcription Kit (Ambion, Austin, TX) per manufacturer's protocol and adjusting the GTP concentration to 1.5 mM and including 6 mM m<sup>7</sup>G(5')ppp(5')G cap analogue (Ambion). Template DNA was digested with RNase free DNase I and RNA was recovered by phenol/chloroform and chloroform extraction followed by isopropanol precipitation. RNA was pelleted by microcentrifugation and the pellet was washed once with 70% ethanol. The pellet was air-dried and resuspended in sterile water.

RNA was incubated for 30 min at 30°C in 20 mM Tris-HCl, pH 7.0, 50 mM KCl, 0.7 mM MnCl<sub>2</sub>, 0.2 mM EDTA, 100  $\mu$ g/ml acetylated BSA, 10% glycerol, 1 mM ATP and 5,000 U/ml

yeast poly(A) polymerase (United States Biochemical, Cleveland, OH). The capped, polyadenylated RNA was recovered by phenol/chloroform and chloroform extraction followed by isopropanol precipitation. RNA was pelleted by microcentrifugation and the pellet was washed once with 70% ethanol. The pellet was air-dried and resuspended in sterile water. RNA was quantitated by measuring OD at 260 and 280 nm and stored at -70°C.

#### Oligodeoxynucleotide Directed Cleavage of OVA mRNA by RNase H

The procedure used for RNase H site-specific cleavage of ovalbumin mRNA was essentially as previously described with minor modifications (31). Briefly, 5–10  $\mu$ g mRNA from E.G7-OVA cells was suspended in 20 mM Hepes, pH 8.0, 50 mM KCl, 4 mM MgCl<sub>2</sub>, 1 mM DTT, 50  $\mu$ g/ml BSA and 2  $\mu$ M of either the oligodeoxynucleotide 5'-CAG TTT TTC AAA GTT GAT TAT ACT-3' which hybridizes to a sequence in OVA mRNA encoding the CTL epitope SIINFEKL or 5'-TCA TAT TAG TTG AAA CTT TTT GAC-3' (Oligos, Etc., Wilsonville, OR) which serves as a negative control. The samples were heated to 50°C for 3 min followed by incubation at 37°C for 30 min. RNase H (Boehringer-Mannheim) was added at 10 U/ml and digestion was for 30 min at 37°C. RNA was recovered by phenol/chloroform and chloroform extraction followed by isopropanol precipitation. RNA was pelleted by microcentrifugation and the pellet was washed once with 70% ethanol. The pellet was air-dried and resuspended in sterile water. Cleavage of OVA mRNA was confirmed by oligo dT primed reverse transcription of test and control samples followed by PCR with OVA-specific primers which flank the cleavage site (data not shown). PCR with actin-specific primers was used to control between test and control samples.

#### Pulsing of Antigen-presenting Cells

Pulsing of DC with RNA was routinely performed in serum-free Opti-MEM medium (GIBCO BRL). APC were washed twice in Opti-MEM medium. Cells were resuspended in Opti-MEM medium at 2–5  $\times$  10<sup>6</sup> cells/ml and added to 15 ml polypropylene tubes (Falcon). The cationic lipid, DOTAP, (Boehringer Mannheim) was used to deliver RNA into cells (32). RNA (in 250–500  $\mu$ l Opti-MEM medium) and DOTAP (in 250–500  $\mu$ l Opti-MEM medium) was mixed in 12  $\times$  75-mm polystyrene tubes at room temperature (RT) for 20 min. The amount of polyA<sup>+</sup> RNA or IVT RNA used was 5  $\mu$ g and the amount of total RNA used was 25  $\mu$ g. The RNA to DOTAP ratio was 1:2. The complex was added to the APC (2–5  $\times$  10<sup>6</sup> cells) in a total volume of 2 ml and incubated at 37°C in a water-bath with occasional agitation for 2–4 h. The cells were washed and used as stimulators for primary CTL induction in vitro.

The synthetic peptide encoding the CTL epitope in chicken ovalbumin OVA, aa 257–264 SIINFEKL (H-2K<sup>b</sup>) (33) was used for peptide pulsing. Peptide was purchased with unblocked (free) amino and carboxyl ends from Research Genetics (Birmingham, AL). Peptides were dissolved in serum-free IMDM and stored at -20°C.

#### Induction of CTL In Vitro and Cytotoxicity Assay

T cells (5  $\times$  10<sup>6</sup> cells/ml) and RNA or peptide pulsed APC (2.5  $\times$  10<sup>5</sup> cells/ml) were cultured in IMDM with 10% FCS, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 5  $\times$  10<sup>-5</sup> M  $\beta$ -mercaptoethanol in 96-well U-bottom plates to give R/S ratio of 20:1. After 5 d the cells were used as effectors in a standard 4-h europium release assay.

5–10  $\times$  10<sup>6</sup> target cells were labeled with europium diethylene-triamine pentaacetate for 20 min at 4°C. After several washes, 10<sup>4</sup>

europium-labeled targets and serial dilutions of effector cells at various effector/target ratios were incubated in 200  $\mu$ l of RPMI 1640 with 10% heat-inactivated FCS in 96-well V-bottom plates. The plates were centrifuged at 500 g for 3 min and incubated at 37°C and 5% CO<sub>2</sub> for 4 h. 50  $\mu$ l of the supernatant was harvested and europium release was measured by time resolved fluorescence (Delta fluorometer; Wallac Inc., Gaithersburg, MD) (34). Spontaneous release was less than 25%. Standard errors (SE) of the means of triplicate cultures were less than 5%.

### Immunotherapy

**E.G7-OVA Model.** C57BL/6 mice were immunized once with irradiated, RNA-pulsed APC ( $2 \times 10^6$  cells/mouse) or  $5 \times 10^6$  E.G7-OVA or EL4 cells. 10–14 d post-immunization mice were challenged with  $2 \times 10^7$  live E.G7-OVA cells subcutaneously in the scapular region. Mice were monitored on a regular basis for tumor growth and size. Mice with tumor sizes  $>3.5$  cm were killed. All survivors were killed 40 d post-challenge.

**F10.9-B16 Melanoma Model.** Mice were injected intrafootpad with  $2 \times 10^5$  F10.9 cells. The post-surgical protocol was used as described previously with a few modifications (27). Legs were amputated when the local tumor in the footpad was 7–8 mm in diameter. Post-amputation mortality was less than 5%. 2 d post-amputation mice were immunized intraperitoneally followed by weekly vaccinations twice, for a total of three vaccinations. Mice were killed based on the metastatic death in the non-immunized or control groups (28–32 d post-amputation). Metastatic loads were assayed by weighing the lungs.

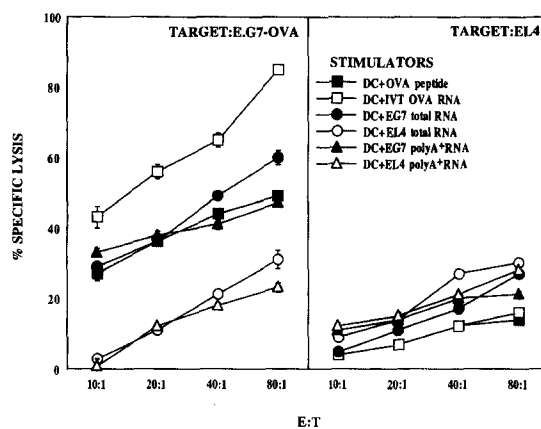
### Statistical Methods

In the B16 melanoma model, the different experimental groups within the study were compared using the Kruskal-Wallis test. Comparisons of significance for differences in lung weights between specific pairs of groups were then compared by the Mann-Whitney U-test. A probability of less than 0.05 ( $P < 0.05$ ) was used for statistical significance.

## Results

**Induction of Primary OVA-specific CTL Responses In Vitro Using Dendritic Cells Pulsed with Chicken Ovalbumin RNA.** Splenic DC derived from C57BL/6 (H-2K<sup>b</sup>) mice pulsed with RNA were tested for their ability to induce a primary CTL response in vitro in the E.G7-OVA tumor system. E.G7-OVA cells are derived from the EL4 tumor cell line (H-2K<sup>b</sup> haplotype) by transfection with the chicken ovalbumin cDNA (29). The chicken ovalbumin protein contains a single dominant epitope (amino acids 257–264) in C57BL/6 mice (33).

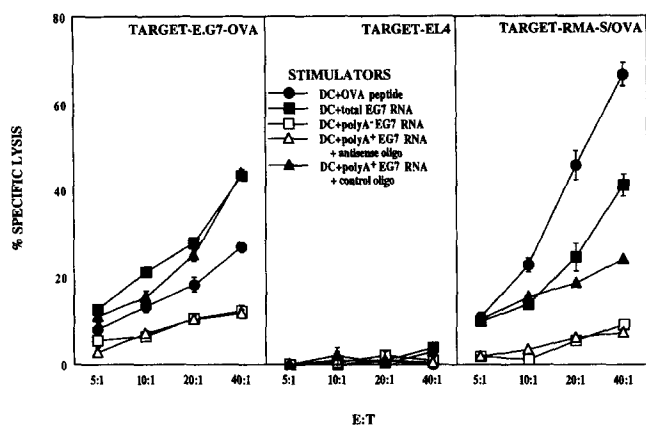
Our primary aim was to determine whether RNA could be used to enable DC to present antigen to CD8<sup>+</sup> T cells. Accordingly, splenic DC were isolated from C57BL/6 mice and pulsed with OVA peptide (amino acids 257–264) or with 5  $\mu$ g of ovalbumin RNA synthesized in vitro (IVT OVA RNA) from a plasmid encoding the chicken ovalbumin cDNA. Based on preliminary studies to optimize the ratio of RNA to lipid, a ratio of 1:2 was used in all experiments (data not shown). The pulsed DC were used to stimulate an OVA-specific, primary CTL response in vitro. As shown in Fig. 1, both OVA peptide and IVT OVA RNA pulsed DC were capable of inducing OVA-specific primary



**Figure 1.** Primary OVA-specific CTL induction in vitro with dendritic cells pulsed with RNA. DC were pulsed with total RNA or polyA<sup>+</sup> RNA isolated from E.G7-OVA or EL4 cells, or with IVT OVA RNA, in the presence of the cationic lipid DOTAP as described in Materials and Methods.  $2 \times 10^6$  DC were pulsed with either 25  $\mu$ g of total RNA or 5  $\mu$ g of polyA<sup>+</sup> RNA or IVT OVA RNA. DC pulsed with OVA peptide were used as positive controls. DC and naive T cells were incubated for 5 d at a R/S of 20:1. Viable lymphocytes were harvested and the CTL activity determined in a europium release assay. E.G7-OVA and EL4 cells were used as targets. This experiment was repeated three times with similar results.

CTL responses, although RNA pulsed DC were consistently more effective than peptide-pulsed DC (Fig. 1 and data not shown). To test whether RNA isolated from E.G7-OVA cells was capable of sensitizing DC to induce primary, OVA-specific CTL, total RNA or polyA<sup>+</sup> RNA was isolated from E.G7-OVA or EL4 cells and incubated with DC.  $2 \times 10^6$  DC were pulsed with either 25  $\mu$ g of total RNA or 5  $\mu$ g of polyA<sup>+</sup> RNA in the presence of DOTAP as described in Materials and Methods. As shown in Fig. 1, DC pulsed with either total or polyA<sup>+</sup> RNA from E.G7-OVA but not from EL4 cells were capable of inducing potent OVA-specific CTL response, comparable to that obtained with DC pulsed with the OVA peptide. Stimulation of a CTL response by (total or polyA<sup>+</sup>) EL4 RNA pulsed DC was low and similar on E.G7-OVA and EL4 targets (Fig. 1), reflecting the immunodominance of the OVA epitope and the relative weakness of the EL4-encoded antigens.

Fig. 2 demonstrates that total as well as polyA<sup>+</sup>, but not polyA<sup>-</sup>, RNA isolated from E.G7-OVA cells is capable of sensitizing DC to stimulate a primary CTL response. To prove that sensitization of DC is indeed mediated by RNA encoding chicken OVA, polyA<sup>+</sup> RNA from E.G7-OVA cells was incubated with either an antisense oligonucleotide spanning the sequence encoding the known H-2K<sup>b</sup> restricted CTL epitope present in the chicken ovalbumin gene or with a control oligodeoxynucleotide, followed by RNase H treatment to remove any RNA sequence to which the oligodeoxynucleotide probe had hybridized. As shown in Fig. 2, induction of primary, OVA-specific CTL responses was abolished when the polyA<sup>+</sup> RNA was incubated with the antisense, but not with the control, oligode-

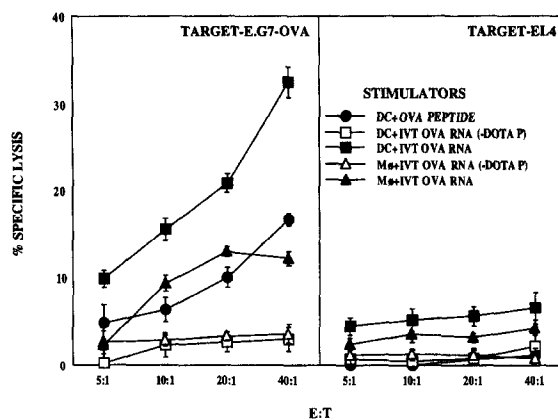


**Figure 2.** Sensitization of total E.G7-OVA RNA pulsed DC for stimulation of OVA-specific CTL responses is mediated by the polyA<sup>+</sup> OVA-specific RNA. DC were pulsed with total RNA, polyA<sup>-</sup> RNA or polyA<sup>+</sup> RNA in the presence of DOTAP and cultured with naive T cells in 96-well U-bottom plates for 5 d. The polyA<sup>+</sup> RNA fraction from E.G7-OVA cells was treated with an antisense oligodeoxynucleotide corresponding to the CTL epitope encoding region of the OVA gene or a control oligodeoxynucleotide followed by RNase H treatment to eliminate the hybridized RNA. DC pulsed with OVA peptide was used to compare the efficacy of the various groups. E.G7-OVA, EL4, and RMA-S cells pulsed with OVA peptide were used as targets.

oxynucleotide. Generation of OVA-specific CTL responses was tested on E.G7-OVA cells, endogenously expressing the ovalbumin gene, and RMA-S cells pulsed with the dominant OVA CTL epitope. Fig. 2 demonstrates that CTL generated by DC pulsed with total or polyA<sup>+</sup> E.G7-OVA RNA lysed both targets to a similar extent. This indicates that the epitope presented by E.G7-OVA RNA pulsed DC corresponds to the previously defined single dominant CTL epitope encoded in the chicken ovalbumin gene. However, DC pulsed with peptide-stimulated CTL lysed the RMA-S/OVA peptide targets more efficiently than the E.G7-OVA targets, suggesting that the CTL generated by DC pulsed with the OVA peptide may have been of lower affinity than CTL generated by RNA-pulsed DC, consistent with the findings of Carbone et al. (35).

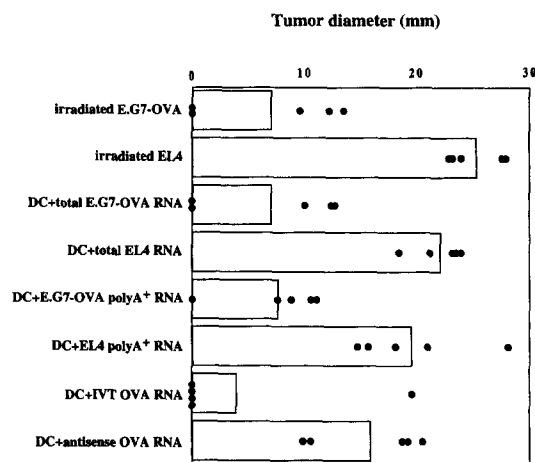
Fig. 3 shows that DC pulsed with IVT OVA RNA are more potent than macrophages (Mø) pulsed with IVT OVA RNA at stimulating primary CTL responses. Fig. 3 also demonstrates the requirement for DOTAP in facilitating sensitization of DC with RNA.

**Induction of Anti-Tumor Immunity by DC Pulsed with Tumor RNA.** We next tested whether vaccination of mice with OVA RNA pulsed DC was capable of providing protection against E.G7-OVA tumor cells. Mice were immunized once with  $2 \times 10^6$  RNA-pulsed DC or with  $5 \times 10^6$  irradiated E.G7-OVA cells and 10 d later, mice were challenged with a tumorigenic dose of E.G7-OVA cells. Appearance and size of the tumor were determined on a regular basis. Fig. 4 shows the size of the tumors 37 d post-tumor implantation. The average tumor size in mice immunized with irradiated EL4 cells was 25 cm while the average tu-



**Figure 3.** RNA pulsed DC are more effective than Mø at stimulating primary, OVA-specific CTL responses in vitro. DC or Mø were pulsed with IVT OVA RNA in the presence or absence of the cationic lipid, DOTAP. Naive T cells were cultured with pulsed APC for 5 d and the viable cells harvested for a CTL assay. E.G7-OVA and EL4 were used as targets.

mor size in animals immunized with the OVA expressing EL4 cells (E.G7-OVA) was only 7.03 cm. Vaccination with  $2 \times 10^6$  DC pulsed with RNA (total or polyA<sup>+</sup> fraction) derived from E.G7-OVA cells was as effective as vaccination with  $5 \times 10^6$  of the highly immunogenic E.G7-OVA cells (average tumor size, 7 cm). Vaccination with DC pulsed with total or polyA<sup>+</sup> RNA derived from EL4 tumor cells had a slight protective effect (average tumor size: 22 cm and 19.5 cm, respectively) which was statistically insignificant, consistent with poor to undetectable im-



**Figure 4.** A single immunization with DC pulsed with OVA RNA induces anti-tumor immunity. DC were pulsed with either total or polyA<sup>+</sup> RNA from E.G7-OVA or EL4 cells, or with IVT OVA RNA or control IVT antisense OVA RNA in the presence of DOTAP. Mice were immunized with  $2 \times 10^6$  DC or  $5 \times 10^6$  irradiated E.G7-OVA or EL4 cells intraperitoneally followed by a challenge with  $2 \times 10^7$  E.G7-OVA cells. Columns represent mean tumor diameter (5 mice/group) and dots represent individual measurements. Mice were periodically examined for tumor growth and were killed when the tumor diameter reached 3–4 cm. All mice were killed 35–40 d post-challenge.

munogenicity of EL4-derived antigens. Consistent with the primary CTL induction data (Fig. 1), vaccination of mice with IVT OVA RNA pulsed DC provided the most effective anti tumor response (average tumor size, 3.9 cm), while vaccination with the control antisense IVT OVA RNA did not elicit a significant protective response.

The potency of DC pulsed with tumor-derived RNA was further evaluated in the B16/F10.9 (H-2<sup>b</sup>) melanoma metastases model. The B16/F10.9 melanoma tumor is poorly immunogenic, expresses low levels of MHC class I molecules and is highly metastatic in both experimental and spontaneous metastasis assay systems (26, 27). Porgador et al. have shown that when vaccinations are carried out after the removal of the primary tumor implant, only irradiated tumor cells transduced with both the IL-2 and the H-2K<sup>b</sup> genes were capable of significantly impacting the metastatic spread of B16/F10.9 tumor cells in the lung (26). Thus, the B16/F10.9 melanoma model and the experimental design used by Porgador and coworkers constitutes a stringent and clinically relevant experimental system to assess the efficacy of vaccination strategies for metastatic cancer.

To test whether immunization with tumor RNA-pulsed DC was capable of causing the regression of preexisting lung metastases, primary tumors were induced by implantation of B16/F10.9 tumor cells in the footpad. When the footpad reached 5.5–7.5 mm in diameter, the tumors were surgically removed and 2 d later mice were immunized with irradiated B16/F10.9 cells, irradiated B16/F10.9 cells

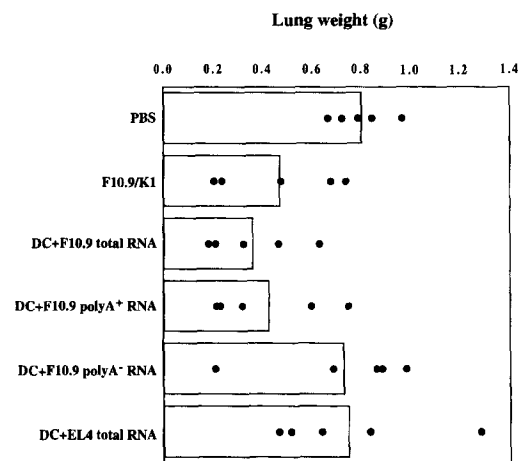
transduced with the H-2K<sup>b</sup> gene (F10.9/K1), or with RNA-pulsed DC preparations (Fig. 5). The mice received a total of three vaccinations given at weekly intervals. The average lung weight of a normal mouse is 0.18–0.22 g. Mice immunized with PBS were overwhelmed with metastases. The mean lung weight of mice in this treatment group was 0.81 g, about three quarters of the weight contributed by the metastases which were too many to count (>100 nodules). A similar metastatic load was seen in animals treated with irradiated B16/F10.9 cells (data not shown), which confirms numerous previous observations that treatment with irradiated B16/F10.9 tumor cells alone has no therapeutic benefit in this tumor model (25, 26). As also previously shown, immunization with H-2K<sup>b</sup>-expressing B16/F10.9 cells (F10.9/K1) had a modest therapeutic benefit, as indicated by a statistically significant decrease in the average lung weight of the animals in this treatment group. A dramatic response was however seen in animals treated with DC which were pulsed with total RNA derived from F10.9 cells. The mean lung weight of mice in this treatment group was 0.37 g. A significant, though somewhat less dramatic response was seen in mice treated with DC pulsed with polyA<sup>+</sup> RNA derived from F10.9 cells (average lung weight, 0.42 g). On the other hand, no statistically significant decrease in metastatic load was seen in mice treated with DC pulsed with either the polyA<sup>-</sup> RNA fraction derived from F10.9 cells or with total RNA isolated from EL4 tumor cells.

## Discussion

In this study we have shown that incubation of murine DC with RNA is an efficient way to introduce antigens into the MHC class I presentation pathway. To confirm that the active component in the preparation was indeed RNA we used a standard procedure for RNA isolation and included extensive incubation with pronase and DNase. Second, the activity was enriched in the polyA<sup>+</sup> RNA fraction (Figs. 2 and 5). Third, in vitro synthesized RNA was highly effective (Figs. 1, 3, and 4). Finally, induction of OVA-specific CTL was specifically eliminated by incubation with a complementary oligodeoxynucleotide and treatment with RNase H (Fig. 2).

RNA pulsed DC stimulated cytotoxic T cells capable of lysing target cells expressing endogenously processed antigen (E.G7-OVA) and cells pulsed with OVA peptide (RMA-S/OVA-peptide) (Figs. 2 and 5). These observations strongly suggest that RNA taken up by APC is translated and the resulting protein is processed and presented via the class I antigen presentation pathway.

We used two stringent experimental systems to assess the antigen-presenting ability of DC incubated with RNA; induction of primary CTL responses in vitro and regression of lung metastases in vivo. DC pulsed with in vitro synthesized OVA-specific RNA were capable of stimulating a primary CTL response in vitro and were reproducibly more effective than DC pulsed with OVA peptide (Figs. 1



**Figure 5.** Regression of lung metastases in mice treated with DC pulsed with F10.9 tumor RNA. F10.9 tumors established in the footpads of C57BL/6 mice were amputated when they reached 5.5–7.5 mm in diameter. 2 d post-amputation, and weekly thereafter, mice were vaccinated intraperitoneally for a total of three times. Mice were killed 28–32 d post-amputation and metastatic loads were determined by measuring lung weights. Columns represent mean lung weight and dots represent individual lung weights (5 mice/group). The results are representative of three different experiments. For more details see Materials and Methods. Relative to the control PBS immunized group, *P* values were 0.056, 0.008, 0.032, 0.841, and 0.421 for F10.9/K1, DC+F10.9 total RNA, DC+F10.9 polyA<sup>+</sup> RNA, DC+F10.9 polyA<sup>-</sup> RNA, and DC+EL4 total RNA immunized mice, respectively. The overall significance of the study as determined by the Kruskal-Wallis test is *P* = 0.034.

and 3). Preliminary results indicate that human precursor-derived DC incubated with RNA transcribed *in vitro* from carcinoembryonic antigen (CEA) cDNA are more potent than DC pulsed with a CEA-specific peptide in stimulating primary CTL *in vitro* (Nair, S.M., and E. Gilboa, unpublished data). A possible reason why RNA-mediated sensitization of DC is more effective than peptide-pulsed DC could be that the transfected RNA serves as a continuous source for the generation of antigenic peptides. The potency of RNA mediated sensitization of DC was further illustrated by the fact that DC pulsed with unfractionated cellular RNA (total or polyA<sup>+</sup>) isolated from OVA-expressing cells were capable of stimulating OVA-specific CTL responses, which were as effective as DC pulsed with OVA peptide (Fig. 1). Immunization of mice with RNA-loaded DC elicited a potent anti-tumor response (Figs. 4 and 5). As shown in Fig. 5, in the poorly immunogenic B16/F10.9 melanoma model, DC incubated with F10.9

tumor RNA were effective at reducing lung metastases. This is especially remarkable in view of the fact that many treatments in this clinically relevant tumor model have failed or were only marginally effective (27).

A potentially unique advantage of using RNA rather than protein or peptides as source of unfractionated tumor Ag is that sufficient amounts of antigen can be generated from very small amounts of tumor tissue using PCR amplification techniques. Moreover, if autoimmunity becomes a problem (17, 18), subtractive hybridization can reduce the contribution of non tumor-specific antigens. The observation that RNA transcribed *in vitro* from cDNA cloned in a bacterial plasmid is effective in sensitizing DC (Figs. 1, 3, and 4) allows the amplification of the antigenic content of a small number of tumor cells thus increasing the applicability of RNA-based vaccines to patients with low tumor burdens.

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