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Antigens

PRINCIPAL INVESTIGATOR: Eli Gilboa, Ph.D.

CONTRACTING ORGANIZATION: Duke University
Durham, North Carolina 27710

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Duke University Durham, North Carolina 27710 E-Mail: e.gilboa@cgct.duke.edu		8. PERFORMING ORGANIZATION REPORT NUMBER	
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13. ABSTRACT (Maximum 200 Words) The focus of this grant is to develop a vaccination strategy for patients with metastatic prostate cancer using DC-based vaccines loaded with normal prostate tissue-derived antigens. Having established a TRAMP colony, protocols for measuring diseases progression in the TRAMP mice we encountered initially two setbacks: The TRAMP colony was severely decimated due to viral infection and generation of dendritic cells was compromised due to lack of access to a critical reagent, GM-CSF. In response to these setbacks we have regenerated the TRAMP colony and developed a GM-CSF producing cell line as a source of GM-CSF. Furthermore we have improved murine DC generation by elimination exposure to foreign substances (antigens)-by replacing FCS with autologous murine serum in a new media, HB104-a significant advance in murine DC culture. We also developed a novel and apparently powerfull vaccination strategy consisting of immunizing mice intradermally with lipid complexed RNA. Two large experiments in the TRAMP model are currently underway-to be completed and analyzed March-April-evaluating the use of normal prostate antigens as well as p53, a prototype for a specific tumor antigen. Successful demonstration of protective immunity in TRAMP mice will trigger preclinical studies in human prostate cancer followed by clinical trials in cancer patients.			
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FOREWORD

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INTRODUCTION

The focus of this grant is to develop a vaccination strategy for patients with metastatic prostate cancer using DC-based vaccines loaded with normal prostate tissue-derived antigens. The key advantage of using normal prostate antigens, as opposed to using tumor-specific antigens, is that it will provide a universal and unlimited source of antigens for vaccination, and in combination with dendritic cells offers a powerful vaccination strategy that will be readily available to all patients with prostate cancer. The hypothesis underlying this, arguably unorthodox, proposal is that it will be possible to induce potent anti tumor immunity in the absence of clinical autoimmunity. While the hypothesis is supported by theoretical considerations and experimental evidence (discussed in the proposal) the primary objective of the studies proposed in this application is to test this hypothesis in a highly relevant animal model for metastatic prostate cancer. The studies described in the grant incorporate our extensive experience in DC vaccine development, including unique methods developed in our laboratory to augment the loading of DC with peptide antigens and the discovery that tumor RNA pulsed DC are highly effective stimulators of T cells and tumor immunity.

The specific goal of this project is to demonstrate in a murine model for prostate cancer that treatment of tumor bearing animals with syngeneic DC loaded with antigens isolated from prostatic tissue is capable of causing the regression or significant delay of metastatic spread in the treated animal without causing harmful autoimmune manifestations. If successful, these studies will set the stage for clinical trials using autologous DC pulsed with normal prostate antigens in patients with prostate cancer at high risk of relapse. The specific objectives of this proposal are:

- (1). To establish and calibrate the TRAMP murine model for prostate cancer.
- (2). To evaluate two promising vaccination strategies in the TRAMP model that were developed in our laboratory.

A. BODY

1. Establishment of the TRAMP model.

The vaccination strategy described in this application will be evaluated in the murine TRAMP model (1). TRAMP is a transgenic line of C57BL/6 mice that develops histologic prostatic intraepithelial neoplasia by 18-22 weeks of age that progresses to adenocarcinoma with metastasis by 24-30 weeks of age. The TRAMP model is arguably the best murine model for prostate cancer as it closely resembles the human disease. The unique feature and special relevance of this animal model for our studies is that we will evaluate the impact of vaccination on autochthonous disease. We have established a colony of TRAMP mice from a breeding pair provided by Dr. Greenberg. We have previously determined the course of disease in our colony which progressed at a rate similar but not identical to what was described by Dr. Greenberg and his colleagues. We have also:

1. In consultation with Dr. Greenberg and his colleagues developed the methodology for measuring and quantitating metastatic spread in the animal.
2. Developed the methodology of surgically removing prostates and have collected >50 normal prostates for antigen preparation.
3. Have calibrated the TRAMP C-1 (C-1) tumor model-determined a dose response curve in C57BL/6 mice, as a prerequisite for the immunotherapy studies. In our hands, C-1 tumors grew very slowly and somewhat erratically. We found that implantation in the ear and use of matrigel improves the efficiency of take, rate of tumor progression and constancy.

In the course of our studies we have encountered considerable delays in establishing the TRAMP colony due to an outbreak of viral infection in the animal facility. The virus was not characterized and was manifested as loss of male fertility (Breeding pairs consist of a male TRAMP and wild type C57BL/6 females of which 25% of offspring are male TRAMP). Dr. Greenberg, the originator of the TRAMP mouse has experienced similar problems (personal communication). After two futile attempts, we were able to rescue two fertile males, establish breeding colonies and regenerate a large cohort of animals for experimentation (see below). Regrettably, this has considerably delayed our progress but, as will be described below, we were able to “catch up” with the main objectives of this research project.

2. Vaccination with DC loaded with prostate antigens

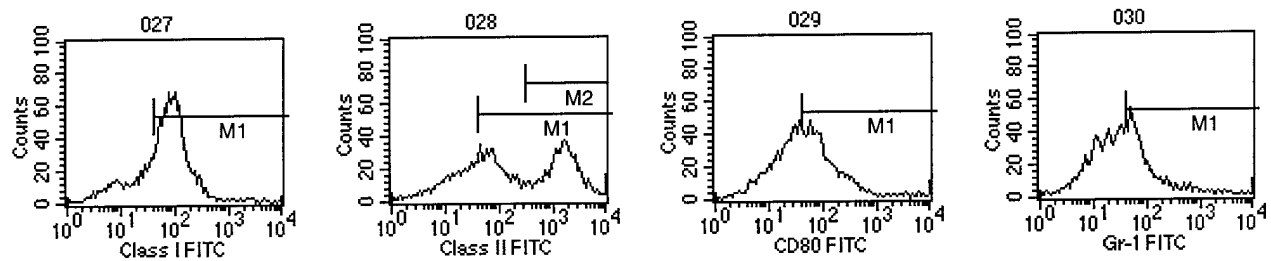
The purpose of these studies was to establish and optimize immunization protocols with DC loaded with antigens isolated from normal prostates.

Dendritic cells are generated from bone marrow of mice in the presence of murine GM-CSF (2). Murine GM-CSF is commercially available but is prohibitively expensive. We have originally obtained GM-CSF for DC preparation as a gift from Amgen Corp. but subsequently have switched (by necessity) to Immunex as a source of GM-CSF. Unfortunately, the lots of Immunex GM-CSF did not support effective DC generation (see below). It, therefore became imperative to develop alternative, cost effective sources of GM-CSF. In brief:

We established a cell line secreting GM-CSF and used the conditioned media as a source of GM-CSF to generate DC. The GM-CSF cell line was established by first constructing a GM-CSF expression plasmid which was introduced into F10.9 cells, a fast growing tissue culture adapted melanoma cell line. Figure 1, upper panel shows that using F10.9 derived GM-CSF we were able to generate DC albeit their phenotypic appearance was compromised as indicated by the moderate to high expression of granulocyte markers (Gr-1) and both low and high MHC class II expressing cells. We therefore embarked on a series of attempts to modify the original DC generation protocol (2) to make it compatible with the new source of GM-CSF. The issue being that the Amgen and Immunex GM-CSF were recombinant product and the new source of GM-CSF provided as conditioned media which is likely to contain other, growth or inhibitory,

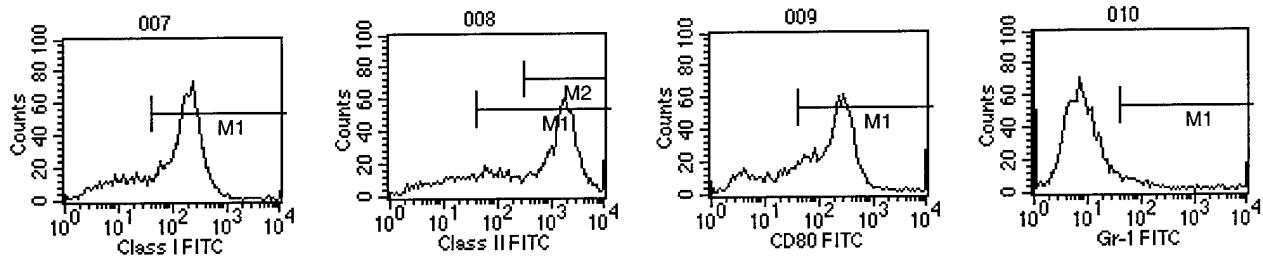
factors. In the original protocol, the bone marrow progenitors are cultured in bovine serum containing medium + GM-CSF. With this in mind, we explored methods which dispense with the use of foreign serum components.

Day 9 BMDCs generated in medium containing FBS and F10.9-derived GM-CSF



Day 9 yield: 8.9% of plated marrow progenitors
 Day 9 viability by trypan blue: 98.4%

Day 9 BMDCs generated in serum-free medium containing F10.9-derived GM-CSF



Day 9 yield: 10.3% of plated marrow progenitors
 Day 9 viability by trypan blue: 91.0%

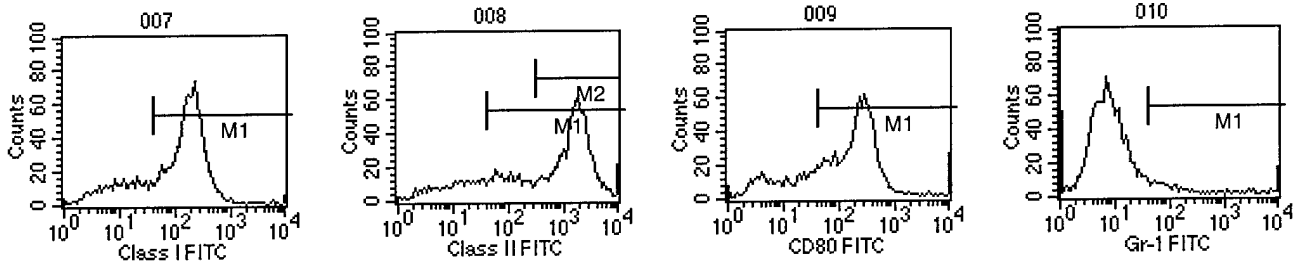
Figure 1. Flow cytometry of bone marrow DC. Murine DC were generated from the bone marrow with F10.9 derived GM-CSF and analyzed by flow cytometry for the expression of class I, class II, B7-1 (CD80) and Gr-1, a granulocyte-specific marker. Upper panels: DC were generated in RPMI-1640 medium with fetal bovine serum (FBS). Lower panels: DC were generated in Sigma serum-free medium.

We have identified a medium composition from Sigma which permitted the generation of DC in the absence of medium. Figure 1, bottom panels, show the phenotype of the serum-free DC derived in the presence of GM-CSF conditioned media. Note by comparison the high yield of class II^{high} cells and the absence of granulocytes.

However, whereas the phenotype of the DC was excellent, their viability was limited. We therefore tested and found that addition of a small amount of syngeneic murine serum (0.3%) was sufficient to improve viability to previous levels.

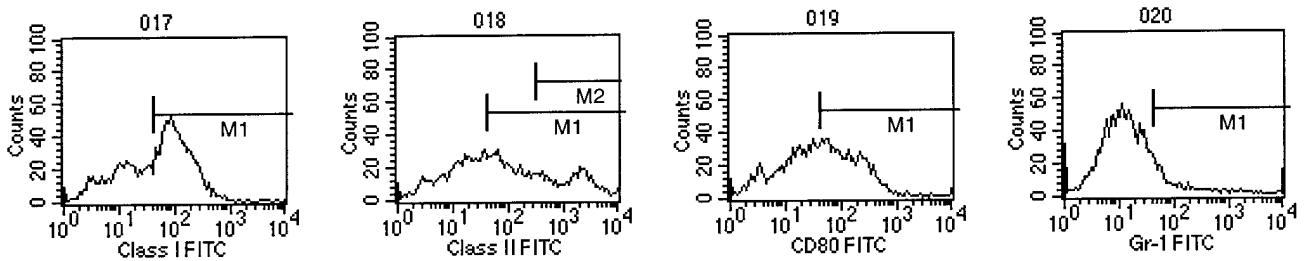
We then compared the F10.9 derived GM-CSF to Immunex GM-CSF in generating DC by the new protocol and as shown in Figure 2, the Immunex GM-CSF was still significantly suboptimal.

Day 9 BMDCs generated in serum-free medium containing F10.9-derived GM-CSF



Day 9 yield: 10.3% of plated marrow progenitors
 Day 9 viability by trypan blue: 91.0%

Day 9 BMDCs generated in serum-free medium containing Immunex GM-CSF



Day 9 yield: 6.3% of plated marrow progenitors
 Day 9 viability by trypan blue: 70.3%

Figure 2. Flow cytometry analysis of murine DC-Comparison of F10.9 derived GM-CSF and Immunex GM-CSF. (See Figure 1 legends for details)

To confirm the functionality of the DC we performed a standard CTL experiment-measuring the ability of OVA peptide pulsed DC to stimulate an OVA CTL in mice. As shown in Figure 3, DC generated in the presence of F10.9 derived GM-CSF were superior to DC generated in the presence of Immunex GM-CSF in priming a CTL response.

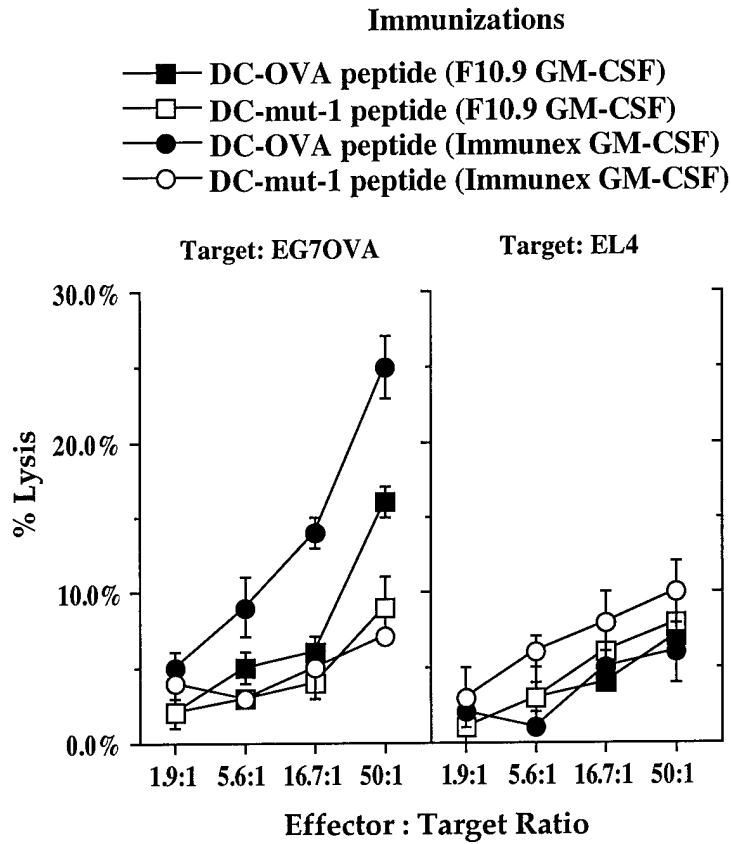


Figure 3: Priming of a CTL response in mice using DC generated with F10.9 derived GM-CSF and Immunex GM-CSF. DC prepared in mouse serum and either F10.9 derived GM-CSF or Immunex GM-CSF were pulsed with OVA peptide and injected into C57BL/6 mice. Splenocytes were removed 5 days later, stimulated in vitro with OVA-expressing E.G7-OVA cells and assayed for OVA-specific CTL using E.G7-OVA or EL4 targets (3, 4).

In summary, we have successfully resolved a serious technical problem and have developed an alternative, inexpensive, and perhaps superior, method of generating murine DC.

To further characterize the system, in Figure 4 we have tested and shown that DC pulsed with OVA peptide were capable of priming a CTL response in mice.

In Vivo OVA CTL: Group O/25
4 hr Eu CTL

Vaccinates (500k M+ SF BMDCS IP once)

▲ DC OVA peptide 1 µg/mL
△ DC mut-1 peptide 1 µg/mL

Restimulation: EG7OVA 1:10 x 5 days (8d after vax)

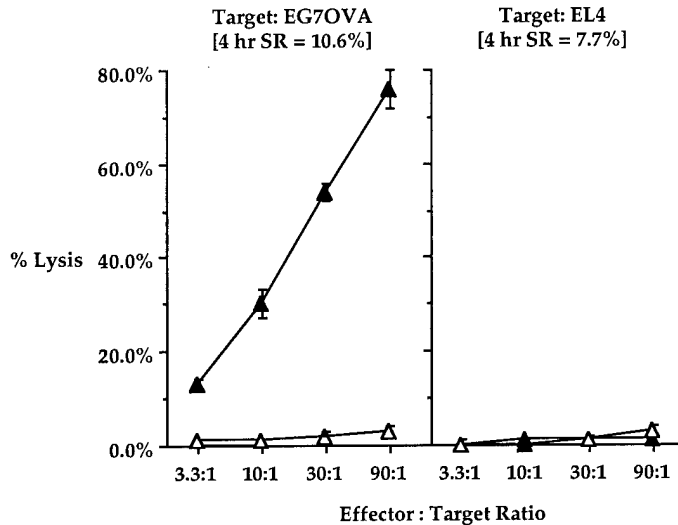


Figure 4: Priming of OVA CTL in mice with OVA peptide pulsed DC generated in autologous mouse serum. Mice were injected with i.v. 2×10^5 OVA peptide or control mut1 peptide, pulsed DC, splenocytes were isolated 9 days later, cultured for 5 days with E.G7-OVA cells and OVA CTL activity was determined using a standard cytotoxicity assay with OVA and mut1 pulsed DC as targets (3, 4).

Next, we assessed the effectiveness of CTL priming by RNA-encoded antigen transfected DC. This is illustrated in Figure 5 where we compared OVA peptide to OVA RNA using mature or immature DC (which may differ in their capability for RNA uptake) and the use of mouse serum (MS) versus fetal calf serum (FCS) generated DC. As shown in Figure 5, RNA transfected DC were able to stimulate CTL responses but were less efficient as compared to peptide transfected DC, mature DC were superior to immature DC and FCS-derived DC were more potent than MS-derived DC. However, this latter findings was not always observed.

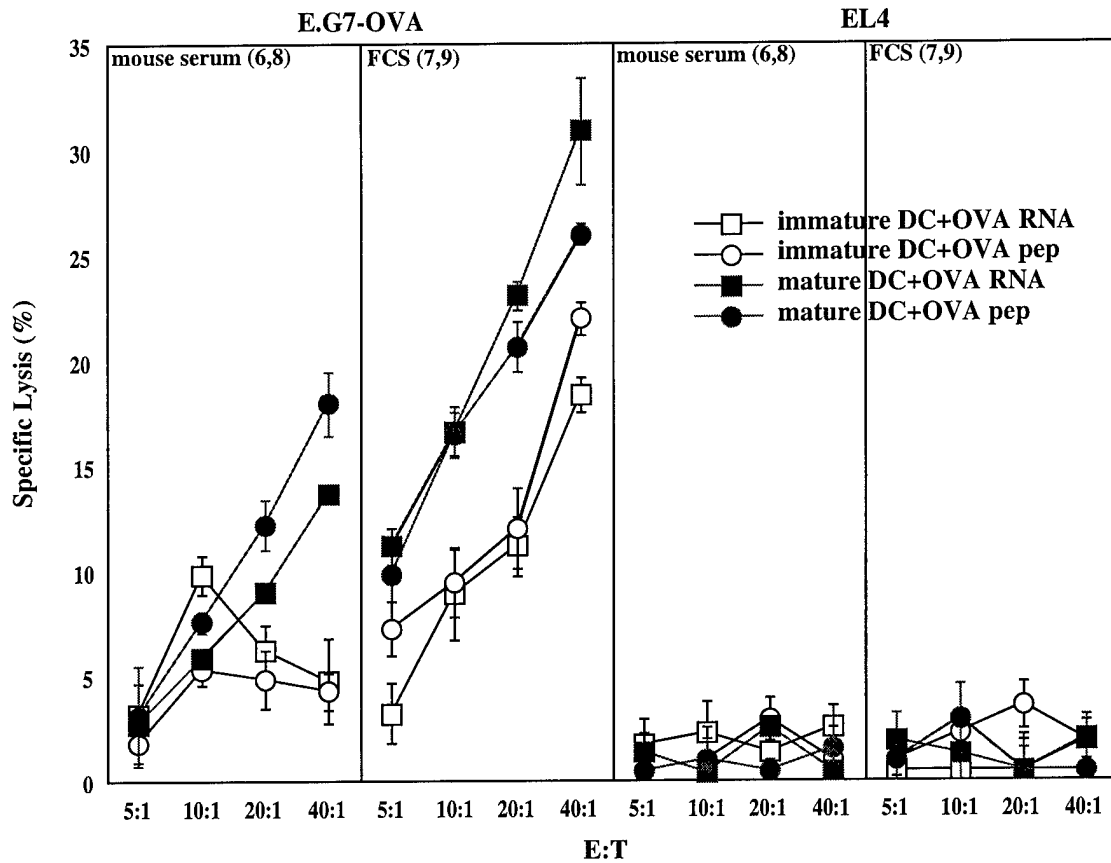


Figure 5. OVA CTL priming in mice immunized with OVA antigen pulsed DC. Antigen was use as RNA or peptide. DC were generated in either mouse serum or fetal calf serum and were used as immature (day 6 for mouse serum DC and day 7 for FCS DC) or mature (day 8 for mouse serum DC and day 9 for FCS DC).

Despite the apparent inefficient priming of CTL responses by mouse serum derived DC transfected with RNA, we proceeded to test whether immunization with C1-TRAMP tumor RNA can confer antitumor protective immunity to mice challenged with C1-TRAMP tumor. As shown in Figure 6, the data are ambiguous. Whereas vaccination with C1 RNA transfected DC appears to have an antitumor impact compared to PBS treated mice or mice treated with irradiated C1 cells, the antitumor effect is not significantly different from that of mice treated with DC loaded with control RNA derived from El-4 tumor cells.

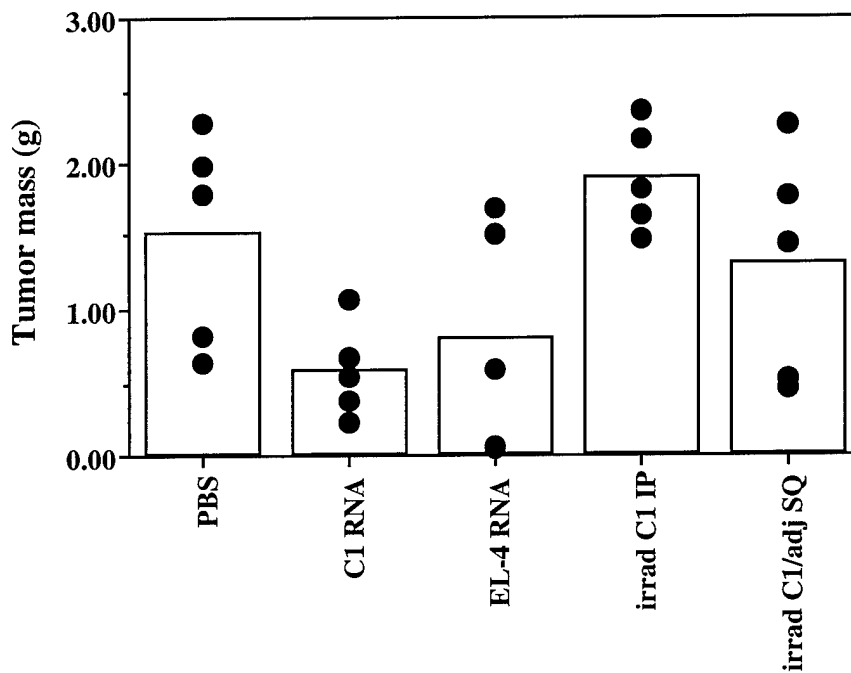


Figure 6. Immunization with dendritic cells transfected with total RNA derived from C1 cells does not completely prevent C1 tumor growth. Male C57BL/6 mice (N=5/group) were given three intraperitoneal (IP) injections (10-14 days apart) of 2.5×10^5 to 5.0×10^5 day 9 BMDCs transfected with $10\mu\text{g}/10^6$ DCs total cellular C1 or EL-4 RNA complexed with DMRIE-DOPE at a 1:2 ratio; mice in other groups were either injected with PBS, 5×10^6 irradiated C1 cells in PBS IP, or 5×10^6 irradiated C1 mixed with Adjuprime® and given subcutaneously (SQ). Two weeks after the last immunization, mice were SQ injected with 1×10^6 C1 cells in the flank. Mice were killed on day 49; tumors were dissected free and weighed. Some mice in the PBS and C1 treatment groups had intra-abdominal metastases; all mice in the DC groups were free of obvious metastases.

3. Vaccination by intradermal injection of lipid complexed RNA

The limited efficacy of vaccination with RNA transfected DC in these models led us to consider an alternative strategy. Two recent studies have suggested that direct injection of RNA into mice is capable of stimulating CTL responses and engender protective tumor immunity (5). We have therefore explored the approach of using lipid complexed DC injected into the dermis (ear pinna) of the mouse. Initial encouraging results has led us to conduct a series of optimization studies in which we have explored a large number of lipid combinations and vaccination regimen (data not shown).

Following is a brief summary of experiments using optimal conditions which demonstrate the utility of this vaccination strategy.

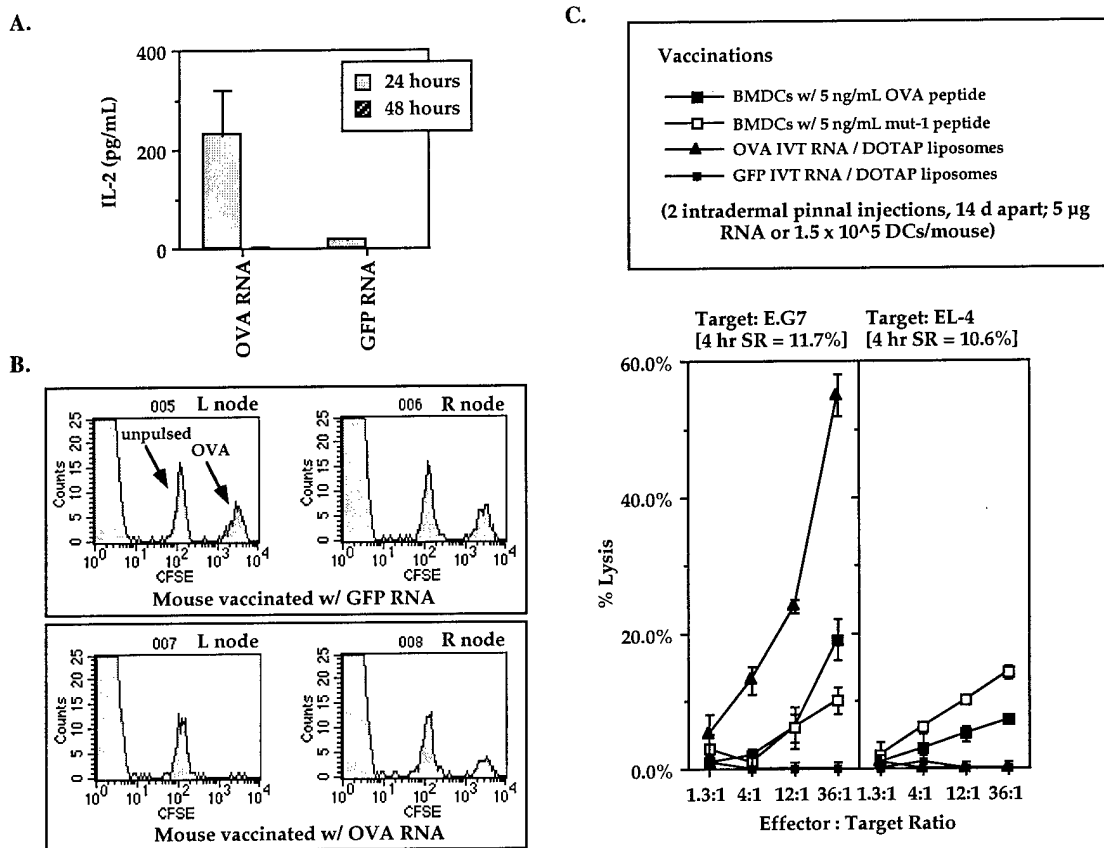


Figure 7: Intradermal injection of OVA RNA stimulate OVA CTL. Panel A, presentation of OVA to class I restricted hybridoma by lymph node cells; Panel B, Disappearance of OVA pulsed targets in vivo in mice vaccinated with OVA RNA; panel C, OVA CTL induction in mice vaccinated with lipid complexed OVA RNA or OVA RNA transfected DC.

Figure 7 shows that following intradermal injection of lipid complexed OVA RNA, OVA epitopes are presented in the draining lymph node for over 24 hours, but not after 48 hours (panel A). Moreover, OVA CTL are functionally active in vivo as evidenced by the disappearance of OVA pulsed targets labeled with CFSE (panel B). Finally, using a standard CTL cytotoxicity assay we have shown that intradermal injection of lipid complexed OVA elicited an OVA CTL response which was superior to that elicited by OVA RNA transfected DC (panel C).

We next tested the antitumor potential of vaccination with lipid complexed RNA. Figure 8A shows that vaccination with lipid complexed OVA RNA, but not GFP RNA, exhibits protective immunity against a challenge with OVA expressing EL4 (E.G7) tumor cells and Figure 8B shows that likewise, intradermal vaccination with OVA RNA confers protection against challenge with F10.9-OVA melanoma cells.

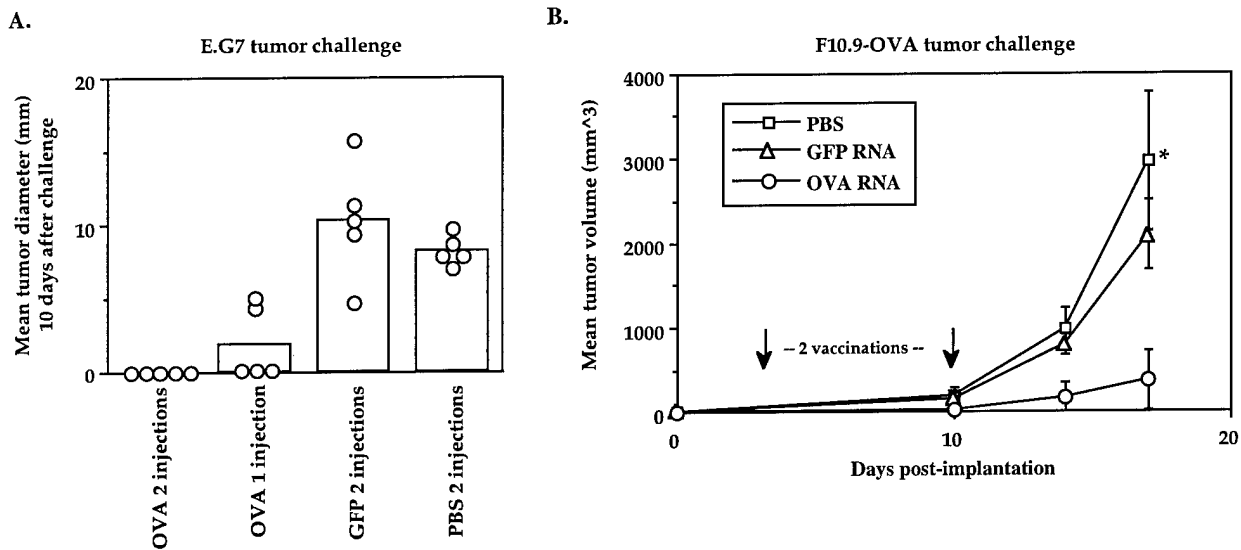


Figure 8: Protective immunity in mice vaccinated with lipid complexed OVA RNA. Mice were injected intradermally (ear pinna) with DOTAP complexed OVA or GFP RNA twice weekly and challenged subcutaneously with OVA-expressing EL4 thymoma (E.G7) (panel A) or F10.9 OVA-expressing melanoma cells (panel B). Tumor growths were determined 10 days after challenge with E.G7 cells (panel A) or at times indicated (panel B).

In order to further improve the potency of vaccination with lipid complexed RNA we tested a variety of adjuvants and cotreatments. Figure 9 shows that inclusion of GM-CSF RNA or IL-18 RNA, but not fMLP peptide, MIP-1 RNA, B7-1 RNA or IL-2 RNA, augmented in a significant manner the CTL response stimulated with OVA RNA.

In summary, we have developed a novel strategy for stimulation CTL and protective immunity which involves the intradermal injection of lipid complexed RNA and have shown that the corresponding CTL response can be further augmented by inclusion of RNA encoding GM-CSF or IL-18.

Vaccinations
 (1 injection of 3 μ g OVA IVT RNA
 complexed w/ DOTAP:DOPE 1:4)

Adjuvant (1 μ g):

- B7.1 RNA
- fMLP peptide
- GFP RNA [control]
- GM-CSF RNA
- ▲— IL-2 RNA
- △— IL-18 RNA
- +— MIP-1a RNA
- *— activated MØ mRNA

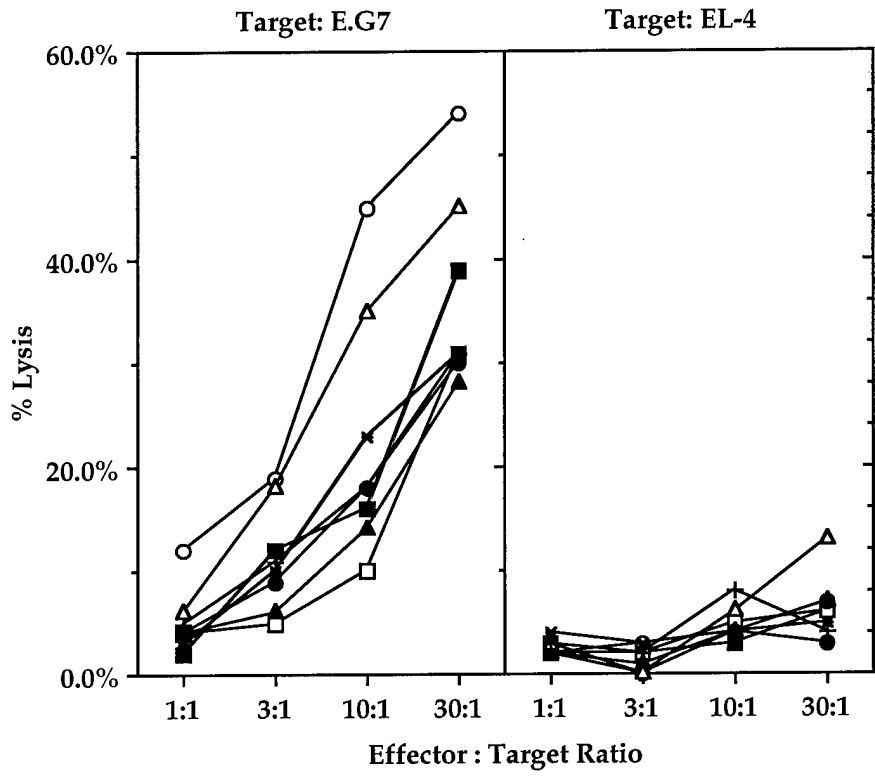


Figure 9: OVA CTL induction in mice immunized by intradermal injection of DOTAP complexed OVA RNA and adjuvants shown in the panel.

4. Antitumor vaccination in the TRAMP model.

Our studies have shown that vaccination with lipid complexed RNA represent the most potent approach to induce CTL and protective immunity in mice and was used to immunize TRAMP mice against spontaneous induction of prostate cancer. Two cohort of breeding pairs were established to provide approximately 300 offspring each, over a period not to exceed 2 weeks (of which 25% of mice will be TRAMP homozygous as determined by PCR analysis). The TRAMP homozygous mice were subjected to vaccination at age 10-12 weeks in groups of 12 per vaccination. Two experiments have been established and are currently under way:

Experiment #1: The vaccination groups consist of mice treated with (a) PBS, (b) C1 TRAMP RNA, (c) spleen RNA and (d) normal amplified prostate RNA. Mice were vaccinated weekly for a total of 5 injections.

In this experiment, we are testing whether direct injections of unfractionated RNAs amplified from normal prostate can protect against local tumor progression and metastatic disease. Data collection at the termination of the experiment will be: local tumor size and histopath grade, presence of draining lymph node metastases, presence and number of (microscopic) lung metastases (ref).

Experiment #2: The vaccination group consist of mice treated with (a) PBS, (b) p53 RNA, (c) p53 RNA+HBVc helper peptide, (d) GFP RNA and (e) SV40 Tag RNA. Importantly, each vaccination also included GM-CSF RNA which was shown to boost the potency of the CTL response (see Figure 9). Mice were vaccinated weekly for a total of 5 injections.

In this experiment, we are using the self antigen p53, which is overexposed in the TRAMP tumor (due to the presence of the SV40 T antigen). In a previous study involving C57BL/6 mice, CTL against p53 was generated by injection of a mixture of p53 peptides and the HBVc helper peptide in adjuvant; we are thus testing a potentially more immunogenic variant of p53 that incorporates the HBVc sequence (~200 bp downstream of the three major H2-b epitopes). As negative controls, groups were injected with PBS or RNA encoding GFP; as a positive control, mice were vaccinated with the Tag antigen. Data collection at the termination of the experiment will be the same as in experiment #1 above.

The two experiments are currently in progress and will be analyzed in March-April. We are cautiously optimistic that conditions will be identified which demonstrate protective immunity against spontaneous induction of prostate cancer in the TRAMP mice.

KEY RESEARCH ACCOMPLISHMENTS

1. An alternative source for murine GM-CSF. We have developed a cell line transfected with a GM-CSF expression plasmid and constitutively secreting GM-CSF in the medium witch is used a source of GM-CSF. This eliminates the dependence on gifts from companies (i.e.

Amgen or Immunex) and reduces expenses since commercially available murine GM-CSF (from R&D systems or Preprotech) is expensive.

2. A novel and potentially superior method of generating murine dendritic cells. We have developed an improved method of generating DC from bone marrow progenitors by culturing cells in autologous murine serum instead of fetal bovine serum. This modification has eliminated the presentation of foreign antigens, present in bovine serum, by the murine DC and reduced the associated background in CTL assays. We believe that this method for DC generation will be adopted by other investigators in the field as the standard protocol of an improved method for murine DC generation.
3. Vaccination with lipid complexed RNA encoded antigens. Arguably, this is the most important, and unexpected, discovery emanating from this research project which may have broad range of applications beyond the scope of this project. Of note, a significant portion of the research emphasis in my laboratory has been redirected as a result to explore the potential of this novel vaccination strategy.
4. The role of GM-CSF and IL-18 as adjuvants in immunotherapy. Incorporation of these reagents, as RNA, in a vaccine formulation may be critical in augmenting the potency of any vaccine to exceed a minimum threshold for clinical efficacy. Ongoing and future studies will focus on optimizing the protocols and identifying additional reagents that can contribute to the outcome of a vaccine regimen.

REPORTABLE OUTCOMES

1. Two manuscripts are being written and a third planned:
 - a. Manuscript describing the generation of murine DC in autologous serum and GM-CSF containing supernatant.
 - b. Manuscript describing vaccination with lipid complexed RNA.
 - c. Manuscript describing the induction of protective immunity in TRAMP mice-predicated on the results of the ongoing experiments described above.
2. Patent application will be submitted for a method for vaccination with lipid complexed RNA and the inclusion of GM-CSF and IL-18 RNA as adjuvants.
3. Two grant applications are being prepared for submission:
 - a. Use of lipid complexed RNA for vaccination.
 - b. Evaluation of feasibility to stimulate immune responses against human prostate cancer in preclinical settings using normal prostate antigens. Submission of this grant to DOD is predicated on the results of the ongoing experiments in the TRAMP mice (see above).

CONCLUSIONS

We have been initially thrown of course due to technical problems: a) viral outbreak in the TRAMP colony and difficulties in reestablishing the colony and b) Lack of availability of GM-CSF for generating DC. The TRAMP colony which was almost wiped out was painstakingly reestablished and we have developed a new, inexpensive and readily available source of GM-CSF from conditioned media of cells transduced with a GM-CSF expression vector. The delays which we have experienced were all but overcome.

Development of efficient vaccination protocols with RNA loaded DC were not altogether satisfactory since the CTL responses and protective immunity seen were generally weak. Following a lead, we have developed a novel and apparently powerful vaccination method involving the intradermal injection of lipid complexed RNA. Moreover, we have introduced a potentially significant improvement by coinjection of RNA encoding two cytokines, GM-CSF and IL-18.

The new and powerful method of inducing immunity were used to immunize large cohorts of TRAMP mice to evaluate two approaches for protecting mice from spontaneous induction of prostate cancer, namely vaccination with normal prostate RNA or with RNA encoding p53, a prototype tumor antigen expressed in this tumor model. The key experiments are in progress at the time of submission of this report (end of February). Mice will be evaluated during March-April and we are cautiously optimistic that protective immunity will be demonstrated using either or both strategies. Successful demonstration of protective immunity will trigger preclinical human studies followed by clinical trials utilizing the infrastructure of clinical vaccine trials ongoing in our Center.

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