AD_____

Award Number: DAMD17-98-1-8304

TITLE: Immunotherapy of Breast with Tumor RNA Transfected Dendritic Cell Vaccines

PRINCIPAL INVESTIGATOR: Eli Gilboa, Ph.D.

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, North Carolina 27710

REPORT DATE: September 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2001	Annual (1 Sep 00 - 31 Aug 01)

4. TITLE AND SUBTITLE
Immunotherapy of Breast with Tumor RNA Transfected Dendritic Cell Vaccines

5. FUNDING NUMBERS
DAMD17-98-1-8304

6. AUTHOR(S)
Eli Gilboa, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University Medical Center
Durham, North Carolina 27710

E-Mail: e.gilboa@cgct.duke.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The major research focus of this grant is to develop effective treatments for patients with low volume metastatic disease using dendritic cell (DC)-based vaccines loaded with tumor RNA. Toward this goal, during the third year of this grant we have made the following progress: 1) We developed a highly improved antigen-presenting platform to stimulate anti-tumor immunity. It involves two major improvements, a) Development of methods to mature DC and b) Electroporation of RNA into DC. 2) Extensive discussions with FDA toward approval of an IND to treat breast cancer patients with amplified tumor RNA-using mature electroporated DC-as described in Specific Aim #4, the major objective of this grant. In the last year of this grant we plan, 1) To obtain approval for the clinical trial with amplified RNA, 2) To carry out clinical validation studies and 3) To treat 3-4 breast cancer patients with the amplified tumor RNA transfected DC protocol.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
12

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....5-11

Key Research Accomplishments.....12

Reportable Outcomes.....12

Conclusions.....12

References.....12

Appendices.....12

INTRODUCTION.

The major research focus of this application is to develop effective treatments for patients with low volume metastatic disease using autologous dendritic cell-based tumor vaccines. The proposed studies are based on our recent discovery that RNA pulsed DC of murine and human origin is highly effective stimulators of T cells and tumor immunity. A key advantage of using tumor RNA as source of antigen is that sufficient antigen can be generated by RNA amplification techniques from small tumor specimens. Ongoing phase I clinical studies with carcinoembryonic (CEA) peptide CEA RNA and PSA RNA transfected DC have demonstrated (so far) the safety of this treatment. Furthermore, preliminary analysis of patients treated with CEA RNA and PSA transfected DC show induction of specific T cell responses in the vaccinated patients. The central hypothesis of this proposal is that vaccination with tumor RNA transfected DC against a broad repertoire of tumor antigens expressed in-patients with breast cancer will constitute an effective therapy for metastatic breast cancer.

Therefore, the primary objective of this research proposal is to develop optimal methods for using DC pulsed with tumor RNA as a broadly applicable treatment for patients with metastatic breast cancer. The specific objectives of the proposed study are:

1. To optimize antigen presentation by DC transfected with tumor RNA isolated from patients with metastatic breast cancer.

2. To perform a phase I clinical trial of active immunotherapy in patients with refractory or recurrent metastatic breast cancer using autologous DC transfected with RNA isolated directly from tumor cells.

3. To develop methods to isolate, amplify, and enrich for biologically active mRNA from breast cancer tissue.

4. To perform a phase I clinical trial of active immunotherapy in patients with refractory or recurrent metastatic breast cancer using autologous DC transfected with amplified tumor RNA.

BODY OF PROGRESS REPORT

According to the Statement of work the original plans for Year 3 are:

1. Optimization of antigen presentation by RNA transfected DC- Enhancing resistance to nuclease degradation (D-2-1).

   A. Use of RNase inhibitors
   B. Use of modified nucleotides

2. Develop methods for amplifying mRNA from a small number of cells (D-4-3).

3. Continue phase I study (D-3); Treat 4 patients.

4. Analyze T cell responses in the vaccinated patients using cytotoxicity assay, ELISPOT and FASTIMMUNE assay.

As discussed in the previous progress reports we have encountered one set back-inability to obtain sufficient tumor cells from patient to conduct the clinical trial proposed in Specific Aim #2. This set back has been however offset by a rapid development of our capability of amplying RNA from tumor cells (Specific Aim #3) which expedited our ability to initiate the clinical trials with amplified RNA as proposed in Specific Aim #4). Therefore, during Year #3 of this grant we focused on a) optimize antigen loading of DC (Specific Aim #1) and set the stage for clinical trials with amplified RNA, (Specific Aim #4).

1. To optimize antigen presentation by DC transfected with tumor RNA isolated from patients with metastatic breast cancer (Specific Aim #1).

a. RNA electroporation and dendritic cells (DC) maturation.

Old and recent studies have shown that mature DC is superior stimulators of T cell responses. The DC that we are currently using-generated in the presence of GM-CSF and IL4-are immature and therefore less effective. Whereas a number of maturation protocols have been described in the literature, in most instances the mature DC also exhibits considerable instability, namely the tendency of loosing viability. Recently, a protocol, which induces maturation without loss of viability, was described () which involves culturing the immature DC in the presence of four agents, TNF, IL-1, IL-6 and PGE2. We have adopted and optimized this maturation protocol for the human monocyte derived DC. Figure 1 shows that DC grown in the maturation cocktail acquire the mature phenotype. Not shown-the mature, but not immature, DC maintain viability for extended period of time.

Recently, Tandeloo et al have described an electroporation-based method, which results in very high levels of RNA transfer into human DC. In the current year we have optimized the combined RNA electroporation and DC maturation to generate superior antigen presenting cells for immunotherapy. Figure 2 shows that immature DC and mature DC cultured in the presence of the 4-reagent maturation cocktail and electroporated with GFP RNA express high levels of GFP, significantly more than generally achieved with or without lipid. This protocol will be further

optimized, the functionality of the RNA electroporated DC confirmed by CTL assays and used in the clinical trial (Specific Aim #4).

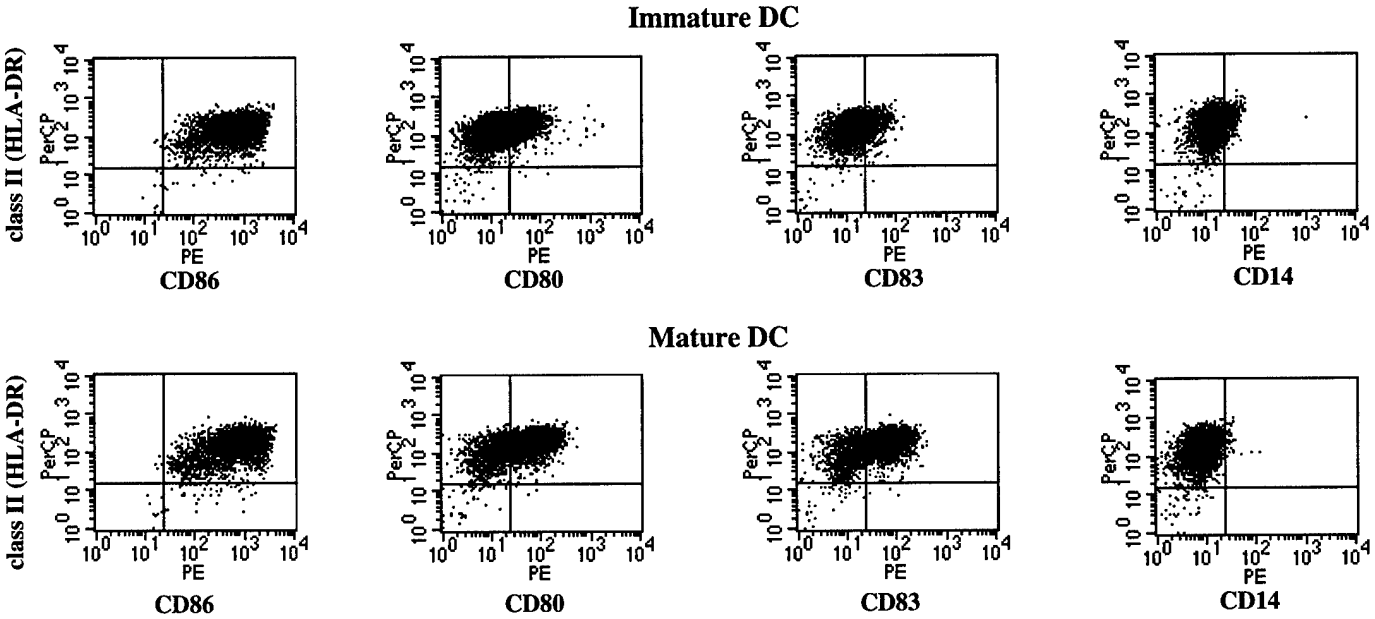

Figure 1: Flow cytometry of mature and immature DC. Immature DC was generated by culturing monocytes in the presence of GM-CSF and IL4 for 7 days. DCs were matured by 24-hour culture in the presence of IL-6, IL-1β, TNF-α and PGE_2. Immature and mature DC were immunostained with antibodies and analyzed by flow cytometry. Both immature and mature DC expressed class II and B7-2 (CD86) but not CD14. Maturation was accompanied by increase in the expression of B7-1 (CD80) and CD83.

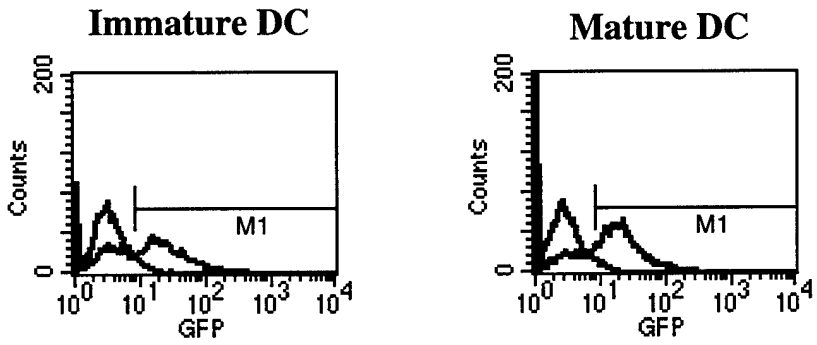

Figure 2: GFP RNA transfers into DC by electroporation. Immature and mature DC (shown in Figure1) were electroporated with RNA encoding GFP and influenza M1 (as negative control) and analyzed by flow cytometry. 60% and 75% of immature and mature DC, respectively have shown expression of GFP

To evaluate the functional consequences of RNA electroporation and DC maturation, immature and mature DC were electroporated with influenza matrix (M1) mRNA and used to stimulate M1 CTL. As shown in Figure 3 and as expected, mature DC was more effective in stimulating a CTL response.

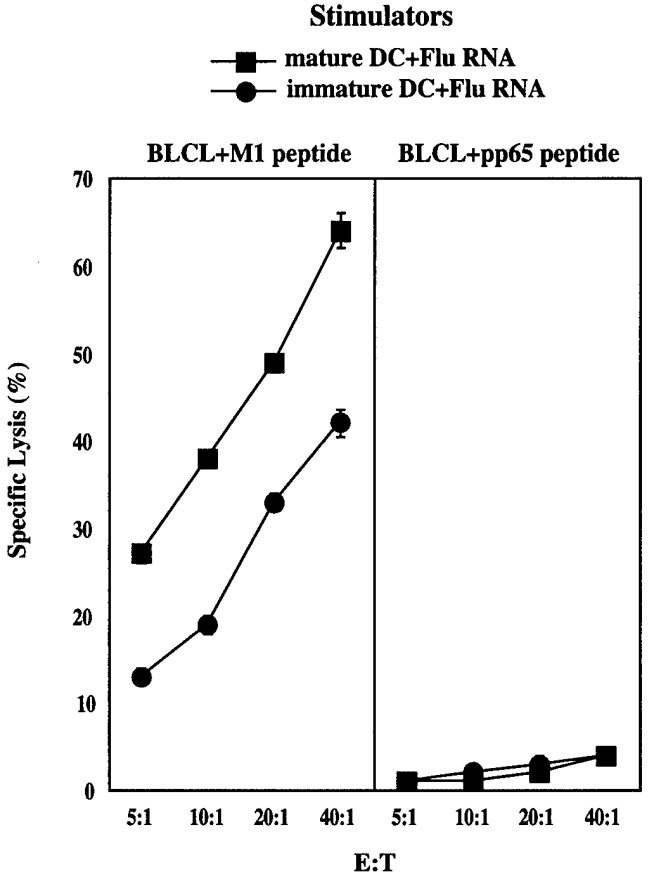

Figure 3: Stimulation of influenza matrix (M1) CTL using immature or mature DC transfected with M1 mRNA. Immature DC was electroporated with M1 mRNA and matured or mock matured. DC were incubated with PBMC from the same volunteer and stimulation of M1 CTL determined using a standard cytotoxicity assay using autologous BLCL pulsed with M1 peptide or control (CMV pp65) peptide.

b. Generation of nuclease resistant "RNA".

As described in the original application, we seek to increase the nuclease resistance of the in vitro generated (amplified) RNA by incorporating modified pyrimidine during the transcription reaction. We chose to make modified GFP RNA and look at protein expression by flow cytometry and compare the results with non-modified GFP RNA. *In vitro* transcription was performed with pyrimidines that had either amino- or fluoro- substitutions at the 2' positions. In addition we tried to incorporate deoxy-pyrimidines into the reaction. Transcription reactions

were carried out using either the MEGAscript kits (Ambion) or R&DNA Polymerase (Epicentre) which is a mutant RNA polymerase that allows higher levels of incorporation of modified nucleotides.

We found that the R&DNA polymerase combined with the buffer from the MEGAscript kits gave the best yields of RNA, however, this was still less than we got with the unmodified nucleotides and normal polymerase. By pooling several reactions together we were able to get enough modified RNA to attempt transfection into F10.9 cells using the liposomal reagent DOSPER (Boehringer-Mannheim). Unfortunately, to date we were unable to detect GFP expression from the modified transcripts.

Plans for next year:

1. Characterize functionally the mature electroporated DC-CTL assays using tumor RNA as source of antigens.

2. Optimize electroporation & maturation and perform large-scale clinical validation studies.

3. Further optimize and improve RNA amplification protocols.

2. To perform a phase I clinical trial of active immunotherapy in patients with refractory or recurrent metastatic breast cancer using autologous DC transfected with amplified tumor RNA.

For the past year we have been attempting to obtain approval from the Food and Drug Administration (FDA) to use total tumor RNA amplified from patients' tumor samples. We had a teleconference call with the FDA on 7/21/00 regarding **IND 6888** and the use of rt-PCR-amplified total tumor RNA-loaded dendritic cells (DC). The teleconference was set up so we could begin exploring the requirements for validating the approach of rt-PCR amplification of total tumor RNA for use in dendritic cell-based immunizations.

1) We were asked why we want to change the DC production methodology?

We responded: There are a number of theoretical advantages to using rt-PCR-amplified total tumor RNA loaded DC. First, this method supplies a larger number of tumor-associated antigens than approaches that use a single antigen; thus, potentially reducing the chance of antigenic escape by tumor cells. Second, this method allows treatment of patients of any HLA-type. However, that is not the case for peptide-based loading strategies. Third, this method does not require knowledge of the antigens actually expressed by a patient s tumor. Therefore, even patients with tumors that do not express known antigens could participate in clinical studies. Fourth, amplification of the RNA permits use of the tiny specimens of tumor that frequently are all that is available from patients. There also appear to be empiric advantages. By using total tumor RNA, data from our laboratories suggests that more potent cytotoxic T-cells are stimulated by amplified total tumor RNA loaded DC.

2) We were asked which components of the total tumor RNA are necessary for greater efficacy?

We responded: This is not known, but this greater efficacy in stimulating T-cells is reproducibly seen for different tumors.

3) We were asked to provide details of the mRNA amplification. These details were provided.

4) FDA reviewers said that the first requirement would be to show reproducibility of the amplification process. Specifically, how reproducible was the amount of RNA obtained? We generated and provided data demonstrating the results of five separate amplifications of total tumor RNA from a breast cancer specimen and reported the amount of RNA produced in each of the five amplifications (Figure 4). The amount of in vitro transcribed RNA produced was similar in each of the amplifications (range 25-28mcg), despite the fact that there was more variability in the amount of cDNA template produced (range 3.8-5.1 mcg). All three mRNAs evaluated (Actin, GAPDH, and CEA) were amplified in the final product of each separate amplification. We believe that the difference in lengths of the mRNA between the total tumor RNA and the amplified-RNA reflect the length of the polyA tails. Native mRNA generally has a PolyA tail of 100-200 bases, but our in vitro transcribed mRNA has 64 bases.

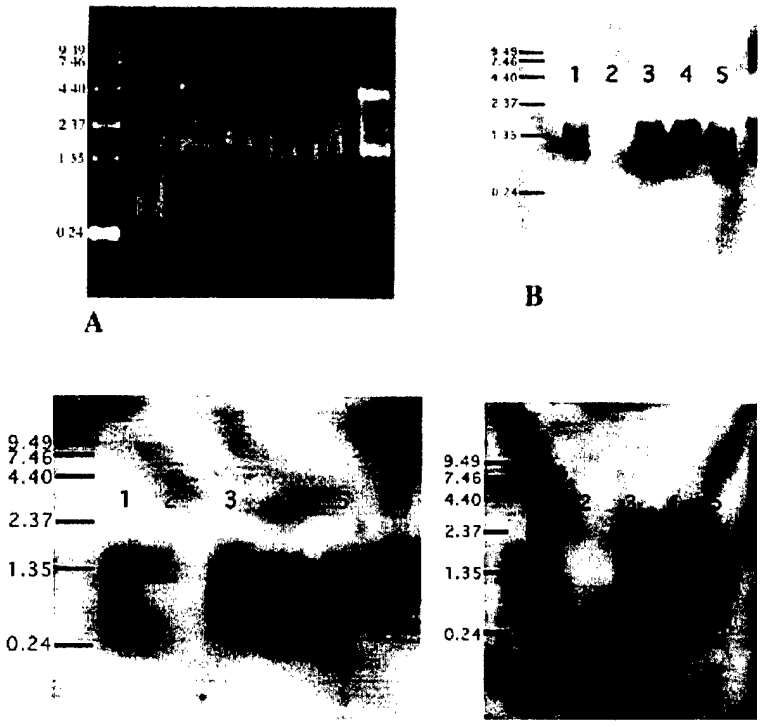

Figure 4: Reproducibility of amplifying RNA from tumor tissue. Total RNA was isolated from 0.9 g of breast tumor tissue using a Qiagen RNeasy Maxi kit and following the manufacturer's protocol. Total RNA (1 μg per reaction) was used in the standard amplification reaction. A total of five reactions were performed on three different days. RNA was produced *in vitro* from 1 μg of each of the amplified cDNAs. The *in vitro* transcribed RNA and total RNA (2 μg of each) was electrophoresed on a formaldehyde/ agarose gel and blotted to nylon membranes. Blots were probed separately with random-primed probes, which hybridize to actin, GAPDH and CEA messages.

5) FDA reviewers asked for our lot release specifications for the amplified RNA.

We responded: In addition to bacterial and fungal sterility and an endotoxin level below 5 EU/kg (patient body weight), we propose that the final product contain detectable CEA mRNA by Northern Blot. The method of RNA purification does not use phenol:chloroform so we will not test for organic solvents.

Based on this information, the FDA responded (March 7, 2001 letter) to our request to use amplified tumor RNA-loaded DC in a vaccine strategy. In the FDA letter it was requested that we provide data to show that CTL can be induced using amplified-RNA obtained from at least three fresh tumor samples, compared to total RNA from the same tumor samples .

We responded: It is not feasible to obtain breast cancer tumor specimens that are large enough to extract the amount of RNA needed to compare with the amplified-RNA. In fact, this is the main reason we wish to proceed with the amplified-RNA approach.

Another conference call was arranged with the FDA to address our response to their March 7, 2001 letter. FDA asked whether an in vitro CTL response could be achieved from mRNA, isolated and amplified from a breast cancer tumor, and how this compared to non-amplified mRNA. We responded: Because of the limited number of cells from breast cancer tumors, there is a need to amplify mRNA. Approximately 10 — 20 micrograms of total tumor mRNA/10^6 DC is used to load in vitro. However, for breast cancer, it is problematic to obtain even 1 — 2 micrograms of total tumor mRNA/10^6 DC. Although various methods are available, it is difficult to isolate purified breast cancer cells from patients.

The FDA requested that we use mRNA from tumor cell lines, not fresh tumor, to respond to their March 7, 2001 letter regarding the use of amplified and non-amplified mRNA. These experiments have been completed and shown in Figure 5. Figure 5 shows that RNA isolated from a tumor of a breast cancer patient and subjected to three separate amplifications stimulated similar levels of CTL responses. This information is being sent to FDA and we are hopeful that the FDA will approve our request and the clinical trials with amplified tumor RNA (Specific Aim #4) will start shortly.

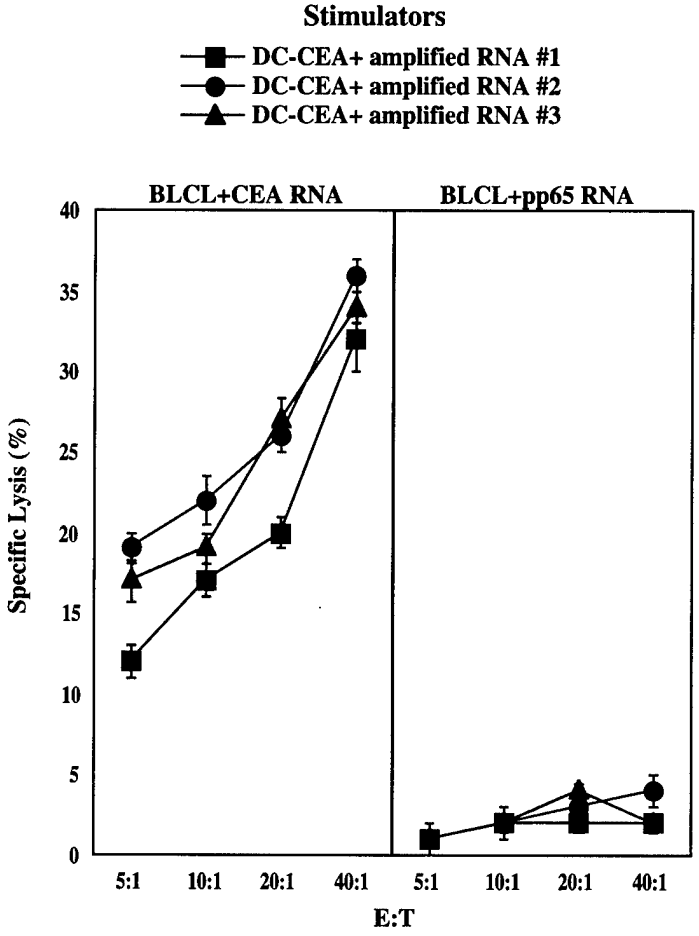

Figure 5. RNA was isolated from a tumor specimen obtained from a patient with breast cancer, divided into three aliquots and amplified. Amplified RNA was transfected into H-2 matched DC and incubated with PBMC. After two cycles of stimulation, the generation of CEA-specific CTL were determined using a standard cytotoxicity assay using H-2 BLCL (autologous to the DC and PBMC) transfected with CEA RNA and control (CMV pp65) RNA.

Plans for next year.

1. Obtain IND approval from FDA.

2. Large scale validation studies under clinical settings.

3. Treatment of 3-4 patients with amplified RNA transfected DC.

KEY RESEARCH ACCOMPLISHMENTS

1. Develop methods for DC maturation.
2. Develop methods for RNA electroporation into mature DC.
3. Addressing FDA requirements for obtaining IND.

REPORTABLE OUTCOMES

N/A

CONCLUSIONS

1. We have developed a superior method of stimulating tumor immunity: a) Use of a more powerful antigen presenting cells-mature DC and b) Improved loading of DC with antigens-electroporation of RNA.
2. Extensive interactions with FDA are likely to lead to approval of the IND to treat patients with amplified tumor RNA tranfected DC (using mature electroporated DC) and initiation of clinical trials as specified in Specific Aim #4, the main objective of this grant.

APPENDICES-NON