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14. ABSTRACT
In this proposal the effectiveness of developing a novelimmunotherapeutic strategy following Radiotherapy for prostate cancer has been explored.The hypothesis was tested using a murine prostate cancer model, RM-1. The study showedthat irradiation induces apoptosis and the irradiated tumor cells were able to activatedendritic cells and stimulate tumor specific immune response in vitro. In vivoimmunization of animals with activated DCs was able to increase their survival againstsubsequent challenge with tumor cells. Treatment with CCL-21 following DC expansion byFL+IL-12 in animals bearing irradiated tumor, significantly increased their survival timewhen they were challenged with tumor cells. This regimen also generated a tumor specificimmune response as evidenced by in vitro studies. Similar protection was observed whenCD40L was used instead of CCL-21 following treatment with FL+IL-12. The study furthershowed that the administration of adenovirus (Ad-CCL-21) to prostate tumor bearinganimals significantly increased their survival time. Collectively, our study provides anovel approach to treat advanced prostate cancer patients following radiotherapy. Aprotocol for treating prostate cancer patients undergoing radiotherapy could be developedimmediately based on these findings.

15. SUBJECT TERMS
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I. INTRODUCTION

The immune surveillance network fails to detect a tumor in most cases mainly due to lack of adequate 'danger' signals available from the tumor to the host. Primal danger signals are mediators that are made available during tissue damage, to the antigen presenting cells (APCs). The APCs does not require previous activation for this response.

Dendritic cells (DCs) are the major APCs that play pivotal role in antigen presentation from damaged/apoptotic cells *([1]Albert et al 1998)*. They can efficiently phagocytose apoptotic cells *([2] Rubertelli et al 1997, [3]Ren et al 1998, [4]Albert et al 1998)* and elicit antigen-specific immune responses *([5]Banchereau et al 1998)*. Immunization of animals with DCs pulsed with apoptotic tumor cells has been shown to successfully prime tumor specific CTLs and confer protection against tumor challenge in a mouse tumor model *([6] Shaifmuthana et al 2000).* This implies that the antigen(s) from damaged/apoptotic and/or necrotic cells can be processed and presented in context of MHC class I by DCs that could subsequently stimulate CD8+ T cells *in vitro* and *in vivo ([7] Bellone et al 1997, [8] Sauter et al 2000, [9] Joke et al 2000).* Once the DCs are activated, they express the lymphoid homing receptor, CCR-7 (*[10] Randolph et al 1999*), and migrate to the secondary lymphoid organ (SLO). CCL-19 & CCL-21 are the two chemokines that bind to CCR-7 and guide mature DC to SLO (*[11]Sozanni et al 1998*). These chemokines also attract naïve T cells to the lymphoid organ (*[11]Sozanni et al 1998*). Once DC and naïve $CD4^+$ T cells come in proximity in the lymphoid tissue, CD40/CD40L interaction between them help to activate DC and cross prime $CD8^+$ T cells (*[12] Bennett 1997*).

Thus DCs play a central role in generating a specific immune response. FLT3 ligand (FL) is a naturally occurring glycoprotein that stimulates the proliferation and

differentiation of a variety of hematopoietic cells including DCs *in vivo*, both in mice and in humans ([13] *Shurin et al 1988*). FL has been shown to retard tumor growth and induce tumor specific immune response ([14] *Lynch et al 1998,* [15] *Chakravarty et al 1999,* [16] *Pawlowska et al 2001*). Interleukin-12 (IL-12) like FL can induce accumulation of functional DC ([17]*Esche et al 2000*) and combination treatment of FL and IL-12 has been reported to result in dramatic increase in the number of DC *in vivo* ([17]*Esche et al 2000*).

Radiation therapy (RT) is one of the major conventional modalities available today to treat cancer, and irradiation (IR) is one among the numerous agents that induces cellular damage *(*[18] *Roth et al 2000,* [19] *Zhou et al 2000).* Unfortunately, RT alone is not very effective in controlling the disease activity *in vivo* especially for the metastatic lesions that are distant from the primary site of irradiation; nor can it induce sufficient immune response in the host that would impact the outcome of the disease. However, it would be a plausible approach to harness the potential of RT for developing immunological strategies for treating cancer. Therefore, it is *hypothesized* that the apoptotic tumor cells that are induced by □-irradiation following RT, could serve as suitable targets for uptake by DCs. Once activated, these DCs should be able to induce the expression of the required stimulatory and migratory molecules to promote tumor specific immune response. For efficient DC amplification *in vivo*, we planned the use of FL alone and in combination with IL-12. To facilitate DC trafficking following activation and to bolster the process of immune response, the use of CCL-21 as well as CD40L was proposed. We tested this *hypothesis* both *in vitro* and *in vivo* using a poorly immunogenic murine prostate cancer model. We addressed the following specific aims: (1) Immunogenic nature of

irradiated prostate tumor cells. (2) The effectiveness of using cytokines like FL, IL-12, CCL-21 & CD40L following irradiation of tumor, in generating tumor specific immune response & (3) The effectiveness of adenovirus, Ad-CCL21 on tumor development.

Here results obtained from experiments carried out both *in vitro* and *in vivo* have been presented and an attempt has been made to explain them in the context of the central theme of the proposal.

[However, major hurdles were encountered to execute the project during the years 2003 and 2004. Some of the on-going *in vivo* experiments using FLT3 ligand (FL) and CD40 ligand (CD40L) were effected as my appointment with the college ended on the 10th of December, due to lack of fund. A request for additional time of six months was not granted. To explain this development in proper context, I should mention that the experiments involving the use of the above mentioned reagents could not be initiated during the second year as the pharmaceutical company that was to provide the reagents did not do so, as agreed upon. The circumstances that led to this situation has been explained in details in the last annual report and the concerned contract specialist and others at the Prostate cancer research program (PCRP) are aware of this development from the beginning. As was suggested by the contract specialist during 2002, I put in a special request to the Army through appropriate authorities in our Institute, for 'additional fund' for the purchase of those reagents. The issue was resolved by the PCRP only in August of 2004 and an amount of $10,000 was received in September 2004. This amount was sufficient to purchase only a small quantity of the reagents. However, only ~$ 4000 was made available to me. The Institute deducted the remaining portion of the money towards 'indirect cost'. Incidentally, I had already spent $ 13,000 towards the purchase of

the two reagents that would only allow experiments with fewer animals (The '*in voice'* was forwarded to the DOD after making the said purchase).

In addition to this setback, from the year 2003 onwards I had at my disposal about $14,000/year from the college towards 'direct' cost though my DOD approved budget had an allocation of $30,000/year towards 'direct' cost. This left me with little or no money even to maintain the laboratory. This coupled with the problem faced for the purchase of the two reagents, left me totally stranded. Some of the expenses were borne out of my pocket. With this acute budget crisis, I was unable to initiate and/or complete many of the intended *in vitro* and *in vivo* experiments. The experiments that needed expensive reagents (growth factors, antibodies etc.) suffered the most. However, I put in my best efforts under the circumstances, to conduct experiments that addressed the central theme of my proposal. Fortunately, I have some exciting data, which should help me acquire funding from PCRP in near future in a better setting]

II. BODY

1. *In vitro studies to establish effect of ionizing radiation on RM-1 cells*

A. Cell lines: i) The mouse prostate cancer cell line, RM-1, that is syngeneic (i.e., same genetic background) to C57BL/6 mice were used. The cells were maintained *in vitro* in D-MEM supplemented with 10% FCS and 10mM Hepes. ii) Immature histo-compatible DC cell line, JAWS-II (ATCC) was maintained *in vitro* in □- MEM (with ribonucleosides) supplemented with 10% FCS and 5ng/ml murine GM-CSF.

B. Determination of RT induced apoptosis of RM-1 cells by electrophoresis, fluorescent microscopy and FACS analysis:

Methods: Exponentially growing RM-1 cells (1x 10^6 cells plated in 25 mm^2 flasks) were irradiated to 10, 20 and 60 Gy at a dose rate of 12 Gy/minute using a cesium source (Cis Bio International). Apoptosis was measured at different time points by the following techniques:

***a). Electrophoresis (DNA ladder)*:** Irradiated cells were harvested at different time points and apoptotic DNA was isolated using the Suicide-Track DNA ladder isolation kit (Oncogene Research Products). The isolated apoptotic DNA was run in 1.5 % agarose gel stained with ethidium bromide and examined under UV light.

b) FACScan analysis of propidium iodide stained cells: Irradiated RMI cells were harvested by incubating in 1% Trypsin/EDTA, washed twice in cold PBS and fixed immediately in 3 ml of 70% cold ethanol. The fixed cells were either immediately stained with propidium iodide or stored at -20° C till further use. Prior to staining, the cells were washed with cold PBS to remove the ethanol. The staining solution containing 8 g/ml of Propidium iodide and 100g/ml of RNAase A, was used. One ml of the staining solution was added to the cell pellet, mixed and incubated for 20 minutes at room temperature prior to analysis by FACScan. The cells in the pre-G1 fraction of cell cycle representing the apoptotic cells were measured.

c) Fluorescent microscopy (Hoechst's staining): RM-1 cells were plated in 6 well tissue culture plates and irradiated with 10 and 60 Gy as described above. The cells were fixed at days 4 and 7 of irradiation with cold 75% methanol/ 25% glacial acetic acid for 10 minutes. The cells were washed with cold PBS and stained with Hoechst (8 g/ml). The apoptotic cells were counted under HPF using fluorescent

microscope. At least 300 cells were counted per HPF and the average of 5 HPF's was taken for analysis.

d) Results: The experiments using different techniques to evaluate apoptosis show that there was a significant degree of apoptosis of RM-1 cells *in vitro* at 10, 20 and 60 Gy of irradiation. The figures -1A, 1B & 1C show the apoptotic DNA using gel electrophoresis of DNA obtained from irradiated cells. A significant fragmentation of DNA was observed at both 10 and 60 Gy of irradiation of RM-1 cells (Figure-1A). The figures 1B & 1C show the pattern of fragmentation at 10 and 20 Gy of irradiation at different time points following irradiation, respectively. The figure 1D shows the extent of apoptosis at 4 and 7 days of 10 Gy irradiation as demonstrated by Hoechst staining. The percent of apoptotic cells were 18.91 ± 1.36 and 37.97 ± 1.51 at 4 and 7 days as compared to their controls (2-3%). Similar results were also observed when the pre-G1 fraction representing the apoptotic population of cells was evaluated at 4 days after irradiation of RM-1 cells (Figure 1E). The percent of apoptotic cells was 22.97 ± 1.31 at 60 Gy and 14.18 ± 0.72 at 10 Gy as compared to controls 7.4 ± 0.79.

The results conclusively show that there was apoptosis of RM-1 cells after irradiation and it increased proportionately with time reaching up to ~60% apoptosis, at day 7 of irradiation.

2. <u>Studies to determine the effect of apoptotic cells on DC activation</u>:

***A) Activation of DC by Irradiated tumor cells*:** The JAWS-II cells ($1x10^6$) were co-cultured with irradiated (4 days post 60 Gy of irradiation) $1x10^5$ RM-1 cells. Prior to incubation, the intact and irradiated RM-1 cells were stained with the red fluorescent dye (PKH-26 cells using the fluorescent cell linker kit protocol provided by the

company (Sigma Chemicals) and the JAWS-II cells were stained for MHC class II with FITC conjugated antibody (Pharmingen). The DCs were also evaluated for co-stimulatory molecules (CD80 & CD86) using specific antibodies. The RM-1 and DCs were co-incubated at 37°C for 4 hours at a ratio of 1:10, harvested, washed in PBS and analyzed by FACScan for the presence of irradiated RM-1 cells/lysate in the gated population of DCs (MHC class II positive).

a). The ***results*** show that the percent of double positive cells representing the phagocytic cells was 20.87 ± 1.0 in the experimental group compared to controls 4.88 ± 0.4 (Figure: 2). The cells were also visualized under fluorescent microscope to confirm the double positive cells.

B. Analysis of the Expression of CCR-7, CCL-19 and CCL-21 by RT-PCR: The DCs were co-cultured with irradiated RM-1 cells as previously described. Total RNA was isolated from naïve DCs, RM-1 and DCs co-incubated with irradiated and non-irradiated tumor cells, using RNAzol (Life technologies, Inc). The RNA was used for cDNA synthesis and as templates for analyzing the expression of CCR-7, CCL-19 and CCL-21 by RT-PCR using the RT-PCR kit (Epicenter). The following primers were used: CCR7 (forward 5'ACAGCGGCCTCCAGAAGAAGAGCGC; reverse-5'-TGACGTCATAGGCAATGTT GAGCTG), CCL-21 (forward- 5' CAACCACAACCATGGCTC, reverse- 5' GGCGGGATCCTGGGCTAT) and CCL-19 (forward- 5' GCCTCAGATTATCTGCCAT, reverse- 5' AGACACAGGGCTCCTTCTGGT). The PCR conditions for CCR-7 was 94°C – 1min, 58°C -1 min, 72°C - one minute and extension at 72°C for 10 minutes. For CCL-21, the conditions were 94°C for one minute, 60°C for one minute, 72°C for 1 minute and extension at 72°C for 7 minutes. For CCL-19, it was 94°C for 1 minute,

59°C for 1 minute, 72°C for one minute and extension at 72°C for 7 minutes ([20] ***Hirao et al 2000***).

a). Results: The DCs when co-cultured with irradiated RM-1 cells, showed an elevated expression of mCCR7 and its ligands (Figure 3). The receptor was not expressed in naïve DCs or when they were exposed to non-irradiated whole live RM-1 cells (Figure 3B). The expression of CCR-7 by the DCs that were pulsed with irradiated tumor cells confirmed that they were activated and were ready to immigrate to the secondary lymphoid organs for initiating the next phase of immune response. The activated DC also produced the two chemokines CCL-19 (Figure 3A) and CCL-21 (Figure 3C) that are specific ligands for CCR-7.The amount of beta actin transcription in the different groups is shown in figure 3D. The DCs pulsed with non-irradiated cells did not secrete the chemokines.

Thus the results showed that irradiated tumor cells appropriately activated DCs so that they could migrate to the lymphoid organs.

C). Analysis of the expression of cell surface molecules on dendritic cells: The DCs were washed twice in ice cold PBS and stained with FITC or PE conjugated antibodies to CD80, CD86, MHC Class I & Class II (BD Pharmingen), in ice for 30 minutes. Following staining, the cells were washed with PBS and analyzed in a cell sorter.

a). Results- The figure-4 shows the results obtained by the FACScan. The intensity of expression of all the cell surface molecules (CD80, CD86, MHC Class I & Class II) were elevated on the DCs exposed to irradiated tumor cells compared to the cells that were not exposed to irradiation.

The results show that the DCs incubated with irradiated tumor cells were activated with respect to the expression of cell surface molecules and were ready for the process of immune response.

3. <u>Effect of activated DC (pulsed with irradiated tumor cells) on lymphocyte proliferation</u>: To study whether the activated DCs could stimulate lymphocyte proliferation, the DCs ($1x10^5$ cells/ml) that were used as stimulator cells, were irradiated (30 Gy) and serially diluted into 96 well microtitre plates and were co-cultured with irradiated RM-1 cells ($1x10^5$ cells/ml) for 1 hr. Subsequently, autologous lymphocytes isolated from naïve C57BL/6J mice (viability > 95%) were added at a concentration of $1x10^5$ cells/ml in a final volume of 200 µl. After incubating cells for 5 days cell proliferation was determined by WST-1 assay as per the manufacturers protocol (Boerhinger Manheim).

a). Results: The figure 5 shows the percent increase in lymphocyte proliferation in the experimental group as compared to controls (The DC exposed to non-irradiated intact live cells). It was observed that at a dilution of 1:256, there was two-fold increase in proliferation in lymphocytes that were co-incubated with DCs pulsed with irradiated tumor cells as compared to controls. Thus there was a significant proliferation of naïve splenocytes when they were co-cultured with activated DCs.

4. <u>Effect of DC pulsed with irradiated tumor cells on the generation of immune response</u>: The dendritic cells ($1x10^6$ cells) were used as stimulator cells. They were irradiated (30 Gy) and co-cultured with previously irradiated RM-1 cells ($1x10^6$ cells) in 100 cm tissue culture dishes for one hour. Subsequently, splenocytes isolated from naïve C57BL/6J mice were added at a ratio of 1:10 and the cell

mixture was further co-cultured for 5 days at 37^0C. The stimulated splenocytes and culture supernatants were collected and tested for their immune activity.

A). Cytotoxicty: The cytotoxicity of the splenocytes was determined in a 24 hr. cytotoxic assay (LDH assay-Boerhinger Manheim). Briefly, $1x10^6$ RM-1 cells were washed and 10^4 tumor cells were co-incubated with 10^5 stimulated lymphocytes, at an effector target ratio of 10:1 in 96 well V-bottom micro-titer plates in final volumes of 200 µl for 24 hrs in RPMI supplemented with 10% FCS and 10mM Hepes. All the samples were in triplicate and controls without lymphocytes were used to assess the spontaneous release. After 24 hrs of incubation at 37^0C, the supernatant (100µl) were harvested and evaluated for cytotoxicity by ELISA (Molecular Devices) using manufacturer's protocol (Boehringer Manneheim). The cytotoxicity was calculated as percent lysis =100x [(Experimental value- low control)/ {high control-low control)]. The results are shown in a target effector ratio of 10:1.

B) Cytokine Assay*:* The culture Supernatants from stimulated splenocytes were collected for estimation of cytokine levels (IL-12 & IFN-γ) using ELISA kits (R & D systems, Minneapolis, MN).

C) Results: 1. The splenocytes that were stimulated with DC pulsed irradiated tumor cells had a significantly higher cytotoxic response (59.68 ± 1.77) against live RM-1 cells (Figure: 6). 2. The culture supernatants evaluated for IL-12 and IFN-γ by ELISA, showed an elevated concentrations of IFN-γ, 668 ± 140.41 (Figure 7A) and IL-12, 317.15 ± 69.55 (Figure 7B) in the experimental group In the controls (splenocytes that were stimulated with DCs pulsed with non-irradiated tumor cells),

the concentration of IFN-γ was 135.44 ± 39.64 and that of IL-12 was 66.50 ± 20.42 respectively.

Thus the significant cytotoxic activity of the splenocytes and their production of Th 1 cytokines, demonstrated their activation in response to activated DC and the data further emphasizes the generation of specific T-cells that are cytotoxic to RM-1 cells.

5. In vivo studies to ascertain the effect of administration of DCs following primary tumor control and immunization by irradiation:

A. Mice: Six to 8 week-old male C57BL/6J (H-2^b) mice that are histocompatible with RM-1 and DCs (JAWS-II) were obtained from Jackson Laboratories (Bar Harbor, MN). Animals were housed in the Albert Einstein College of Medicine, Animal Resources facilities under controlled conditions (temperature, humidity, and a 12h light: dark cycle with food and water ad libitum).

B. Cell lines: i) The mouse prostate cancer cell line, RM-1, that is syngeneic (i.e., same genetic background) to C57BL/6 mice. ii) Immature histo-compatible DC cell line, JAWS-II (ATCC) iii) Bone marrow DC (BM-DC)**:** DCs were obtained using methods described previously *([21]Inaba et al 1994)*. In brief, the BM cells were harvested from the femur and tibia of sacrificed mice. Contaminating erythrocytes were lysed with 0.83 M NH_4Cl buffer and lymphocytes depleted with a cocktail of antibodies (RA3-3A1/6.1 anti B220; 2.43 anti-lyt2; GK1.5, anti-L3T4 (ATCC, Rockville, MD) and rabbit complement on day 0. The cells were cultured over-night in complete medium to remove the adherent macrophages, and the non-adherent cells were placed in fresh medium containing murine GM-CSF (1000units/ml- Sigma Chemicals) and mIL-4 (1000units/ml- Sigma Chemicals). The cells were

cultured for 6 days and used for experiments.

C). Immunization with DC pulsed with irradiated tumor cells increases survival and generates tumor specific immune response:

a). Experimental design: Naive C57BL/6J mice were immunized (*ip*) with $1x10^6$ DCs (non-activated) or DCs activated with irradiated RM-1 cell, once a week for three consecutive weeks. Prior to *in vivo* use, the DCs were exposed to 30 Gy of irradiation. One week following last immunization, the animals were partitioned for immunological and survival studies. For survival study, a lethal dose of $5x10^4$ RM-1 cells were given on the dorsum of the foot of the experimental animals and their appropriate controls. The animals were monitored for survival. For immunological studies, the spleen was removed under aseptic condition and the splenocytes were separated by standard procedure.

b) Immune response studies: The isolated splenocytes were re-stimulated with irradiated RM-1 cells (60 Gy) at an effector and target ratio of 10:1 for five days and were tested for i) proliferation, ii) cytotoxicity, and iii) cytokine profile.

i) Lymphocyte proliferation: Following co-culture for 5 days, the proliferation was determined by WST-1 assay by procedure as described by the manufacturer ((Boehringer Mannheim).

ii) Cytotoxicity assay: The splenocytes were stimulated as previously described and they were incubated with fresh RM-1 cells at an effector and target ratio of 30:1 for 24 hrs. The cytotoxicity was determined by LDH assay as per manufacturer's protocol (Boehringer Mannheim).

iii) Cytokine profile: The culture supernatants from the stimulated splenocytes were tested for the presence of cytokines (TNF-□, IFN-□and IL-12) by using ELISA kits

(R &D systems). The results were read using ELISA reader (Molecular Devices).

c). Results: 1. The control animals died by ~3 weeks of tumor cell transplantation (median -24 days). The figure 8 shows that the survival of immunized animals was more than 8 weeks (median-56 days) with 20% surviving for more than 16 weeks (log rank test- $p = 0.01577$).

2. The figure 9 shows that the splenocytes from the animals immunized with activated DCs had an increase in proliferation compared to controls ($p < 0.01$).

3. The figure-10 shows that immunized splenocytes effectively lysed fresh RM-1 cells (56.2%±5.1) at an effector and target ratio of 30:1 in a 24 hrs. Cytotoxic assay ($p < 0.01$).

4. The culture-supernatant secreted high amounts of IFN-γ (690.5± 13 pg/ml), Tnf-α (153.8 ±35.3 pg/ml) & IL-12 (286.2± 40 pg./ml), as compared to controls (Figure-11). The results were statistically significant for all the three cytokines when compared to controls ($p < 0.01$).

D. In vivo administration of DCs following primary tumor control by irradiation increases survival.

a) Experimental design: *Six* to 8 week-old male C57BL/6J ($H\text{-}2^b$) mice obtained from Jackson Laboratories (Bar Harbor, MN) were inoculated with 10^5 RM-1 cells subcutaneously in the dorsal aspect of the foot. Two weeks after tumor inoculation, all animals developed primary footpad tumors (50-60mg). If left untreated the tumor grew progressively and the animals succumbed to tumor burden by ~ 3 weeks post tumor cell transplantation. Animals with established two week-old tumors (50 - 60 mg) were given a single dose of localized irradiation (60 Gy) by procedure described earlier *([15] Chakravarty et al 1999).* Following irradiation, the animals received 10^5

DCs or PBS through the tail vein once a week for three consecutive weeks. One week following the last injection, the animals received $1x10^5$ tumor cells sub-cutaneously and were followed for survival.

b) Results: The animals receiving DC had significantly higher survival compared to the controls (Figure-12). The combination of RT + DC significantly increased survival when compared to controls. The median survival time was 17 days for control (naïve animals), 43 days for RT group and 66 days for the RT+DC group. The survival time between the RT and RT+DC group was statistically significant ($p< 0.05$).

6. Construction of selectively replicating E1B deleted adeno virus with cytokine gene for in vivo gene delivery: To deliver a gene into somatic cell with high efficiency, two major genetically engineered vector systems, retrovirus and adenovirus, have been developed and well characterized. The retrovirus-mediated gene transfer system has limited use especially in prostate cancer gene therapy because the integration of retrovirus into the host genome could result in activation of cellular oncogenes and they have been associated with several malignancies in both mammal and vertebrates. In general, the titer of retrovirus is 100 to 1000-fold lower than that of adenovirus. Along with the advantage of producing high-titer virus stocks, adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. Adenovirus produces little morbidity and has not been associated with human malignancies (*[22] Fan et al 2000, [23] Mukherjee et al 2002).* We used replication conditional adenovirus (RCA) that only replicate in tumor cells *([24] Heise et al 1999).* The discovery of replication conditional adenovirus has opened a new avenue for developing novel therapeutics for treating different forms of cancer. A replicating vector is able to deliver a therapeutic gene more efficiently into a tumor

than a non-replicating vector because of its ability to spread from initially transduced cells. The replication-competent adenovirus-derivative (E1B attenuated/deleted) was developed to specifically replicate in tumor cells that lack functional p53 protein and causes cyto-pathigenicity in certain cancer cells *([25] Bischoff et al 1996).* The RCA achieves intra-tumoral titers of 100- to 1,000-fold over the course of 72 hours after intravenous administration *([24] Heise et al 1999).* It also dramatically increases the expression of a transgene in transduced cells, relative to a replication-defective version *([26] Freytag et al 1998).*

A). Materials: ***a) Shuttle vectors***: The pCA14-E1a/E1b plasmid used as the left hand shuttle vector for constructing replication competent adeno virus was received as a gift from Svend O. Freytag, Detroit, MI. The pCA14-E1A/E1B plasmid contains the complete E1A gene, but has point mutations (introduced by PCR) at bases 2253 (C= > T) and 2262 (G = > T) to generate two premature stop codons in the 55kDa E1B gene that renders it ineffective, followed by a sole BglII site for introduction of the expression cassette. In this E1B attenuated (replication–competent) adenovirus construct, CMV promoter with the multiple cloning site (MCS) was introduced by blunt end ligations at the NOT1 and R1 site in the E1B deleted region, in our laboratory *([23] Mukherjee et al 2002).* The MCS has the R1 and HIND-III restriction sites where the gene of interest can be inserted. This vector (Ad-E1B□-MCS) was amplified by routine procedure of transformation and bacterial growth. The plasmid DNA was isolated using Qiagen plasmid purification kit. ***b) CCL21 cDNA***: The pORF-CCL-21 vector containing the cDNA for the chemokine CCL21) was purchased from Invivogen (San Diego, CA). The pORF plasmid has an ECORV site (compatible to ECORI site) upstream of the CCL-21 gene and a HINDIII site down

stream of the gene. We have used these two restriction enzyme sites for our cloning purposes. ***c)***. The pJM-17 vector used as the right hand vector for recombination is available in our laboratory**.** The cell line, HEC-293 cells was used for the recombination of the vectors and for production & propagation of the adenovirus.

B) Procedure: The two plasmids (pAd-E1B□-MCS and pORF-CCL-21) were digested with the restriction enzymes, ECOR1, ECO-RV and HINDIII respectively, by procedure provided by the manufacturer (Boehringer Manheim). The CCL-21 fragment was sub-cloned in to the compatible restriction (ECO-R1 and HIND-III) site of the Ad-E1B□-MCS by standard procedure. A schematic representation of the strategy has been outlined in Figure: 13. The resulting plasmid (pAd-E1B□-CCL-21) was transformed into bacteria. The resulting clones were amplified and plasmid DNA was purified by standard procedure. The Figure: 14 shows some of the clones obtained by this procedure. The clones were screened for the presence of the insert (CCL-21) by PCR, using specific primers. One of the clones (clone #4) was further amplified and plasmid DNA was purified using a Qiagen kit.

C) Virus generation: To generate replication-conditional recombinant viruses, the pAd-E1B□-CCL-21 was co-transfected with pJM17 into 293 cells to generate recombinant viruses, using the LipofectAMINE PLUS Reagent (GibcoBRL Life Technologies) according to the manufacturer's protocol. The presence of CCL-21 in the adenovirus lysates were identified by PCR using an upstream primer from the CMV promoter region (5'CACCAAAAT CAACGGGAC-3') and downstream primers from CCL21 (5' GGCGGGATCCTGGGCTAT-3'). For purification, the recombined viruses were harvested from tissue culture plates of 293 cells, after 36h of infection and purified using the virakit purification kit (*[27]Virapur, LLC, San*

Diego, CA.). Briefly, after multiple cycles of freezing and thawing, the cellular debris was removed by centrifugation and the clear supernatant with viable virus particles, was first treated with DNAse and further cleared by passing through 0.54 micron filter. After adding a buffer, the virus solution was slowly passed through a treated filter, which adsorbs the virus particles. The virus was then eluted with elution buffer. The procedure is simple and gives good yield. The virus particle was quantified by procedure described in the kit. Briefly, 50μl of purified virus was diluted in SDS buffer (0.1% SDS in PBS) and the OD was recorded in 260 and 280nm. The particle number was calculated as follows: OD 260 reading X 10 (dilution factor) X $1.1X10^{12}$ particles = the number of particles per ml of sample. The 260/280 reading ratio gives an indication of the purity of the preparation. Ratios in the range of 1.17 to 1.35 were considered acceptable purity level. The stock of viruses was aliquoted and stored at -80c at 10^{10} particle/ml in glycerol until use. The adenovirus containing beta-gal (ad5-CMV-beta-gal) was kindly provided by Dr.Roy Chowdhury (liver center, AECOM). The adenovirus infection efficiency was determined by the method described elsewhere *([22] Fan et al 2000).* Briefly, RM-1 cells (10^4) were seeded in 35 mm tissue culture plate and different concentrations of ad5-CMV-beta-gal was added for 5 hours and washed with culture medium and incubated for another 24 hr. Cells were then fixed with 2% formaldehyde and 0.05% glutaraldehyde for 5 min at room temperature. The cells were washed with PBS twice and stained in PBS containing 1mg/ml X-gal, 2 mM MgCl2 and 5mM potassium ferrocyanide at 37*C for overnight. The cells were then washed with PBS. Individual dark blue cells were counted to estimate the infectivity of RM-1 cells. A significant infectivity of RM-1 cells was observed in our experiments.

D) Cytopathic effect of Ad-CCL-21: For cytopathic studies, RM-1cells (10^5/well, 24 well plate) were either mock infected or infected with increasing multiplicity of infection (MOI) of the Ad-CCL-21 virus in 0.2-0.4 ml of DMEM containing 10% FCS. The plates were fixed and stained with crystal violet 5 days post infection.

a) Results: The figure 15 shows that there was significant cytopathicity of RM-1 cells at the different concentration of adeno-virus used.

E) Secretion of CCl-21 by RM-1 cells infected with Ad-CCL-21: RM-1cells (10^5/well, 24 well plate) were infected with 1 MOI of Ad-CCL-21 virus in D-MEM medium containing 10% FCS for 48 hours. Appropriate controls were used for the study. Following incubation, the cell supernatant was collected and estimated for the presence of CCL-21 using an ELISA kit (R & D systems).

a) Results: There was a significant amount of CCL-21 produced by the RM-1 cells that were infected with Ad-CCL21 as compared to controls (Figure-16). Similar events were expected when the virus was used for *in vivo* studies.

7. In vitro chemokine assay to demonstrate the mobility of activated DC. The assay was performed using trans-well membrane, 0.4□m (***[20] Hirao et al 2000)***. Aliquots (100 □l) of activated DC or naïve DC ($5x10^6$ /ml) were added to the upper chamber of the trans-well membrane fitted in a 24 well tissue culture plate and to the lower chamber either recombinant CCL21 (20 ng/ml) or culture supernatants from RM-1 cells infected with Ad-CCL21 were added. The DCs were allowed to migrate for 24 hours at 37^0C in a 5% $C0_2$ incubator, after which the filters were fixed with 1% glutaraldehyde in PBS for 30 minutes and stained with 0.5% toludine blue overnight. Cell migration was quantified by counting cells in each lower chamber**.**

a) Results: The Figure: 17 show the migration of activated DC (DC exposed to irradiated tumor cells) across the membrane in response to either rCCL-21 (20 ng/ml) or supernatant of RM-1 cells infected with Ad-CCL21 (as described above). The result shows that there was a significant migration of activated DC across the membrane in response to the cell supernatant. This implies that the recombinant virus would be suitable for *in vivo* use to study the effect of its administration on tumor development.

8) Administration of CCL-21 following FL+IL-12 treatment increases survival and generates tumor specific immune response:

A). Experimental design: ***a) Tumor transplantation***: Six-eight week old C57BL/6J male mice were injected with exponentially growing $5x10^4$ RM-1 cells on the dorsum of the foot. Two weeks post inoculation of tumor cells, all mice developed primary footpad tumors (~50-60 mg). Animals with established tumors were given a single dose of localized primary tumor irradiation (60 Gy) with or without intra-peritoneal recombinant cytokines (PeproTech, NJ), The irradiation dose of 60 Gy was selected after pilot experiments of 40-60 Gy showed that this fraction size was optimal for primary tumor regression and control.

b) Irradiation: Briefly, anesthetized (nembutal-45mg/kg) animals were placed into a Lucite jig with 0.5cm lead body protection and individualized compartments through which a circular port was accessible for localized leg irradiation (60 Gy). A 40 MGC Philips orthovoltage unit, operating at 320 kVp, 5mA and 0.5 mm Cu filtration (2.60 Gy/min exposure to the dorsum of the footpad within the jig at 31-cm SSD) was used. Thermo luminescence dosimetry (TLD) was used to a midline phantom within the jig and was the basis for all dose calculations.

c) Cytokine treatment: From the day of RT, the animals were administered with intra-peritoneal recombinant human FL (10 g/day/mouse x 10 days), and recombinant human IL-12 (1 g/day/mouse x 5 days). Following this treatment, recombinant CCL-21 was administered (0.5 g/day/mouse x three times a week - 5 doses).

The animals were partitioned for survival and immunological studies, For survival studies, animals received $5x10^4$ RM-1 cells (sc) on the dorsal aspect of upper hind limb three days prior to RT of footpad tumor. Animals were observed for survival. One week following last treatment with cytokines, the animals were used for immunological studies. The spleen was removed from the different groups of animals under aseptic condition.

C). Immune response studies: The isolated splenocytes were re-stimulated with irradiated RM-1 cells (60 Gy) at an effector and target ratio of 10:1 for five days and were tested for a) proliferation b) cytotoxicity c) cytokine profile.

i) Lymphocyte proliferation: Following co-culture for 5 days, the proliferation was determined by WST-1 assay by procedure described by the manufacturer ((Boehringer Mannheim).

ii) Cytotoxicity assay: The splenocytes were stimulated as described before and they were incubated with live RM-1 cells at an effector and target ratio of 10:1 for 24 hrs. The cytotoxicity was determined by LDH assay as per manufacturer's protocol (Boehringer Mannheim).

iii) Cytokine profile: The culture supernatants from the stimulated splenocytes were tested for the presence Th-1 cytokines (TNF- , IFN- and IL-12) by using ELISA kits (R &D systems; PeproTech). The results were read using ELISA reader

(Molecular Devices).

D). Results: The untreated tumor bearing animals succumbed to the tumor burden by ~3 weeks of tumor cell transplantation. The results obtained from the survival studies have been shown in figure 18. The animals in the FL+IL-12+CCL-21 group (D) had increased survival (median- 87 days) compared to RT controls (A), median- 25 days; long rank p = 0.02), as well as to the FL+IL-12 group (C), median- 64 days log rank- p= 0.018).

The stimulated splenocytes from group D had two folds increase in proliferation compared to RT controls and their cytotoxicity against fresh RM-1 cells was significantly higher (45.2 ± 7.2%) compared to RT controls. The cytokine profile of the group D splenocytes reflected an increase in Th1 cytokines [IL-2 (904.8 ± 30.6 pg/ml), and IFN-□(1020.7± 41.3 pg/ml)] compared to controls.

The results show that CCL-21 treatment of animals bearing irradiated tumor following DC expansion by FL+IL-12, significantly protected the animals and increased their survival time when given tumor cells (figure 18). This regimen also generated a tumor specific immune response as reflected by cytotoxic activity of the stimulated splenocytes. The long term *in vivo* experiments following the treatment with cytokines were not undertaken as there were no long-term surviving animals (disease free). However, the efficacy of the regimen was amply addressed with the immunization experiments as well as with the above experimental model wherein the live tumor cells were inoculated at a secondary site prior to irradiation of the primary tumor. This mimicked the *in vivo* metastatic experimental model.

9. ***Histology/Immunohistochemistry***

The tissues were collected from experimental animals and they were either formalin

fixed (10% buffered formalin) or cryo preserved in –80 ^{0} C. The tissues were processed and sectioned at the Central Histology Facility of the institute. Hematoxylin and eosin (H&E) staining was performed on the tumor tissue sections to ascertain the prostate cancer. Formalin fixed and cryo-sections were also used for staining with CD4+ and CD8+ T cells using HRP-conjugated antibodies (Pharmingen). The results showed that there were infiltrating T cells in the tumor tissue following treatment with the different regimens.

10. ***Intra-tumoral administration of Ad-ccl-21 increases survival in mice bearing prostate tumor:***

A). Experimental design: The tumor was developed in the dorsum of the foot of the C57BL/6J mice by procedure as described elsewhere in this report. The two-week old tumor (50-60 mg) received either 20 μl of PBS or $1x10^{10}$ particles of control adenovirus or the recombinant Ad-CCL-21 intra-tumorally thrice a week for two week. The animals were partitioned for survival and immune response studies.

B). Immune response studies: The isolated splenocytes from the different cohorts were re-stimulated with irradiated RM-1 cells (60 Gy) at an effector and target ratio of 10:1 for five days and were tested for a) proliferation b) cytotoxicity c) cytokine profile as described before.

i) Lymphocyte proliferation: Following co-culture for 5 days, the proliferation was determined by WST-1 assay by procedure described by the manufacturer ((Boehringer Mannheim).

ii) Cytotoxicity assay: The splenocytes were stimulated as described before and they were incubated with live RM-1 cells at an effector and target ratio of 10:1 for 24 hrs. The cytotoxicity was determined by LDH assay as per manufacturer's protocol

(Boehringer Mannheim).

iii) Cytokine profile: The culture supernatants from the stimulated splenocytes were tested for the presence Th-1 cytokines (TNF-α, IFN-γ and IL-12) by using ELISA kits (R &D systems). The results were read using ELISA reader (Molecular Devices).

B) Results: The figure 19 shows the results obtained from this study. The untreated tumor bearing animals succumbed to the tumor burden by ~3 weeks of tumor cell transplantation. The animals in the Ad-CCL21 group (C) had increased survival (median- 52 days) compared to controls (A), median-22 days; long rank p = 0.009), as well as to the adeno virus group (C), median- 35 days log rank- p= 0.006). The splenocytes from the Ad-CCL21 had significant proliferation ($p < 0.01$) and Cytotoxicity ($p < 0.01$) compared to controls. The cytokines TNF-α, IFN-γ, IL-2 and IL-12 from the stimulated splenocytes were also significantly higher than the controls ($p < 0.001$). The results showed that the administration of Ad-CCL21 was able to increase survival time in the tumor bearing animals and induce a tumor specific immune response in the murine prostate cancer model.

11. Effect of administration of CD40L following FL+IL-12 treatment on tumor development

A). Experimental design: a) Tumor transplantation: Six-eight week old C57BL/6J male mice were injected with exponentially growing $5x10^4$ RM-1 cells on the dorsum of the foot. Two weeks post inoculation of tumor cells, all mice developed primary footpad tumors (~50-60 mg). Animals with established tumors were given a single dose of localized primary tumor irradiation (60 Gy) with or without intraperitoneal recombinant cytokines (PeproTech, NJ),

b). Cytokine treatment: From the day of RT, the animals were administered ip with recombinant human FL (10 g/day/mouse x 10 days), and recombinant human IL-12 (1 g/day/mouse x 5 days). Following this treatment, recombinant CD40L was administered (1 g/day/mouse x 5 days). The animals were partitioned for survival and immunological studies. One week following last treatment, animals were used for immunological studies. For survival studies, animals received $5x10^4$ RM-1 cells (sc) on the dorsal aspect of upper hind limb three days prior to RT of footpad tumor. Animals were observed for survival. The immunological response studies were performed by the same procedure as described earlier in the text. As the experiments were performed with recombinant CD40L, it was not necessary to perform similar experiments with Ad-CD40L.

c). Results: The untreated tumor bearing animals succumbed to the tumor burden by ~3 weeks of tumor cell transplantation. The results are shown in figure: 20. The animals in the FL+IL-12+CD40L group (D) had increased survival (median- 92 days) compared to RT controls (A), median-25 days; long rank p = 0.02), as well as to the FL+IL-12 group (C), median- 64 days log rank- p= 0.0216).

The stimulated splenocytes from group D had two folds increase in proliferation and their cytotoxicity against fresh RM-1 cells was significantly higher (55.6 ± 9.4%) compared to controls ($p < 0.05$). The cytokine profile of the group D splenocytes reflected an increase in Th1 cytokines [IL-2 (728.8 ± 21.5 pg/ml), and IFN- (920.7± 33.7 pg/ml)] compared to controls ($p<0.05$).

The results showed that CD40L administration following DC expansion by FL+IL-12 in mice bearing irradiated tumor, significantly increased their survival when transplanted with live tumor cells. This regimen of FL+IL-12+CD40L following

irradiation of primary tumor was able to generate a tumor specific immune response in the murine prostate cancer model.

11. Statistics: The survival data was analyzed using statistica software (Statsoft Inc., Tulsa, OK). Log-rank test was performed after Kaplan-Meier analysis of survival data with Statistica 4.1 software for Macintosh. The results are expressed as mean ± SEM. The results were statistically evaluated by student T-test and were considered significant when $p<0.05$.

III. <u>Key Research Accomplishments</u>:

- Irradiation induced apoptosis in tumor cells.
- This is the first demonstration to show that irradiated prostate tumor cells can be used to load dendritic cells to elicit tumor specific immune response.
- The immature DCs that exhibit low levels of class II and other co-stimulatory molecules were able to take-up irradiated tumor cells more efficiently than the intact tumor cells.
- Activated DC had increased expression of CD86, MHC class-I & Class-II on the cell surface, which are required for immune response.
- The DCs pulsed with irradiated tumor cells expressed migratory receptor CCR-7 and its ligands CCL-19 and CCL-21.
- DCs pulsed with □-irradiated tumor cells were able to stimulate naïve splenocytes *in vitro*.
- Animals immunized with DCs that were previously exposed to irradiated prostate tumor cells showed increase in survival time when challenged with live tumor cells. The splenocytes from the immunized animals showed a tumor specific immune response.

- The replication conditional adeno-virus containing the CCL-21 gene (Ad-E1B□-CCL-21) was successfully generated using standard molecular Biology techniques.
- Administration of recombinant CCL-21 following DC expansion by FL+IL-12 and primary tumor irradiation significantly increased survival time when transplanted with live tumors. This regimen also generated a tumor specific immune response as evidenced from *in vitro* immunological studies.
- Administration of Ad-CCL-21 increased survival time in the animals with established primary tumor.
- CD40L treatment following DC expansion by FL+IL-12 and primary tumor irradiation significantly increased survival time when transplanted with live tumors. This regimen also generated a tumor specific immune response as evidenced from *in vitro* immunological studies

IV. Reportable outcomes:

1. Chakravarty P.K**,** Zoya Niazova. Dendritic cells pulsed with irradiated tumor cells increases survival and generate tumor specific immune response in a murine model of prostate cancer. Proceedings of the American Association of Cancer Research, abstract # 4765, 2003.

V. *Conclusions/significance*

The present study confirms and extends the earlier findings *([1]Albert et al 1998, [4] Albert et al 1998)* that i) DC that capture killed influenza virus infected cells can activate lymphocytes to generate virus specific immune responses; and 2) Dc that capture killed tumor cells over expressing tumor antigens can present antigens to specific T cells. One primary observation in this study is the ability of irradiated

tumor cells and not whole tumor cells to efficiently activate DCs. And these activated DCs can stimulate splenocytes that respond by killing the tumor cells.

Major problems in tumor immunotherapy are 1) The paucity of defined Tumor Associated Antigens (TAA) and 2) the lack of evidence that show that the known TAAs actually represent rejection antigen *in vivo*. Furthermore, the use of MHC class I binding peptides is associated with 1) the HLA restriction and 2) the limitation of induced immune responses to CD8+ T cells. In this context, unfractionated antigenic material in the form of irradiated tumor cells, which could provide both MHC class I and MHC class II epitopes could lead to a diversified immune response. This supports the 'danger signal' hypothesis, which emphasizes that DCs are required to receive adequate 'signals' from the effected tissues in order for them to acquire tumor antigens *([28] Metzinger 1994)*. In our experiments irradiation provided the danger signals. The DCs were able to take up irradiated tumor cells (un-fractionated tumor antigens) and transit through maturation process to evolve in to potent antigen presenting cell (APC) that presented antigens to naïve splenocytes resulting into significant proliferation of the splenocytes compared to the unpulsed DC or DC pulsed with non-irradiated tumor cells. Thus the DCs appear to play a pivotal role in acquisition and presentation of exogenous antigens from irradiated tumor cells. The un-fractionated antigenic material in the form of irradiated tumor cells provided both MHC class-I and MHC class-II epitopes that should be able to initiate a diversified immune response. The migration of activated DCs is dependent on the expression of CCR-7 and its ligands CCL-19 and CCL-21 (*[11] Sozanni et al 1998*). The two novel chemokine cytokines also have a preferential expression in T cell areas of lymph nodes, HEV & lymphoid tissue *([29] Willimann et*

al 1998). Recent studies have shown that ectopic expression of the murine chemokine CCL21 induces the formation of lymphnode-like structures in pancreas *([30] Chen et al 2002)* suggesting its central role in lymphocyte migration and for lymhoid architecture. Upregulation of receptors on mature DCs for chemokines that are expressed in secondary lymphoid organs allows DCs to leave the sites of inflammation and antigen uptake, and to migrate to regional lymph nodes. The mature DCs once in the T cell area, present antigens to the naïve CD4+ and CD8+ T cells for their activation and proliferation. In this study, we investigated the expression of chemokine receptors on DCs after they were pulsed with irradiated tumor cells. And we found that CCR-7 mRNA was upregulated along with the mRNA for CCL-19 & CCL-21. However, the DCs that were co-cultured with the supernatant of recently irradiated tumor cells did not express mCCR-7. Thus the immature DCs when in contact with apoptotic irradiated tumor cells acquired altered expression of chemokine receptors and its ligands that would help them to migrate to lymphoid organs. This suggested the necessity for DCs to directly interact with irradiated tumor cells to induce changes in chemokine receptor usage. This has been confirmed by a study wherein CCR-7 gene transduction augmented the migratory ability of DC (*[31] Okada et al 2005*).

Consistent with our *in vitro* findings, the *in vivo* studies showed that DCs co-cultured with irradiated tumor cells efficiently immunized naïve animals against challenge with RM-1 cells and these animals lived longer than the non-immunized animals. The splenocytes from the immunized animals had significant immune response when tested *in vitro*.

The DCs that play such an important role in generating immune response can be amplified *in vivo* by a cytokine, FLT3 ligand, FL (*[13] shurin etal 1997*). Studies have shown that administration of FL following hematopietic cell transplantation increased circulating DC precursors (*[32] Chen et al 2004*). FL also expands DC in normal and malignant prostate (*[33] Moghaddani et al 2002*). A recent study showing increased plasma FL level following radiation in mice (*[34] Prat et al 2005),* raises the assumption that FL production could also take place in response to some kinds of stress. If such events occur *in vivo*, our proposed regimen using RT along with FL would be further bolstered. In our regimen, we used FL in combination with IL-12 to expand DC *in vivo* following a single dose of localized irradiation (RT). And subsequently CCL-21 or CD40L was administered to ensure the development of appropriate immune response. We observed that administering either CCL-21 or CD40L following *in vivo* DC expansion, to irradiated tumor bearing animals significantly increased their survival time.

Studies have shown that in MHC negative prostate tumors, the micro-environment profoundly diminishes the molecules critical for normal DC and T cell function, thus limiting the efficacy of FL and CD40L immunotherapy (*[35] Ciavarra et al 2003*, *[36] Ciavarra et al 2004*). Thus immunotherapy using FL & CD40L require adjuvant modalities to achieve strong immune response in such tumors (*[37] Nagayama et al 2003*). However, this is not to undermine the importance of cytokines especially CD40L for immune response development because CD40L ligand enhances APC capacity to stimulate specific CD4+ & CD8+ T cell response (*[38] Feder-Mengus et al 2005*). Their impairment may cause defective IL-12 & TNF-□ production (*[39] French et al 2005*). Thus using an additional regimen that involves the destruction of the

tumor along with the cytokines would affect the tumor microenvironment. In this protocol, RT could achieve this goal.

In both our *in vitro* as well as *in vivo* studies using either activated DC or their expansion by cytokines, the splenocytes showed a significant immune response when tested *in vitro*. However, the immune response observed *in vitro* was not translated *in vivo* in terms of survival. This could be due to various reasons. One of the major reasons being the slower rate of destruction of tumor cells by CTLs in comparison to the tumor progression.

In our studies, the splenocytes involved in the immune response possibly are representative of both CD4+ and CD8+ T cell populations functionally as demonstrated from the high levels of IFN-γ and IL-2 in the supernatants of naïve splenocytes that were stimulated with DC pulsed with irradiated tumor cells as well as those obtained from the *in vivo* experiments. Both these cytokines have been implicated as important for favoring the development of a Th1 immune response *(*[40] *Fields et al 1998)*. The activated splenocytes were also significantly cytotoxic against the prostate tumor cells suggesting its specificity.

Immunotherapy with DCs is extensively being used for treating various diseases including cancer *(*[41] *Banchereau et al 2000).* Availability of activated DCs *in vivo,* therefore, appears to be central to the development of an effective immunotherapy against prostate cancer ([42] *Chakravarty et al 2003*). Our study has shown that either immunization of animals with DCs pulsed *in vitro* with irradiated tumor cells or their *in vivo* expansion using cytokines (FL+IL-12) followed by CCL-21 or CD40L administration to bolster the response, should be an effective tool for developing robust immunotherapy in patients with prostate cancer. We have demonstrated by an earlier study the efficacy of CD40 ligand following irradiation for the

treatment of lung cancer (*[43] Chakravarty et al 2002*). The present study also demonstrated the efficacy of using RT on established tumor burden for developing effective immunotherapeutic strategy. Similar approach of immunotherapy following tumor cell killing by herpes simplex virus thymidine kinase/ganciclovir suicide gene therapy has shown promise in a recent study using murine prostate cancer (*[44] Pandha et al 2005)*. One major criticism of targeting irradiation for generating immune response has been the possible development of auto immunity. However, development of immunotherapy for prostate cancer based on the induction of autoimmunity to prostate tissue could be a very attractive strategy because prostate is not a vital organ beyond the reproductive years.

Collectively, this study provides a novel immunotherapeutic strategy to treat advanced prostate cancer patients following radiotherapy. A protocol for treating prostate cancer patients undergoing radiotherapy could be developed immediately based on these findings.

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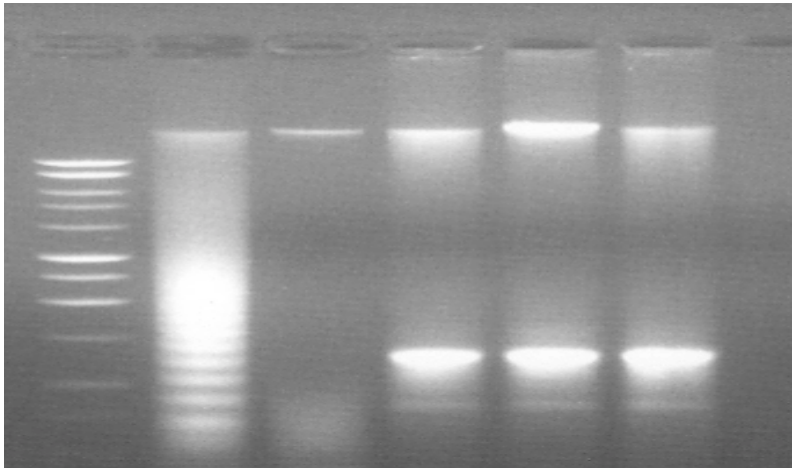
APPENDIX-A

FIGURES 1 THROUGH 20

DNA Ladder- 60 Gy

DNA LADDER



A B C D E

A - (+) CTR - U937 cells

B - RMI non irradiated

C - RM-1 60 Gy/4 day

D - RM-1 10 Gy/4 day

E - RM-1 10 Gy/7 dy

Figure:1A

DNA Ladder-10 & 20 Gy

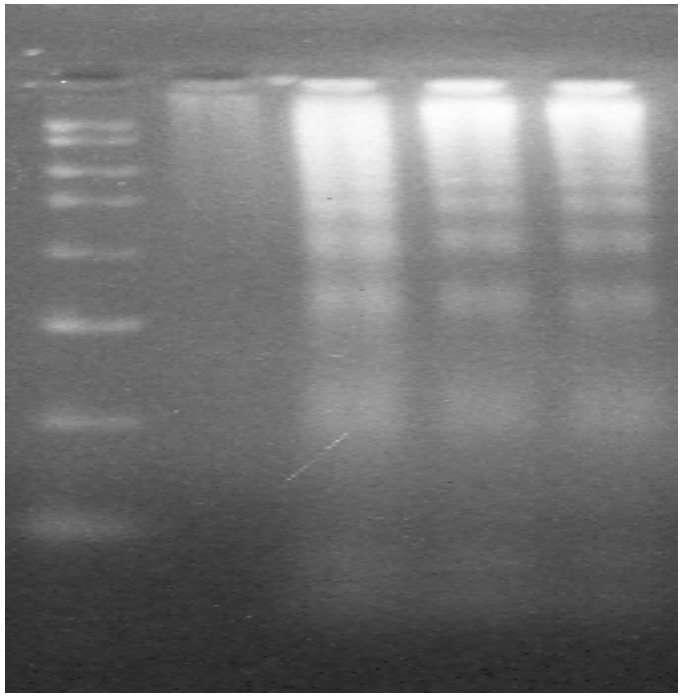


Figure:1B

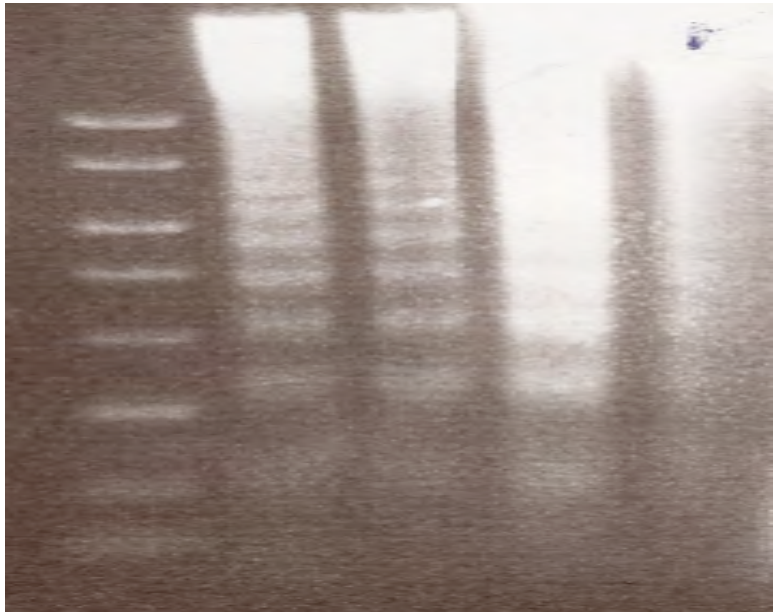


Figure:1C

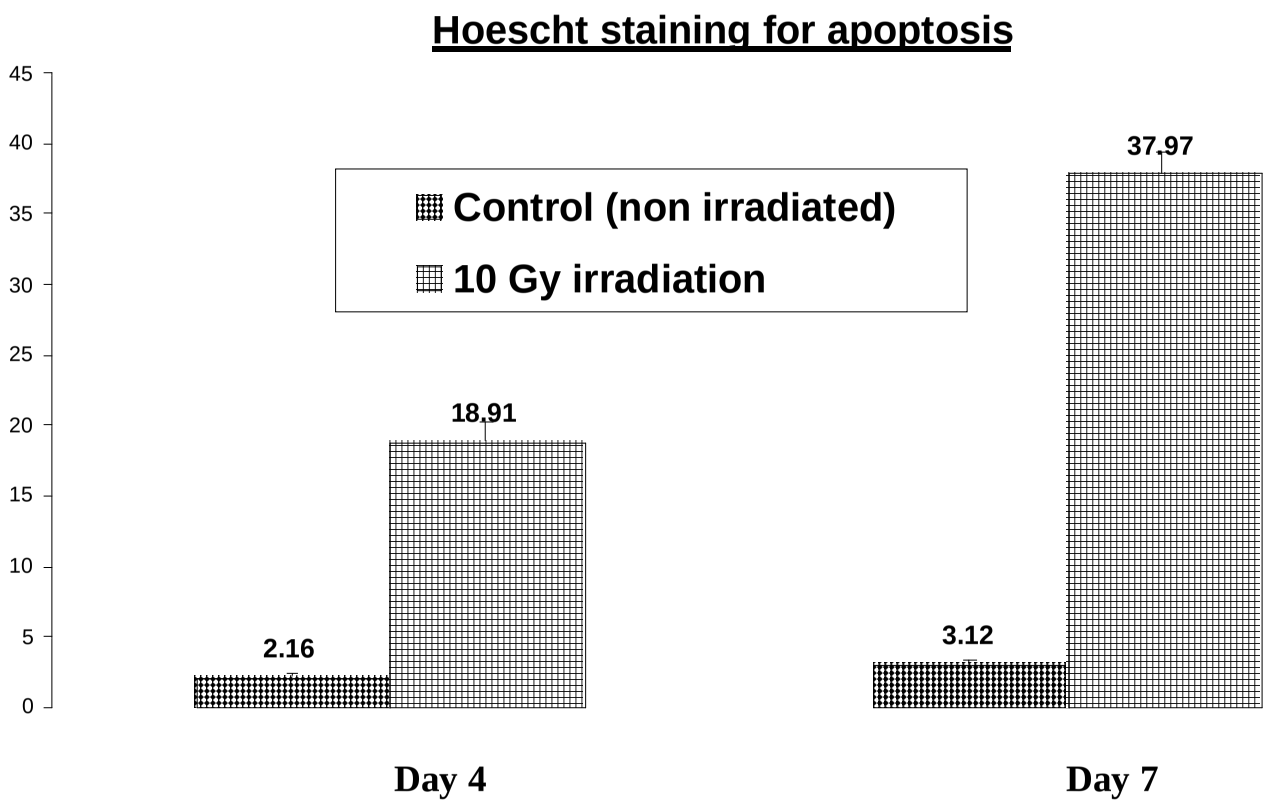
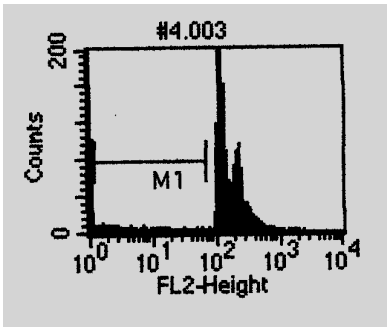
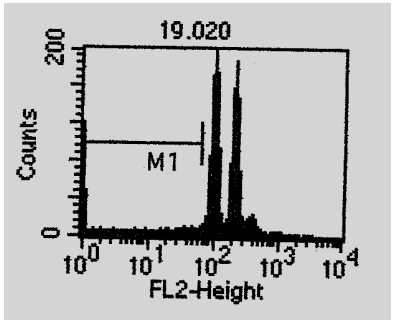

Hoescht staining for apoptosis
45
40
35
30
25
20
15
10
5
0
Control (non irradiated)
10 Gy irradiation
2.16
18.91
3.12
37.97
Day 4
Day 7

Figure:1D

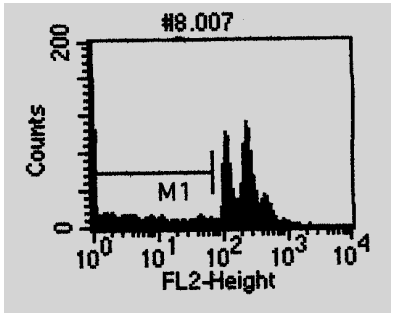
Pre- G1 fraction (Apoptosis) in Irradiated RM-I cells as demonstrated by FACScan



Control
4 day



10 Gy
4 day



60 Gy
4 day

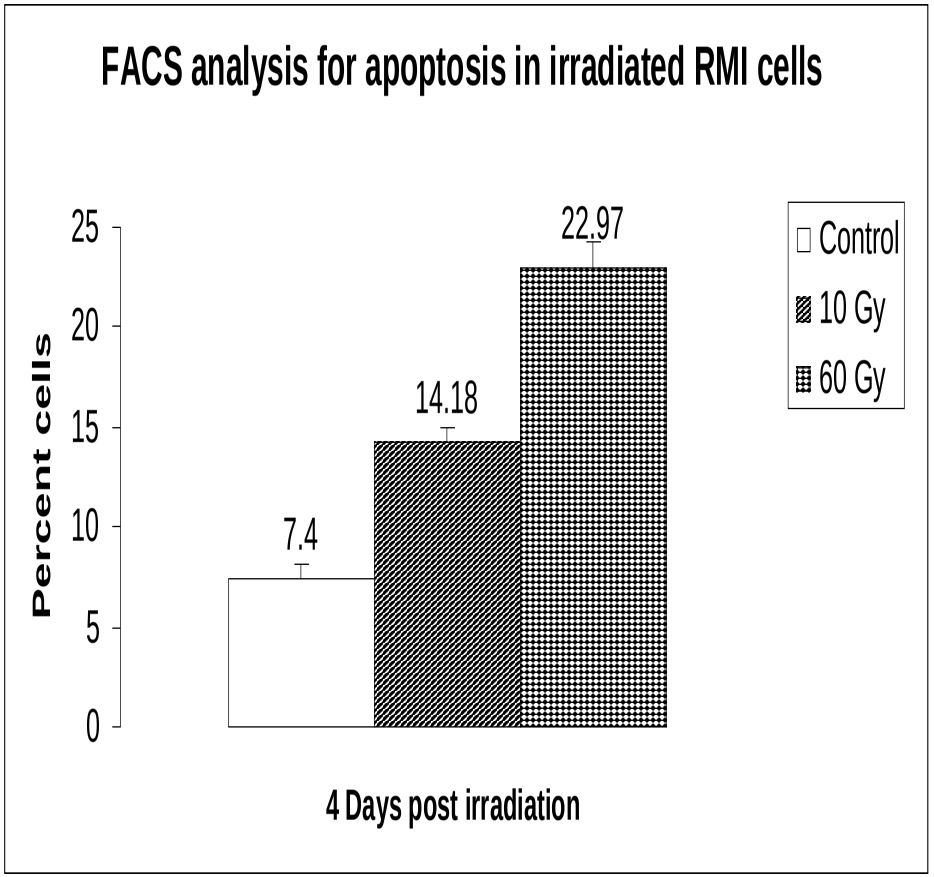
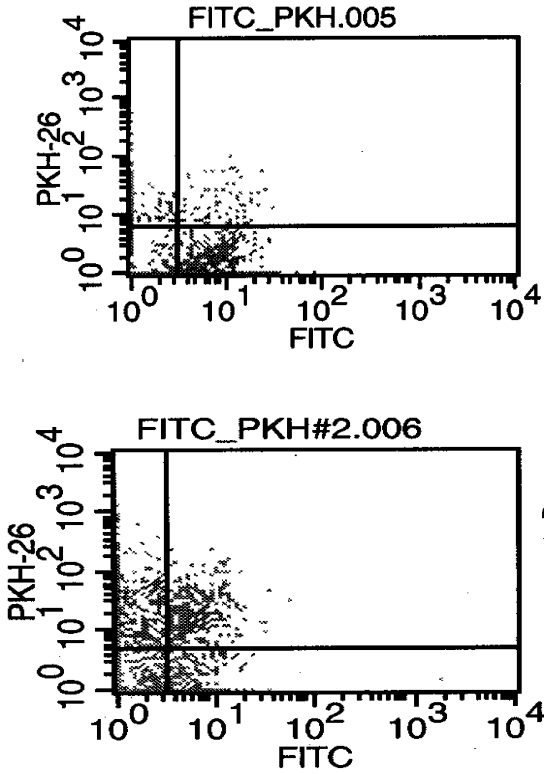


Figure:1E

DC Take up irradiated RM-1 Cells



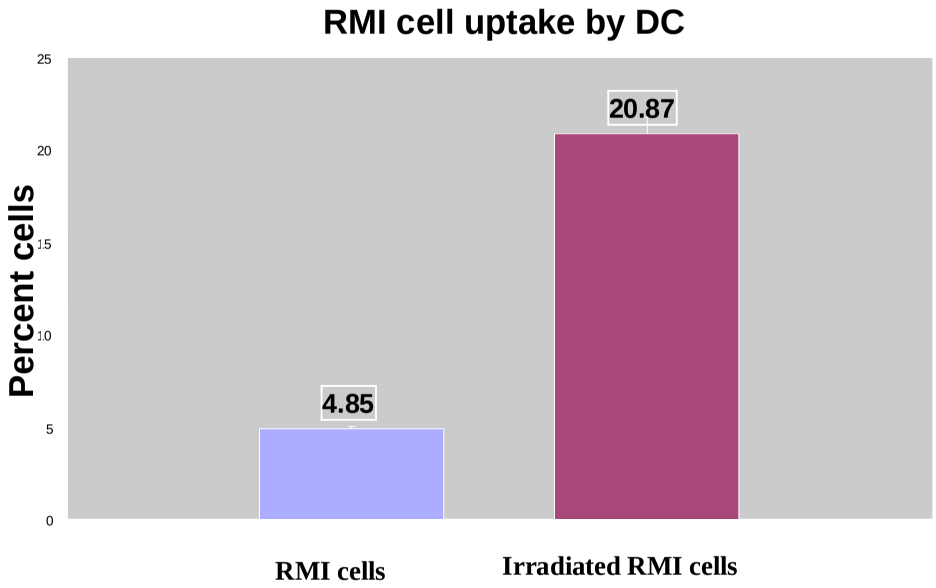


Figure: 2

Expression of CCL-19 and CCR-7

Figure 3A

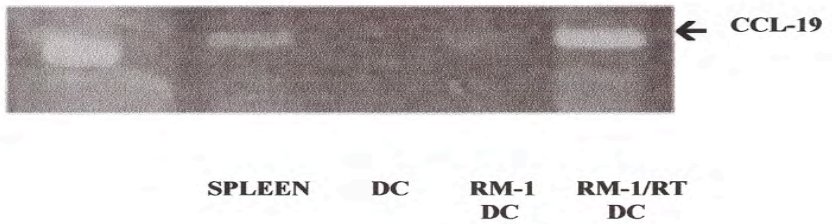
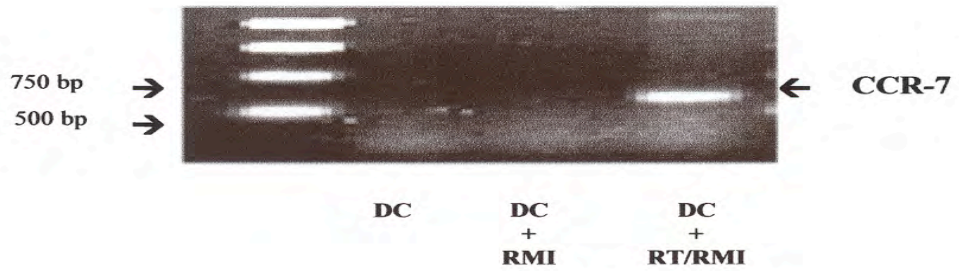


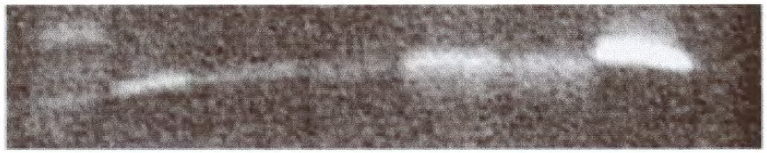
Figure 3B



Expression of CCL-21 and □- actin

Figure 3C

Figure 3D



FACSCAN OF CELL SURFACE MOLECULES ON DC

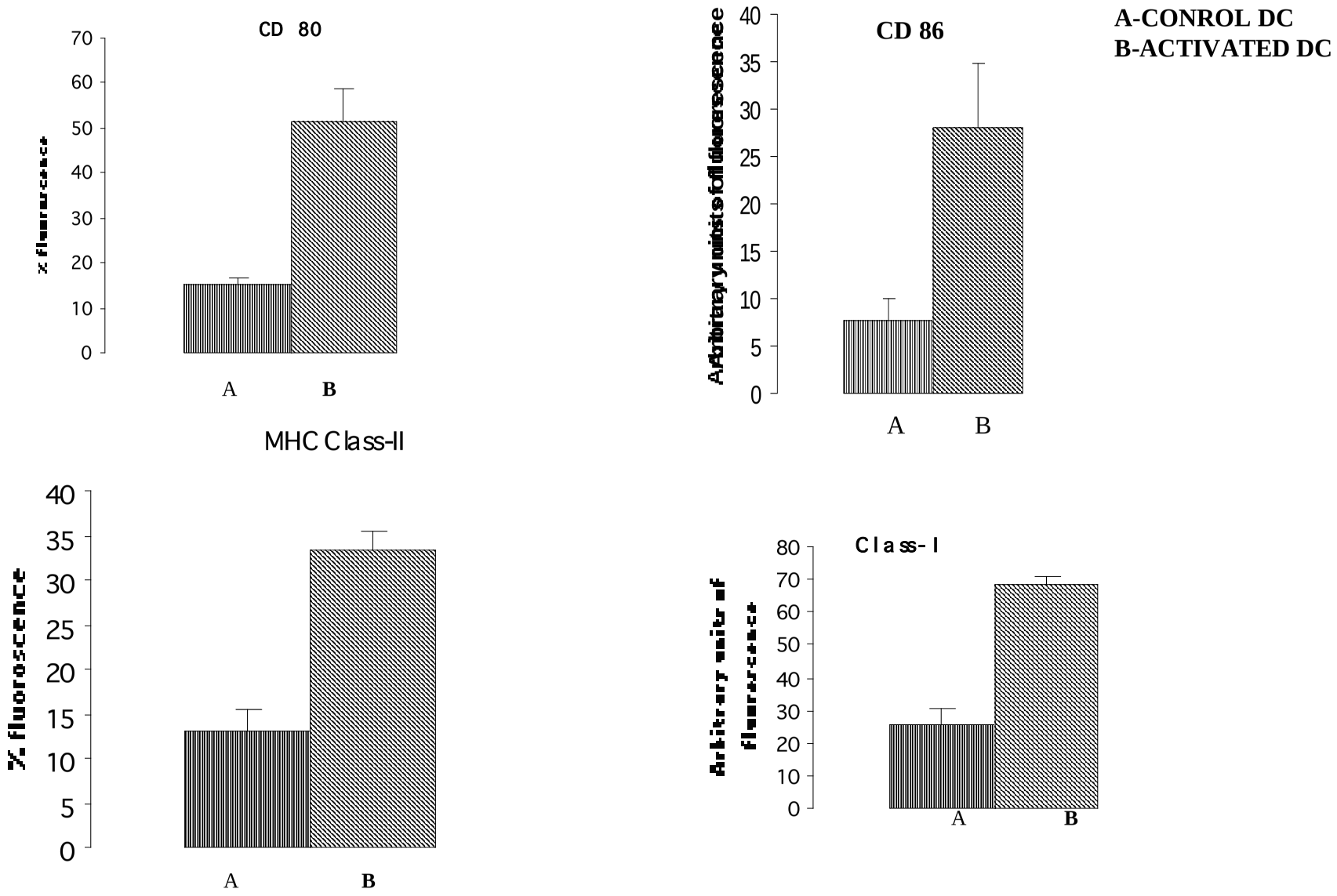


Figure: 4

Lymphocyte proliferation (WST assay)

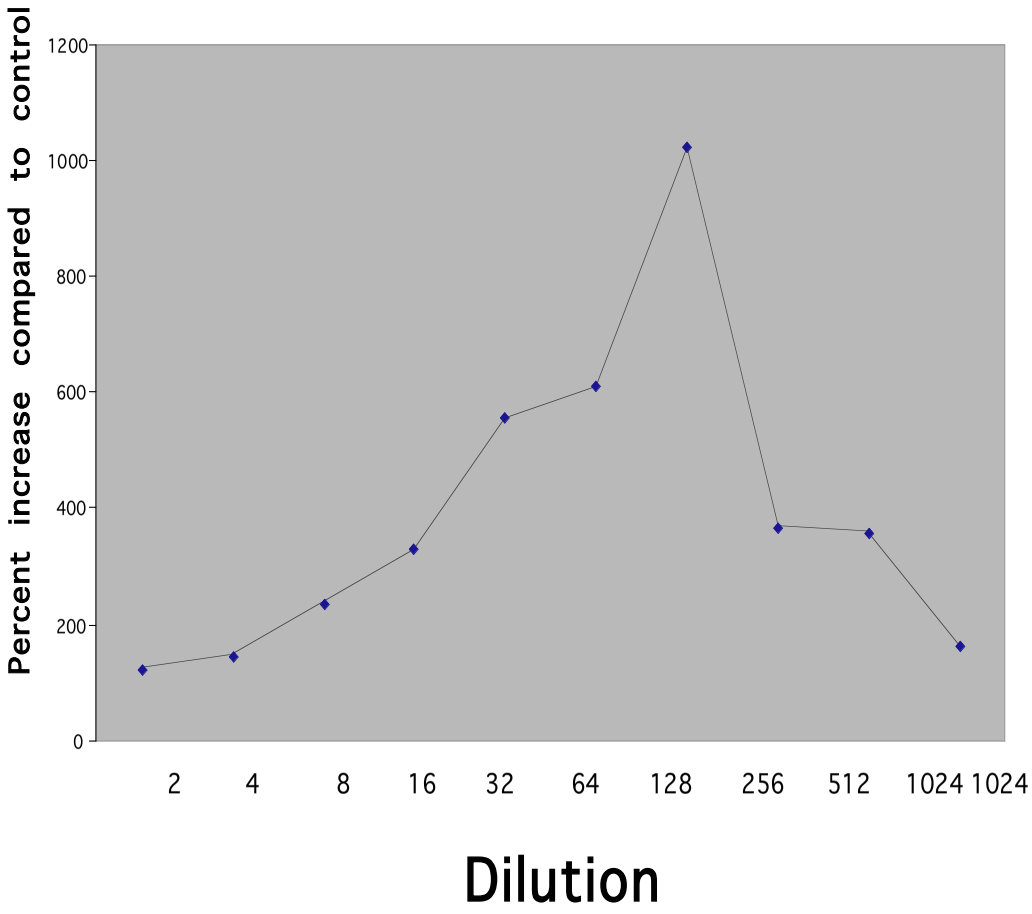


Figure: 5

Cytotoxicity Assay

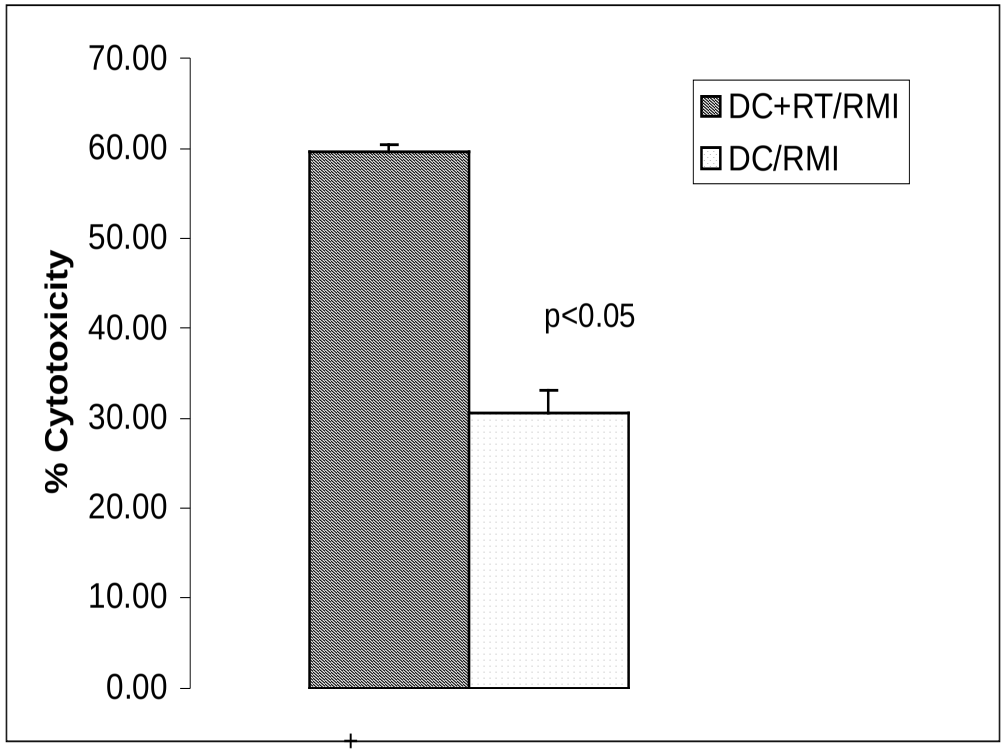


Figure: 6

Cytokine profile

IFN-g

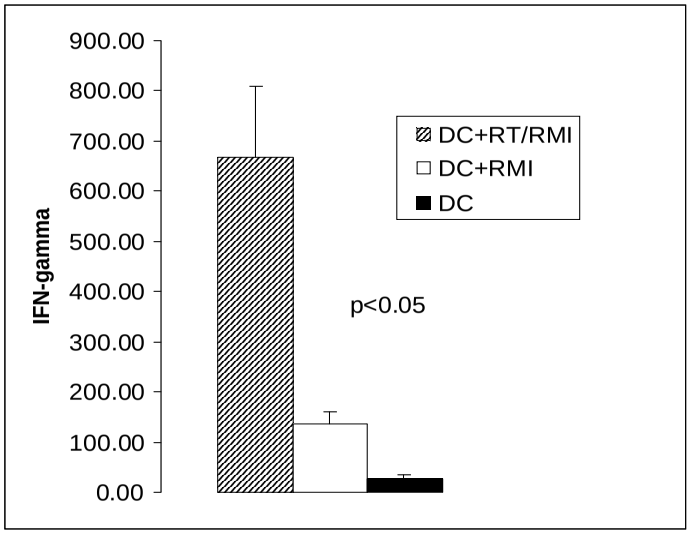


Figure:7A

IL-12

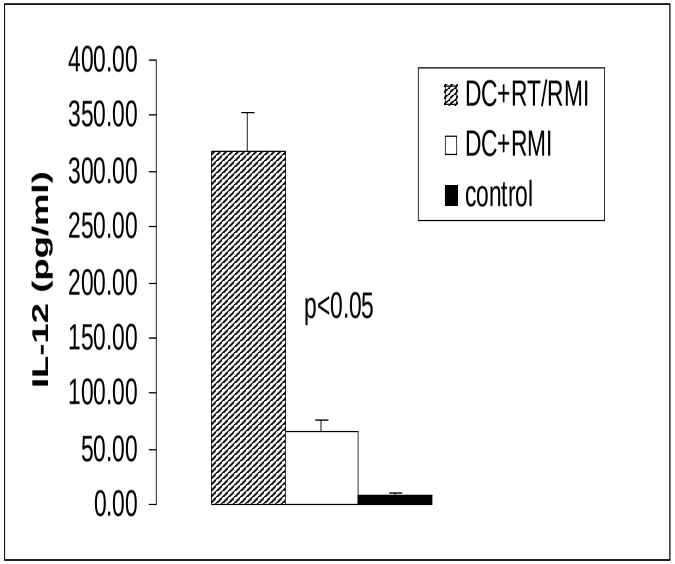
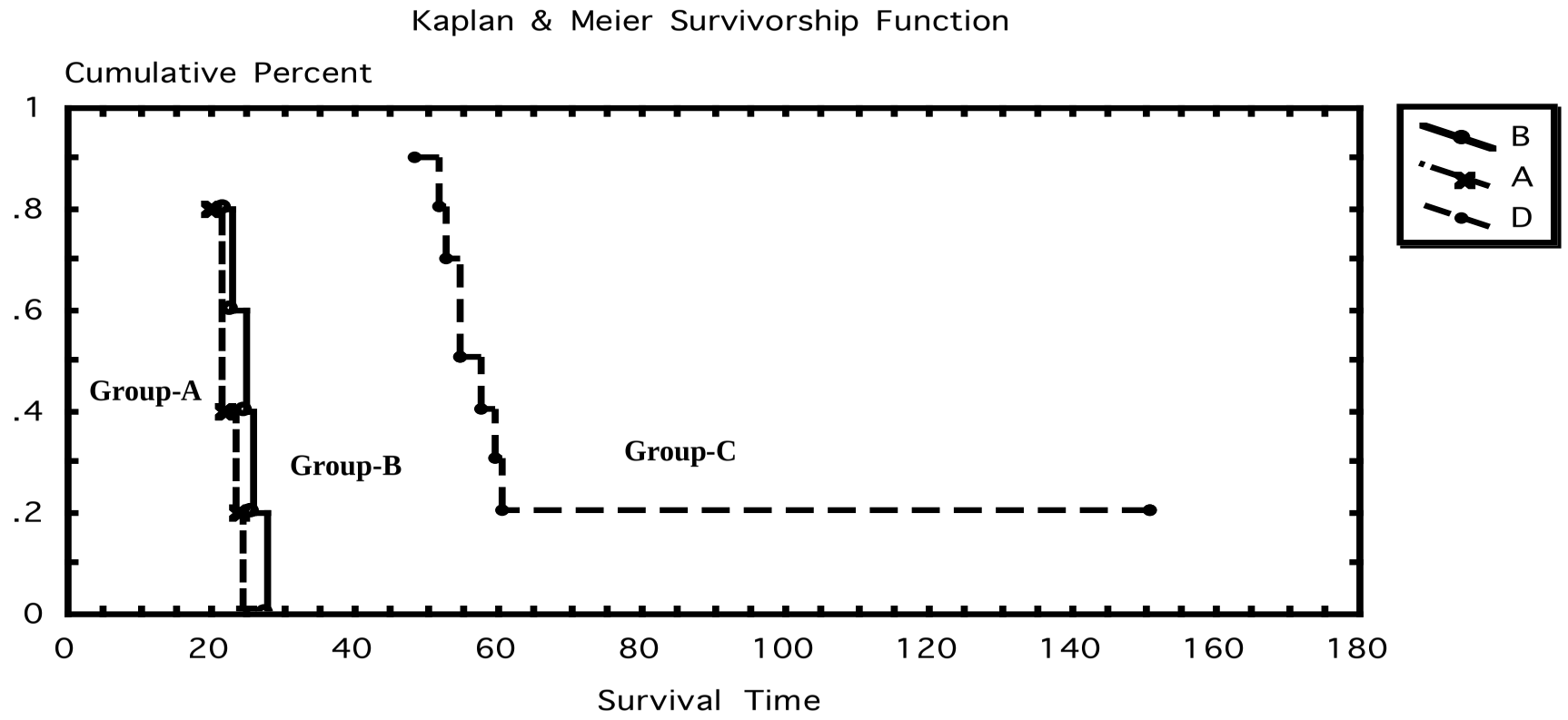


Figure:7B

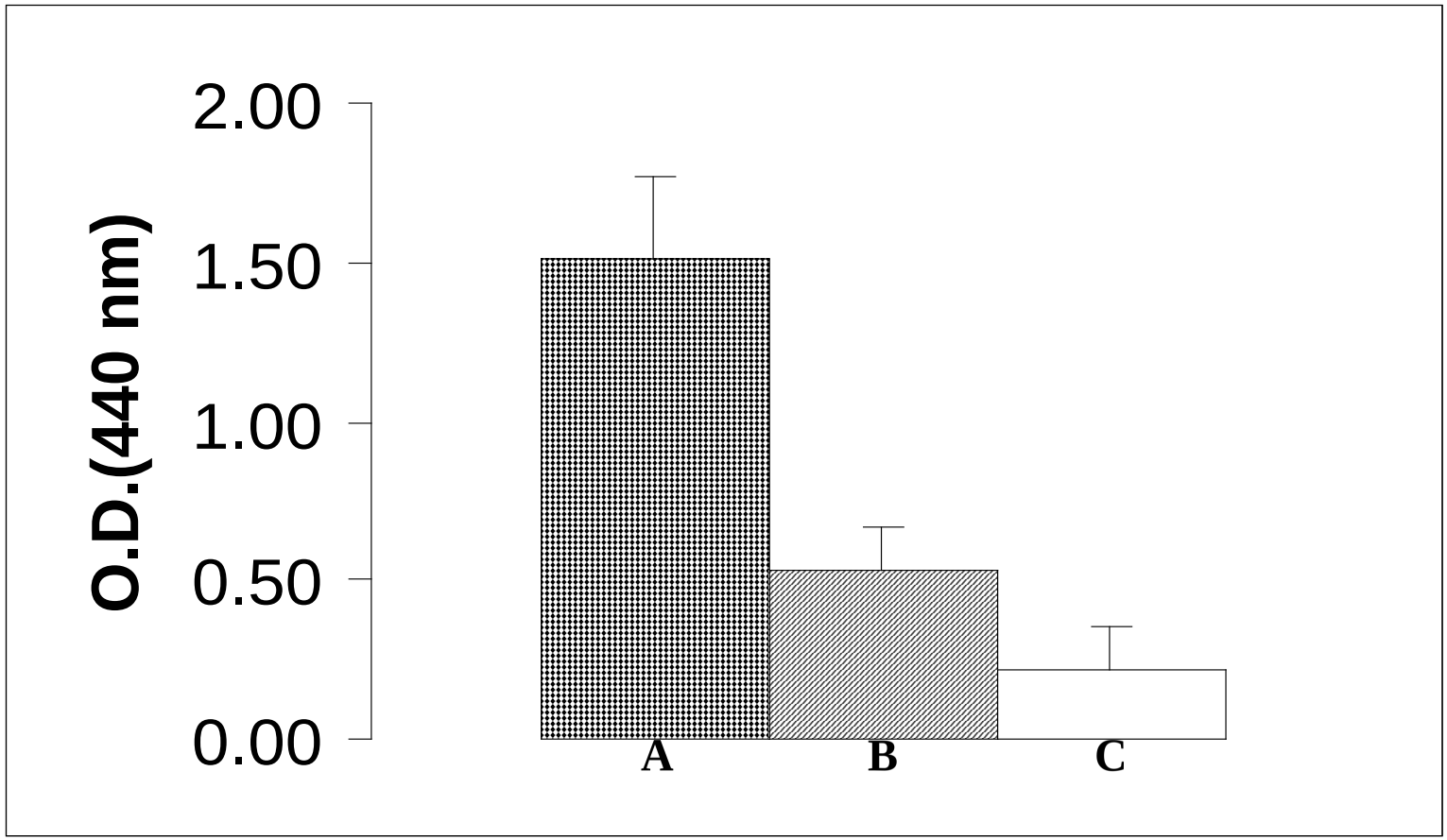
Survival Graph



A-CONTROL, B- DC ONLY, D- DC+IR-RM-1 CELLS

Figure: 8

Proliferation Assay



Splenocytes obtained from animals (A) immunized with DC+ irradiated RM-1 cells, (B) withDC alone and (C) tumor bearing animals.

Figure:9

Cytotoxicity Assay

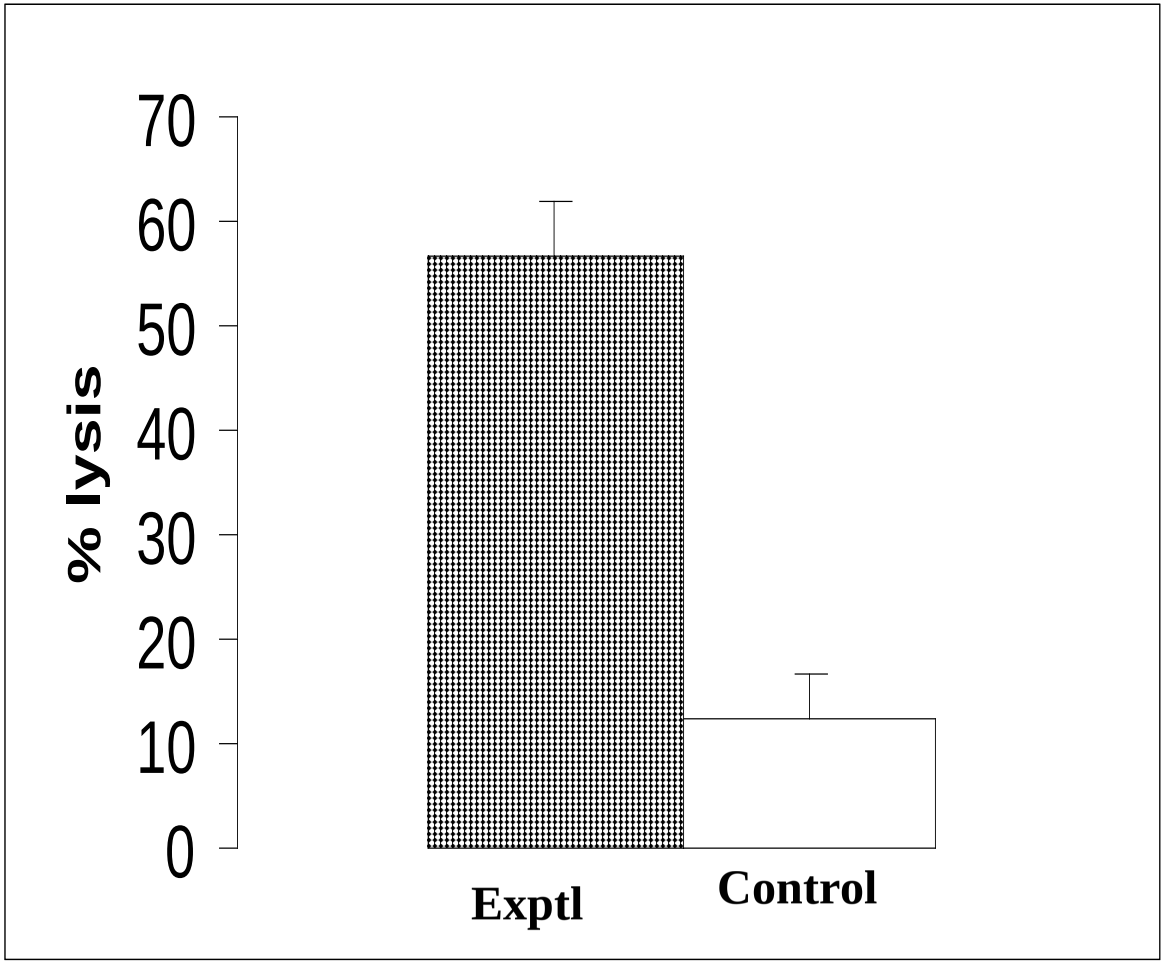
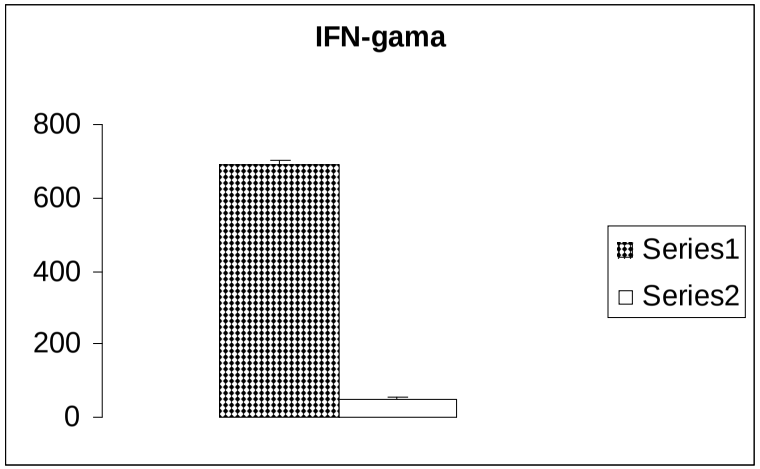
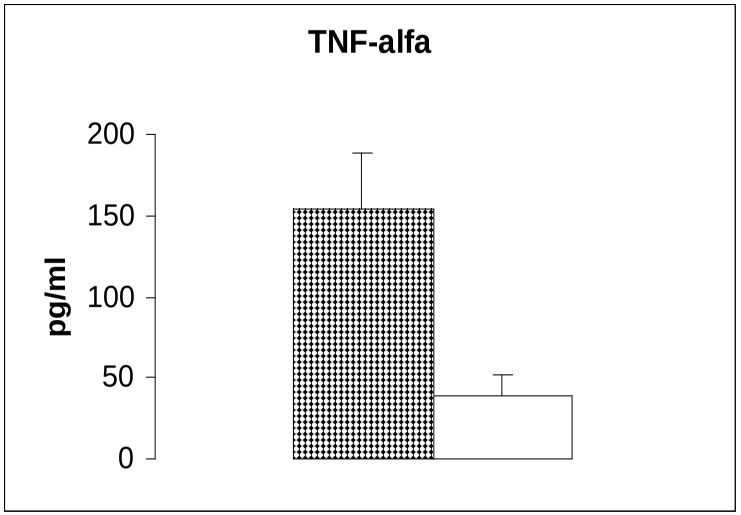
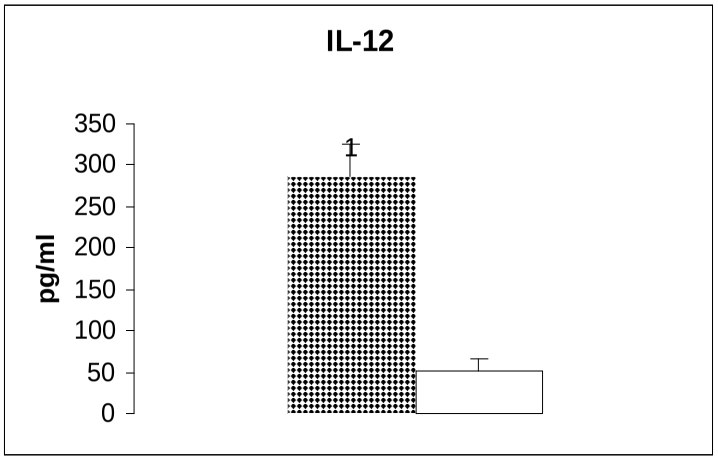


Figure:10

Cytokine Profile







Series 1- experimental (activated DC)
Series 2- controls (non- activated DC)

Figure:11

Survival graph

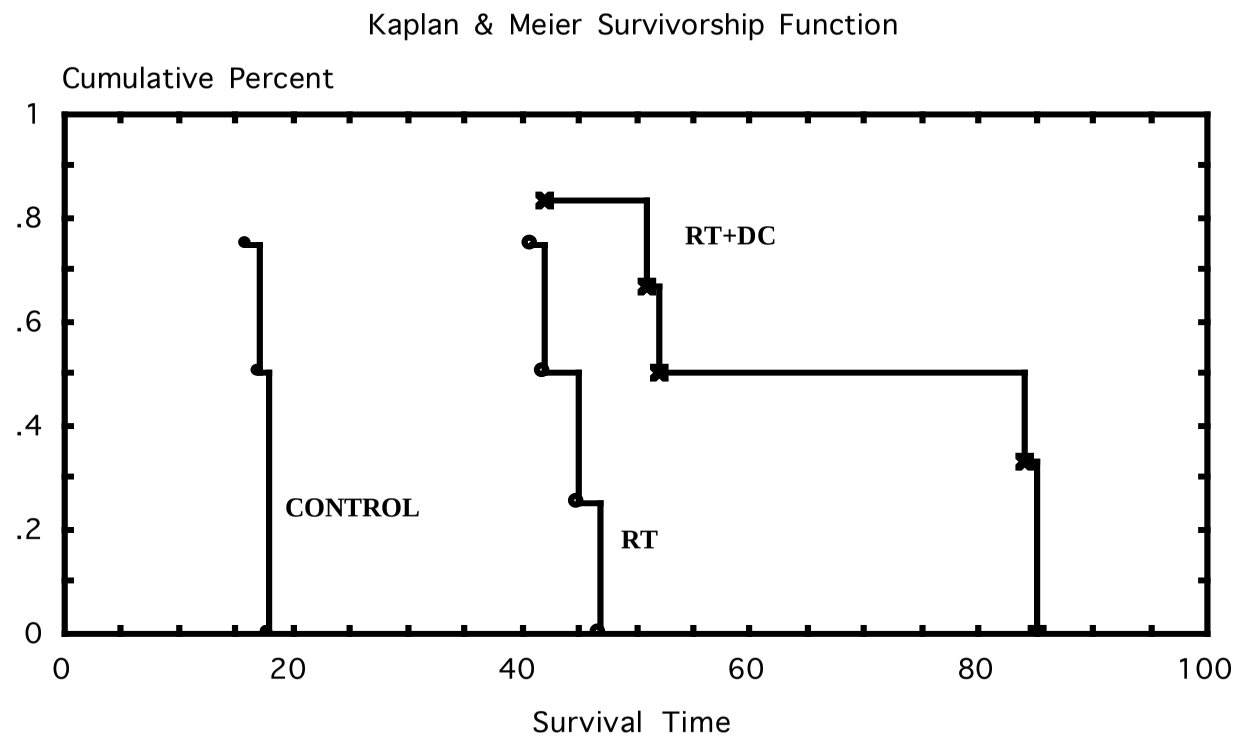
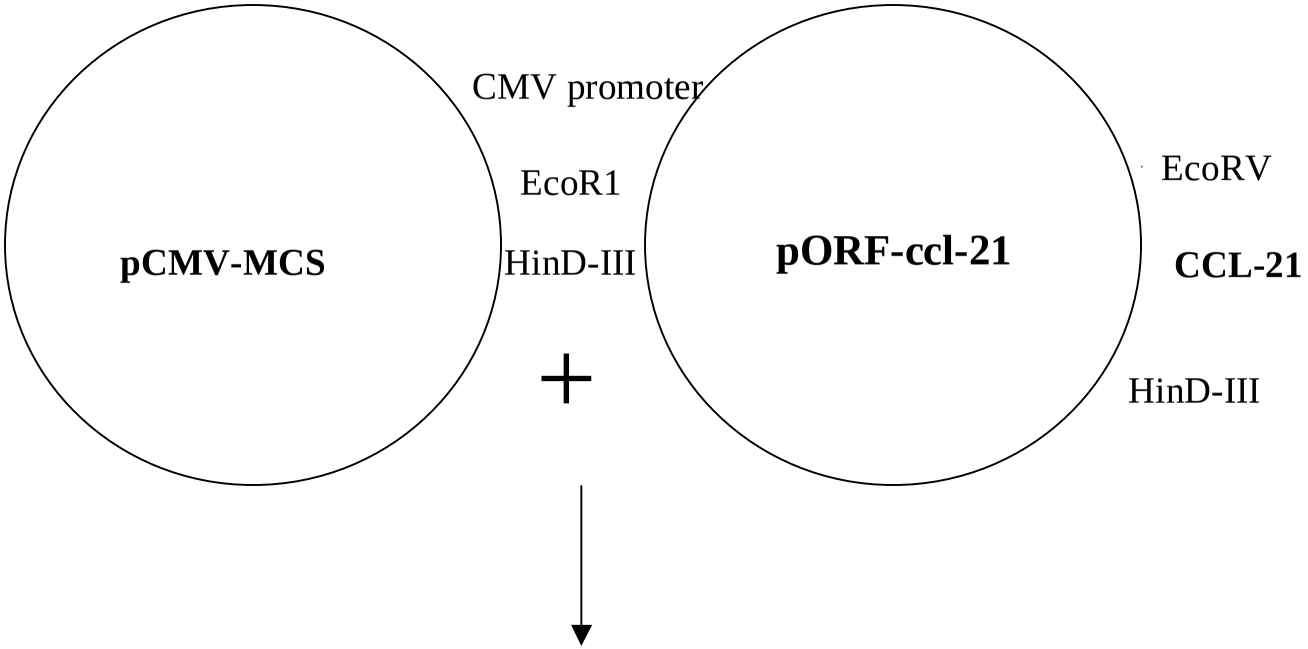


Figure:12

Schematic presentation of construction of p Ad-CCL-21



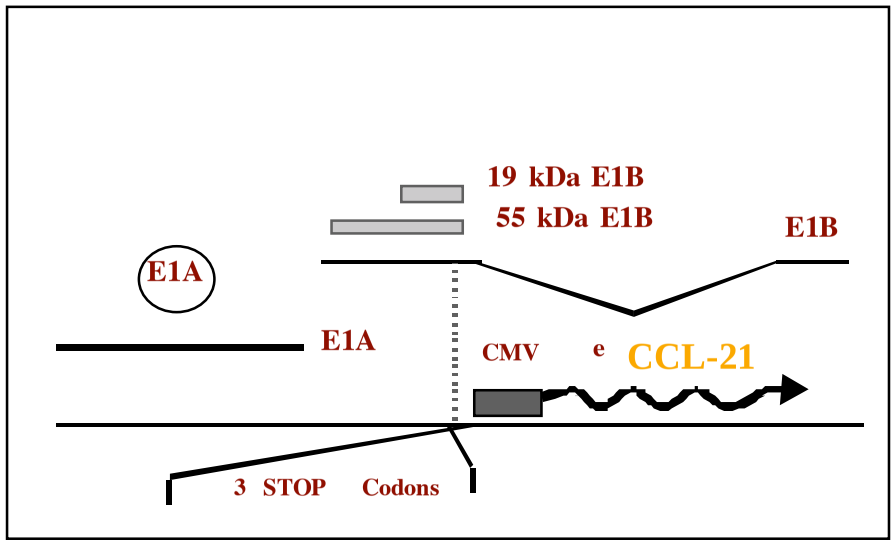
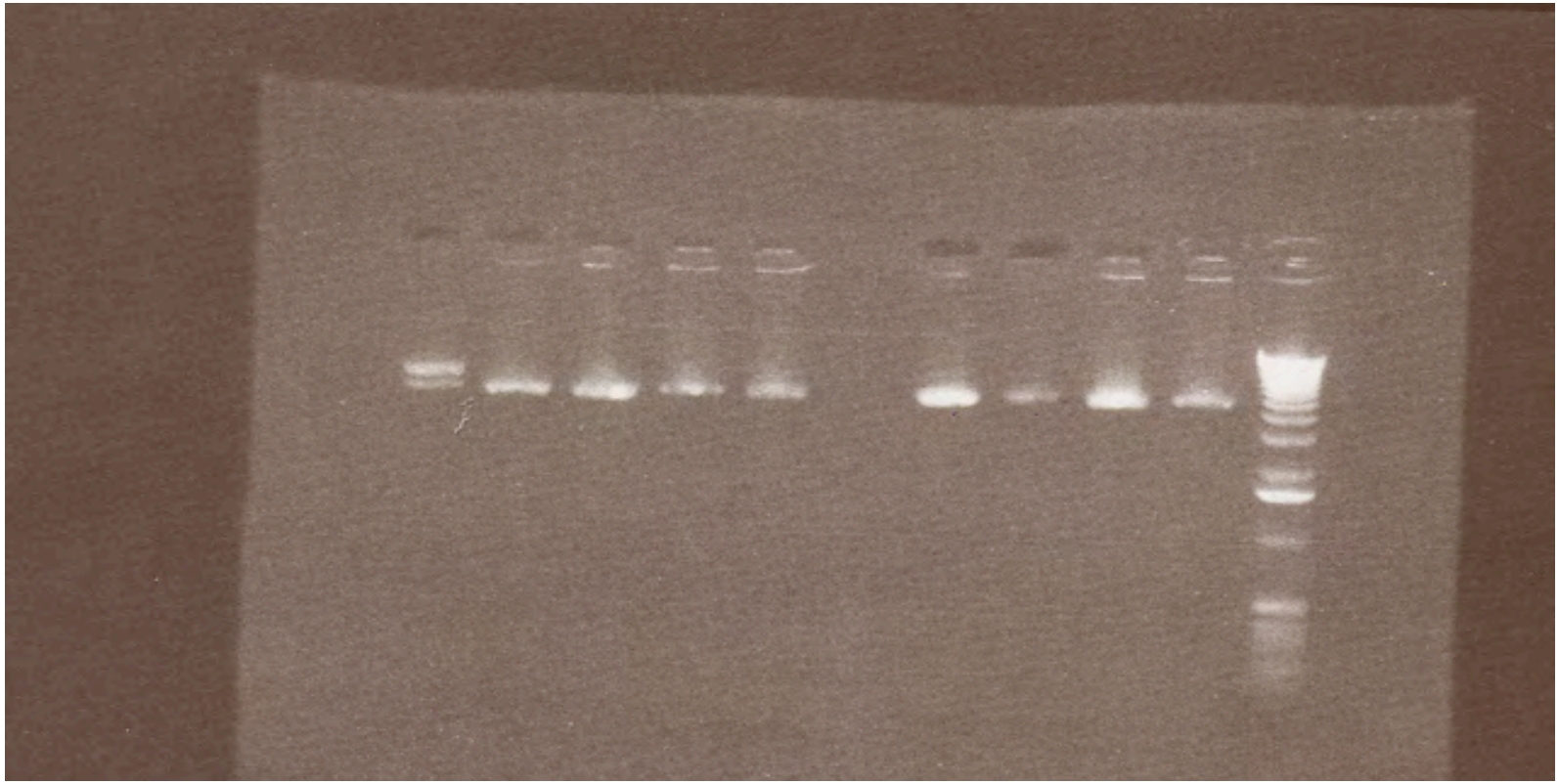


Figure:13

Plasmid DNA Of Different Clones OF pAD-CCL-21

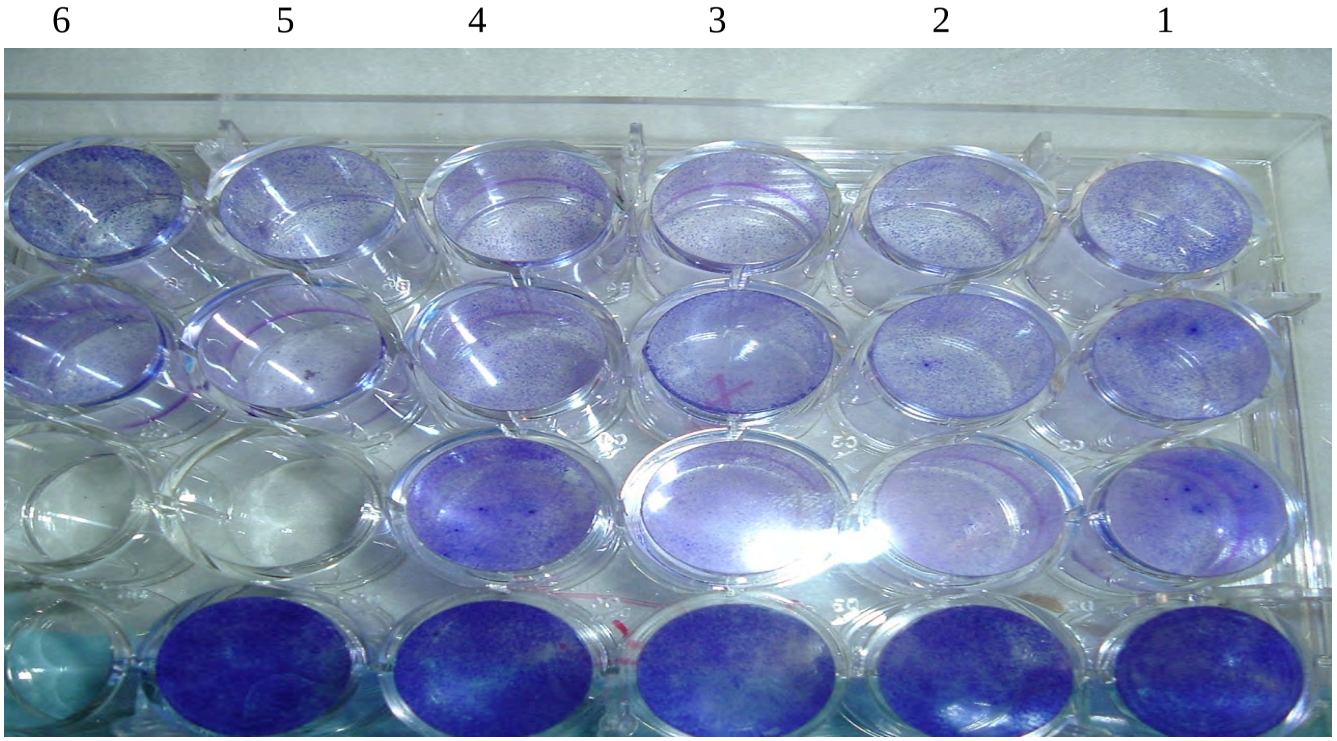


NR 1 2 3 4 M

NR- NOT RELEVANT; CLONES: 1-4 pAD-CCL-21; M-1 KB LADDER

Figure:14

Cytopathic assay of RM-1 cells



A 1- 4 1MOI, A5-6 & B1-2 - 2 MOI, B 3-6 4 MOI, C--Not Relevant, D-control cells

Figure 15

CCl-21 Assay

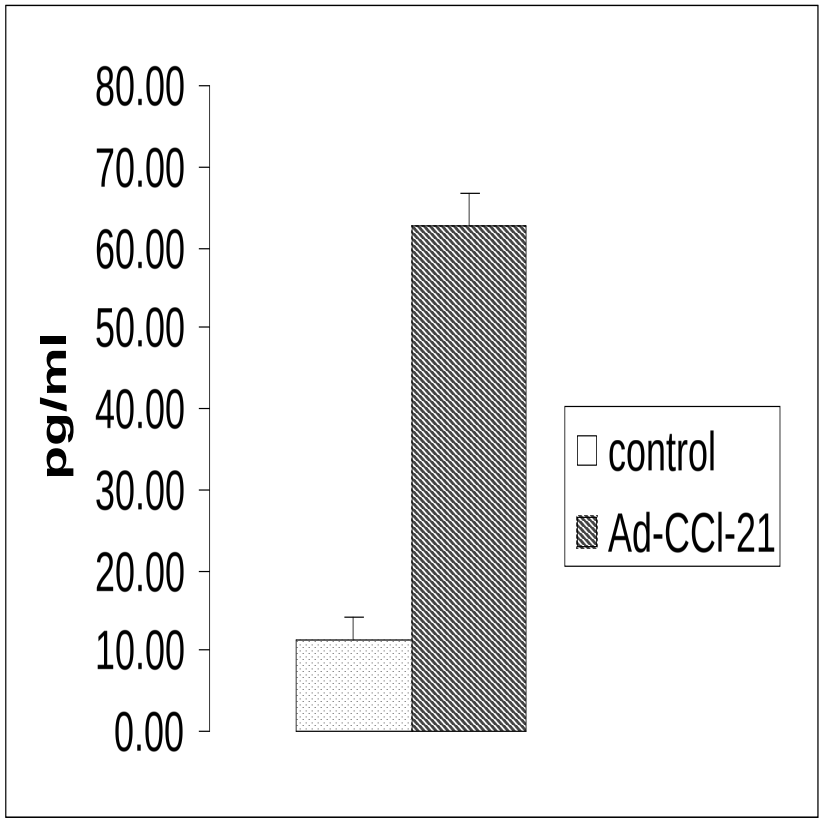


Figure 16

DC Migration assay

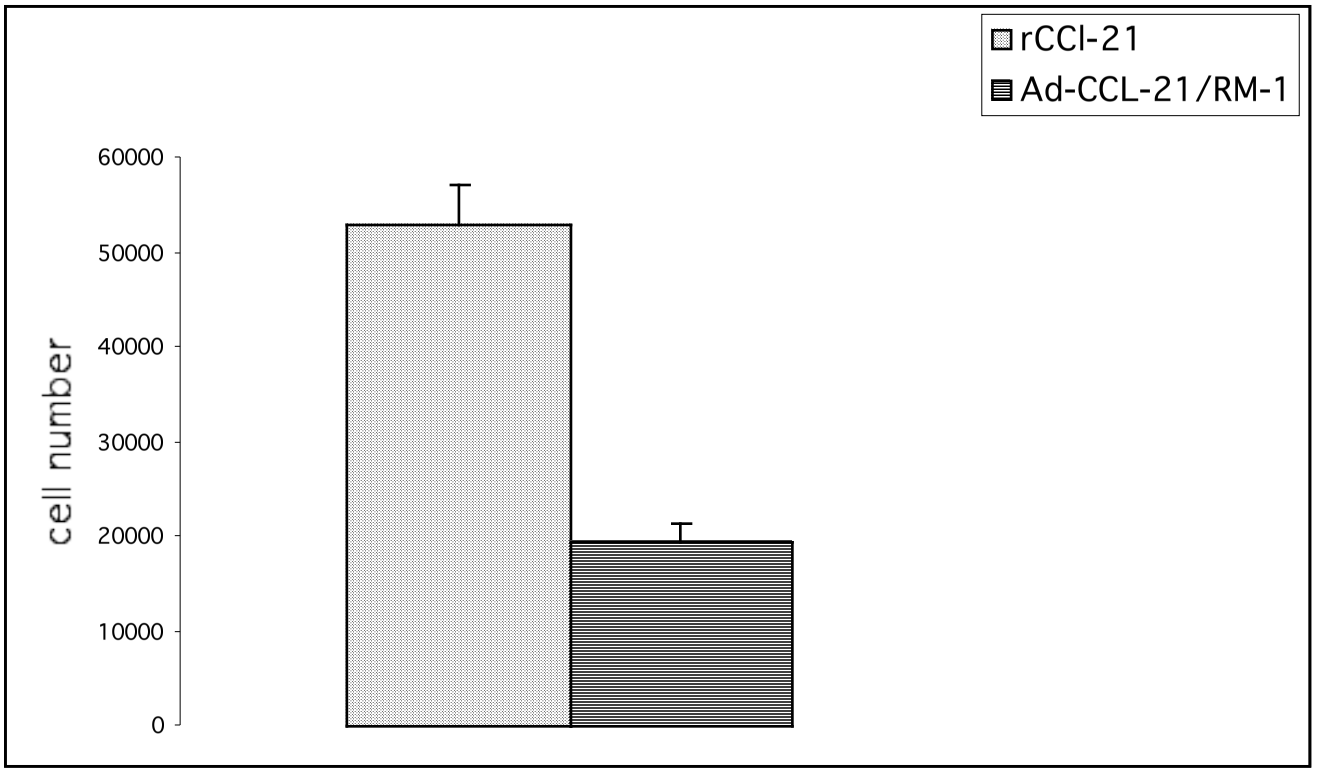


Figure 17

Survival study with CCL-21

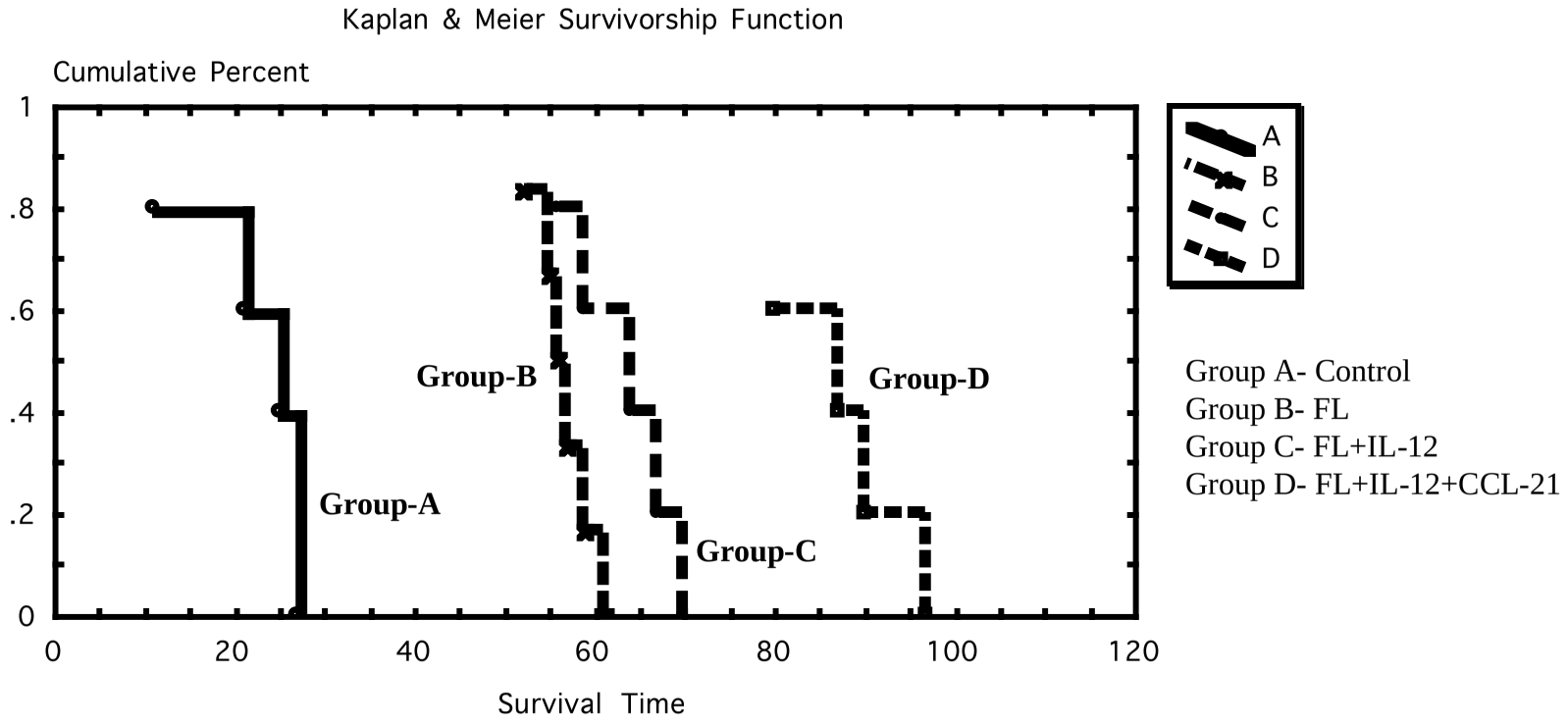


Figure: 18

Survival study with Ad-CCL21

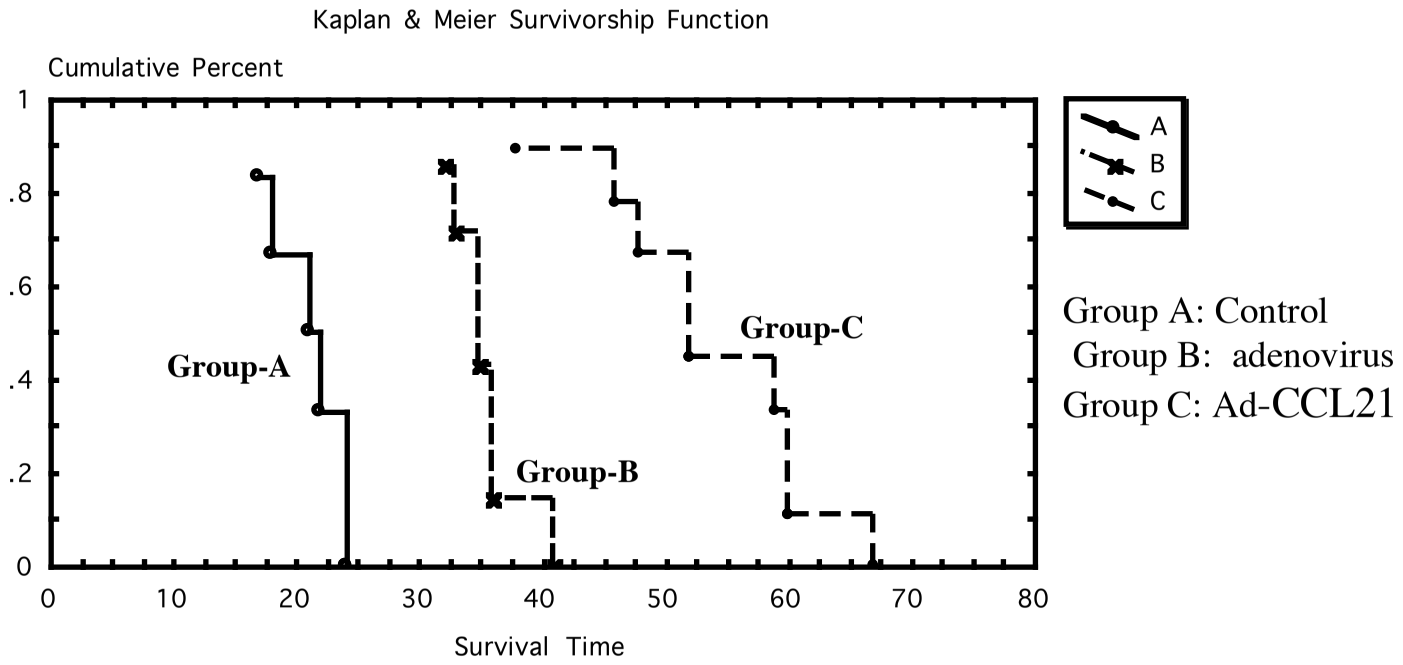


Figure:19

Survival study with CD40 Ligand

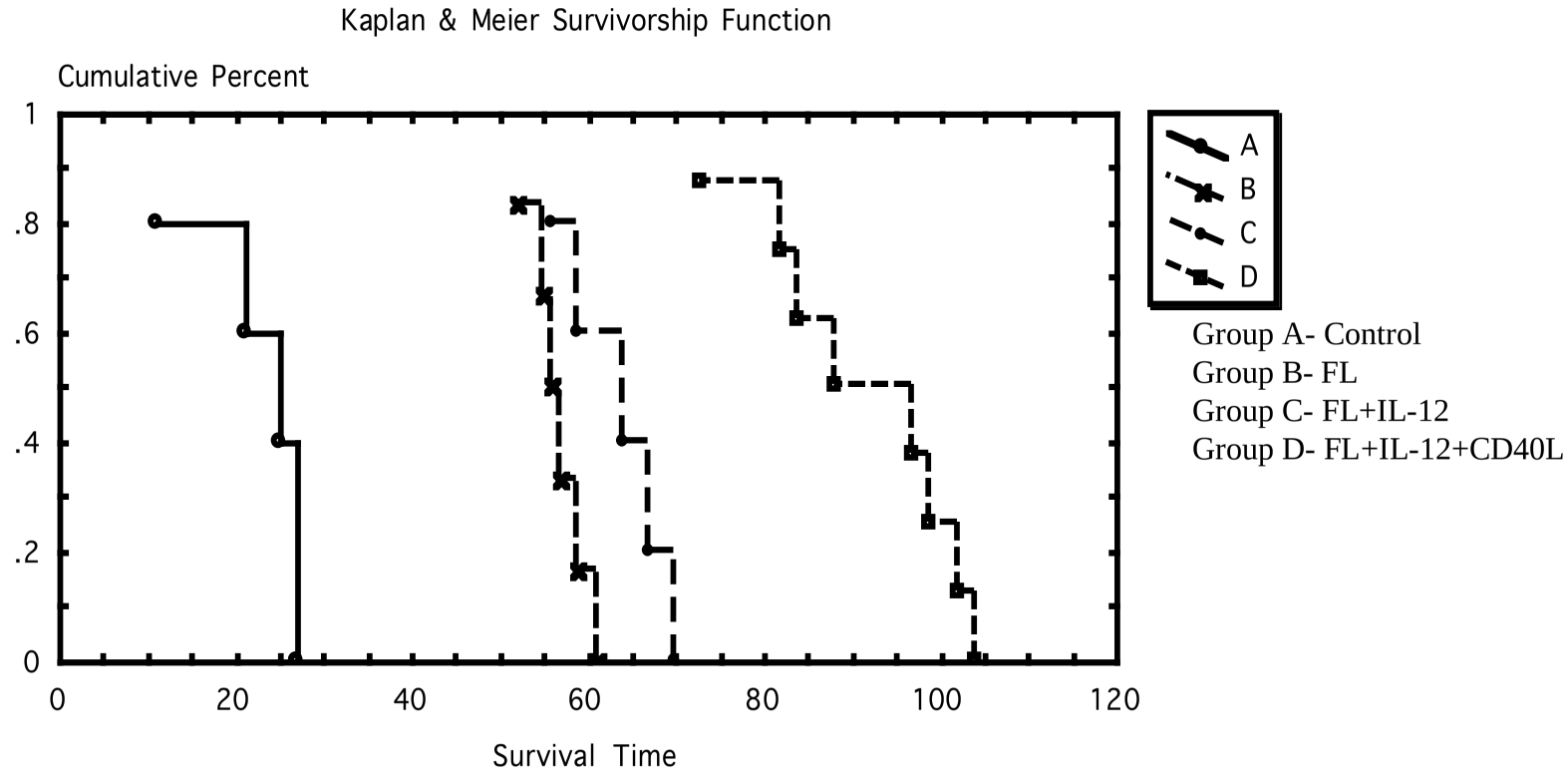


Figure:20

APPENDIX- B

LEGEND TO FIGURES

Figure 1: Radiation causes apoptosis in RM-1 cells. *Figure 1A:* This figure shows apoptosis in the form of DNA ladder. DNA was extracted from both irradiated and control RMI cells and run on a 1.5% agarose gel. The different samples in the lanes are as follows. **Lane A:** Positive control – U937 cells, **Lane B:** RMI non-irradiated cells, **Lane C:** RM-1 cells + 60 Gy – 4 days, **Lane D:** RM-1 + 10 Gy – 4 days, **Lane E:** RM-1 cells + 10 Gy – 7 days. The *figure 1B* shows the effect of 10 Gy of irradiation on RM-1 cells at different time points following irradiation (6hr, 24 hr and 48 hrs respectively). The *figure 1C* shows the effect of 20 Gy of irradiation on RM-1 cells at different time points following irradiation (6hr, 24 hr and 48 hrs respectively). *Figure 1D:* Hoechst staining- RMI cells were radiated to 10 Gy and allowed to grow in culture. Cells were fixed, stained with Hoechst and scored for apoptotic bodies. The results are shown as percent of cells in culture. The graph shows that the irradiated cells showed increased apoptotic death compared to control cells. *Figure 1E:* Apoptosis in irradiated RM-1 cells was also evaluated by evaluating the pre-G1 population of the cells by FACScan analysis. Irradiated cell were grown in culture and analyzed at 4 days. In the left panel the real graph depicting the cells in different stages of cell cycle. The proportion of pre-G1 fractions of control and irradiated cells are compared in the bar graph to the right.

Figure 2: DC take up irradiated RM-1 cells: The figure-2 shows the uptake of irradiated RM-1 cells by activated DCs. The irradiated (four day post 60 Gy RT) and non-irradiated RM-1 cells were stained with PKH red fluorescent dye. DCs that were being grown exponentially in culture were stained with a FITC MHC II fluorescent antibody. The stained RM-1 cells were incubated with stained DCs at a

concentration of 1:1 (1x 10^6 each) for 4 hours. The cells were then harvested and analyzed for double positive cells, which reflect uptake of RM-1 cell fragments by DCs. The cells were also visualized under fluorescent microscope to confirm the nature of double positive cells. The graphs show that there was an increase in the number of double positive cells in the sample collected from DCs incubated with irradiated RM-1 cells (20.86 vs. 4.85).

Figure 3: Expression of CCR-7 & its ligands in activated DC: The figures show the results of RT-PCR on RNA samples. ***A) Activated DC expresses CCL-19:*** DCs were incubated with RM-1 cells as described earlier. Their RNA was collected and probed for the presence of CCL-19 using specific primers. As seen in the figure the DCs that were incubated with irradiated RM-1 cells expressed CCL-19. ***B) Activated DC express CCR-7****:* DCs were incubated with both non-irradiated RM-1 cells and irradiated RM-1 cells (60 Gy). RNA was extracted from the cells and RT-PCR using specific primers for CCR-7 was carried out. The DCs that were incubated with irradiated RM-1 cells expressed CCR-7 suggesting their activation. ***C) Activated DC express CCL-21:*** The DCs were incubated with both non-irradiated and irradiated RM-1 cells (60 Gy) respectively and RNA was extracted from the cells and RT-PCR using specific primers for CCL-21 was carried out. The CCL-21 transcription was up regulated in the DCs exposed to irradiated RM-1 cells. ***D) Expression of Actin*:** The figure 3D shows the results of RT-PCR for actin RNA in the different experimental samples. NR – represents two samples that were non relevant to these set of experiments.

Figure 4: Activated DC expresses accessory surface molecules: The figure shows the expression of CD80, CD86, MHC- class I and MHC-class II molecules on the

cell surface of DCs. The primary antibodies against the surface molecules and the secondary anti- mouse fluorescent antibodies were used for the study (Pharmingen). The DCs were exposed to irradiated tumor cells and the activated Dcs were analyzed for the intensity of fluorescence using cell sorter (FACScan). All the four surface molecules showed higher intensity in DCs that were incubated with irradiated tumor cells compared to controls.

Figure 5: Lymphocyte proliferation assay: The proliferation of naïve splenocytes that were co-cultured with serially diluted mixture of DC and irradiated tumor cells, was assessed by WST-1 assay. The graph is shown as percent increase in proliferation of lymphocytes. A significant increase in proliferation was observed when splenocytes were co-cultured with activated DCs.

Figure 6: Cytotoxicity assay: The figure shows that splenocytes stimulated with DCs pulsed with irradiated tumor cells had significant cytotoxic activity *in vitro*. The splenocytes were stimulated with DCs pulsed with irradiated and non-irradiated tumor cells *in vitro* for five days at 37^{o}C in CTL media. The cytotoxic activity of the activated splenocytes was determined by LDH assay (Boehringer Mannheim).

Figure 7: Cytokine assay: The culture supernatants from splenocytes stimulated with DCs pulsed with irradiated tumor cells *in vitro* for five days at 37^{o}C, had significantly higher cytokine levels. The figure 7A shows the IFN-γ level in splenocytes that were co-cultured with either 1) DCs pulsed irradiated cells or 2) DCs pulsed with non-irradiated cells. 3) Non-pulsed DCs. The figure 7B shows the IL-12 concentration in the same groups The cytokine levels were elevated significantly ($p < 0.05$) in the experimental group compared to controls and the non-pulsed DCs.

Figure 8: Immunization with DC pulsed with irradiated tumor cells increases survival: Naïve animals were immunized with DC pulsed with irradiated tumor cells and subsequently challenged with RM-1 cells. The figure shows that survival was significantly increased in the immunized group compared to non-immunized group ($p=0.015$).

Figure 9: Immunization with DC pulsed with irradiated tumor cells increases lymphocyte proliferation: The proliferation of splenocytes obtained from different cohort of animals (immunized and non-immunized) were determined following their stimulation *in vitro* with heavily irradiated tumor cells (60Gy) by WST-1 assay. There is a significant increase in splenocyte proliferation in samples that were obtained from animals that were immunized with DCs pulsed with IR cells compared to controls ($p<0.05$).

Figure 10: Immunization with DC pulsed with irradiated tumor cells increases tumor specific cytotoxic immune response. The splenocytes isolated from the different cohort of animals (immunized and non-immunized) were stimulated *in vitro* with heavily irradiated (60Gy) RM-1 cells for five days at 37^0C in CTL media and cytotoxicity was determined by LDH assay (Boehringer Mannheim). The splenocytes from the immunized cohort were significantly ($p< 0.01$) cytotoxic at a effector /target ratio of 10:1 when compared to the splenocytes of controls. Exptl- Immunized, contronon-immunized.

Figure 11: Immunization with DC pulsed with irradiated tumor cells induce the production of Th1 cytokines: The splenocytes from different groups were assessed for the secretion of IL-12, IFN-□and TNF-□ following stimulation. The levels of all

three cytokines were significantly ($p < 0.05$) elevated in the immunized group compared to controls.

Figure 12: In vivo administration of DCs following primary tumor irradiation increases survival: Animals with established three week old tumors were given a single dose of localized primary tumor irradiation (60 Gy) and followed by DC administration. The combination of RT + DC significantly increased survival when compared to controls. The median survival time was 17 days for control, 43 days for RT group and 66 days for the RT+DC group. The survival time between the RT and RT+DC group was statistically significant ($p< 0.05$).

Figure 13: Schematic representation of the cloning of CMV promoter driven CCL-21 gene in replication conditional adenovirus (RCA): The Eco-RV and HIND-III fragment containing the CCL-21 gene from pORF-CCL-21 was sub cloned in the EcoR-1 and HinD-III restriction sites present in multiple cloning site (MCS) of the adeno-viral vector, in a sense orientation driven by CMV promoter. The lower panel shows the resultant vector with a CCL-21 gene driven by a CMV promoter.

Figure 14: **Gel electrophoresis of the pAdE1B□-CCL21 plasmid DNA:** Following cloning, the plasmid was transformed into competent bacteria and amplified in antibiotic resistant media and several clones were selected and amplified. The figure shows four clones of the vector DNA along with a marker (1-KB ladder). The plasmids were found to be positive for CCL-21, by PCR. One of these vectors was subsequently used for co-transfection with the pJM-17 in to the 293 cells to get infective adeno-virus.

Figure 15: Cytopathic assay of RM-1 cells: For cytopathic studies, RM-1cells (10^5/well, 24 well plate) were either mock infected or infected with increasing

multiplicity of infection (MOI) of the Ad-CCL-21. After five days of infection, the plates were fixed and stained with crystal violet.

Figure 16: CCL-21 Assay: The supernatant from RM-1 cells infected with Ad-CCL-21 were collected after 48 hrs of viral infection and assayed for CCL-21 using ELISA kit (R&D system). The Ad-CCL21 infected RM-1 cells produced significant amount of CCL-21 compared to controls.

Figure 17: DC motility Assay: Aliquots (100 μl) of activated DC ($5x10^6$ /ml) were added to the upper chamber of a trans-well membrane in a 24 well of tissue culture plate, and to the lower chamber either recombinant CCL21 (20 ng/ml) or supernatant from recombinant adeno-viral cultures (Ad-CCL21) were added. Cell migration was quantified by counting cells in each lower chamber**.**

Figure 18: **Survival study with FL, IL-12 & CCL-21**: Six-eight week old C57BL/6J male mice were injected with exponentially growing $5x10^4$ RM-1 cells on the dorsum of the foot. Animals with established tumors were given a single dose of localized primary tumor irradiation (60 Gy). Irradiated animals were treated with cytokines (FL, IL-12 and CCL-21. The animals were challenged with live RM-1 cells and followed for survival.

Figure 19: Survival study with Ad-CCL21: Six-eight week old C57BL/6J male mice were injected with exponentially growing $5x10^4$ RM-1 cells on the dorsum of the foot. The tumor bearing animals were injected with Ad-CCL21 and followed for survival. The animals in the Ad-CCL21 group (C) had increased survival (median-52 days) compared to controls (A), median-22 days; long rank p = 0.009), as well as to the adeno virus group (B), median- 35 days log rank- p= 0.006).

Figure 20: Survival study with FL, IL-12 & CD40L: Six-eight week old C57BL/6J male mice were injected with exponentially growing $5x10^4$ RM-1 cells on the dorsum of the foot. Animals with established tumors were given a single dose of localized primary tumor irradiation (60 Gy). Irradiated animals were treated with cytokines (FL, IL-12 and CD40L. Treated animals were challenged with live RM-1 cells, and were followed for survival. The group D animals (FL+IL-12+CD40L) had significantly higher survival time compared to other groups.