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14. ABSTRACT
The goal of this research is to logically integrate immunotherapy (IT) with conventional radiotherapy (RT) to improve the treatment of men with advanced or recurrent prostate cancer. The initial aim is to determine whether local RT of prostate tumors in a preclinical and clinical setting leads to measurable tumor-specific immune responses and whether tumor vaccination can boost these responses leading to better tumor control. Survivin is our tumor antigen of choice, because it seems superior to other prostate tumor antigens. We humanized mouse prostate cancer cell lines (TRAMP C1 and TRAMP C2) to express human HLA-A2.1 and confirmed that these cells express survivin. Interestingly, the tumor cell lines failed to form tumors in transgenic HLA-A2.1 mice and seemed to be immunologically rejected. Nevertheless, we were able to use these humanized TRAMP cells to examine the vaccination responses to human survivin epitopes and to other clinically relevant human prostate cancer associated antigens. Clinical samples were analysed by tetramers to show that circulating survivin-specific CD8$^+$ T lymphocytes were present in prostate cancer patients that they were increased further upon completion of RT. We hypothesize that this is due to increased liberation of antigenic peptides and that IT will not be hindered by RT. In further support of this conclusion, circulating T regulatory cells were not influenced by RT. It is clear is that RT does not induce immune tolerance to survivin nor does it increase suppressor T cells making IT approaches feasible in combination with RT for prostate cancer.

15. SUBJECT TERMS
prostate cancer, radiation therapy, immunotherapy, survivin, tetramer

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1. INTRODUCTION

This proposal was to improve the treatment of advanced or recurrent prostate cancer, which so often fail conventional treatment modalities, such as Radiotherapy (RT). We proposed that therapies tackling prostate cancer from multiple angles, are more promising in achieving local and systemic control. We believe, that harnessing the immune system by immunotherapy (IT) alongside RT to further eliminate cancer cells, is an attractive alternative in these patients.

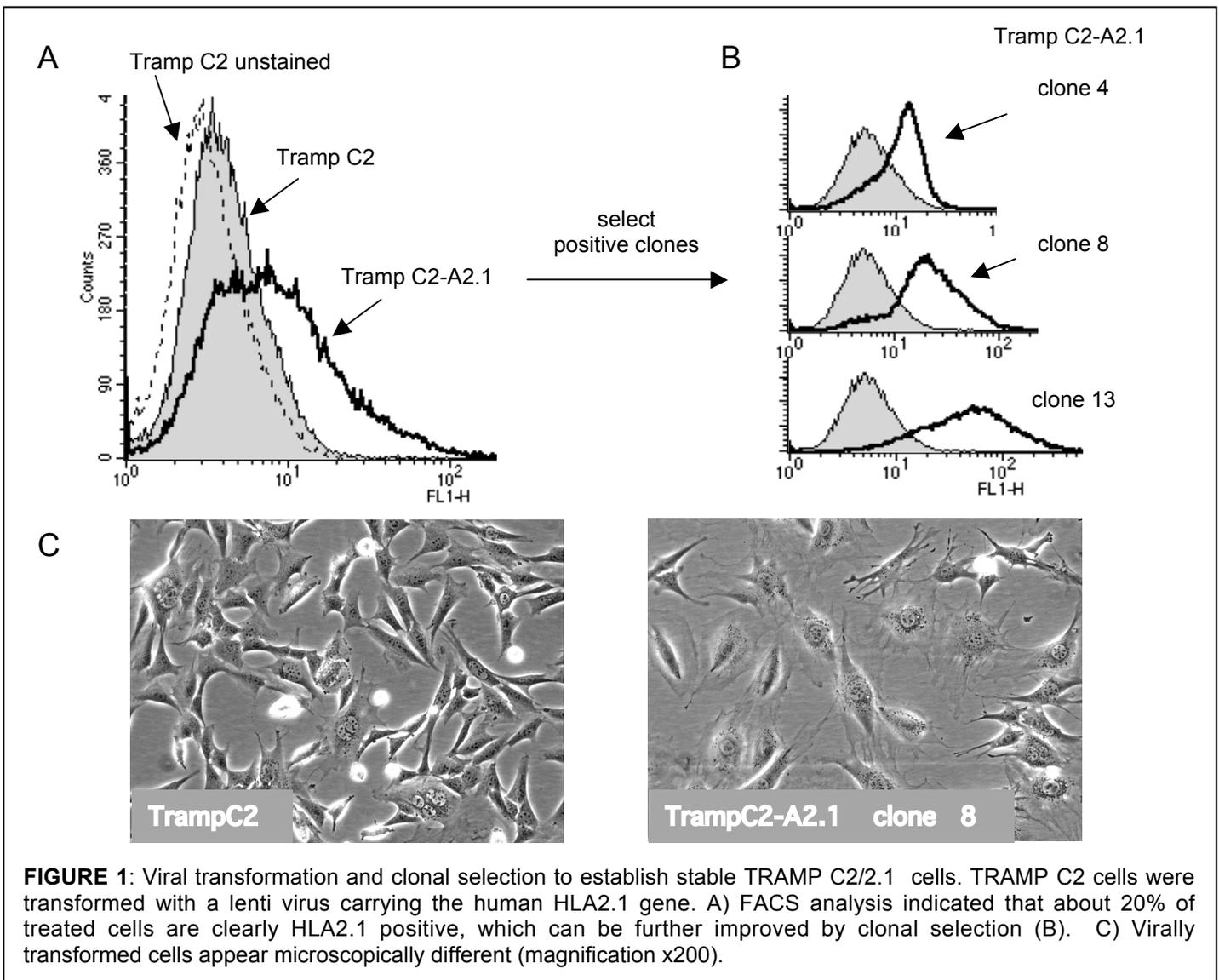Our goals as stated in the grant are as follows:
The overall aim of this proposal is to determine whether local irradiation of prostate tumors in a preclinical and clinical setting leads to measurable tumor-specific immune responses. We address the issue as to whether tumor vaccination can boost these immune responses possibly leading to better tumor control. Survivin is our tumor antigen of choice, because it seems superior to other prostate tumor antigens in that it is clearly overexpressed in tumors of the prostate, it controls its growth (anti-apoptotic & pro-proliferative), and it is immunogenic in these patients. The preclinical studies in this proposal use TRAMP C1 and TRAMP C2 mouse prostate cell lines, that were originally derived from the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice that spontaneously develop prostate cancer and closely mimics the development of the human disease [1]. Tumor bearing mice will receive local RT with or without a dendritic cell-based vaccine. Survivin-specific immune responses in these animals will be monitored using enzyme-linked immunospot assay (ELISPOT), flow-cytometric tetramer staining, T cell proliferation assay and CTL assays. Additionally, the number of $CD4^+ CD25^+ FoxP3^+$ T regulatory cells in the tumors, lymph nodes and spleens will be enumerated by FACS. Whether or not this translates directly into tumor cell killing will be determined in vivo by observing tumor growth. These animal experiments will allow us to investigate the possibility that local irradiation affects overall anti-tumor immune-responses, something that has been difficult to assess in the clinic. If this proves to be correct, we will determine whether or not the combined application of radiation and dendritic-cell vaccination can increase specific anti-tumor immune responses and whether this effectively translates into tumor rejection. Some of the in vivo studies will include the systemic application of a COX-2 inhibitor (Celecoxib, oral), a proteasome-inhibitor (PS-341, i.v.) or cytokines (GM-CSF, s.c.). These agents are already in the clinic with and without RT. They are relevant to our choice of tumor antigen. Survivin is a proteasome target during interphase but proteasome inhibitors paradoxically decrease survivin expression and in turn induce apoptosis [2]. COX-2 inhibition also augments the ubiquitination of survivin, ultimately leading to its proteasome-dependent degradation and feeding it into the endogenous antigen presentation pathway [3]. GM-CSF will enhance endogenous survivin expression. The consequences of these intercepting pathways, in terms of the presentation of survivin will add another dimension to our studies on combining RT with IT. Another powerful feature of our study is reflected by the fact that the results of our preclinical studies will be directly comparable to equivalent studies that we will perform on prostate cancer patients undergoing Radiotherapy.

2. BODY

The first aim of this proposal was to determine whether local irradiation of prostate tumors in mice caused measurable tumor-specific immune responses. The preclinical studies in this proposal used TRAMP C1 and TRAMP C2 mouse prostate cell lines that express human HLA-A2.1 (TRAMP C/2.1) and grow them s.c. in transgenic humanized C57BL/6 (C57Bl/6-K^{b}2.1) mice that express the chimeric mouse/human class I MHC, which would allow us to examine the responses to the <u>human</u> survivin epitopes that are clinically most relevant.

Initial experiments focused on the generation of stable TRAMP C1 and TRAMP C2 cell lines that have been transfected to express HLA2.1. We used a recently developed Lentivirus introducing the HLA2.1 gene (kindly provided by Drs T Ribas and R Koya, UCLA) to establish stable TRAMP C/2.1 cell lines.

TRAMP C2 cells were treated by spin-inoculation twice on two consecutive days and left to recover for 3 days. Antibody-staining for the human HLA2.1 antigen (clone BB7.2, BD Pharmingen) and FACS analysis revealed low expression levels with only 15-20% truly positive cells (Figure 1A). Because this Lentivirus does not contain a selection marker to prevent possible anti-viral immune responses in vivo, we selected for HLA2.1-positive cells by cloning. 100 cells in single-cell suspensions were plated in 10cm dishes. 5 out of 20 clones stained clearly positive, clone 13 with the highest level of expression (Figure 1B showing 3 of the 5 positive clones). Interestingly, cells from all of these clones appeared to be bigger or more spread than the parental Tramp C2 cells, at least initially (Figure 1C). Whether this has any significance in terms the viability, growth rate and HLA2.1 expression remains unclear, but was also observed in transduced Tramp C1 cells (see below) and is therefore likely to be of significance.

FIGURE 1: Viral transformation and clonal selection to establish stable TRAMP C2/2.1 cells. TRAMP C2 cells were transformed with a lenti virus carrying the human HLA2.1 gene. A) FACS analysis indicated that about 20% of treated cells are clearly HLA2.1 positive, which can be further improved by clonal selection (B). C) Virally transformed cells appear microscopically different (magnification x200).

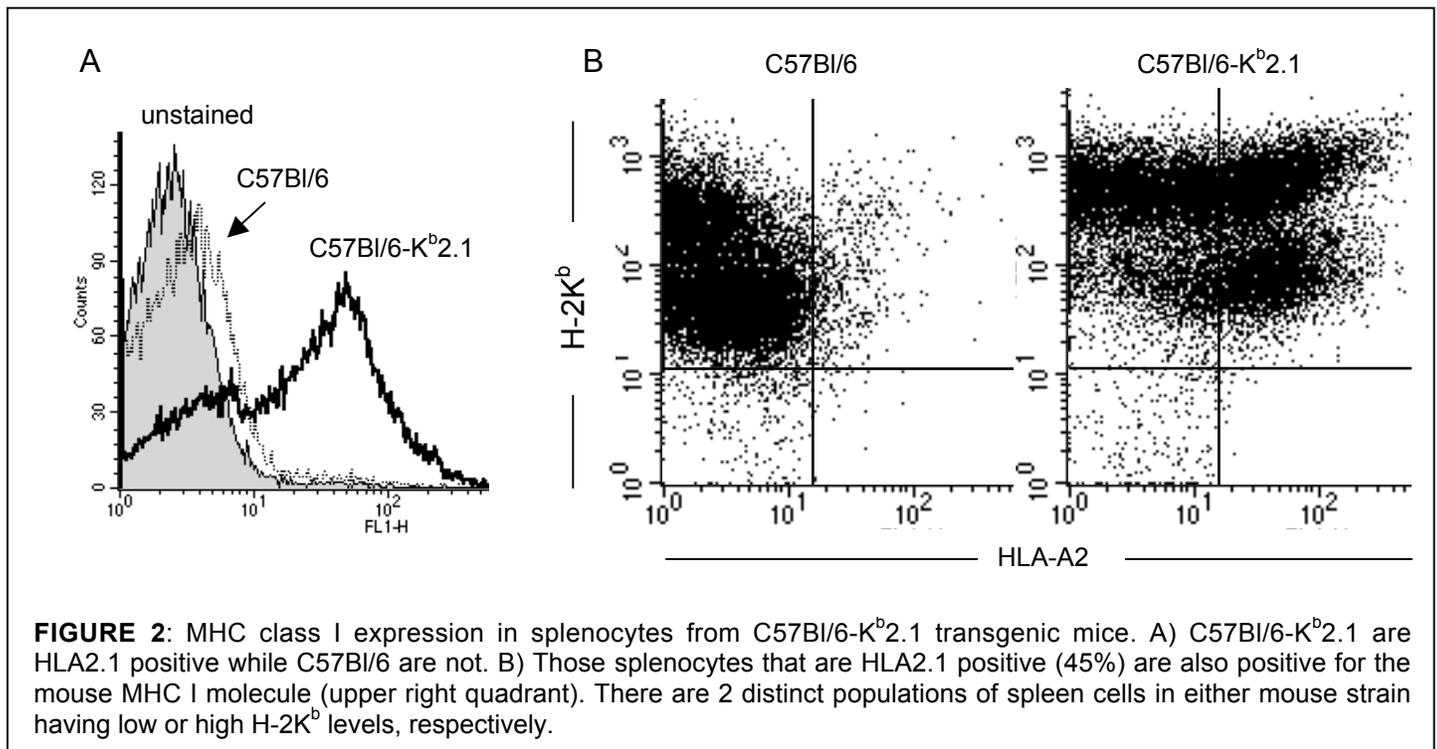Tramp C1 cells were also treated with the HLA2.1 lentivirus as above. In an attempt to improve the selection of TRAMP C1 cells that are HLA2.1positive, cells were stained 5 days post transduction with the anti-HLA-A2 antibody and then sorted through magnetic separation using the MACS system (Miltenyi Biotec Inc.). After one week in culture, only 10% of sorted TRAMP C1/2.1 cells were HLA2.1 positive which prompted us to clonally
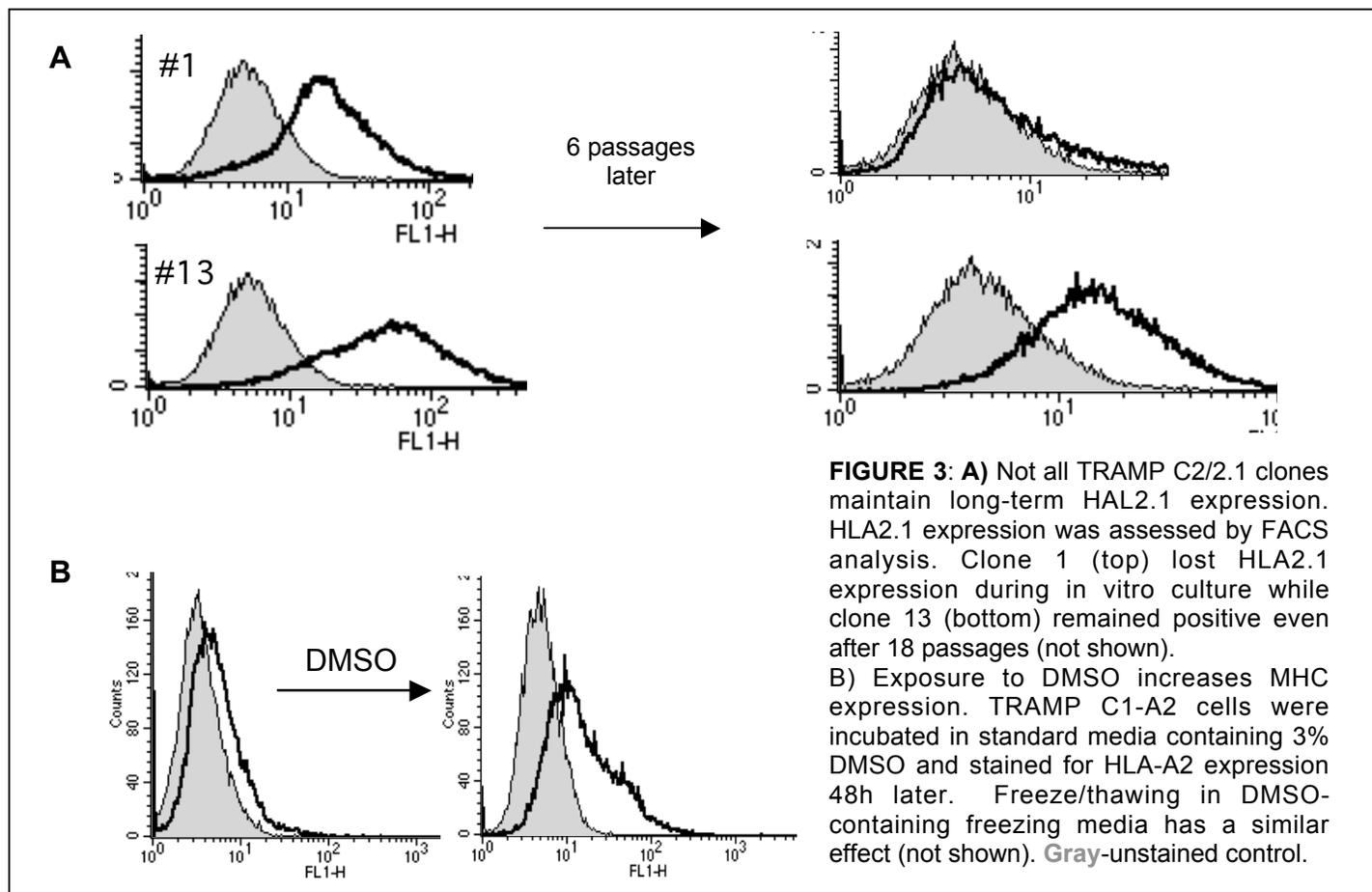
select those positive cells as above. 6 out of 30 clones had high levels of HLA2.1 expression (not shown). Again, HLA2.1 positive cells appeared bigger, more spread, and tended to grow slower in culture.

We then aimed to establish the tumorigenic potential of these HLA2.1 positive clones in vivo. All positive clones of TRAMP C1/2.1 and TRAMP C2/2.1 were injected into transgenic humanized C57BL/6 (C57Bl/6-K^b2.1) mice that express a chimeric class I molecule composed of the HLA-A2.1 extracellular domain while the transmembrane and intracellular parts remain murine H-2K^b [4]. 1×10^6 cells were injected s.c., however none of the clones formed tumors. In comparison, parental TRAMP C2 tumors were palpable after 4 weeks in C57BL/6 mice, while TRAMP C1 tumors tended to form tumors even faster. We subsequently increased the inoculums up to 5×10^6 cells without success. Lympho-suppressive preconditioning with 5.5-6Gy sublethal whole-body irradiation prior to tumor injection to enhance tumor take was also fruitless. Similarly, cell suspensions within Matrigel and/or irradiated parental TRAMP C1 tumor cells as feeder layer alongside TRAMP C1/2.1 also failed. More than 100 mice have been injected over the course of 8 months. This is certainly surprising since Drs Koya and Ribas have used this virus extensively in lymphoma and melanoma cell lines with high efficiency and have not experienced any problems regarding tumor formation in the very same transgenic humanized C57Bl/6-K^b2.1 mice and we have performed similar studies successfully using B16 melanoma. The fact that TRAMP C2/2.1 did form tumors in 4 out of 4 injected SCID/beige mice suggests to us that immune rejection might play a role. We harvested those tumors and re-injected them as smaller pieces into C57Bl/6-K^b2.1 mice. At the time of the first year report we were awaiting the results. Unfortunately, these C57Bl/6-K^b2.1 mice also remained tumor-free for several months.

We ruled out the HLA status in C57Bl/6-K^b2.1 mice (Figure 2a) as being a factor. In fact, the transgenic mice express both human HLA2.1 and murine H-2K^b while C57Bl/6 have the mouse MHC class I, only (Figure 2b).

FIGURE 2: MHC class I expression in splenocytes from C57Bl/6-K^b2.1 transgenic mice. A) C57Bl/6-K^b2.1 are HLA2.1 positive while C57Bl/6 are not. B) Those splenocytes that are HLA2.1 positive (45%) are also positive for the mouse MHC I molecule (upper right quadrant). There are 2 distinct populations of spleen cells in either mouse strain having low or high H-2K^b levels, respectively.

We also checked the stability of HLA2.1 expression in individual clones. We noticed that some but not all clones maintain long-term expression; only long-term positive clones were used for in vivo studies (Figure 3A). Accidentally, we also noticed that freeze/thawing cells appeared to increase levels of HLA-A2 and as it turns out, there have been reports in the literature that exposure to DMSO can increase levels of MHC expression [5]. Indeed, exposure of TRAMP C1/2.1 to 3% DMSO increased the expression of HLA-A2 (Figure 3B).

FIGURE 3: **A)** Not all TRAMP C2/2.1 clones maintain long-term HAL2.1 expression. HLA2.1 expression was assessed by FACS analysis. Clone 1 (top) lost HLA2.1 expression during in vitro culture while clone 13 (bottom) remained positive even after 18 passages (not shown).
B) Exposure to DMSO increases MHC expression. TRAMP C1-A2 cells were incubated in standard media containing 3% DMSO and stained for HLA-A2 expression 48h later. Freeze/thawing in DMSO-containing freezing media has a similar effect (not shown). **Gray**-unstained control.
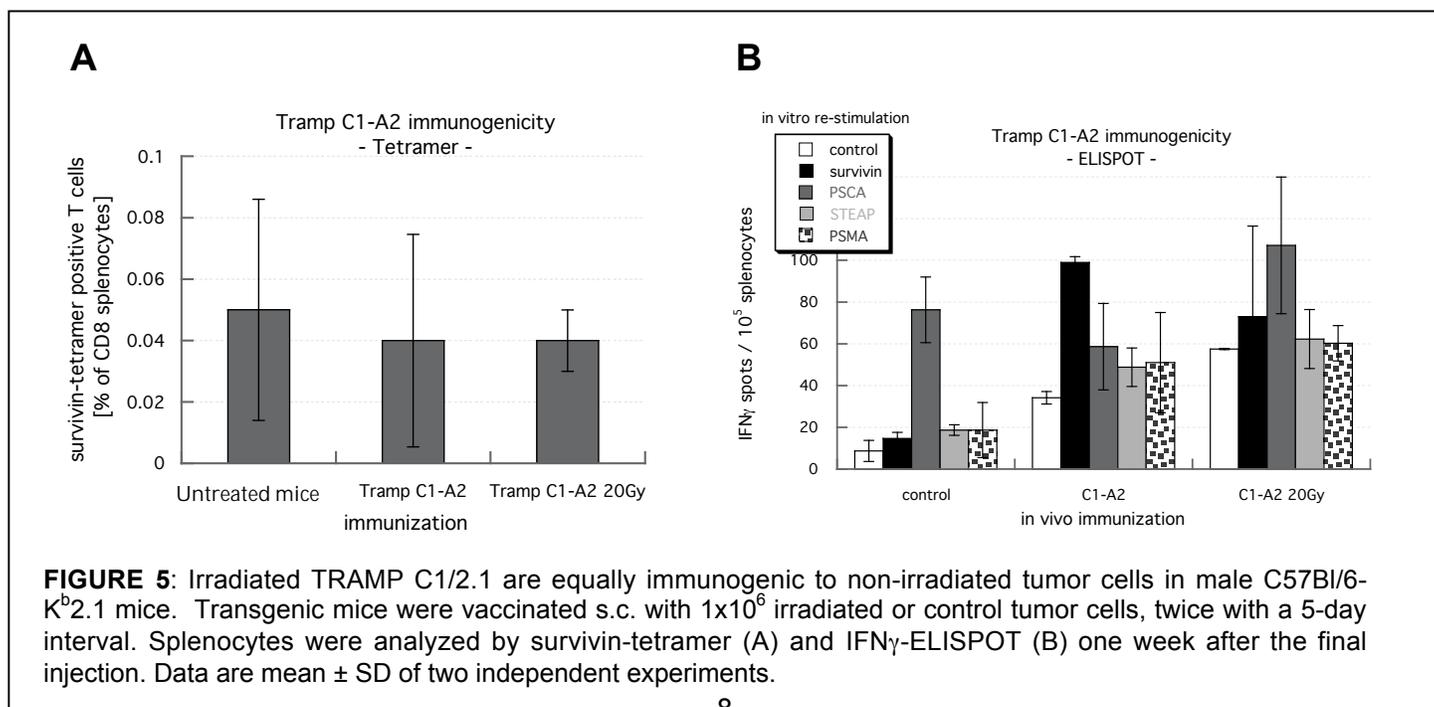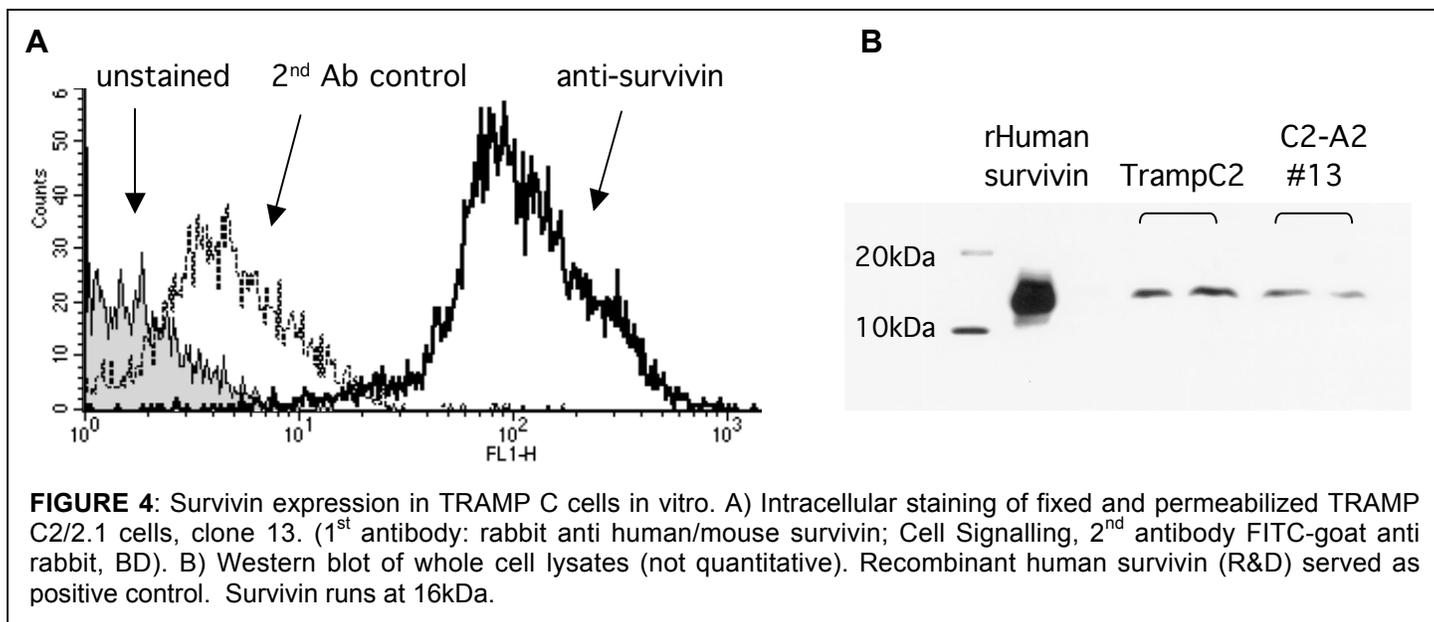
Our backup plan was to return to our original TRAMP C1/2.1 and TRAMP C2/2.1 cells (uncloned population) and inject those into transgenic animals. More than 20 mice (male and female) were injected with $3\text{-}5\times10^6$ tumor cells but remained disease-free during the time of this study. Ultimately, we returned to our original tumor cells lines TRAMP C1 and TRAMP C2 and confirmed tumor take in standard C57Bl/6 mice in 80-90% of cases. This indicates to us that either the transduction or altered immunogenicity plays a role in the rejection of these tumors in our transgenic mice.

As part of the fulfillment of Task 1 in our proposal we addressed the issue of survivin expression in vitro. There are indications that TRAMP tumor cells express survivin [6]. We were able to confirm that this is the case for our TRAMP C cells and their HLA2.1 derivatives (Figure 4), which showed strong expression.

We hypothesized that our humanized prostate cancer cells did not grow in vivo because of their immunogenicity and that they would present survivin as antigen, which we could test by using them as a vaccine. We repeatedly injected 1×10^6 TRAMP C1/2.1 cells into C57Bl/6-K^b2.1 mice and measured human survivin-specific immune responses by tetramer staining and IFNγ-ELISPOT. We also monitored ELSIPOT-responses to other tumor-specific antigens that are relevant in human prostate cancer and that are known to

be expressed in the murine system, namely prostate stem cell antigen (PSCA), six-transmembrane epithelial antigen of the prostate (STEAP) and prostate-specific membrane antigen (PSMA) [7-9]**.** To address the issue as to whether radiation affect tumor immunogenicity and the immune-responses that ensues we compared responses to 20Gy-irradiated tumor cell vaccine with responses seen to sham-irradiated tumor cells. By tetramer staining, the number of survivin-specific splenic CD8 T cells remained low in these mice regardless the vaccination strategy chosen (Figure 5A). By ELISPOT, control mice had low background IFNγ-responses to a panel of prostate tumor-specific HLA-A2 antigenic peptides, with the exception of PSCA (Figure 5B). Interestingly, ELISPOT showed significant responses on vaccination, especially in the case of survivin. There appeared to be no benefit from irradiating the tumor cells prior to vaccination, in fact anti-survivin responses even declined by about 50%.

FIGURE 4: Survivin expression in TRAMP C cells in vitro. A) Intracellular staining of fixed and permeabilized TRAMP C2/2.1 cells, clone 13. (1st antibody: rabbit anti human/mouse survivin; Cell Signalling, 2nd antibody FITC-goat anti rabbit, BD). B) Western blot of whole cell lysates (not quantitative). Recombinant human survivin (R&D) served as positive control. Survivin runs at 16kDa.

FIGURE 5: Irradiated TRAMP C1/2.1 are equally immunogenic to non-irradiated tumor cells in male C57Bl/6-K^{b}2.1 mice. Transgenic mice were vaccinated s.c. with 1x10^6 irradiated or control tumor cells, twice with a 5-day interval. Splenocytes were analyzed by survivin-tetramer (A) and IFNγ-ELISPOT (B) one week after the final injection. Data are mean ± SD of two independent experiments.

Because of the failure of humanized TRAMPC1.2.1 to grow in vivo, we examined the effect of irradiation to translate tumor cell kill into immunity in a B16-OVA tumor model. We were aware of the fact that this took us away from the prostate tumor environment, but at the same time it would allow us to get a step closer to the clinic. The B16-OVA tumor model is a well-defined and widely-used melanoma model that uses the ovalbumin antigen to track immune-responses following cancer treatment in the animal system [10, 11]. We implanted mice s.c. into the right leg with B16-OVA (up to 1×10^6 per mouse) and started local radiation treatment when tumors were small (4mm diameter). In addition to regular tumor size measurements, OVA-specific anti-tumor immunity was assessed in spleens at 5 days following treatment. 10Gy, 20Gy and 4x4Gy significantly delayed tumor growth, 5Gy did not (Figure 6A). B16-OVA tumor-bearing mice overall had more IFNγ spots than mice without tumors (Figure 6B). Mice whose tumors received 10Gy, 20Gy and 4x4Gy, but not 5Gy had more responses than non-irradiated, tumor-bearing mice. Changes in responses involved OVA-specific spots (targeted at EG7.OVA) as well as non-specific responses (control and EL4). All mice apart from the 20Gy-irradiated mice were also analyzed with OVA tetramers. Overall, the levels of OVA-specific CD8$^+$ T cells in spleens were higher than expected, though, surprisingly, tumor-bearing mice had the highest percentage of all of them (Figure 6C). Furthermore radiation appeared to dose-dependently decrease the occurrence of these OVA-specific T cells. In contrast, the intensity of the tetramer staining tended to increase upon the presence of a tumor with or without treatment, indicating possibly higher levels of OVA-T cell receptors on the surface of these T cells (Figure 6D). This indicates a problem with the assay, that we are still working on but the fact that the avidity of the T cells increased suggests that the data may eventually support the ELISPOT findings.
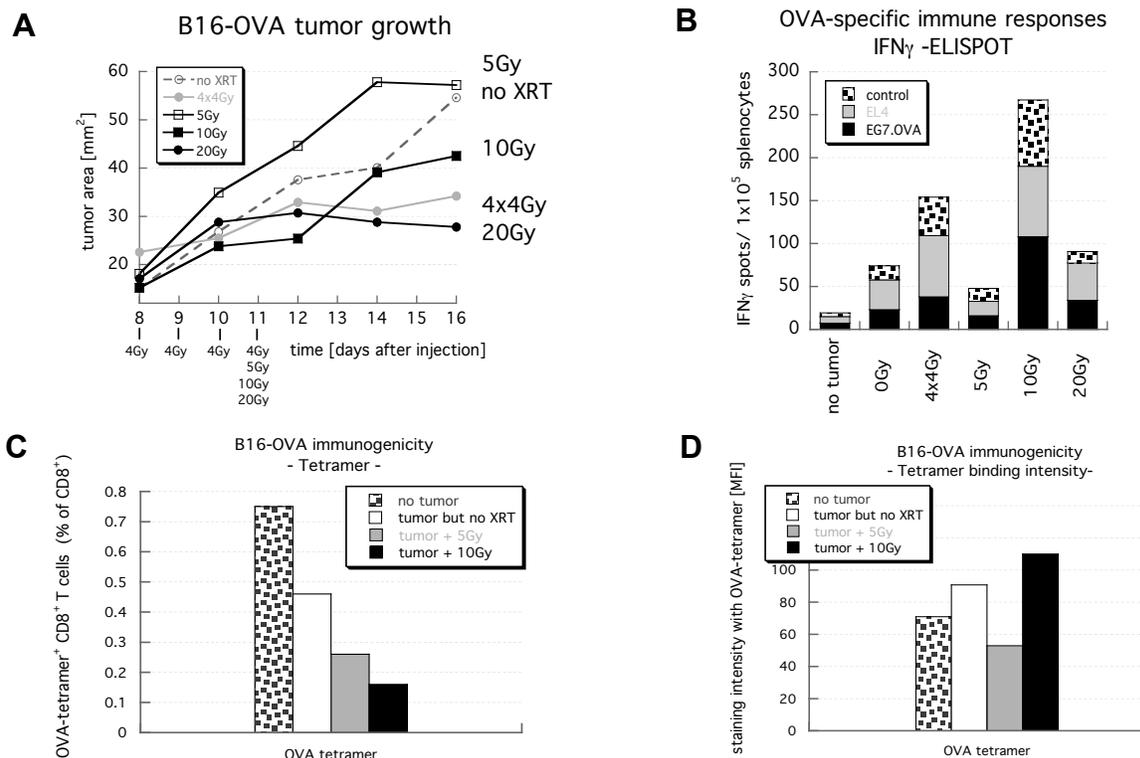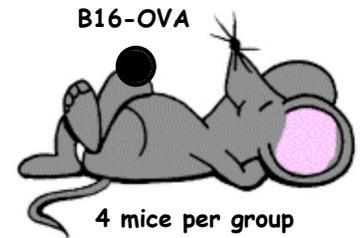

FIGURE 6: Tumor irradiation of 10Gy and above delayed tumor growth and increased immune-recognition. Subcutaneous B16-OVA tumors were irradiated with different doses of radiation and followed for 5 days. A) 10Gy, 20Gy and 4x4Gy significantly delayed tumor growth as measured with calipers. Data are mean of n=4 mice. Splenocytes were analyzed 5 days after XRT by B) IFNγ-ELISPOT and C+D) survivin-tetramer. The number of IFNγ-producing T cells increased in tumor-bearing mice, especially following 10Gy irradiation and following 16Gy given in 4 daily fractions (B). The number of OVA-receptor+ CD8 T cells declined with tumor growth, and even further following treatments (C) while the OVA-tetramer staining intensity per T cells increased.

We also addressed the question as to whether there was a correlation between the delay in tumor growth and the development of anti-tumor immunity as detected by ELISPOT. On day 12 of the experiment, I.e. one day after treatment there was a good inverse correlation between tumor size and the anti-tumor immune-response (ELISPOT) measured at the end of the experiment (Figure 7). This was lost by day 16 (not shown) but it is likely that larger tumors simply overwhelmed the immune system with negative regulatory mechanisms. Taken together, B16-OVA tumor-bearing mice whose tumors received 10Gy, 20Gy and 4x4Gy, but not 5Gy had delayed tumor growth and showed more OVA-specific IFNγ-responses than non-irradiated, tumor-bearing mice. We think this is circumstantial evidence for our hypothesis that ionizing radiation might have an immune-modulatory component that could help eradicate a tumor outside the radiation field.
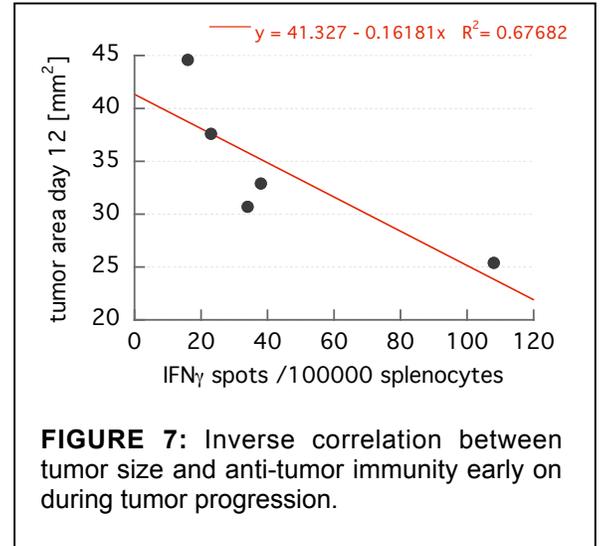

FIGURE 7: Inverse correlation between tumor size and anti-tumor immunity early on during tumor progression.

To build on the B16-OVA model and in keeping with the original idea of this proposal to combine radiotherapy with immunotherapy to improve the treatment of prostate cancer, we decided to incorporate the novel immune-modulator MIS416 (VIRIONYX Corporation Limited, Auckland, New Zealand) into our studies. MIS416 is a microparticle immune-stimulant that targets Toll-like receptors. It is especially interesting in this context because of its apparent radioprotective and chemoprotective potency by supporting normal bone-marrow function and counteracting myeloidsuppression that often accompanies cytotoxic caner therapies. Indeed, in our B16-OVA model, tumor growth delay by radiation was as potent with the drug as it had been previously without (Figure 8A). 50µM MIS416 had been given i.v. 1 day prior to the start of the radiation regimen. Again, OVA-specific CD8 T cells decreased following treatment, especially following the combined treatment, yet the tetramer binding intensity on the surface of these cells increased (Figure 8B). Mice whose tumors received MIS416 alone had more responses than non-irradiated, tumor-bearing mice (Figure 8C). MIS416 with radiation or radiation alone had the opposite effect. Again, non-specific responses were higher than the OVA-antigen specific responses indicating problems of specificity of the assay.
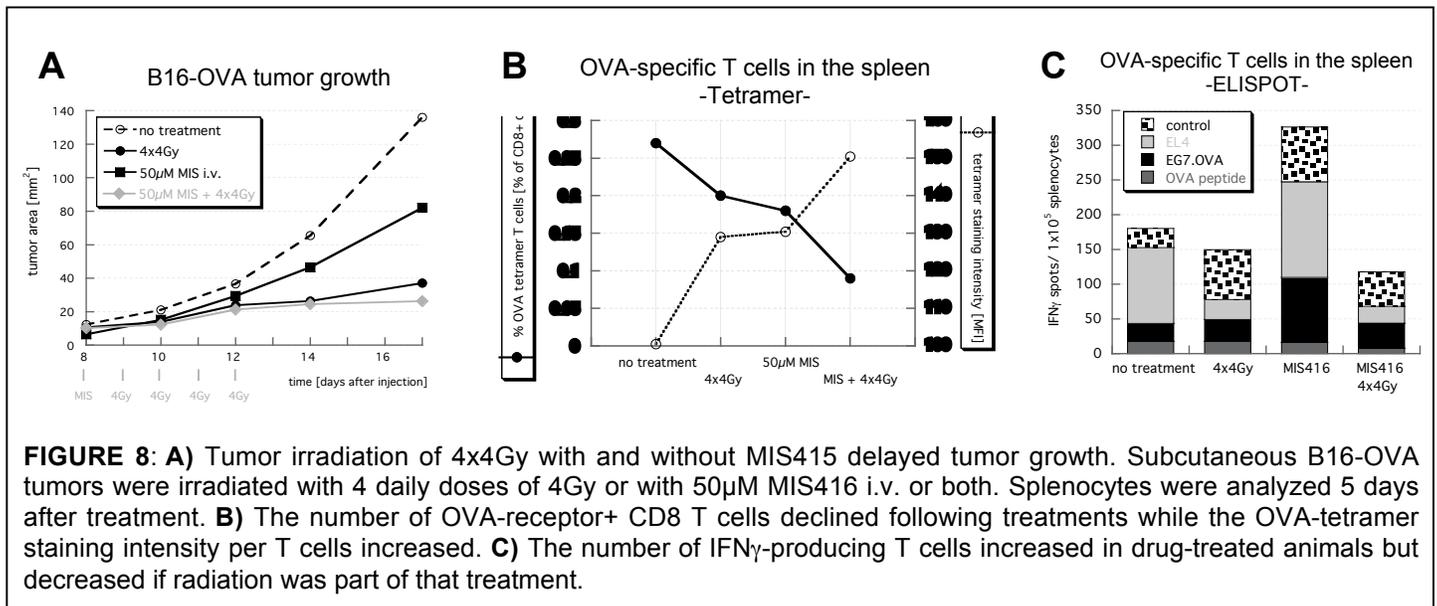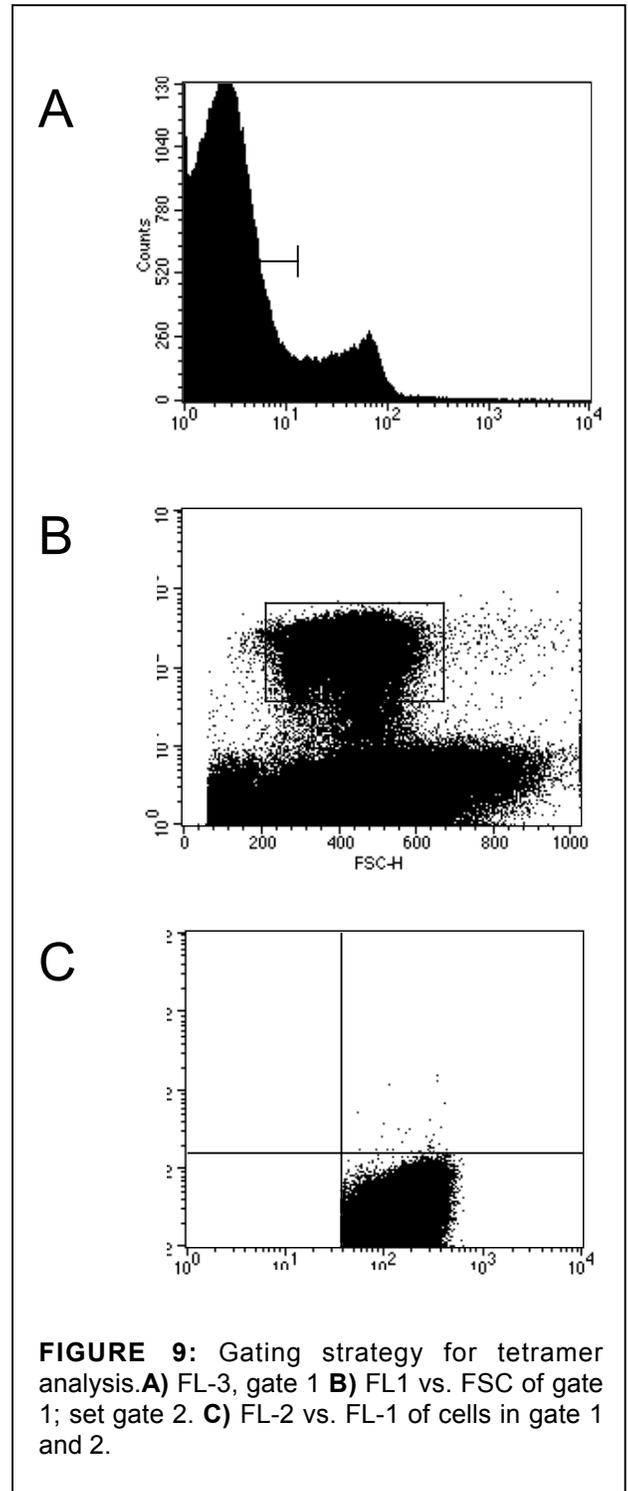

FIGURE 8: **A)** Tumor irradiation of 4x4Gy with and without MIS415 delayed tumor growth. Subcutaneous B16-OVA tumors were irradiated with 4 daily doses of 4Gy or with 50µM MIS416 i.v. or both. Splenocytes were analyzed 5 days after treatment. **B)** The number of OVA-receptor+ CD8 T cells declined following treatments while the OVA-tetramer staining intensity per T cells increased. **C)** The number of IFNγ-producing T cells increased in drug-treated animals but decreased if radiation was part of that treatment.

The third and clinical task of our proposal aims to evaluate immune responses to tumor antigens in prostate cancer patients undergoing RT and to find out whether or not they are amplified by RT. We received ficoll-gradient isolated peripheral blood mononuclear cells (PBMC) from 13 patients with prostate cancer before, during and after they had undergone radiation therapy (Dr. Haustermans, University Hospital Leuven,

Gasthuisberg, Belgium. We confirmed the HLA-A2 haplotype in 10 of these patients (HLA-A2 antibody clone BB7.2, BD Pharmingen) and these patients were therefore eligible for tetramer analysis of survivin-specific CD8$^+$ T lymphocytes that have the T cell receptor for the survivin peptide Sur1M2 (LMLGEFLKL, Beckman Coulter) (28 samples). Sample volume permitting a negative tetramer determined background PE fluorescence. Ficoll-Paque^{TM}-isolated PBMCs from a single volunteer was internal control for each assay. 1-2x10^5 events were accumulated. The gating strategy was (Figure 9):

1) plot FL3 , set viability gate (gate 1); (fixed cells served as control)
2) plot FL1 vs. FSC of population in gate 1; set gate 2 for CD8^{high} lymphocytes, excluding NK cells (CD8^{low})
3) plot FL-2 vs. FL-1 of cells in gate 1 and 2 (viable CD8^{high} lymphocytes). Samples of the healthy volunteer stained with negative tetramer were used to set an arbitrary FL-2 lower limit of 0.03% double positives [12].

Quality control required ≥10,000 viable events and ≥2,000 CD8$^+$ T cells. However, one sample did not meet the minimum standards of no less than 10,000 viable events with at least 2,000 CD8$^+$ T cells and was therefore excluded. Sufficient material from 7 patients was available for tetramer analysis at all 3 time points, with 3 being tested at 2 time points,. Overall there were 9 samples before RT, 10 samples during RT and 8 samples after RT (Table 1).

In most cases, staining of CD8$^+$ cells with the survivin tetramer was significantly higher than with the irrelevant, negative control tetramer indicating the presence of antigen-specific T cells (Figure 10, p=0.002). The HLA-A*0201$^+$ healthy volunteer had 0.11% ±0.04 of CD8$^+$ T cells reactive. First, we determined whether or not a sample was positive for survivin-specific T cells. The low-limit of detection (LLD) was taken to be the mean ± 2SD of the values for th9 healthy volunteer, i.e. 0.19%. By this criterion, 6 of 9 (66.7%) patients before treatment, 4 of 10 (40%) patients during treatment, and 6 of 8 (75%) patients after treatment were positive (Table 1). There appeared to be a trend towards an initial drop during RT with increased responses upon completion of therapy.
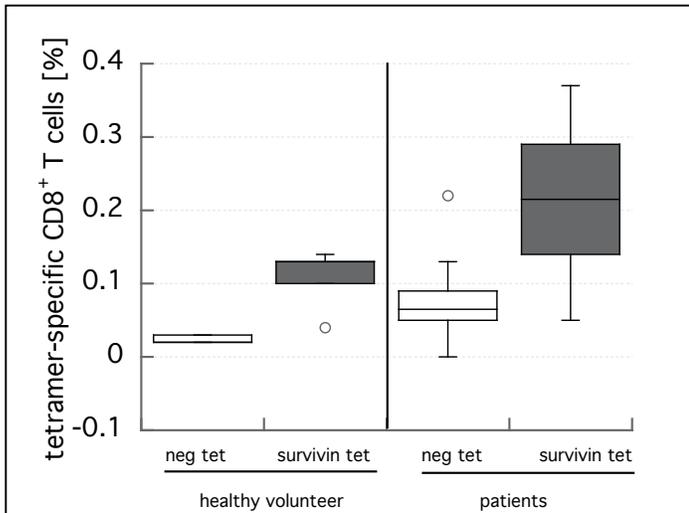

FIGURE 9: Gating strategy for tetramer analysis.**A)** FL-3, gate 1 **B)** FL1 vs. FSC of gate 1; set gate 2. **C)** FL-2 vs. FL-1 of cells in gate 1 and 2.

FIGURE 10: Specificity of the survivin tetramer. Human samples stain relative higher with the tetramer for survivin when compared to staining with the irrelevant, negative tetramer (p<0.005). Data are percent CD8$^+$ T cells staining positive with the tetramer for survivin or the negative tetramer, respectively.

TABLE 1: Increasing percent of cancer patients show survivin-reactive CD8+ T cells in peripheral blood upon completion of radiation treatment. Data are Data are CD8+ T cells staining positive with the tetramer for survivin [%] from 10 HLA-A2 positive patients with prostate cancer. Gray fields highlight those samples that stained above the background level (LLD = mean ± 2x SD of % survivin-reactive CD8+ T cells in the healthy volunteer).

patient	radiotherapy		
	before	during	after
prostate 1	0.23	0.29	0.37
prostate 2	0.12	0.05	0.25
prostate 3	0.28	0.23	0.53
prostate 4	1.33	0.16	x
prostate 5	0.22	0.12	x
prostate 6	0.15	0.15	0.26
prostate 9	0.46	0.16	0.15
prostate 11	0.32	0.31	0.21
prostate 12	0.15	0.5	0.32
prostate 13	x	0.1	0.14

Obviously, the number of samples is small but the power of the study is in its longitudinal nature. When patient responses were examined individually over time the tendency of circulating tumor-specific T cells to decrease during RT became very evident (Figure 11B). The number of survivin-specific CD8$^+$ T cells decreased in 66.7% patients (66.7%) during the course of radiotherapy (from *before → during*). Intriguingly, the majority of patients (5 out of 7 patients) ended up with more survivin-specific CD8$^+$ T cells in their circulation than before they had started treatment (*before → after* RT). These are very preliminary data and will have to be evaluated further. We are hoping to extend this study group to 30 more patients.
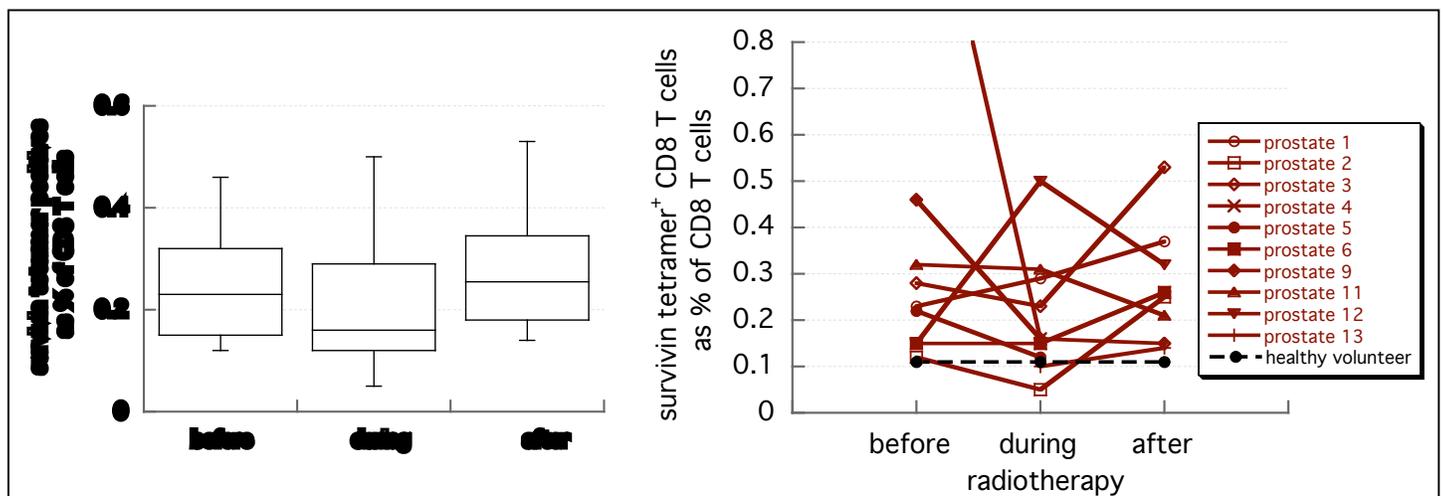

FIGURE 11: Levels of circulating survivin-reactive CD8$^+$ T cells initially drops during the course of radiotherapy but increases upon completion of radiotherapy. Data are shown percent CD8$^+$ T cells staining positive with the tetramer for survivin of **A)** median data in box plot or **B)** as time courses for each individual patient.

As outlined in Task 3.5 we also monitored $T_{regulatory}$ cells in these patients. This allowed us to get an idea regarding immune suppression in these patients. PBMC from 19 prostate patients regardless their HLA-type were analyzed for the expression of $CD4^+$, $CD25^{high}$, with intracellular $Foxp3^+$ as follows: For most samples, 1×10^6 cells were stained in 100µl human AB serum with 20µl FITC-anti-human CD4 (clone OKT4, eBioscience) and 20µl PE-anti-human CD25 (clone BC96, eBioscience) and incubated for 30 minutes on ice. Cells were washed and incubated in 1ml of fixation/permeabilization buffer for 45mins on ice, washed twice and re-suspended in 2% v/v normal rat serum in 100µl of permeabilization buffer. 20µl of PE-Cy5-anti-Foxp3 (clone PCH101) was added followed by 30mins on ice. Cells were washed, re-suspended in 200µl of 10% FBS and analyzed by flow cytometry. In an earlier protocol, the antibody cocktail containing PE-Cy5™-anti-CD25, R-PE-anti-CD4 and the first generation FITC-anti Foxp3 antibody (clone hFOXY) were applied simultaneously after fixation & permeabilization. PBMCs from one volunteer served as an internal control for each assay. If possible, 1×10^5 events were accumulated. Quality control required all acquired data to be ≥70% viability and ≥2,000 $CD4^+$ T cells. The gating strategy was: 1) FL-1 vs. FSC of all events, set gate 1 for $CD4^+$ cells, excluding debris and monocytes ($CD4^{low}$). 2) FL2 vs. FL1 of population in gate 1, set gate 2 for $CD4^+CD25^{high}$ double positive lymphocytes. 3) Plot FL-3 of cells in gate 1 and 2 to determine fraction of $CD4^+CD25^{high}Foxp3^+$ triple positive cells. The number of $T_{regulatory}$ cells varied in 8 healthy volunteers (aged 30-64) from 1%-4.6% of total $CD4^+$ (mean 2.8±1.1% $CD4^+CD25^{high}Foxp3^+$) cells, which compares well with the literature, indirectly validating our protocol. Most patients (13 of 17; 76%, p=0.083) started treatment with less $T_{regulatory}$ cells in their circulation than the volunteers (Figure 12A, table 2). There was no notable trend for the number of circulating Tregs to change during therapy in most (8 out of 13) prostate patients with matched samples from before and after treatment (Figure 12B). This suggests that Treg may not be a problem for immunotherapy in prostate cancer.

TABLE 2: The frequency of circulating $T_{regulatory}$ cells in prostate cancer patients were generally less than those observed in 8 healthy volunteers (2.8 ±1.1). Data are the % of $CD4^+$ cells that are $CD25^{high}$ and $Foxp3^+$. Levels of $CD4^+$ PBMCs were also reduced these cancer patients compared to healthy controls (34% ± 6.5%).

|	Treg [% of CD4]			CD4 [% of PBMC]		
	before	during	after	before	during	after
prostate 03	4.3		3.4	28.5		37.7
prostate 04	0.7	0.7	0.6	37	39.8	30.4
prostate 05	0.4			34		
prostate 06	0.4		0.1	45		53
prostate 07	3.6		3.8	19		13
prostate 08	2.5	1.6	2.5	54	47	49
prostate 1	2.5	2	2.3	23	18	24
prostate 2	2.2	3.1	3.3	28	19	20
prostate 3	1.1	2.9	2.2	22	14	11
prostaet 4	1.8	2.3		19	24	
prostate 5	2.8	2.5	3.3	32	25	12
prostate 6	4.7			20		
prostate 7	2.5	1	2.2	34	16	9
prostate 8	1	0.8	1.6	14	13	20.4
prostate 9			0.5			35
prostate 10	1.2	0.8		24	19	
prostate 11	2.5	0.5	0.7	17	18	22
prostate 12	1.1		0.9	14		19
prostate 13		1.5	0.7		36	34
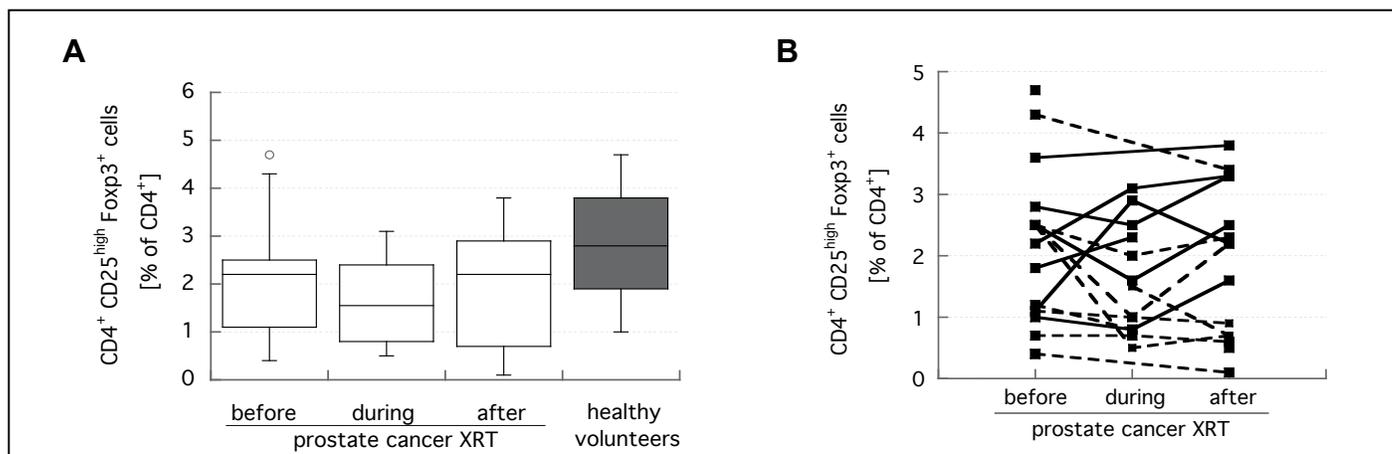
FIGURE 12: The frequencies of circulating $T_{regulatory}$ cells in prostate cancer patients were generally less than those observed in 8 healthy volunteers with no apparent trend to change during radiation therapy. Data are the % of $CD4^+$ cells that are $CD25^{high}$ and $Foxp3^+$ presented as **A)** box and whisker diagram and **B)** as time course for individual patients with solid lines indicating an upward trend, dashed lines do not.

3. KEY RESEARCH ACCOMPLISHMENTS

- Generation of stable TRAMP C1 and TRAMP C2 mouse prostate cell lines that express human HLA-A2.1 (TRAMP C1/2.1 and TRAMP C2/2.1).
- Confirmation of HLA-A2 expression in TRAMPC/2.1 cells after transduction by antibody staining and FACS analysis.
- Isolation of individual TRAMP C1/2.1 and TRAMP C2/2.1 clones that maintain long-term expression, but with altered morphology.
- Successful growth of TRAMP C2/2.1 clone 13 in SCID/beige mice.
- Confirmation of survivin expression in vitro in parental TRAMP C2 and in their HLA2.1 derivatives using Flow cytometry and Western blotting.
- Observation of an increase in HLA expression on TRAMP C1/2.1 and TRAMP C2/2.1 after freeze/thawing of cells and pinpointing the possible involvement of exposure to DMSO in this.
- Induction of prostate tumor-specific immune responses in C57Bl/6K^{b}2.1 transgenic mice following vaccination with irradiated or non-irradiated TRAMP C1/2.1 cells on the basis of IFNγ-ELISPOTs.
- Correlation of tumor-immune responses with slowed tumor growth in animals whose B16-OVA tumors were treated with local radiotherapy.
- Inverse correlation of the number of OVA-tetramer positive cells and the intensity of the staining per cells indicating treatment-related changes in the T cells receptor density.
- Isolation of peripheral blood mononuclear cells (PBMC) from blood samples from 20 patients with prostate cancer before, during and after they had undergone radiation therapy and from 8 healthy control.
- Confirmation of the MHC haplotype in 10 patients with a HLA-A2 specific antibody, and subsequent FACS analysis.
- Enumeration of circulating tumor-specific CD8$^+$ T lymphocytes in HLA-A2 positive prostate cancer patients using tetramers specifically binding T cells that have the T cell receptor for the survivin peptide Sur1M2 (LMLGEFLKL, Beckman Coulter). Preliminary data suggest that there are radiation therapy-related changes in this patient population.
- Enumeration of circulating T regulatory cells expressing CD4, CD25 and FoxP3 in prostate cancer patients and preliminary data suggesting that the number of these suppressor T cells does not change during radiation therapy.

4. REPORTABLE OUTCOMES, MEETING ABSTRACTS & PUBLICATIONS

- Development of mouse prostate cancer cell lines TRAMP C1/2.1 and TRAMP C2/2.1, that stably express the human MHC I molecule HLA-A2.1.
- **D. Schaue**, Y. Liao, E. L. Kachikwu, B. Comin-Anduix, A. Ribas, L. Goodglick, A. Debucquoy, K. Haustermans, and W. H. McBride: Human T Cell Responses to Survivin in Cancer Patients Undergoing Radiation Therapy. In: Proceedings of the American Association for Cancer Research in Los Angeles, CA, 2007.
- Kachikwu, E., **Schaue, D**., Liao, Y-P., Iwamoto, K.S., Economou, J.S., and McBride, W.H.: The Role of Naturally-Occurring T Regulatory Cells in Radiation-Induced Immune Modulation in Murine Prostate Cancer. Manuscript in preparation.
- **Schaue D.**, Comin-Anduix B., Ribas A., Zhang L., Goodglick L., Sayre J., Debucquoy A., Haustermans K., and McBride W. H. (2008): T-Cell Responses to Survivin in Cancer Patients Undergoing Radiation Therapy. Clin Can Res 14, 4883-4890.
- Kachikwu E.L., **Schaue D**., Liao Y-P., Economou J. McBride W.H.: Naturally-occuring Tregs and their role in radiation-induced immune suppression in murine prostate cancer. In: Proceedings of the American Association for Cancer Research in San Diego, CA, 2008.

> **Schaue, D**., McBride W.H.: Ionizing radiation does not compromise the cross-presentation of antigen by dendritic cells. In: Proceedings of the American Association for Cancer Research –Tumor Immunology in Miami, Fl, 2008.
> Collaboration with Dr Michael Steinberg at Radiation Oncology at UCLA: Recruitment of 30 more prostate patients undergoing radiation therapy at UCLA and their monitoring regarding tumor immune responses to tumor-associated antigens and levels of suppressor T cells.

5. CONCLUSION

The first aim of this proposal is to determine as to whether local irradiation of prostate tumors in mice alters measurable tumor-specific immune responses. The preclinical studies in this proposal use TRAMP C1 and TRAMP C2 mouse prostate cell lines, that express human HLA-A2.1 (TRAMP C/2.1) and grow them s.c. in transgenic humanized C57BL/6, which will allow us to examine the responses to <u>human</u> survivin epitopes that are clinically relevant.

In summary, we were able to generate stable TRAMP C1 and TRAMP C2 cell lines that express the human MHC class I molecule HLA2.1. using a recently developed Lentivirus. This is an important step, firstly because TRAMP C1 and TRAMP C2 are generally known to be difficult to transfect and secondly because this will allow us to examine the responses to <u>human</u> antigenic epitopes (in our case: survivin) that are clinically relevant in our humanized mouse model (C57Bl/6-K^b2.1), that express the chimeric mouse/human class I MHC. The fact that these transfected cell lines form tumors in SCID/beige mice but not in C57Bl/6-K^b2.1 is interesting and challenging. It suggests to us that they have maintained their tumorigenic potential but that immune-mediated mechanisms might be in place that prevents tumor formation in C57Bl/6-K^b2.1 mice. Faced with the problem of having no tumor formation in these mice we adjusted our approach and used these cells as tumor vaccines. We were able to observe prostate tumor-specific immune-responses in C57Bl/6K^b2.1 transgenic mice following vaccination with irradiated and non-irradiated TRAMP C1/2.1 cells on the basis of IFNγ-ELISPOTs. This in itself is not new since irradiated tumor cells have been used as vaccines before [13]. But the fact that survivin-specific responses are measurable with prostate cancer cells is a novel finding and is an important one especially with regards to radiation having no adverse effect in this context. We were also able to observe tumor regression in the B16 melanoma-OVA system and could demonstrate a simultaneous increase in tumor-immunity. This supports our hypothesis that the radiation therapy of some cancers may have an immune-modulatory component that translates into increased tumor cell eradication or inhibition of tumor progression.

We were clearly able to detect survivin-reactive T cells by tetramer binding in more than half of prostate patients when compared to binding of a negative tetramer or to our healthy control. This is in agreement with others [14, 15] and it suggests that anti-survivin responses do indeed occur in cancer patients. This is important because although reverse immunology has clearly established that survivin is immunogenic and that survivin-specific responses can be elicited in vitro, it had been uncertain whether anti-survivin responses occur in vivo in untreated cancer patients. More importantly, we were able to show that the level of these tumor-specific T cells changed as patients underwent radiation treatment, initially declining but eventually rising so that most patients had more of these T cells in their circulation after completion of RT. Ultimately, whether this immune response is associated with tumor down-staging awaits further analysis, since this trial is still under way.

It is tempting to ascribe the increase in tumor-specific T cells to a radiation-induced increase in antigenic peptide liberation and dendritic cell activation [16, 17], and we have shown that presentation of exogenous antigen by dendritic cells can be boosted by radiation [18]. Among possible mechanisms by which RT might stimulate immune responses are radiation-induced up-regulation of molecules such as Fas, MHC I and II and co-stimulatory CD80 molecules on tumor and dendritic cell surfaces that can enhance both immunogenicity and susceptibility to T cell-mediated attack [19-21], improved T cell trafficking [22], and altered proteasome expression/function that might modify the antigens presented [23]. Further work is necessary to elucidate

which of these might operate functionally in a clinical setting and to answer the all-important question as to whether the anti-survivin response that is generated by RT assists tumor regression.

Furthermore, in our study levels of $T_{regulatory}$ cells in prostate patients do not change following radiation therapy, which is drastically different to colorectal patients that presented to us with increasing Treg level upon completion of chemoradiation. It is feasible that systemic effects induced by the chemoradiation more so than by local radiotherapy could have contributed to such a selective increase in $T_{regulatory}$ cells in colorectal cancer patients by affecting the balance in lymphocyte subpopulations [24].

Perhaps the most important point from this study is that RT does not induce immune tolerance to survivin making immunotherapy approaches feasible in combination with RT, as has also been suggested from the recent clinical vaccination trial in prostate cancer [25]. Furthermore, tetramer technology coupled with other flow-based methods provide us with powerful tools to study the anti-tumor immune status in patients undergoing such treatments.

6. REFERENCES

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7. APPENDIX

N/A

8. SUPPORTING DATA

N/A