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14. ABSTRACT Immunotherapy (IT) has become popular as an alternative treatment for late-stage and metastatic prostate cancer, however, IT alone is not a very effective modality due to multiple tumor escape mechanisms and would benefit from combination with other therapies, such as Radiation Therapy (RT). Our working hypothesis is that while radiation induces danger signals or alarmins in the tumor microenvironment up-regulating co-stimulatory molecules and hence promoting T cell activation, it also affects antigen processing through the proteasome. We believe that radiation also affects antigen presentation by inducing cellular maturation. We observed increases in cell surface markers on irradiated dendritic cells such as MHC II and CD86 in vitro and we were able to show immature dendritic cells appeared to have their propensity to degrade antigen enhanced when they had received radiation prior to antigen exposure. This is important because the state of maturation is in turn known to affect the composition of proteasomes, and hence the antigen repertoire presented. One goal of the proposal is to determine if radiation affects the hierarchy of antigenic peptide presented by DCs and tumor cells. We used murine prostate tumor cells TRAMP but with a human MHC class I molecule, which allowed us to monitor responses to tumor specific antigens that are relevant in the clinical settings. We show that irradiating tumor cells prior to vaccination does not alter their antigenicity. Whether this holds true for local tumor irradiation in vivo will have to be determined. Overall, we conclude from these experiments that the effects of radiation on antigen cross-presentation by dendritic cells in vitro and in vivo are encouraging and give us no reason to believe that radiotherapy is not a good candidate to be used in conjunction with cancer IT, although immune deviation may result in a slightly different response.					
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INTRODUCTION

Radiation therapy (RT) is a very effective treatment for early stage cancer but not for large tumors nor for distant micrometastatic disease. Recently, immunotherapy (IT) has gained in popularity by finding some patients have specific T cell response to prostate tumor-associated antigens such as prostate specific antigen (PSA) and this can be boosted by vaccination. Yet, IT given alone is not a very effective clinical option in prostate cancer. Combining IT with RT is an attractive concept as it might improve the therapeutic effects of both treatments. There are studies, including our own, showing that radiation up-regulates the expression of MHC class I and immune co-stimulatory molecules. We proposed that radiation generates these “danger” signals and modulates the tumor microenvironment. We were the first to show that radiation inhibits dendritic cell (DC) endogenous processing of MART-1 antigen but enhances exogenous MART-1 peptide presentation. We hypothesized that the inhibitory effect of radiation is due to inhibition of 26S proteasome function, which is critical for the generation of immunopeptides. This large multi-subunit protein is composed of core 20S and 19S regulator structures. IFN- γ treatment of cells causes replacement of constitutive 20S enzymes subunits with LMP7, LMP2, and MECL-1 and the 19S regulator with an 11S complex, forming an immunoproteasome that favors cleavage of proteins into peptides better able to bind MHC class I molecules. However, most cells do not express immunoproteasome with the notable exception of DC. If tumors express different epitopes to the one that DC express, then the responses that are generated are unlikely to be effective. In this proposal, we will examine how RT affects the immunological landscape of anti-tumor immunity by altering antigenic epitope presentation by DC and tumor.

BODY

Our hypothesis is that radiation affects proteasome function and modifies peptides presented by DC and tumor. Our previous study on MART-1 system has shown that irradiated DC presented exogenous peptide more efficiently and antigen that was processed endogenously less efficiently. Therefore irradiation skews the immune system. One obvious question was whether or not this applied to prostate tumor antigens. To examine PSA protein processing and presentation, we had to develop a humanized mouse model. We placed PSA within an adenoviral delivery vehicle to express it within DCs and used these DCs to immunize transgenic, humanized C57BL/6 (C57BL/6-K^b2.1), that express the chimeric mouse/human class I MHC, which will allow us to examine the responses to human PSA epitopes that are clinically relevant. Irradiated DCs transduced with AdVPSA were compromised in presenting PSA within the context of HLA-A2/Kb. T cell stimulation as judged by IFN- γ ELISPOT assay was decreased in mice injected with irradiated DC. We were also able to show that the exogenous antigen presentation pathway was differentially affected. PSA-3 peptide-pulsed DC showed enhanced IFN- γ and IL-4 expression following 10Gy radiation treatment. This clearly indicates that it is not a question of cell viability, but an alteration in DC function following irradiation. More importantly, it shows that our observation is not an artifact of the presentation of melanoma antigen, but it is also highly relevant within the context of prostate tumor associated antigens.

Given the stark contrast between radiation effects on endogenous vs exogenous PSA presentation, we asked whether irradiation affects antigen uptake and degradation by DCs. In order to dissect these specific DC functions we utilized the well-defined ovalbumin (OVA) system. Bone-marrow dendritic cell cultures (C57BL/6) were mixed with either OVA-FITC (uptake) or DQ-OVA (degradation). We noticed that the uptake (Figure 1) and degradation (Figure 2) of the ovalbumin antigen was not adversely affected in irradiated, mature dendritic cells. However, immature dendritic cells appeared to have their propensity to degrade antigen enhanced when they had received radiation prior to antigen exposure (Figure 2C). The degradation of DQ-OVA by both mature and immature DCs was inhibited in the presence of the proteasome inhibitor MG132 (40 μ M) (not shown). This is important because it indicates – against general wisdom- that the proteasome is involved in the degradation of exogenous antigen in addition to the endogenous pathway. It also implies that the differences in radiation effect on exogenous and endogenous pathway of antigen presentation may be mediated by elements other than changes in proteasome function. Finally, it seems that radiation may affect dendritic cells maturation. Overall, we conclude from these experiments that the effects of radiation on antigen cross-presentation by dendritic cells in vitro are encouraging with respect to prostate anti-tumor immunity after local radiotherapy.

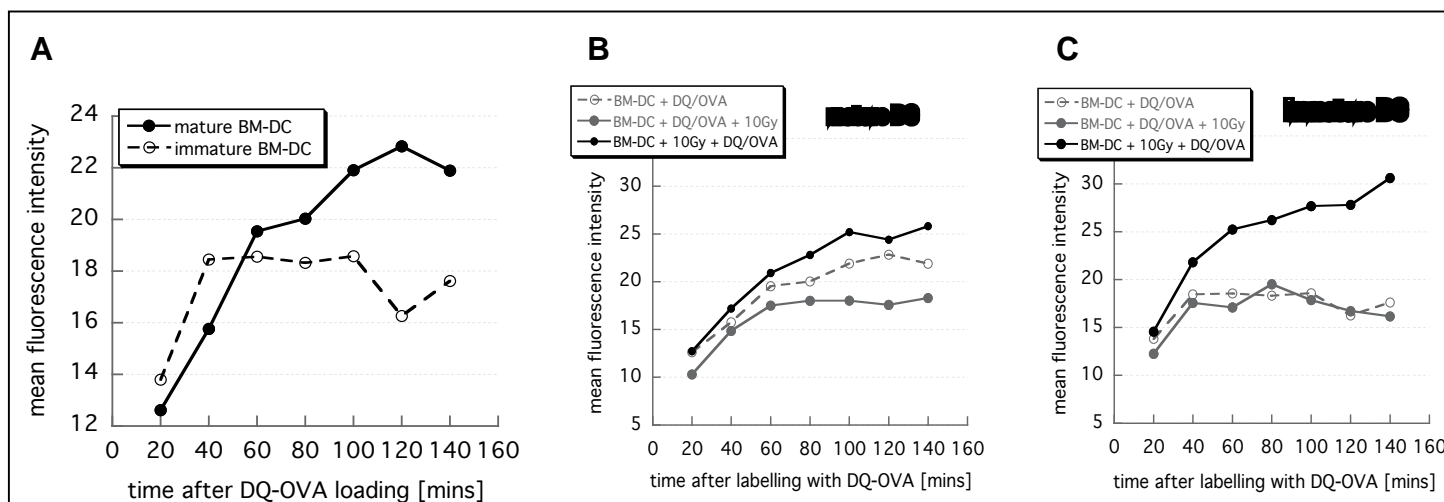
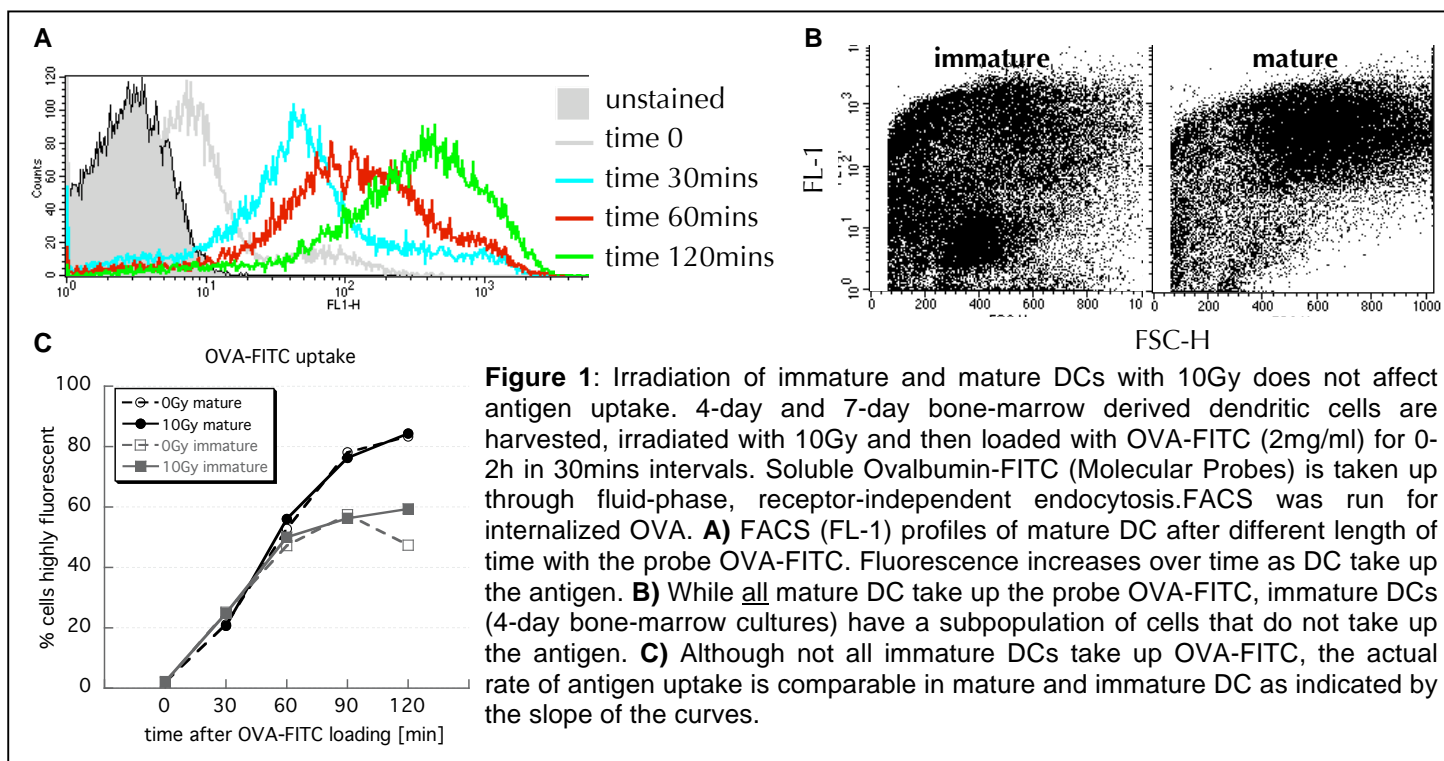
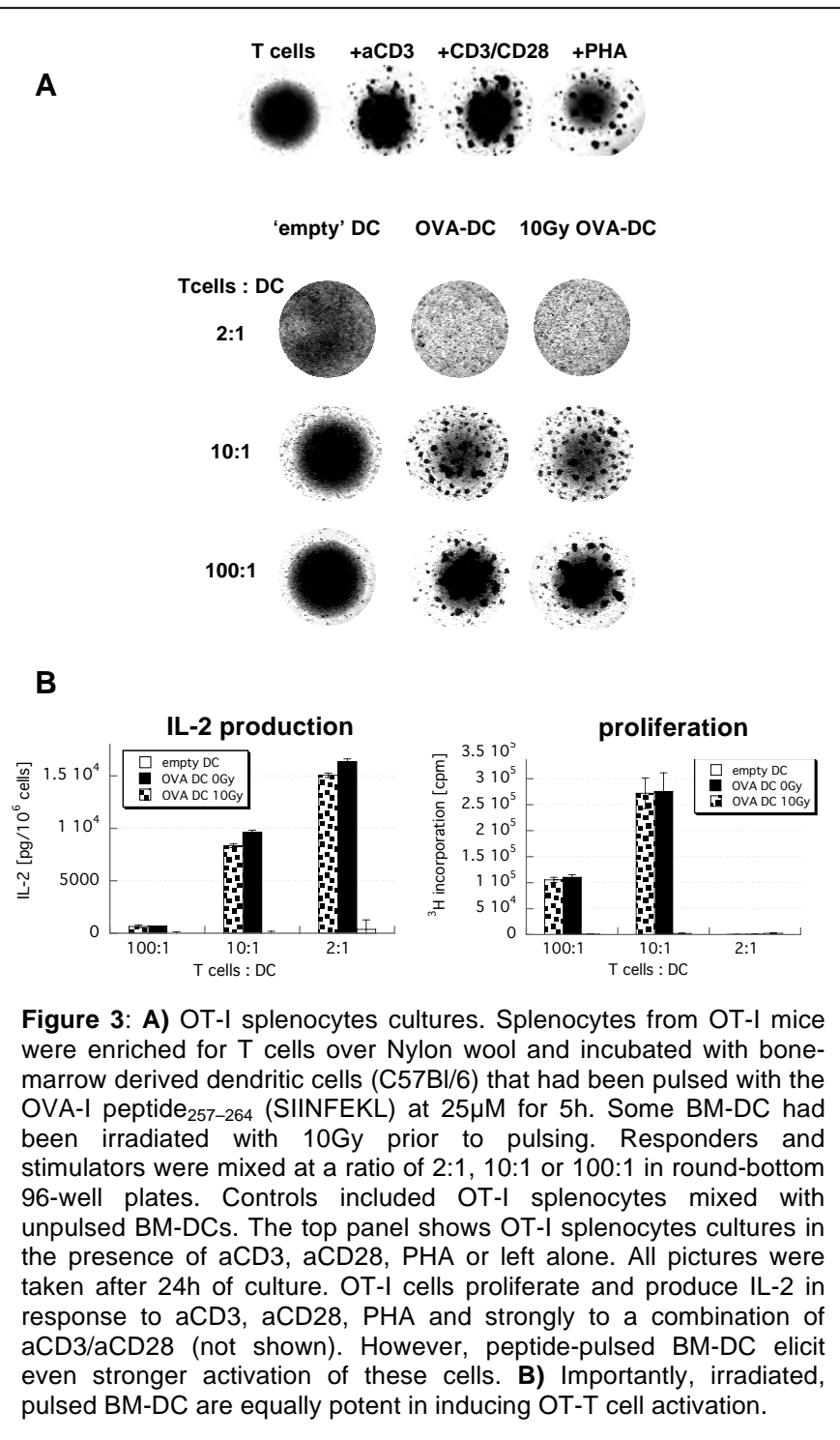


Figure 2: 10Gy radiation given to mature DC before or after antigen exposure does not greatly impact the rate of antigen degradation. DQ-OVA (Molecular Probes) is a highly self-quenched conjugate of OVA. DQ-OVA trafficking to the lysosomes. DQ-OVA is highly saturated with BODIPY which start to fluoresce once they are sufficiently apart such as during proteolytic degradation. With time cells degrade DQ-OVA and become more fluorescent. **A**) Initially, the degradation of antigen by immature and mature DC is comparable, yet immature DC plateau out earlier than mature DC. **C**) 4-day (immature) and **B**) 7-day (mature) old bone-marrow derived dendritic cells were harvested and loaded with DQ-OVA for 20mins at 37°C (10µg/ml). Some samples were irradiated with 10Gy before or after DQ-loading. Degradation as increase in fluorescence was monitored by FACS analysis in 20mins intervals.



The question of how antigen uptake and degradation might translate into changes in antigen cross-presentation was addressed in the following set of experiments using CD8+ T-cell hybridoma cells, B3Z, or nylon wool-enriched splenocytes from T cell receptor transgenic mice, OT-I (C57Bl/6 background) as responders. Both responders are specific for the chicken OVA₂₅₇₋₂₆₄ epitope presented within H-2K^b. B3Z (N.Shastri) utilize a reporter construct with the β-galactosidase gene (lacZ) under the control of the NF-AT element of the IL-2 enhancer. Appropriate T cell receptor engagement triggers IL-2 production, accumulation of β-galactosidase and hence blue X-gal staining. The uptake of exogenous antigen was initiated by pulsing the dendritic cells with the OVA-I peptide₂₅₇₋₂₆₄ (SIINFEKL). Engagement of the OVA-specific T cell receptors by mixing the responders with peptide-pulsed dendritic cells led to strong activation as measured by IL-2 production, β-galactosidase activity (B3Z, Figure 4) and proliferation (OT-I T cells, Figure 3). Irradiation of BM-DC prior to ovalbumin peptide pulsing somewhat reduces their ability to present antigen to B3Z or to OT-I T cells. But overall the effect of radiation on antigen presentation appears to be only a minor one and the question remains as to whether these differences have any importance at the physiological relevant (i.e. much lower) antigen concentrations. Overall, we conclude from these experiments that the effects of radiation on antigen cross-presentation by dendritic cells in vitro are -if any- minor.

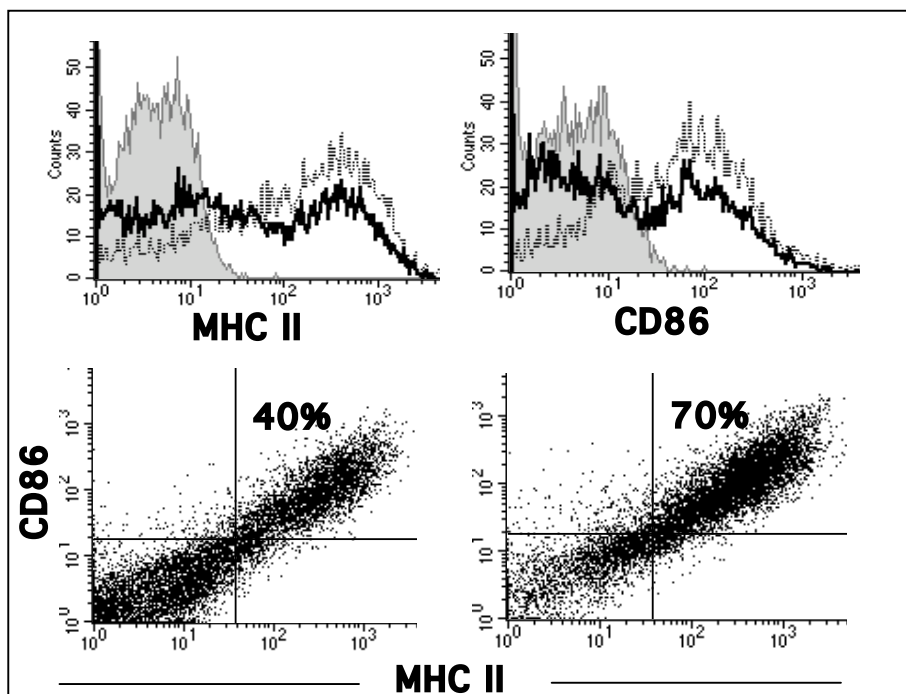
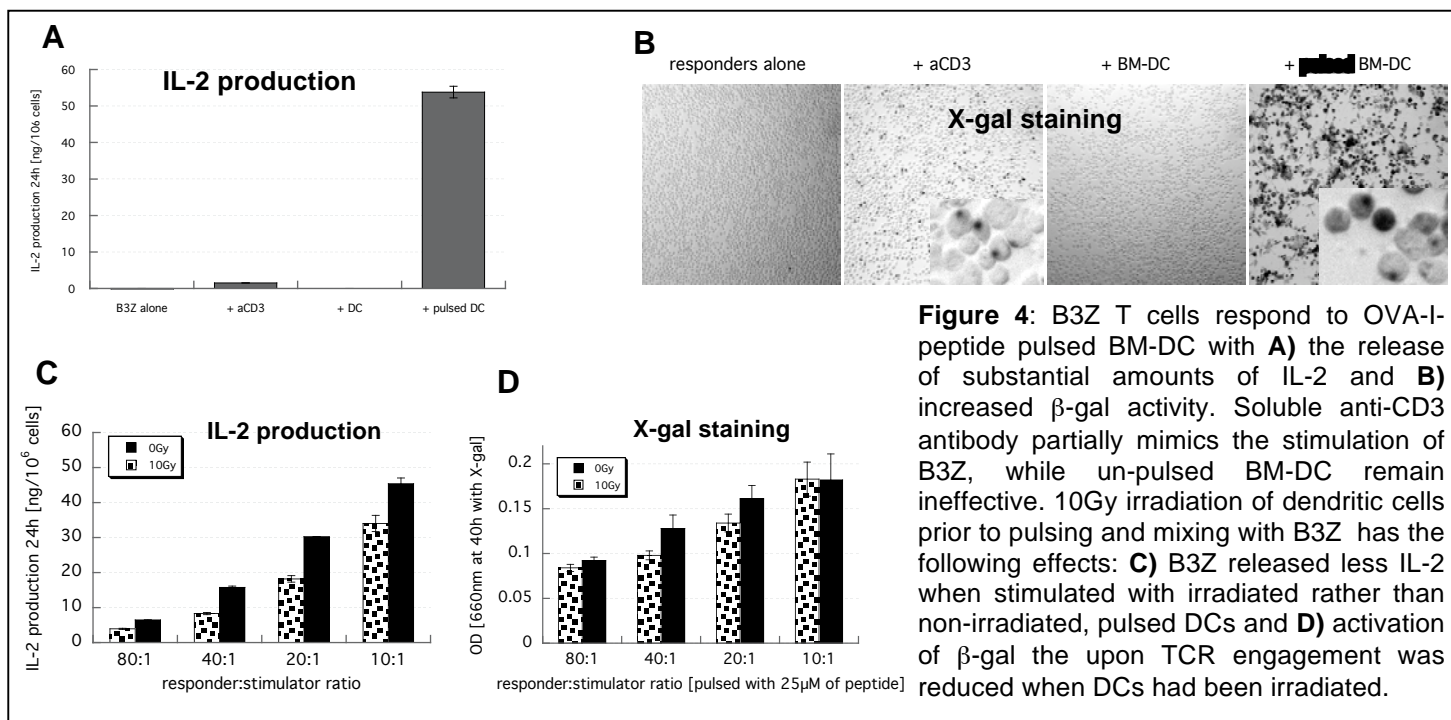


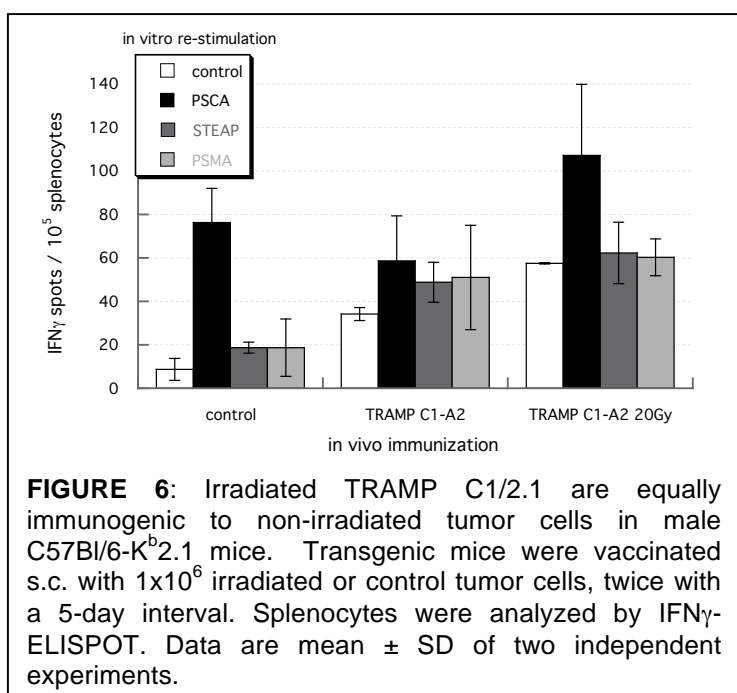
Figure 5: Radiation causes an increase in cell surface markers in dendritic cells. BM-DCs from 8-day bone-marrow cultures of C57Bl/6 mice were irradiated with 10Gy and incubated for 72h prior to staining for MHC II and CD86 and FACS analysis. Dead cells were excluded with 7AAD (gray = unstained; black line = 0Gy; dotted line = 10Gy). The expression of MHCII and CD86 increased following XRT and/or a loss in the low staining population occurred. Double positive cells increased from 40% to 70% as result of radiation treatment.

Our main goal was to develop PSA-expressing murine tumor lines to determine if tumor rejection is affected. An important aspect of this is the stability of MHC class I molecules, to be addressed in aim 1. Previously, We examined the effect of RT on MHC stability using the classic T2 cell model, the hypothesis being that radiation stabilizes MHC class I expression on cells. Indeed we demonstrated that irradiation with 2Gy and 10Gy appeared to increase the levels of stable MHC I complexes over a 24 h period suggesting a stabilization effect by radiation. When studying the radiation effects on the dendritic cell membranes we also observed MHC class I upregulation (shown in our original proposal). We have taken this one step further and we are now able to report that surface molecules other than MHC I are also increased following radiation, e.g. MHC class II and CD86 (Figure 5). All of this indicates to us that the radiation-induced changes maybe membrane-associated. In fact, this ties into our

previous observations on radiation effects on membrane lipids, which are important for signaling proteins and receptors and hence cellular responses to radiation. Radiation enhanced the clustering of lipid rafts but further studies will have to determine exactly how this translates to effects further downstream.

We focused most of our efforts on the generation of TRAMP cells stably expressing hPSA. We had previously prepared both TRAMP C1 and TRAMP C2 with the pSecTag2 construct containing hPSA (Invitrogen). However any attempt to detect hPSA in vitro in any meaningful amounts failed (not shown). As a backup strategy we used a retroviral system to introduce hPSA, again without any PSA production. Also, when injected in vivo, PSA could not be detected. The fact that we could not detect PSA production by these cells forced us to alter our approach regarding radiation responses and the ratio of constitutive proteasomes to immunoproteasomes. As partial fulfillment of aim 2, we examined the radiation effects on proteasome function of the parental cells before inserting PSA. As expected and as shown in our previous report, radiation treatment of TRAMP C1 decreased proteasome chymotrypsin-like activity by ~40% by 10Gy. However, we decided not to pursue this question further until we can confirm the expression of hPSA in these cells.

In light of absent hPSA detection we changed our approach to address aim 4 and used TRAMP cells stably expressing the human HLA-A2.1 gene and examined if responses to other prostate tumor-specific antigens that are known to have human homologs would be processed by murine TRAMP cells and expressed in the clinically relevant context HLA-A2.1. We looked for prostate stem cell antigen (PSCA), six-transmembrane epithelial antigen of the prostate (STEAP) and prostate-specific membrane antigen (PSMA). We used these humanized prostate cancer cells (TRAMP C1/2.1) and repeatedly injected 1×10^6 cells into C57Bl/6-K^b2.1 mice and measured tumor-specific immune responses by IFN γ -ELISPOT. To address the issue as to whether radiation affect tumor immunogenicity, the hierarchy of antigens and the immune-responses that ensues we compared responses to 20Gy-irradiated tumor cell vaccine with responses seen to sham-irradiated tumor cells. Overall, control mice had low background in IFN γ -responses to the panel of prostate tumor-specific HLA-A2 antigenic peptides, apart from PSCA (Figure 6) but responses increased significantly upon tumor vaccination. There appeared to be no significant benefit or detriment from irradiating the tumor cells prior to vaccination, apart for anti-PSCA responses, which were increased.



KEY RESEARCH ACCOMPLISHMENT

- Development of PSA-TRAMP C1, PSA-TRAMP C2 -100% completed.
- Study of the radiation effect on MHC class I expression extended to MHC II and CD86 upregulation.
- Study of radiation effects on proteasome and immunoproteasome function – 50% completed.
- Study of radiation effects on antigen uptake and degradation by DCs – 100% completed.
- Radiation studies on antigen cross-presentation using OVA-specific T cells as a surrogate antigen.
- Radiation effects on antigen hierarchy involving prostate-specific antigens in vivo.

REPORTABLE OUTCOMES

- ❑ **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-occurring 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.

CONCLUSIONS

Our primary focus this year was to develop our hybrid murine-human prostate tumor model further. The generation of murine prostate tumor cells expressing the human form of PSA is completed but initial results regarding the production of this protein within the murine system proved challenging. We therefore followed an alternative approach using the same parental murine prostate tumor cells but with a human MHC class molecule which allowed us to monitor responses to prostate tumor specific antigens other than PSA that are relevant in the clinical settings.

We were also able to show that radiation does not substantially impact the uptake and degradation of antigen by mature dendritic cells. However, immature dendritic cells appeared to have their propensity to degrade antigen enhanced when they had received radiation prior to antigen exposure. This is important because the state of maturation is known to affect the composition of proteasomes, and hence the antigen repertoire and might therefore be differentially targeted by radiation. We have also reasons to believe that radiation affects antigen presentation independently from effects on proteasome antigen degradation, e.g. by inducing cellular maturation. Additionally, we observed increases in cell surface markers on irradiated dendritic cells such as MHC II and CD86, which should further add to their functional integrity. Overall, we conclude from these experiments that the effects of radiation on antigen cross-presentation by dendritic cells in vitro and in vivo are encouraging and give us no reason to believe that radiotherapy is not a good candidate to be used in conjunction with cancer immunotherapy.

APPENDIX – see publication attached.

T-Cell Responses to Survivin in Cancer Patients Undergoing Radiation Therapy

Dörthe Schaeue,¹ Begonya Comin-Anduix,² Antoni Ribas,² Li Zhang,³ Lee Goodglick,³ James W. Sayre,⁴ Annelies Debucquoy,⁵ Karin Haustermans,⁵ and William H. McBride¹

Abstract Purpose: The goal of this study was to determine if radiation therapy (RT) of human cancer enhances or diminishes tumor-specific T-cell reactivity. This is important if immunotherapy is to be harnessed to improve the outcome of cancer radiotherapy.

Experimental Design: Lymphocytes were isolated from colorectal cancer (CRC) patients before, during, and after presurgical chemoradiotherapy. Similar samples were taken from prostate cancer patients receiving standard RT. The level of CD8⁺ T cells capable of binding tetramers for the tumor-associated antigen survivin, which is overexpressed in both cancer types, was enumerated in HLA-A*0201 patient samples. CD4⁺, CD25^{high}, Foxp3⁺ cells were also enumerated to evaluate therapy-induced changes in T_{regulatory} cells. For CRC patients, most of whom were enrolled in a clinical trial, pathologic response data were available, as well as biopsy and resection specimens, which were stained for cytoplasmic and intranuclear survivin.

Results: Survivin-specific CD8⁺ T lymphocytes were detected in the peripheral blood of CRC and prostate cancer patients and increased after therapy in some, but not all, patients. Increases were more common in CRC patients whose tumor was downstaged after chemoradiotherapy. Biopsy specimens from this cohort generally had higher nuclear to cytoplasmic survivin expression. T_{regulatory} cells generally increased in the circulation following therapy but only in CRC patients.

Conclusion: This study indicates that RT may increase the likelihood of some cancer patients responding to immunotherapy and lays a basis for future investigations aimed at combining radiation and immunotherapy.

Management of cancers of the rectum and the prostate relies heavily on radiation therapy (RT), but later-stage disease is often hard to control. Harnessing the immune system to assist in the elimination of cancer cells within, and outside, the radiation field could be beneficial in such situations but this will require knowledge of the effects of RT on tumor-specific immunity in humans, about which little is known. Preclin-

ical data are not very helpful, suggesting consequences ranging anywhere from favoring tolerance to enhancing immunity (1–4).

Here, we used a sensitive tetramer assay for the tumor-associated antigen survivin to ask what happens to tumor-specific T-cell responses in colorectal and prostate cancer patients during and after RT. Survivin is highly expressed in many human cancers but is largely undetectable in most normal tissues (5, 6). It augments cell proliferation and survival (7), either by inhibiting caspase-9 and hence apoptosis (8) or by directing chromosome movement during mitosis (9). Its location in the cytoplasm or nucleus may be crucial in determining its function as well its prognostic potential (10–12). Importantly, it is associated with resistance to therapy, including RT (7, 13).

The evidence that survivin is immunogenic is strong. In preclinical models, survivin-reactive CD8⁺ CTLs can be generated *in vitro* that efficiently lyse target cells and confer tumor protection on adoptive transfer *in vivo* (14–16). In humans, survivin-reactive T cells can be detected in primary breast cancer and melanoma lesions and in lymph nodes and blood of cancer patients (17, 18), who also develop anti-survivin antibodies (19). Both animals and patients respond to vaccination with this antigen (20–22).

Unfortunately, clinical experience indicates that adaptive antitumor immune responses generally fail to translate into measurable tumor regression. This has been ascribed to a variety of immune escape mechanisms, one of which is the

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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presence of $T_{\text{regulatory}}$ cells (23). Different types of $T_{\text{regulatory}}$ cells have been described (24–26) but the $CD4^+$, $CD25^{\text{high}}$, Foxp3⁺ subsets are generally considered important in suppressing antitumor immunity (27). Human tumors are frequently infiltrated with such cells (28–30), which suppress effector T cells through multiple mechanisms (31, 32). Although older studies in mice showed that tumor-induced suppressor T cells were more radiosensitive than other T-cell subsets (33), there is little data on the radiosensitivity of $T_{\text{regulatory}}$ subsets in humans.

This study asks whether survivin-specific cytotoxic $CD8^+$ T cells can be detected in patients with prostate or colorectal cancer (CRC), whether cancer treatment with RT or chemoradiotherapy (CRT) alters the tumor-specific immune status in these patients, whether the level of circulating $T_{\text{regulatory}}$ cells is affected, and whether any immune variables correlate with pathologic tumor regression.

Materials and Methods

Chemicals. The following were used: Ficoll-Paque (GE Healthcare Bio-Sciences); human AB serum (OmegaSci.); DMSO and DNase (Sigma); RPMI 1640 with L-glutamine and antibiotics (Fisher); tetramers and anti- $CD8$ antibody (T8-FITC; clone SFCL21Thy2D3; Beckman Coulter, Inc.); 7-aminoactinomycin D, FITC-anti-human HLA-A2 (clone BB7.2), phycoerythrin (PE)-Cy5-anti-human $CD25$ (clone M-A251), and R-PE-anti-human $CD4$ (clone RPA-T4; PharMin-

gen); FITC-anti-human Foxp3 (clone hFOXY), PE-Cy5-anti-human Foxp3 (clone PCH101), FITC-anti-human $CD4$ (clone OKT4), and PE-anti-human $CD25$ (clone BC96; eBioscience); rabbit anti-human survivin (clone NB500-201; Novus Biologicals); and biotinylated anti-rabbit (BA-1000; Vector).

Patients and sample collection. Blood samples came from patients with colorectal cancer (CRC; $n = 28$) or prostate cancer ($n = 20$) in the University Hospital Gasthuisberg (Leuven, Belgium), with local Institutional Review Board approval and consent. Prostate patients received conventional RT. All but three with CRC were part of a phase II randomized, double-blind, placebo-controlled clinical trial with the cyclooxygenase-2 inhibitor celecoxib described previously (34). Preoperative CRT was 45 Gy in 25 fractions with continuous 5-fluorouracil infusion. Patients were randomized to celecoxib (2×400 mg/d) or placebo before surgery, which was on week 6. Blood samples were taken into Vacutainer CPT tubes (Becton Dickinson) before, during (week 3), and after CRT (week 5). Peripheral blood mononuclear cells (PBMC) were isolated following gradient centrifugation and frozen in human AB serum containing 10% (v/v) DMSO. Frozen blood samples were shipped on dry ice and stored in liquid nitrogen on arrival in the United States. Serial samples of individual patients were assayed for tetramer and $T_{\text{regulatory}}$ cell staining (see below) on the same day. PBMCs from eight healthy volunteers were isolated on Ficoll-Paque at the University of California at Los Angeles and stored as above. Pretreatment biopsies (21) and residual tumor surgery specimens (10) of CRC patients were fixed and processed for immunohistology. This trial was delayed because of cyclooxygenase-2 inhibitor safety issues, resulting in some incompleteness of data.

Table 1. CRC patients show an increasing number of survivin-reactive $CD8^+$ T cells in peripheral blood on completion of radiation treatment

	Tetramer (% of $CD8^+$)			Tetramer set	$CD8^+$ (% of PBMC)		
	Before	During	After		Before	During	After
CRC 7		0.04	0.09	1		14.9	14.1
CRC 19		0	0.18	1		3.2	5.4
CRC 21	0.1		0.16	1	15.5		1.6
CRC 22		0.76		2		1.2	
CRC 23	1.57	1.93		2	12.8	10.9	
CRC 24	1.16	0.27	0.97	2	5.78	15.5	12.1
CRC 27		1.67	0.75	2		1.9	4.8
CRC 28			0.42	2			9.4
CRC 29	0.68		2.25	2	18.1		24.5
CRC 30		1.48	1.46	2		7.24	10.1
CRC 33	0.12	0.17		2	28.2	20.8	
CRC 34	0.23	0.15	0.12	2	8.4	7.6	5.3
Rectum 5	0.02		0.18	1	11.9		14.9
Rectum 8	0.04	0.03	0.07	1	14.7	11.5	10.5
Rectum 10	0.01		0.07	1	18.2		8.95
Prostate 05	0.1	0.11		1	15.2	21.6	
Prostate 1	0.23	0.29	0.37	2	16.4	13.7	13.8
Prostate 2	0.12	0.05	0.25	2	17	18.7	17.7
Prostate 3	0.28	0.23	0.53	2	4	4.1	3.3
Prostate 4	1.33	0.16		2	17.7	18.5	
Prostate 5	0.22	0.12		2	8.9	8.1	
Prostate 6	0.15	0.15	0.26	2	13.9	15.7	7.3
Prostate 9	0.46	0.16	0.15	2	5.4	7.7	9
Prostate 11	0.32	0.31	0.21	2	28	40	36
Prostate 12	0.15	0.5	0.32	2	6	2.5	5.5
Prostate 13		0.1	0.14	2		5.4	5.5

NOTE: Data are from 15 HLA-A2–positive patients with CRC and 11 patients with prostate cancer. CRC indicates patients that were part of a cyclooxygenase-2 inhibitor clinical trial. Data are $CD8^+$ T cells staining positive with the tetramer for survivin (%) and levels of $CD8^+$ PBMCs (%). Two batches of survivin tetramer were used and are indicated. Five healthy volunteers served as control. Gray fields highlight those samples that stained above the background level ($\geq \text{mean} \pm 2 \times \text{SD}$ of % survivin-reactive $CD8^+$ T cells in the healthy volunteers for that tetramer batch).

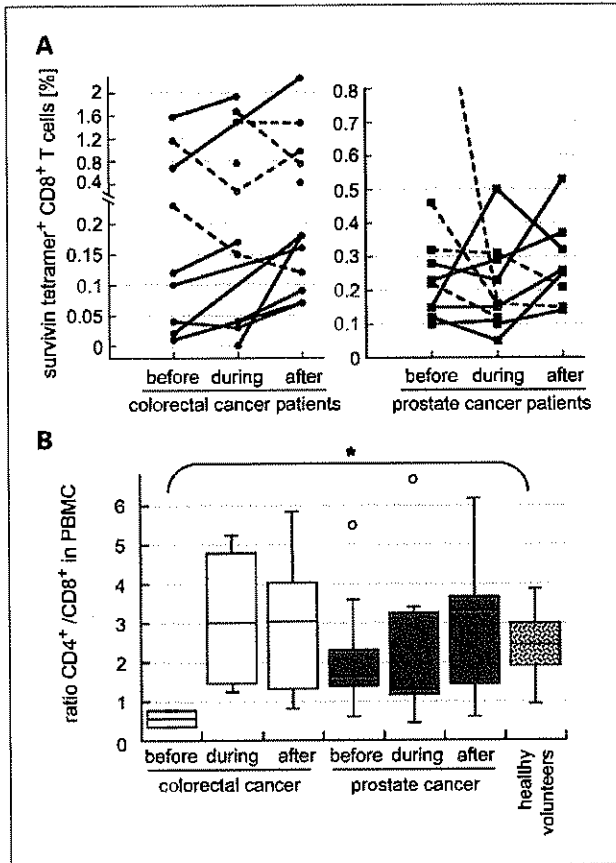


Fig. 1. A, levels of circulating survivin-reactive CD8⁺ T cells for individual CRC (left) and prostate cancer (right) patients before, during, and after treatment. Solid lines, overall upward trend; dashed lines, downward trend. B, ratios of CD4⁺ to CD8⁺ increase in patients before, during, and after treatment. Box and whisker diagrams summarize individual CD4⁺ to CD8⁺ T-cell ratios in cancer patients and in five healthy controls. *, $P = 0.008$.

HLA-A2 testing. PBMCs from patients and healthy subjects were thawed by dilution in prewarmed RPMI 1640 with 10% (v/v) human AB serum. Cells were treated with DNase, washed, and resuspended at $5 \times 10^6/\text{mL}$ in human AB serum. Cells (1×10^5 ; 20 μL) were stained with 1 μL of FITC anti-HLA-A2 antibody for 30 min at 4°C, washed, and resuspended in 300 μL PBS for flow cytometry (FACSCalibur, BD Biosciences).

Tetramer-binding assay. Cells (1×10^6) in human AB serum (200 μL) from HLA-A*0201-positive subjects were stained with 8 μL of the MHC tetramer for the HLA-A2-restricted survivin epitope Sur1M2 (LMLGEFLKL; ref. 18) along with 8 μL of anti-CD8 antibody. Sample volume permitting (28 samples) a MHC class I human negative tetramer with no known specificity that does not bind CD8⁺ T cells of any HLA allele (Beckman Coulter) was used to determine background PE fluorescence. After incubation for 30 min at room temperature and washing, samples were resuspended in PBS. 7-Aminoactinomycin D was added to detect nonviable cells 5 to 10 min before flow cytometry. PBMCs from a single HLA-A*0201-positive volunteer were run as an internal control for each assay. Events (1×10^5 to 2×10^5) were accumulated. Quality control required $\geq 10,000$ viable events and $\geq 2,000$ CD8⁺ T cells.

The gating strategy was

1. plot FL3, set viability gate (gate 1); (fixed cells as control);
2. plot FL1 versus FSC of population in gate 1; set gate 2 for CD8^{high} lymphocytes, excluding natural killer cells (CD8^{low}); and

3. plot FL-2 versus FL-1 of cells in gates 1 and 2 (viable CD8^{high} lymphocytes). Samples of one healthy volunteer stained with negative tetramer were used to set an arbitrary FL-2 lower limit of 0.03% double positives (35).

Two batches of survivin tetramer were used and correction had to be made for differences in binding. Positivity was based on a HLA-A*0201⁺ healthy volunteer having $0.053 \pm 0.023\%$ reactive CD8⁺ T cells for one batch and five HLA-A*0201⁺ healthy volunteers having $0.100 \pm 0.075\%$ for the other (Table 1). The low limit for a positive value was taken to be the mean ± 2 SD of these values (i.e., 0.099% for batch 1 and 0.25% for the second batch).

T_{regulatory} cell staining. CD4⁺, CD25^{high} cells with intracellular Foxp3⁺ were examined. For most samples, 1×10^6 cells were stained in 100 μL human AB serum with 20 μL FITC-anti-human CD4 and 20 μL PE-anti-human CD25 and incubated for 30 min on ice. Cells were washed and incubated in 1 mL of fixation/permeabilization buffer for 45 min on ice, washed twice, and resuspended in 2% (v/v) normal rat serum in 100 μL of permeabilization buffer. PE-Cy5-anti-Foxp3 (20 μL ; clone PCH101) was added followed by 30 min on ice. Cells were washed, resuspended in 200 μL of 10% fetal bovine serum, 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 and 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 $\geq 70\%$ viable and $\geq 2,000$ CD4⁺ T cells.

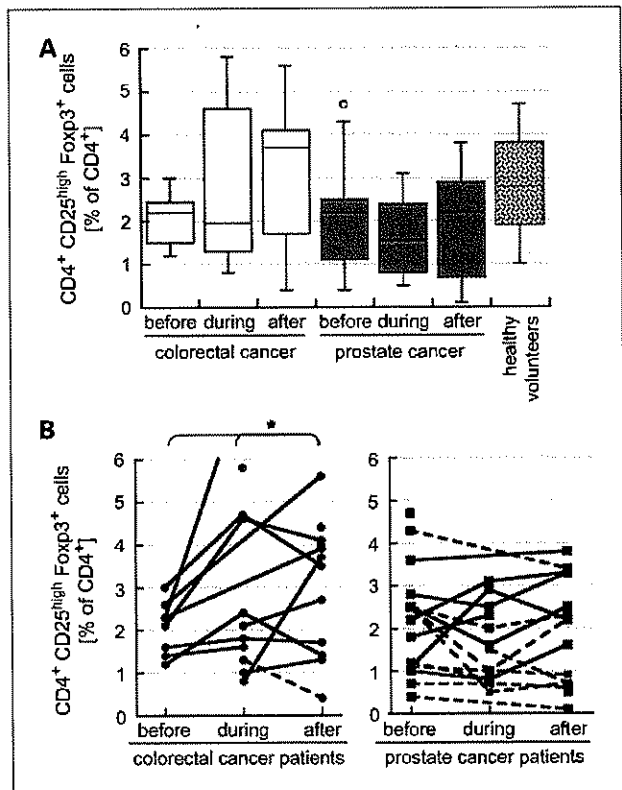


Fig. 2. The frequencies of circulating T_{regulatory} cells in CRC and prostate cancer patients were generally less than those observed in eight healthy volunteers and seemed to rise toward completion of CRT in CRC patients but not after RT in prostate cancer patients. Data are the % of CD4⁺ cells that are CD25^{high} and Foxp3⁺ presented as box and whisker diagram (A) and as time course for individual patients (B), with solid lines indicating an upward trend (*, $P = 0.039$), dashed lines do not.

The gating strategy was

1. FL-1 versus FSC of all events, set gate 1 for CD4⁺ cells, excluding debris and monocytes (CD4^{low});
2. FL2 versus FL1 of population in gate 1, set gate 2 for CD4⁺CD25^{high} double-positive lymphocytes; and
3. plot FL-3 of cells in gates 1 and 2 to determine fraction of CD4⁺CD25^{high}Foxp3⁺ triple-positive cells.

Immunohistochemistry for survivin. Tissues were deparaffinized at 75°C (30 min) in xylene and decreasing percentages of alcohol and washed five times in water. Sections were steamed in citrate buffer (100 mmol/L, pH 6.0; 25 min) and washed five times with PBS. Endogenous peroxidase was blocked with 3% H₂O₂ in methanol (15 min), washed, and incubated with 5% normal goat serum in 0.05% Tween 20 in PBS. Polyclonal rabbit anti-human survivin (1:200) was added and slides were incubated (30 min, room temperature; overnight at 4°C). Biotinylated anti-rabbit Ig (1:200) in 5% normal goat serum in 0.05% Tween 20 was added (40 min) followed by 3,3'-diaminobenzidine (3 min). After counterstaining with hematoxylin (10 s), slides were dehydrated and mounted. The identity of slides was blinded through a number code and scored in Belgium. Cytoplasmic survivin was scored for percentage tumor tissue staining positive and for intensity on a scale from 0 to 3. For the nuclear staining, we only scored the percentage because the intensity did not vary.

Statistics. All but one set of data were analyzed for statistical significance with the sign test and, after a square root transformation, with a Student's *t* test (36). Whether the level of circulating T_{regulatory} cells was significantly different from control levels was determined with the Wilcoxon signed rank test (36). Statistical significance was at the 5% level. In general, pooled patient data sets were compared as cohorts to the healthy control levels. Longitudinal responses for each patient compared outcome values (during or after) to individual baseline levels (before) and were then summarized for the whole cohort.

Box plots are box whisker diagrams summarizing the distribution of data as (a) the box spanning the 75% to 25% percentile and (b)

the median (line), (c) the minimum and maximum (whiskers above and below the box), and (d) individual outlying data points (open circles).

Results

Survivin-specific CD8⁺ T cells. Of 49 patients, 28 (57%) were HLA-A*0201 positive and eligible for tetramer analysis. However, two samples did not meet the quality control standards and were excluded from analyses.

Levels of survivin tetramer-reactive CD8⁺ cells were significantly higher in patients than five healthy controls ($P < 0.001$; Supplementary Figure), indicating the presence of antigen-specific T cells, and exceeded those for the negative control tetramer for all cohorts ($P < 0.001$).

Samples for tetramer analysis were available for 10 patients before, during, and after treatment, for 14 patients from two time points, and for 2 patients at a single time point. Overall, there were 19 lymphocyte samples before RT, 21 during RT, and 20 after RT (Table 1).

Samples from four of nine (44%) CRC patients before CRT treatment were positive for survivin tetramer binding (>2 SD from the mean of healthy controls), 5 of 10 (50%) during treatment, and 8 of 12 (67%) after treatment (Table 1). This trend toward increased responses on completion of CRT in CRC patients ($P = 0.499$) was not apparent in prostate cancer patients ($P = 0.352$) who exceeded the criterion of positivity in 5 of 10 (50%) cases before RT, 4 of 11 (36%) during RT, and 4 of 8 (50%) after RT.

Treatment-dependent responses to survivin. Because samples were taken before, during, and after treatment, we were able to evaluate individual patient responses over time (Fig. 1A). The percent survivin-specific CD8⁺ T cells increased in 9 of 13

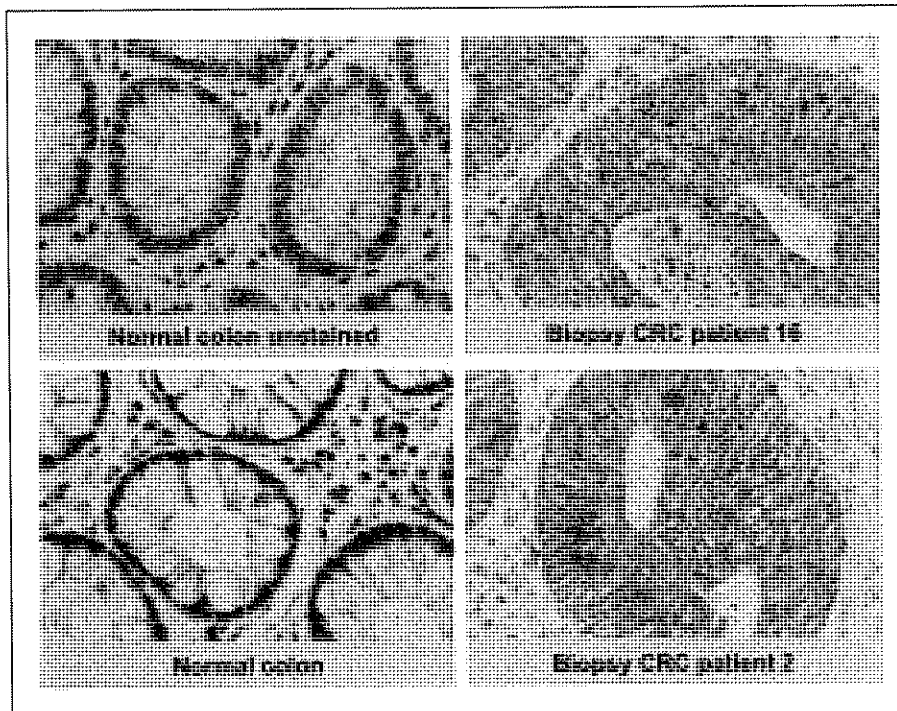


Fig. 3. The tumor antigen survivin was highly expressed in CRC patient specimens, whereas it remained mostly undetectable in normal colon tissue. Survivin seemed to be primarily located in the cytoplasm but occasionally nuclear staining was evident. Magnification, $\times 800$.

Table 2. Summarized data from 30 CRC patients that were part of the clinical trial

CRC patient	T downstaging	N downstaging	HLA-A2	Tetramer increasing	T _{regulatory} increasing	Survivin expression in biopsy			Survivin expression in resect		
						% cyto.	intens. cyto.	% nuclear	% cyto.	intens. cyto.	% nuclear
11	0	0				74	3	14	52.5	2	3.75
12	0	1	0		1	80	1	10			
16	0	1	0			90	2	0	100	3	5
17	0	0				73.3	1	3.3			
19	0	N/A	1	1		77.5	2	3			
24	0	1	1	0	1				92	2	24
26	0	1	0		1	0	0	20	34.2	1	1.4
27	0	0	1	0	N/A	58	1	0	77	3	7
33	0	1	1	1	1						
1	1	1	0			66.7	2	6.7			
2	1	1	0			70	2	7.5			
3	1	1				74	2	12			
4	1	0				90	3	2			
7	1	1	1	1		50	1	N/A	55.4	2	0.38
8	1	N/A			N/A	88	3	1			
9	1	1				68.3	3	28.3			
10	1	1				88	2	40			
18	1	N/A				70	3	1.25			
20	1	1	0			32.8	2	37.14	100	3	0
21	1	0	1	1		65	1	2.5			
22	1	1	1	N/A							
23	1	1	1	1					30	1	0
28	1	1	1	N/A	N/A	10	1	40			
29	1	1	1	1	1	40	1	0			
30	1	1	1	0	1	75	2	0			
31	1	1	0		1						
32	1	1	0		1						
34	1	1	1	0	0						
35	1	1	0		1						
36	1	1	0		1						

NOTE: "1" is a positive indicator for the respective variable, whereas "0" specifies a negative response. Tumor ($n = 21$) and lymph node ($n = 22$) stage decreased or did not ($n = 9$ and 5 , respectively). Twelve patients were HLA-A2 positive. The number of survivin-reactive CD8⁺ T cells increased in the peripheral blood in four of six responding patients and in two of four nonresponders. The frequency of systemic T_{regulatory} cells increased in six of seven responding patients and in four of four nonresponders. All biopsies tested stained positive for survivin, which was mostly in the cytoplasm and lesser so in the nucleus. In most samples, nuclear but not cytoplasmic survivin staining decreased and, in all resection specimens from the three responders, was essentially zero.

Abbreviations: % cyto., percent tumor staining positive for survivin in cytoplasm; intens. cyto., intensity of the cytoplasmic staining; % nuclear, percent tumor staining positive for survivin in the nucleus; N/A, not available.

(69%) CRC patients as a result of treatment, including before → during RT (2 of 5 cases), during → after RT (4 of 7), and before → after RT (5 of 7). Statistical significance was not reached ($P = 0.267$), but in only 2 of 13 cases (15%) was there a clear decrease in survivin-specific CD8⁺ T cells after CRT. Seven of 11 (64%; $P = 0.599$) prostate cancer patients also responded to RT with an increase in survivin-specific CD8⁺ T cells, including before → during RT (3 of 10 cases), during → after RT (5 of 8 cases), and before → after RT (5 of 7 cases). Hence, CRT of CRC and RT of prostate cancer generally increased the percent of circulating survivin-specific T cells in the CD8⁺ pool ($P = 0.076$).

Interestingly, the ratio of CD4⁺ to CD8⁺ T cells increased in most patients from before to after completion of therapy ($P_{\text{CRC patients}} = 0.071$ and $P_{\text{prostate}} = 0.47$; Fig. 1B). This was most marked for CRC patients, who had a low CD4 to CD8 ratio before therapy compared with all other cohorts ($P = 0.008$, compared with healthy volunteers). Overall, cancer patients tended to have less CD4⁺ in their circulation than healthy

control subjects ($33.6 \pm 6.5\%$) at the beginning of therapy (Supplementary Table).

Time course of response of circulating T_{regulatory} cells. The number of T_{regulatory} cells varied in eight healthy volunteers (aged 30-64) from 1% to 4.6% of total CD4⁺ (mean, $2.8 \pm 1.1\%$ CD4⁺CD25^{high}Foxp3⁺) cells, which compares well with the literature, indirectly validating our protocol. Most patients started treatment with less T_{regulatory} cells in their circulation than the volunteers (Fig. 2A; Supplementary Table). This was true for 7 of 8 (88%; $P = 0.182$) CRC patients and for 13 of 17 (76%; $P = 0.083$) prostate cancer patients. All seven CRC patients with matched samples from before and after treatment ended the study with more T_{regulatory} cells than they had initially; hence, there was an overall trend for these values to increase ($P = 0.039$; Fig. 2B). Notably, the majority of prostate cancer patients (8 of 13) did not adhere to this trend (Fig. 2B).

Intratumor survivin expression. Tumor biopsies and resections from patients who were part of the CRC trial were stained

with an antibody that recognizes both cytoplasmic and nuclear survivin. Examples are shown in Fig. 3. All biopsies, with one exception, tested positive for cytoplasmic survivin, in most cases covering an extensive area of the tumor, whereas survivin was mostly undetectable in normal colon tissue, with the exception of crypts. Nuclear staining was seen more sporadically and in a smaller area of the tumor (Table 2).

Clinical responses. For patients in the clinical CRC trial, Table 2 summarizes the data on clinical tumor and lymph node stage and immunologic variables of survivin-specific CD8⁺ T cells, T_{regulatory} cells, and intratumoral survivin expression. The patient numbers and subgroups were too small and the responses were too variable to derive statistical significance, but there were indications that a larger study could valuably explore.

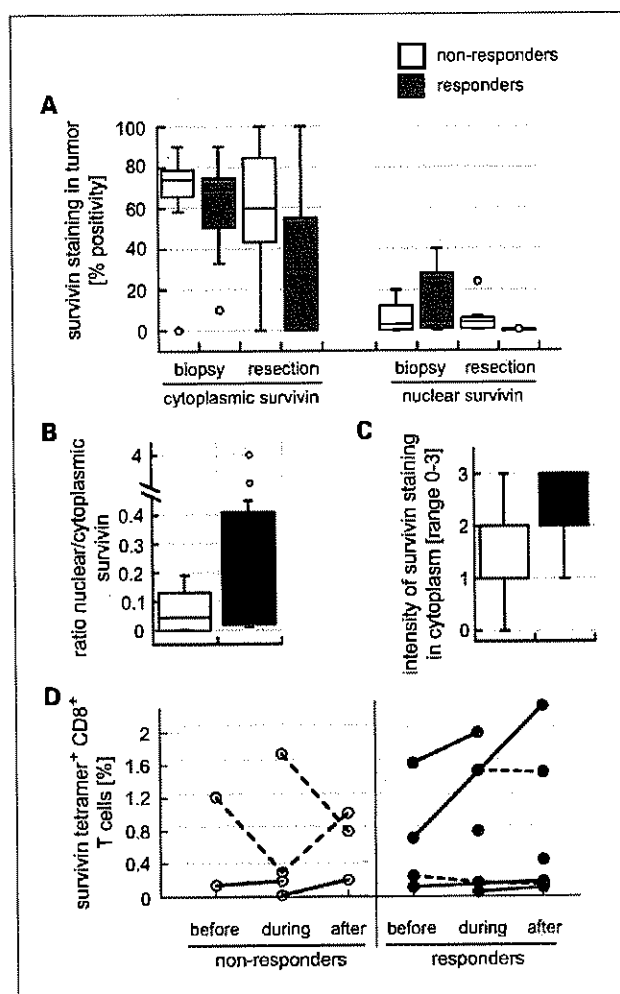


Fig. 4. The expression of survivin in CRC tissue sections (21 biopsies, 10 resections). Responders (T downstaging) had higher nuclear survivin levels at biopsy and less at resection. Cytoplasmic survivin levels also were lower in resection specimens from responders. **A**, box and whisker diagram showing data as median percent tumor staining positive for cytoplasmic (left) and nuclear (right) survivin. Biopsies of patients who responded to preoperative CRT had higher ratios of nuclear to cytoplasmic survivin (**B**), express more survivin (**C**), and have more promising changes in circulating survivin-specific CD8⁺ T cells than biopsies from nonresponders (**D**). Solid lines indicate an upward trend; dashed lines do not.

Biopsy specimens from patients whose tumors were downstaged tended to have higher nuclear survivin levels at biopsy than those who were not downstaged (Fig. 4A, right). Both the ratio of nuclear to cytoplasmic survivin and staining intensity in biopsy specimens were higher for the responders (Fig. 4B and C). The number of resection specimens was inevitably low, but both cytoplasmic and nuclear survivin were decreased over the comparable biopsy specimens, particularly for nuclear staining (Fig. 4A and B). Only six patients who responded with T downstaging and four who did not respond could be evaluated for therapy-related changes in survivin tetramer-positive T cells, with the former showing more promising changes (Fig. 4D).

Discussion

Although reverse immunology has clearly established that survivin is immunogenic, it remains uncertain whether anti-survivin responses occur in untreated cancer patients. We were clearly able to detect T cells binding survivin tetramer in almost half of CRC and prostate patients before treatment when compared with healthy controls. This agrees with Coughlin et al. (37), who measured similar frequencies in pediatric cancer patients using the same tetramer, and Grube et al. (38), who detected survivin-reactive T cells in 40% of patients with multiple myeloma using IFN- γ mRNA as a readout. It contrasts Casati et al. (15), who, using a different tetramer, reported that <0.1% of CD8⁺ T cells from a CRC patient bound survivin, although this level could be boosted by *in vitro* stimulation.

From a phase I clinical vaccination trial, we know that anti-survivin responses are not easily induced in CRC patients with only 1 of 15 responding (39). Assessing whether responses increase or decrease following RT or CRT alone therefore inevitably stretches the sensitivity and reproducibility of the assays being used. Nonetheless, we observed that tumor-specific T cells clearly increase in most CRC patients after completion of CRT and in most prostate cancer patients after RT. Perhaps more important is that only a few patients showed a decrease in survivin-reactive CD8⁺ T cells, which suggests that their ability to respond is not compromised by treatment. This was also suggested in a randomized phase II clinical trial in prostate cancer patients where RT did not obstruct T-cell responses to prostate-specific antigen when given at the end of a cancer vaccination regimen (40).

It is tempting to ascribe the increase in tumor-specific T cells to a radiation-induced increase in antigenic peptide liberation (41) and presentation by dendritic cells, which we have shown can be boosted by radiation (42). The rate of tumor regression (i.e., tumor kinetics) may modulate this and hence the generation of immunity. The fact that this was not detected (40) by enzyme-linked immunospot in patients with prostate cancer receiving RT could be due to the relative sensitivity of the assays. We have not been able to distinguish the two treatment arms of the CRC cohort with some patients receiving the cyclooxygenase-2 inhibitor celecoxib because this trial is still ongoing. There are suggestions in the literature that celecoxib might further enhance the development of antigen-specific T_{effector} cells (43, 44), but this will not alter the conclusions from the study. The fact

that CRC patients who responded with T downstaging tended to have higher levels of nuclear survivin suggests that this is a potential predictive marker of response, with the understanding that nuclear survivin relates more to proliferation and hence may signal a more rapid response (11, 45).

There are several ways to interpret radiation-induced antitumor-reactive CD8⁺ T-cell levels. Loss of immune suppression due to decreased tumor burden is one. In recent years, T_{regulatory} cells have gained prominence as a powerful suppressive mechanism. The frequency of T_{regulatory} cells that we detected in the majority (80%) of our patients was actually lower than in healthy subjects, who were well within the published range for normal individuals (30, 46). This contrasts to several reports of high levels of circulating T_{regulatory} cells in cancer patients (30, 46, 47).

In our study, levels of T_{regulatory} cells in CRC patients increased on completion of CRT, whereas this did not happen in prostate cancer patients. It may be that in CRC T_{regulatory} cells, and perhaps other CD4⁺ T-cell subsets, relocate to tumor sites, for which there is evidence (28), and are remobilized by therapy-induced changes in the tumor microenvironment. RT-induced adhesion molecule and chemokine expression could alter migratory behavior of T_{regulatory} cells

(30, 48). It is also feasible that systemic effects induced by the CRT more so than by RT could have contributed to such a selective increase in T_{regulatory} cells in CRC by affecting the balance in lymphocyte subpopulations (49). Such increases in circulating T_{regulatory} cells may therefore be more apparent than real. Even local irradiation can affect the lymphocyte balance because lymphocyte subsets have differential sensitivity to radiation, with the simplified picture being that naive T cells are more sensitive than effector cells (50), whereas antigen-induced T_{regulatory} cells gain in radiosensitivity (33). The fact that we observed an increase in CD4⁺CD25^{high}Foxp3⁺ Tregs might simply reflect changes in other CD4⁺ subpopulations.

Perhaps, the most important point from this study is that CRT and RT do not induce immune tolerance to survivin, making immunotherapy approaches feasible in combination with RT. Furthermore, tetramer technology coupled with other flow-based methods provides us with powerful tools to study the antitumor immune status in patients undergoing such treatments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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