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Defining Molecular Signature of Pro-Immunogenic Radiotherapy Targets in Human Prostate Cancer Cells

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To understand the impact of clinically relevant radiation therapy (RT) on tumor immune gene expression and to utilize the changes that occur during treatment to improve cancer treatment outcome, we examined how immune response genes are modulated in prostate cancer cells of varying p53 status. LNCaP (p53 wild-type), PC3 (p53 null) and DU145 (p53 mutant) cells received a 10 Gy single dose or 1 Gy × 10 multifractionated radiation dose to simulate hypofractionated and conventionally fractionated prostate radiotherapy. Total RNA was isolated 24 h after multifractionated radiation treatment and single-dose treatments and subjected to microarray analysis and later validated by RT-PCR. RT-PCR was utilized to identify total-dose inflection points for significantly upregulated genes in response to multifractionated radiation therapy. Radiation-induced damage-associated molecular pattern molecules (DAMPs) and cytokine analyses were performed using bioluminescence and ELISA. Multifractionated doses activated immune response genes more robustly than single-dose treatment, with a relatively larger number of immune genes upregulated in PC3 compared to DU145 and LNCaP cells. The inflection point of multifractionated radiation-induced immune genes in PC3 cells was observed in the range of 8–10 Gy total radiation dose. Although both multifractionated and single-dose radiation-induced proinflammatory DAMPs and positively modulated the cytokine environment, the changes were of higher magnitude with multifractionated therapy. The findings of this study together with the gene expression data suggest that cells subjected to multifractionated radiation treatment would promote productive immune cell–tumor cell interactions.

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INTRODUCTION

Ionizing radiation is a standard modality of treatment for many solid tumors, with the goal of eliminating tumor cells through extensive DNA damage leading to growth arrest, apoptosis and clonogenic death (*1*). However, the high frequency of malignancies in immune-compromised patients supports a crucial role of the immune system in controlling tumorigenesis (*2*). Recent studies have emerged highlighting the importance of the immune response elicited by tumoricidal effects of radiation therapy (RT). The immune system can participate in antitumor mechanisms by eliminating transformed and premalignant cells, often observed in viral-induced cancers, which are mostly dependent on immune response stimulators such as stress or necrosis or those induced by radiation exposure (*3*). It has been demonstrated that melanoma mouse models release tumor antigens upon tumor cell death in response to the direct effects of radiotherapy on the tumor tissue. Antigen-presenting cells prime effector cells in the lymph nodes that travel to the tumor site and trigger malignant cell lysis (*4*).

Ionizing radiation triggers the release of various inflammatory cytokines, causing an overall antitumor effect on the tumor cell stroma (*5*). It is believed that inflammatory cytokines released from both cancer cells and non-cancer cells form a radiation-induced bystander/abscopal response, in which signals are released from irradiated cancer cells to neighboring normal cells (bystander) or to distant tumor cells (abscopal) and aid immunomodulatory response. These events are often caused by release of cytokines such as IL-6, IL-8, TGF-β1 and TNF-α, among others (*6*). Other studies indicate that $CD8^+$ T cells play a role in orchestrating radiation-related therapeutic effects, when comparing tumor growth in immunocompetent versus T-cell-deficient mice (*7*).

Radiotherapy has the ability to make dendritic cells (DCs) capable of producing lymphocyte responses involving adaptive antitumor immune attack by taking up tumor antigens, consequently presenting them to effector T cells and thereby inhibiting tumor growth (*8, 9*). Moreover, recent studies have shown the use of radiotherapy in

combination with Th1 cell therapies can enhance the production of cytotoxic-T-lymphocytes specific for the tumor malignancy, thereby actively participating in the regression of such cancers (*10*). Thus, radiation therapy can increase the T-cell response for antitumor effects, suggesting that radiation therapy has a direct link to the induction of immune modulation genes that participate in the overall immunological cascade to elicit a robust immunogenic tumor cell death (*11*).

Previous studies from our group demonstrated that the PC3 prostate carcinoma cell line showed a significant upregulation of immune-related genes after multifractionated treatment (*12*), suggesting that radiation therapy has the potential to elicit an immune response that will initiate a cascade of events leading to immunogenic antitumor effects. Corroborating evidence based on gene expression studies in prostate and breast cancers exhibited a distinctive upregulation of interferon-related genes after multifractionated therapy when compared to single-dose treatment (*13*). The current study was undertaken to identify the immunoregulatory role of such differentially expressed genes in prostate cancer cells that were subjected to different radiation exposure schemes such as multifractionated or single dose. Further, multifractionated and single-dose mediated immune gene modulation in these prostate cancer cells was evaluated by analyzing the damage-associated molecular pattern molecules (DAMPs) such as HMGB1 along with evaluation of cytokines at the protein level. Overall, the data presented here suggest that although both single dose and multifractionated dose altered DAMPs and cytokine levels, in general the effect was of greater magnitude with multifractionated treatment.

MATERIALS AND METHODS

Cell Lines and Radiation Treatments

PC3, DU145 and LNCaP human prostate carcinoma cells were obtained from the American Type Culture Collection (ATCC®, Rockville, MD). PC3 cells are null for p53 function and DU145 harbors homozygous p53 mutation (*14*), whereas LNCaP cells contain intact p53 function (*15*). Cells were maintained and grown according to ATCC recommendations.

Radiation Treatment

Cells were irradiated in a PANTAK high frequency X-ray generator (Precision X-ray Inc., North Bedford, CT), operated at 300 kV and 10 MA. The dose rate was 1.6 Gy per min and this protocol was used for all experiments.

Sample Collection for Microarray Analysis

Cells were plated into T75 cm^2 flasks ($1–1.5 \times 10^6$ for single-dose treatment and $0.8–1 \times 10^6$ for fractionated treatment). After 24 h, cells were exposed to a total of 10 Gy, administered either as a single fraction or as a 1 Gy × 10 multifractionated treatment. These non-isoeffective doses were selected to simulate clinical hypofractionated and conventionally fractionated radiotherapy regimens. For the multifractionated protocol, cells were exposed to 1 Gy, twice a day, at 6 h intervals for 5 consecutive days. The cells were approximately 90% confluent at the time of harvesting. For both protocols, radiation-induced changes were analyzed at 24 h after the final dose of radiation. Separate controls were maintained for single-dose and multifractionated treatment protocols.

Inflection Point after Multifractionated Radiation Treatment

To investigate the number of 1 Gy fractions required to elicit a cellular adaptation response, PC3 and DU145 cells were plated into T75 cm^2 flasks ($1.5–2 \times 10^5$) and exposed to 1 Gy, twice a day, for a total of 1–10 fractions. RNA was collected 24 h after each of the indicated number of fractions (Fig. 1). Each fraction was compared to its own control collected at the same time.

Cytokine Study

Cells were plated into T75 cm^2 flasks ($1.5–2 \times 10^5$) and after 24 h, cells were exposed to 10 Gy in multifractionated doses of 1 Gy, twice a day, for 5 days (1 Gy × 10), with 6 h between each dose. For the single dose a total of 10 Gy was administered (10 Gy × 1) during the last day of the multifractionated radiation treatment. For both single and multifractionated radiation-treated cells, culture supernatant was collected 48 and 72 h after the final radiation dose, and cells were counted using a Countess® Automated Cell Counter (cat. no. C10227, Invitrogen™ Life Technologies Inc., Grand Island, NY). Separate controls were maintained for single-dose and fractionated radiation treatment protocols.

Microarray Analysis

Microarray analysis was performed in all three cell lines using RNA isolated from three separate experiments. Cells were pelleted at 24 h after the final dose of the single or multifractionated radiation treatment and stored in liquid nitrogen. Total RNA, including small RNAs, was isolated using miRNeasy Mini Kit (cat. no. 217004, QIAGEN, Germantown, MD). The concentration and purity of total RNA was measured by spectrophotometry at OD260/280 and the quality of the total RNA sample was assessed using an Agilent Bioanalyzer with the RNA6000 Nano Lab Chip (Agilent Technologies Inc., Santa Clara, CA). The microarray analysis was done using CodeLink™ Whole Genome Bioarrays representing 55,000 probes (Applied Microarrays Inc., Tempe, AZ). CodeLink Expression Analysis software 5.0 (Applied Microarrays Inc.) was used to process the scanned images from arrays (gridding and feature intensity) and the data generated for each feature on the array were analyzed with GeneSpring® software (Agilent Technologies). Raw intensity data for each gene on every array were normalized to the median intensity of the raw values from such arrays. Data for all arrays were filtered for intensity values that were above background in at least two of any set of three replicates for any condition within each radiation-treated protocol. Unsupervised hierarchical clustering of all probes present (significantly above background detection levels) in at least one condition (control or radiation treatment) from all three replicates indicated that expression profiles clustered by biological replicate treatments, rather than by any technical condition of the experimental design. To ensure that genes were measured reliably, ANOVA was used to compare the means of each condition ($n = 3$). Cutoff ratios were greater than 2.0 and less than 0.5 with a P value <0.05 relative to the respective control group were selected for this study.

Real-Time RT-PCR

Immune response genes that exhibited significant differential expression patterns were further confirmed and analyzed for inflection point by RT-PCR using Taqman® Custom Express Plate (cat. no. 4413264, Applied Biosystems, Foster City, CA) and ABI PRISM® 7500 Sequence Detection System instrument equipped with the Sequence Detection Software (SDS) version 1.4. RNA (2 μg) were

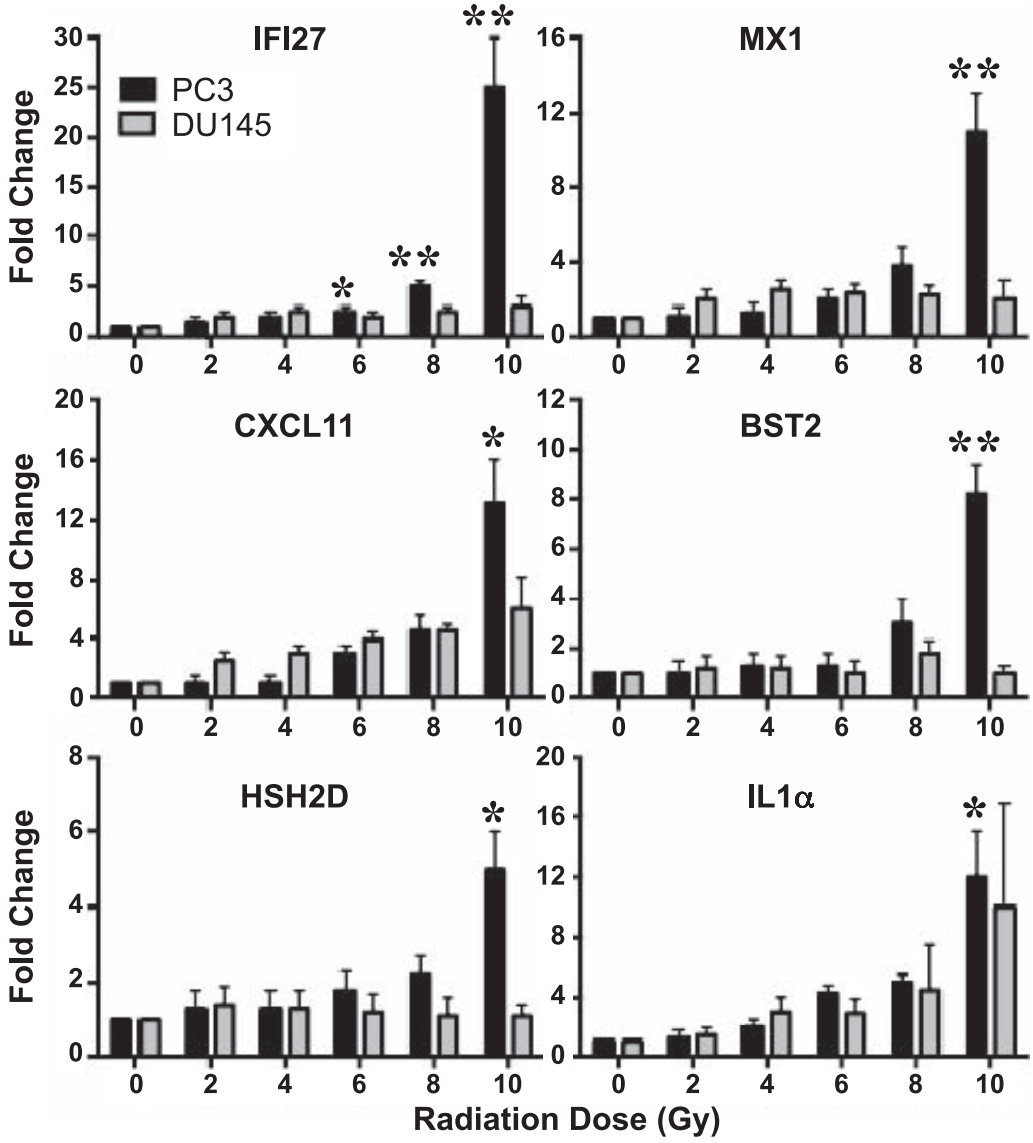

FIG. 1. Inflection point kinetics of immune genes in multifractionated treated PC3 and DU145 cells as assessed by real-time RT-PCR. PC3 and DU145 cells were exposed to 1–10 Gy of radiation delivered as 1 Gy multifractionated treatment. Fold change in IFI27, MX1, CXCL11, BST2, HSH2D and IL1A expression 24 h after cells received 1–10 Gy of radiation was determined by RT-PCR. Data shown are fold change (AV ± SEM) of 3 biologically distinct experiments. $*P < 0.05$, $**P < 0.01$.

reverse transcribed to synthesize single-stranded cDNA using the High-Capacity cDNA Reverse Transcription Kit (cat. no. 4368814, Applied Biosystems). The threshold cycle (C_t) of the endogenous control (18S) was used to normalize target gene expression (ΔC_t). The relative change in gene expression ($\Delta\Delta C_t$) was used for comparison of the gene expression in irradiated samples versus nonirradiated control.

Ingenuity® Pathway Analysis (IPA)

The functional significance of differentially expressed mRNAs (twofold change and $P < 0.05$) after single-dose and multifractionated radiation treatment was evaluated using IPA software (version 8.7–3203, Ingenuity Systems, Redwood City, CA). Data sets were uploaded into the IPA, then mapped to the functional networks available in the Ingenuity Pathway Knowledge Base and ranked by score as described previously (*16*).

Analysis of Radiation-Induced Damage-Associated Molecular Pattern Molecules

Prostate cancer cell lines (DU145, PC3, LNCaP) were mock irradiated (0 Gy), 1 Gy irradiated in 10 fractions or 10 Gy single-dose irradiated. Cell culture supernatants were collected after 48 and 72 h were analyzed for ATP by bioluminescence (Sigma-Aldrich LLC, St. Louis, MO) and high-mobility group box 1 (HMGB1) protein content by ELISA (IBL International, Hamburg, Germany), according to the manufacturer's instructions.

Radiation-Induced Modulation of Tumor Cell Cytokine Production

Tumor cells (DU145, PC3, LNCaP) were mock irradiated (0 Gy), 1 Gy irradiated in 10 fractions or 10 Gy single-dose irradiated. Supernatants were analyzed after 48 and 72 h for GM-CSF, IFN-γ, IL-12p70, IL1β, IL-2, IL-6, IL-8 and TNF-α by 9-plex using the

electrochemiluminescence detection system (Meso Scale Discovery®, Rockville, MD). The detection limit for all cytokines was 2.4–10,000 pg/ml.

Statistical Analysis

Tests of significance are reported as *P* values, derived from Student's *t* test using a two-tailed distribution and calculated at 95% confidence using GraphPad Prism 4.0 for Macintosh (GraphPad Software Inc., La Jolla, CA).

Due to the specificity and sensitivity of the electrochemiluminescence assay utilized for the quantitation of cytokines, changes greater than or equal to twofold vs. control (0 Gy) were considered relevant.

RESULTS

Multifractionated Radiation Treatment Activates Immune Response Genes More Robustly than Single-Dose Radiation Treatment with a Relatively High Number of Genes in PC3 Followed by LNCaP Cells

Current radiation therapy clinical protocols encompass a wide range of radiation doses, delivered as single dose or in multiple fractions of variable size (*17*). There is limited understanding about the mechanism of induction of immunogenicity in tumors irradiated with low-dose or high-dose radiotherapy. This includes the impact of radiation dose as well as fractionation schedules on stimulating immunity, on immune effector function and survival. This current study builds on previous work from our laboratory (*12, 16, 18*) and is designed to more fully understand the immune gene induction cascade in tumor cells, in response to 10 Gy single dose or 10 Gy given in 1 Gy fractions (multifractions) in three prostate cancer cell lines, PC3, DU145 and LNCaP.

PC3 Cells

Multifractionated radiation treatment showed greater than twofold changes in 31 immune-related genes in PC3 cells 24 h after the cells were irradiated (Table 1). Out of these 31 immune-related genes, seven genes were upregulated more than 10–34-fold. These include *IFI27* (34-fold), *MX1* (23-fold), *BST2* (22-fold), *IFITM1* (18-fold), *IFIT1* (12-fold), *OASL* (12-fold) and *CXCL11* (11-fold) (Table 1). These seven highly expressed genes in response to multifractionated treatment are mainly involved in sensing viral/pathogen infection to trigger an immune response (*19–23*). No immune-related genes were downregulated in PC3 cells treated with multifractionated radiation treatment. In response to 10 Gy single radiation exposure, five immune genes were also upregulated to two- to threefold (Table 1). Out of these 5, two genes, *IFITMI* and *OASL* (*19, 20*), were upregulated in multifractionated treatment. The remaining three genes, *S100A9, FABP4* and *HLA-DMA*, are involved in inflammatory secretions by T cells or macrophages and peptide exchanges (*24–26*) and were unique for single-dose treatment. One gene, PDL-1 (CD274), which was downregulated by twofold, is an inhibitor of T-cell receptor signaling (*27*). To better evaluate the immune response gene expression changes specific for each radiation protocol, the specific genes were mapped to the functional networks in the IPA database and ranked by score. Only the networks with a score of 10 or more are shown (Table 4). Out of the three cell lines, in PC3 cells fractionated radiation treatment had a greater number of networks in comparison to single-dose radiation treatment. These findings demonstrate that for PC3 cells, 1 Gy multifractionated radiation treatment is able to trigger a high incidence of activation of immune genes, potentially eliciting a more robust immune response.

TABLE 1
List of Immune Genes Modulated Greater than Twofold in PC3 Cells 24 h after Single-Dose or Multifractionated Treatment

10 Gy (single dose)		1 Gy × 10 (multifractionated)
Up	Down	Up
OASL*,§	PDL1	IFI27
IFITM1*,¶		MX1
S100A9		BST2
FABP4		IFITM1*
HLA-DMA		IFIT1
		OASL*
		CXCL11
		IFIT2
		HSH2D
		IFIT3
		DDX58
		IL1A
		IFI6
		ISGF3G
		PTX3
		ISG15
		CCL5
		CCL20
		CXCL5
		IRF7
		IL23A
		IL32
		LY96
		IFI35
		CTSS
		RELB
		APOL3
		MX2
		TAPBPL
		ZFP36
		PSMB9

* Denotes genes that are commonly upregulated in both single-dose and multifractionated treatment.

§ Upregulated in both PC3 and LNCaP treated with multifractionated radiation and DU145 treated with single dose radiation.

¶ Upregulated in PC3 treated with multifractionated radiation but downregulated in LNCaP treated with single-dose radiation.

DU145 Cells

Multifractionated radiation treatment of DU145 cells resulted in greater than twofold upregulation in four genes

TABLE 2
List of Immune Genes with Greater than Twofold Upregulation in DU145 Cells Treated with Single-Dose or Multifractionated Radiation

10 Gy (single dose)	1 Gy × 10 (multifractionated)
CFB	IL29
OASL*,§	HSH2D
APOBEC3G	IFI16
AZU1	PDL1
IFIT3	
IFIT1	
HDAC5	

* Denotes genes that are commonly upregulated in both single-dose and multifractionated treatment.

§ Upregulated in both PC3 and LNCaP treated with multifractionated radiation and DU145 treated with single-dose radiation. None were downregulated.

TABLE 3
List of Immune Genes with Greater than Twofold Changes in LNCaP Cells Treated with Single-Dose or Multifractionated Radiation

10 Gy (single dose)		1 Gy × 10 (multifractionated)	
Up	Down	Up	Down
TRIM22	PRG3	CFB	FCGRT
C4B	CKLF	IFI27	
TNFRSF14	IFITM1¶	OASL*,§	
	TCF7	MAF	
	EXO1	LY96	
		NMI	
		PLA2G4C	
		GBP3	
		IFIT3	
		LGALS3BP	
		IFIT1	
		EFNB1	

* Denotes genes that are commonly upregulated in both single-dose and multifractionated treatment.

§ Upregulated in both PC3 and LNCaP treated with multifractionated radiation and DU145 treated with single-dose radiation.

¶ Upregulated in PC3 treated with multifractionated radiation but downregulated in LNCaP treated with single-dose radiation.

(Table 2) that include *IL29, HSH2D* and *IFI16* (*28, 29*), which are important for antitumor T-cell activation. Whereas PDL1, an inhibitor of T-cell receptor signaling (*27*), was upregulated, which indicated that both activation as well as inactivation of immune response exist in multifractionated radiation treatment. Interestingly, *PDL1* was downregulated to single-dose radiation treatment in PC3 cells. Single-dose radiation treatment of DU145 cells caused a greater than twofold increase in seven genes, three of which were unique to single-dose treatment compared to multifractionated treatment. These include OASL, AZU1 and APOBEC3G (Table 2). OASL and AZU1 are directly involved in immune activation (*20*), whereas *APOBEC3G* promotes DNA repair (*30*). *IFIT1* and *IFIT3* are robust activators of immune response (*31*), whereas HDAC5 regulates the expression of oncoproteins of T-cell leukemia virus (*32*). Together, in DU145 cell type, IPA findings (Table 4) indicated that 10 Gy single-dose treatment had a response similar to an immunological regulation of hematological function and hematopoiesis; whereas multifractionated treatment response was similar to an antimicrobial and inflammatory response.

LNCaP Cells

Interestingly, functional p53 harboring LNCaP cells responded differently to single-dose treatment when compared to PC3 and DU145 cells, by downregulation of a greater number of immune-related genes. In response to multifractionated treatment, LNCaP cells increased expression of 12 genes (Table 3). Of notable, increased expression was observed in *IFIT1, IFIT3, OASL, LY96, PLA2G4C, GBP3, CFB, EFNB1* and *NM1* (*19, 20, 33–37*). One gene, *FCGRT*, which was downregulated by twofold, is involved in regulation of pharmacokinetics of therapeutic antibodies (*38*) and this may have implications particularly when combining a multifractionated treatment schedule with radio-immunotherapy. In response to the 10 Gy single-dose treatment, five genes were significantly downregulated and three genes were upregulated. It is clearly evident from IPA findings (Table 4) that 1 Gy multifractionated treatment has higher potency for eliciting a pathogenic immune response similar to immune regulation of hematopoiesis coupled with infectious states, and that infectious disease immune response was absent in single-dose treated LNCaP cells.

Inflection Point of Multifractionated Radiation Treatment-Induced Immune Genes in PC3 Cells were Observed in the Range of 8–10 Gy Total Dose of Radiation

Figure 1 shows the kinetics of induction of genes in response to 1 Gy fractionated treatment in PC3 and DU145 cells. Highly expressed genes from the microarray data were selected for inflection point analysis, and included *IFI27, MX1, CXCL11, BST2, HSH2D* and *IL1α*. In PC3 cells, *IFI27, MX1, BST2* and *HSH2D* showed an increasing trend in gene expression at a total dose of 8 Gy and were significantly upregulated at a total of 10 Gy (Fig. 1), whereas *CXCL11* and *IL1α* started to increase expression from 6 Gy total dose treatment. In DU145 cells, *IFI27, MX1, BST2* and *HSH2D* failed to show any significant increase at any total radiation dose point (Fig. 1). Although *CXCL11* and *IL1α* levels were increased at 10 Gy total dose, the increase was not statistically significant. Overall, these findings suggest that multifractionated treatment is effective in inducing critical immune function genes, particularly after adaptation at around 6–10 Gy total dose (>10 Gy was not analyzed in this study). Hence, this inflection point window could potentially be utilized for adjuvant immunotherapy agents.

TABLE 4
Networks and Associated Functional Categories Identified by IPA for PC3, DU 145 and LNCaP Cells 24 h after Exposure to 10 Gy of Radiation Administered as a Single-Dose or Multifractionated Regimen (1 Gy × 10)

Cell type	Treatment (24 h)	Score	Functions (number of genes in the category implicated)
PC3	Single dose	18	Connective tissue disorders, inflammatory disease, skeletal and muscular disorders (6)
	Multifractionated	57	Infectious disease, dermatological diseases and conditions, antimicrobial response (21)
		17	Hematological system development and function, tissue morphology, inflammatory response (8)
		10	Cell-to-cell signaling and interaction, metabolic disease, infectious disease (5)
DU145	Single dose	21	Cellular development, hematological system development and function, hematopoiesis (7)
	Multifractionated	12	Infectious disease, antimicrobial response, inflammatory response (4)
LNCaP	Single dose	23	Cellular development, cellular growth and proliferation, hematological system development and function (8)
	Multifractionated	27	Cellular development, hematological system development and function, hematopoiesis (10)
		15	Infectious disease, carbohydrate metabolism, lipid metabolism (6)

Notes. IPA score refers to statistical significance, with all immune response genes with at least a twofold change and $P < 0.05$, which are mapped to the functional networks available in the Ingenuity Pathway Knowledge Base. Focus molecules indicate the number of genes that could be mapped to molecules out of a possible 35 molecules in each network.

Radiation Treatment Induces Proinflammatory DAMPs and Positively Modulates the Cytokine Environment

Tumor exposure to diverse sublethal doses of radiation has been demonstrated to induce immunogenic modulation in a wide variety of carcinoma types by altering the biology of surviving tumor cells to render them more susceptible to T-cell-mediated killing (*39, 40*). Having observed induction of genes that function to regulate immune activation, in this study it is pivotal to ascertain which immunogenic cell death events occur in response to single-dose or multifractionated treatment. The cardinal signs of immunogenic cell death include DAMPs, also know as danger-associated molecular pattern molecules, which can initiate and perpetuate immune responses. DAMPs include: 1. release of ATP, and 2. secretion of high-mobility group box 1 protein (HMGB1) (*41*). To examine the biologic significance of multifractionated vs. single-dose exposure observed as per gene expression analysis (Tables 1–3), we first sought to examine *in vitro* the effect of radiation as fractionated dose or single dose on the induction of cardinal DAMPs in human prostate carcinoma cell lines, DU145, PC3 and LNCaP. Cells were mock irradiated (0 Gy) or 10 Gy irradiated as either fractionated (1 Gy × 10, multifractionated) or single dose (10 Gy). Exposure of cells to 10 Gy single-dose treatment was sufficient to promote significant ATP release in all three prostate cell lines, while exposure to multifractionated treatment elicited significant ATP release in DU145 and LNCaP (Fig. 2A). Interestingly, exposure of DU145 and PC3 to multifractionated radiation treatment induced a greater level of HMGB1 than an equivalent dose delivered as a single fraction (Fig. 2A). These trends were seen at both 48 and 72 h after radiation exposure. Additionally the endogenous cytokine levels of 9 cytokines were analyzed after radiation exposure. No changes were seen in the IL-8 expression from DU145 cells after radiation exposure. PC3 cells exposed to the 10 Gy multifractionated treatment significantly decreased the endogenous expression of IL-8 while exposure of these cells to 10 Gy single-dose radiation treatment failed to lower the expression of this cytokine at 48 h (Fig. 2B). At 72 h post exposure, both single-dose and multifractionated treatments reduced expression of IL-8 in PC3 cells. Multifractionated and single-dose treatment induced increased levels of GM-CSF in both DU145 and PC3 cells, however, the multifractionated treatment induced greater levels of GM-CSF than single-dose treatment (Fig. 2B). Similarly, IL-6 was induced in DU145 and PC3 lines after exposure to radiation, with multifractionated treatment in PC3 cells inducing much greater levels of IL-6 than an equivalent single dose (Fig. 2B). Similar results were seen at 48 and 72 h after irradiation. There were no differences seen postirradiation for these three prostate cell lines for the cytokines for *IFNγ, IL-12p70, IL1β, IL-2* and *TNF-α*. Taken together, these results indicate that exposure to radiation causes proinflammatory DAMPs and positively modulates the cytokine environment, particularly downregulation of IL-8, which can lead to curtailing of the pro-immunogenic tumor growth events.

DISCUSSION

We hypothesized that radiation exposure (either multifractionated or single dose) would modulate the tumor secretome in a manner that would potentially promote productive immune cell–tumor cell interactions. DAMPs are molecules that can initiate and perpetuate immune responses.

Gene expression studies clearly demonstrated that multifractionated treatment modulates genes directly involved in the activation of the immune system, similar to a response to a pathogenic infection irrespective of cell lines with different genotype. The following pivotal genes support the proposal that 1 Gy fractions are highly immunogenic in its effect: BST-2 functions not only as an effector of the interferon-induced antiviral response but also as a negative

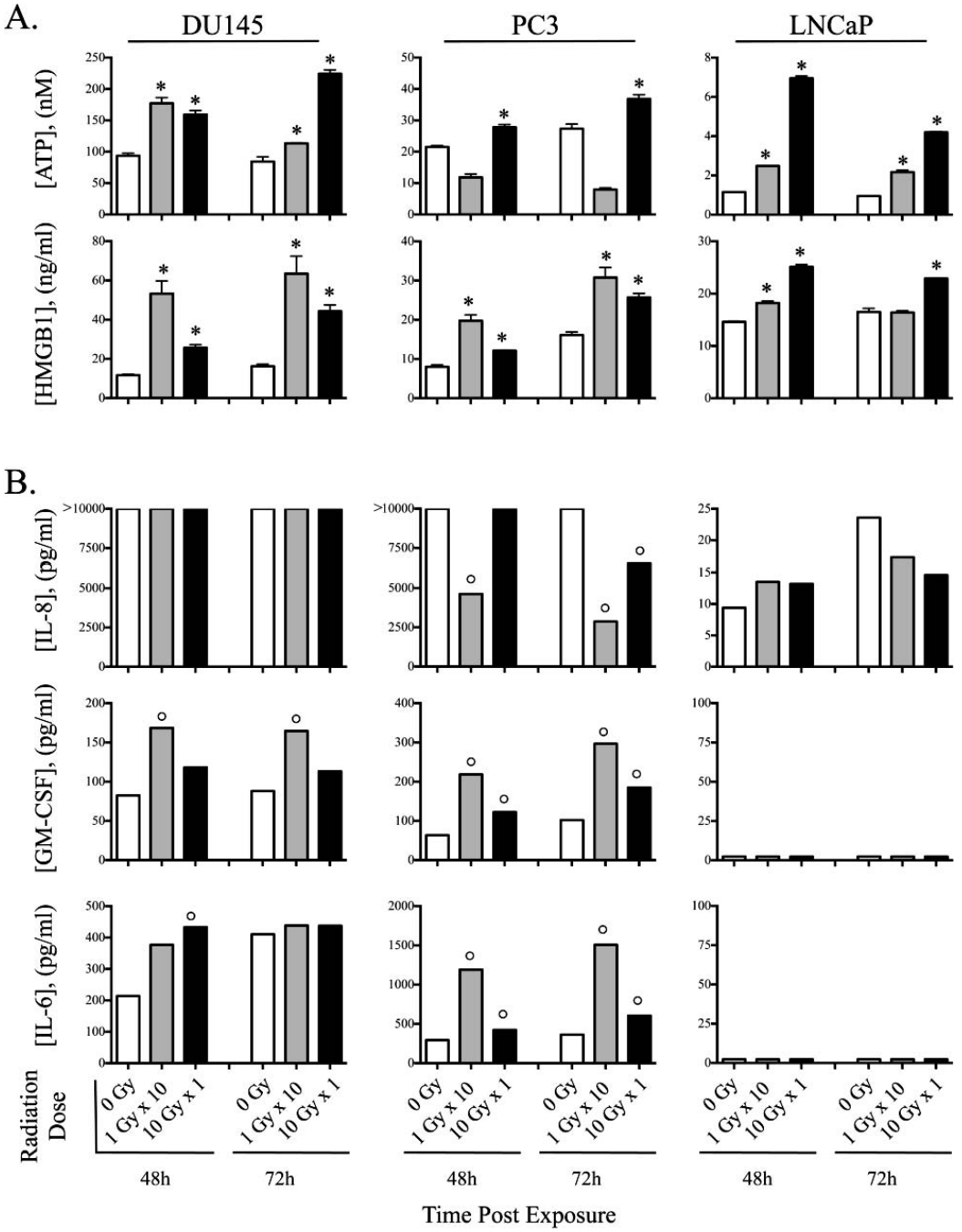

FIG. 2. Radiation induces proinflammatory DAMPs and positively modulates the cytokine environment. Prostate carcinoma cells (DU145, PC3, LNCaP) were exposed to 10 Gy fractionated treatment (1 Gy × 10, multifractionated, gray bars) or 10 Gy single-dose treatment (10 Gy, single dose, black bars) or were mock irradiated (0 Gy, open bars). Culture supernatant was analyzed in triplicates after 48 and 72 h. Panel A: secreted DAMPs ATP and HMGB1, and panel B: modulation of tumor derived cytokines IL-8, GM-CSF and IL-6. *Denotes statistical significance vs. control (0 Gy). °Denotes marked modulation (≥twofold) relative controls (0 Gy).

feedback regulator of interferon production by plasmacytoid dendritic cells (*23*); DDX58 functions as a pattern recognition receptor (*42*); ISG15 is a type I interferon (*43*); IFI6 is IFN-inducible protein that recognizes dsDNA (*44*); IL23A specifically acts on memory $CD4^+$ T cells (*45*) to enhance T-cell priming that stimulates the production of proinflammatory cytokines; HSH2D is an important target involved in the T-cell activation (*28*). IL29 is implicated in antiviral activity, anti-proliferative activity and *in vivo* antitumor activity (*29*); IFIT1, IFIT3 and OASL (*19, 20*) are genes that respond to viral infection to activate several components of immune system; LY96 (also called as MD2)

is required for activating TLR4 signaling as a part of innate immune activation against bacterial infection (*33*); PLA2G4C is an enzyme induced in response to cytokine induction cascade as well as inflammation (*34*); guanylate-binding proteins (GBPs) belong to the family of large GTPases that are induced in response to interferons, specifically, hGBP-3 possess anti-influenza viral activity (*35*); CFB acts as a downstream effector of TLR signaling and plays a critical role in the pathogenesis of severe bacterial sepsis (*36*); Efnb1 proteins have been reported to regulate thymocyte development, peripheral T-cell differentiation and antiviral immune responses and are essential for interleukin-6 (IL-6) signaling (*37*) and NMI augments STAT-mediated transcription in response to IL6 and IFNγ cytokines (*46*).

The functions of the above genes, which were modulated predominantly in response to multifractionated treatment in this study, strongly indicate that 1 Gy dose fractions have the potency to induce a variety of immune regulatory genes in these tumor cells that will facilitate the recognition by the host immune machinery. It has been well documented that during total-body irradiation, doses in the range of 0.01–0.25 Gy fractions lead to immune stimulation that can produce high remission rates (*47*) in hematological malignancies, and therefore, the host immune system appears to play an important role in positive clinical outcomes.

The findings of the current study demonstrate the activation of immune response genes in tumor cells exposed to 1 Gy fractions, four times higher than low-dose total-body irradiation used for the treatment of chronic lymphocytic leukemia and non-Hodgkin lymphoma (0.1–0.25 Gy), suggests that solid tumors (unlike lymphoid malignancies) may need a higher threshold total dose to activate its own immunogenic environment. In such a scenario, multifractionated treatment can be effectively combined with immunotherapy to harness the immunogenic induction programs intratumorally, and may also produce an abscopal effect. The sequence of combination therapy with immune modulating agents can potentially be utilized at a cumulative tumor dose of 6–8 Gy in multifractionated treatment settings based on the observation of an inflection point in PC3 and DU145 cells.

The gene expression data revealed that 10 Gy single-dose treatment was generally less pro-immunogenic than 1 Gy multifractionated treatment as very few genes were classified by IPA as being related to lymphocyte development or infectious disease, and also because overall multifractionated treatment modulated more immune genes than single-dose treatment. To interpret the functional impact of the changes observed it is important to keep in mind that: IFIT1 and IFIT3 are both implicated to function against viral infections (*19*); OASL functions in antiviral response (*20*); AZU1 serves as an important mediator during the initiation of the immune response (*30*); and APOBEC3G promotes cytidine deaminase-dependent DNA repair to render radiation resistance in lymphoma (*48*). In addition, IFN-inducible IFITM proteins (IFITM1, 2 and 3) inhibit the replication of various viruses including HIV-1 (*21*). Therefore, functions of the single-dose treatment gene signature support the fact that few genes are implicated in eliciting an immune response that could possibly be exploited with immunotherapy or vaccination to harness the tumor cell mediated immunogenic events.

The most notable inference from the microarray findings is the downregulation of PDL-1 in response to single-dose radiation treatment in PC3 cells (Table 1). Recently, we reported the downregulation of PDL-1 protein in both PC3 and DU145 cells treated with a 10 Gy single-dose treatment (*49*). At the RNA level in PC3 cells, this was similar to what we reported at the protein level in PC3 cells, however, we did not observe downregulation of PDL-1 in DU145. On the contrary, we found that DU145 cells showed upregulation of PDL-1 in response to multifractionated treatment (Table 2). These observations suggest that combining anti-PDL1 immunotherapy with single-dose treatment will be more effective than combining with multifractionated treatment.

The above gene expression findings were further tested to compare how certain cardinal immunogenic responses differed between multifractionated vs. single dose by functional assays. DAMPs have been shown to stimulate dendritic cells to facilitate the presentation of tumor antigens to the immune system (*50*). ATP binds purinergic PRX7 receptors on DCs, further supporting T-cell activation (*7, 51*). HMGB1, a nonhistone chromatin binding protein, promotes DC maturation through TLR-4 signaling (*7*). We found that irradiation of tumor cells induced these DAMPs (Fig. 2A). Also playing a role in DC function, GM-CSF and IL-6 have a well-defined role in the recruitment and maturation of DC (*52, 53*). We found that although both multifractionated and single-dose treatment induced elevated levels of GM-CSF, multifractionated treatment induced the greatest level of GM-CSF in PC3 but LNCaP had lower levels. Similar results were seen with radiation-induced secretion of IL-6 (Fig. 2B). Finally, a strong correlation has been observed between the metastatic phenotype of a cell and its IL-8 expression, suggesting a role for IL-8 in promoting the metastatic potential of tumor cells (*54*). We found that in PC3 cells, multifractionated treatment was more effective in reducing the levels of IL-8 (Fig. 2B). In summary, the analysis of DAMPs and cytokines together with the gene expression data suggest that cells subjected to multifractionated radiation treatment could indeed promote productive immune cell–tumor cell interactions.

Several preclinical studies have demonstrated that ionizing radiation induces pro-immunogenic and inflammatory changes *in vivo* and that the response varies with the size of the dose (*55–57*). In B16/OVA murine melanoma model, a 15 Gy single-dose treatment resulted in greater numbers of host immune cells infiltrating tumors than a 5 × 3 Gy fractionated-dose treatment (*55*). In the same B16/OVA model radiation doses of 7.5 Gy/fraction gave the best

tumor immune activation compared to a 15 Gy single-dose treatment (*56*). Similarly, in terms of fractionation our previous *in vitro* multifractionated radiation treatment studies have shown that even 1 Gy fractions differentially expressed immune response genes in prostate carcinoma cells (*12*). In an earlier study, DU145 xenografts revealed very different gene expression patterns compared to the DU145 cells irradiated *in vitro* (*13*). Radiation-induced gene expression changes in tumor cells can vary depending on whether the cells are grown in *in vitro* or *in vivo* settings suggesting the importance of tumor microenvironment and tumor-host interaction in radiation-induced gene expression changes.

Much work remains to be done and studies currently in progress include additional radiation fractionation schemes with a broader range of cell types including cells with normal and dysfunctional p53, as well as *in vivo* studies. In addition, investigations are underway of the induction of susceptibility to molecular targeted therapy after fractionated radiation treatment using the wide range of available molecular therapeutics. Exploiting the phenotype of cells surviving fractionated treatment offers great potential for combined modality therapy to both enhance tumor cell killing by immunological approaches and to reduce normal tissue toxicity. This approach could not only improve outcomes but provide additional benefit from the investment in molecular imaging, radiation therapy technology and drug development.

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