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Citation for final published version:

Al-Taei, Saly, Salimu, Josephine, Lester, Jason F., Linnane, Seamus, Goonewardena, Madusha, Harrop, Richard, Mason, Malcolm David and Tabi, Zsuzsanna 2012. Overexpression and potential targeting of the oncofoetal antigen 5T4 in malignant pleural mesothelioma. *Lung Cancer* 77 (2) , pp. 312-318. 10.1016/j.lungcan.2012.03.008 Item availability restricted.

Publishers page: <http://dx.doi.org/10.1016/j.lungcan.2012.03.008>

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## **Cross-presentation of the oncofoetal tumor antigen 5T4 from irradiated prostate cancer cells – a key role for Hsp70**

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Running Title: Antigen cross-presentation from irradiated tumor cells

Financial support: JSal is a recipient of a PhD scholarship from Cardiff University and Cancer Research Wales; AC and ZT are supported by a Cancer Research Wales programme grant.

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Key words (5): prostate cancer, 5T4, antigen cross-presentation, ionizing radiation, hsp70

Conflict of interest: The authors have no conflicting interest to declare

Word count: 5,010  
Abstract: 248 words  
Figures: 5  
References: 49

**Abstract**

Immune responses contribute to the success of radiation therapy of solid tumors; however, the mechanism of triggering CD8<sup>+</sup> T cell responses is poorly understood. Antigen cross-presentation from tumor cells by dendritic cells (DC) is a likely dominant mechanism to achieve CD8<sup>+</sup> T cell stimulation. We established a cross-presentation model in prostate cancer in which DC present a naturally expressed oncofetal tumor antigen (5T4) from irradiated DU145 tumor cells to 5T4-specific T cells. Ionising radiation (12 Gy) caused G2/M cell cycle arrest and cell death, increased cellular 5T4 and high-mobility protein group-B1 (HMGB1) levels and upregulated surface calreticulin and Hsp70 expression in DU145 cells. Co-culture of DC with irradiated tumor cells lead to efficient phagocytosis of tumor cells and upregulation of CD86 and HLA-DR on DC. CD8<sup>+</sup> 5T4-specific T cells, stimulated with these DC, proliferated and produced IFN $\gamma$ . Inhibition of HMGB1 decreased T cell stimulation but not DC activation, while TRIF/MyD88 inhibition only had a marginal effect on T cell stimulation. Unlike previous reports, we found no functional evidence that DC with Asp299Gly toll-like receptor-4 (TLR4) single nucleotide polymorphism had impaired ability to cross-present tumor antigen. However, we observed a highly significant and robust prevention of antigen cross-presentation when tumor cells were pre-treated with the novel Hsp70 inhibitor, VER155008. The inhibitor also prevented CD86 upregulation on DC co-cultured with irradiated tumor cells. Together, our study demonstrates that radiation induces immunologically relevant changes in tumor cells, which can trigger CD8<sup>+</sup> T cell responses via a predominantly Hsp70-dependent antigen cross-presentation process.

## Introduction

Traditional treatments of cancer such as surgery, chemotherapy and radiotherapy (RT) have been shown to trigger immune responses, which may contribute towards treatment outcome. Radiation is curative in up to 40% of patients with early stage (localized) prostate cancer (PCa) but it is yet unclear what the predictors of complete responses are. RT in PCa has been shown to be associated with increased frequencies of tumor antigen-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cells (1). The abscopal effect of radiation (tumor regression at a distant site following localized radiation) has been shown to be immune-mediated as demonstrated not only in mouse tumor models (2, 3) but also in patients with metastatic melanoma and lung adenocarcinoma (4, 5). Furthermore, CD8<sup>+</sup> T cell infiltration in the irradiated tumor tissue serves as a prognostic factor (4-8) indicating that radiation can switch the immunosuppressive tumor milieu to a pro-immune environment.

For solid tumors, tumor antigen-specific CD8<sup>+</sup> T cell responses can be induced either by tumor cells entering lymph nodes and presenting antigens directly (9) or by dendritic cells (DC) cross-presenting tumor antigens, either in the lymph node or in ectopic lymphoid tissues, as observed in melanoma (10, 11). Efficient cross-presentation requires tumor cell damage or cell death, associated with upregulation and/or release of danger signals or damage associated molecular patterns (DAMP). The precise nature of immunogenic cell death (ICD) is not well defined but generally involves surface translocation of “eat me” signals such as calreticulin (CRT) and stress-associated proteins such as Hsp70, release of chemoattractant molecules such as ATP and DAMP such as high-mobility protein group-B1 (HMGB1). However, there seems to be considerable plasticity in the combination and extent of these changes. The type of trigger causing cell damage and cell death may influence the relative proportions of key ICD events (12). Our study focuses on ionising radiation, which is known to cause DNA damage, cell cycle arrest and cellular damage response triggering either DNA repair or cellular senescence and apoptotic, necrotic or necroptotic cell death. Irradiated cells initiate post-phagocytic immune responses (13). The early release of IFN $\alpha/\beta$  by irradiated tumor cells can polarize antigen-presenting cells and aids their cross-presentation function (14). High dose (10-100 Gy) in vitro radiation of human tumor cells enhances CRT translocation to the cell surface and dose-dependent release of HMGB1 and ATP by breast, colon and prostate cancer cell lines (15). These typical ICD markers facilitate phagocytosis of damaged/dead cells and provide maturation signals for DC. Hsp70 translocation and release are also well-documented consequences of radiation contributing to radiation resistance (16) with potential immunological effects (17).

The aim of our study was to determine the mechanism of radiation-mediated antigen cross-presentation in PCa. As antigen cross-presentation studies often use artificially overexpressed antigens which may provide false positive results, we established a model focussing on a naturally expressed oncofetal antigen, 5T4 (18), which is expressed in most solid tumors, including PCa. The cross-presentation model we established enabled us to study the effect of irradiated tumor cells on DC phenotypic and functional maturation and the use of specific inhibitors to reveal the mechanism of the cross-presentation process. We show here that unlike in cross-presentation induced by anthracyclines (19), in radiation-induced tumor antigen cross-presentation the toll-like receptor-4 (TLR4) agonist-triggered TRIF/MyD88 pathway (20) plays only a partial role and the Asp299Gly TLR4 single nucleotide polymorphism (SNP) is not associated with any impairment of CD8<sup>+</sup> T cell responses. Instead, we found that Hsp70 is crucially important both in activating DC and

triggering CD8<sup>+</sup> T cell responses via DC co-cultured with irradiated tumor cells. Our results highlight the plasticity of the tumor antigen cross-presentation process and demonstrate the important immunological role of Hsp70 following tumor radiation.

## Materials and Methods

### Media and Reagents

RPMI 1640 (Lonza, UK) was supplemented with low endotoxin fetal bovine serum (FBS, PAA Laboratories, Austria) 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (all from Gibco, UK), 25 mM Hepes buffer and 1 mM sodium pyruvate (both Sigma, UK). 1% AB-serum (Sigma) was added where indicated. LPS and Glycyrrhizin were obtained from Sigma, VER155008 from Tocris Bioscience (R&D Systems, UK), MyD88 inhibitory peptide (and control) from ProImmune (Oxford, UK) and TRIF inhibitory peptide (and control) from Invivogen (San Diego, CA).

### Tumor cells and irradiation

DU145 human prostate cancer cells were obtained from ECACC and maintained in adherent cultures with regular passaging following trypsinization for less than 6 months. The cell line was authenticated by the supplier using cytogenetic, isoenzymatic and DNA profile analyzes. HLA typing results show that DU145 cells are HLA-A2<sup>-</sup> (and encode for HLA-A03, A33, B50, B57) (Welsh Blood Transfusion Service, Cardiff). The cells were mycoplasma-free, as tested monthly using a MycoAlert Mycoplasma Detection Kit (Lonza). Cells used for experiments were in exponential phase of growth. Irradiation was carried out using a <sup>137</sup>Cs-source (with dosimetry quality assurance) at a rate of 0.627 Gy/min.

### Donors, dendritic cell preparation

Ethical approval was granted for the study and informed consent was obtained from healthy volunteer donors. HLA Class I and II DNA typing was carried out as above. PBMC from venous blood collected in EDTA vacutainers was prepared by density gradient centrifugation. CD14<sup>+</sup> monocytes were isolated by negative selection using the EasySep Human Monocyte Enrichment Kit without CD16 Depletion (Stem Cell Technologies, Grenoble, France) according to the manufacturer's protocol. The average purity of CD14<sup>+</sup> cells was 70-80%. The isolated cells were incubated at 5x10<sup>6</sup> cells/well in a 6 well tray and grown in 5ml/well in 10% FBS-RPMI in the presence of 500 ng/ml human recombinant (hr) GM-CSF (ProSpec, Israel) and 500 U/ml hr IL-4 (Gentaur, Belgium) for 5-6 days.

### T cell and B cell line

A CD8<sup>+</sup> T-cell line was developed from a HLA-A2<sup>+</sup> healthy donor by repeated stimulation of non-adherent PBMC with autologous DC, loaded with 2 µg/ml 5T4<sub>17-25</sub> peptide (RLARLALVL; 90.4% purity; ProImmune), as described previously (21). For the experiments, T-cells (1–2×10<sup>6</sup>) were expanded using a mixture of 5×10<sup>6</sup> peptide-pulsed autologous B lymphoblastoid cells (BLCL) irradiated with 40 Gy; 5×10<sup>7</sup> allogeneic PBMC mixed from 2 to 3 donors and irradiated with 30 Gy; 50 U/ml IL-2 and 1 µl/ml OKT3 hybridoma supernatant (MRC Cooperative, Cardiff University) in 50 ml RPMI supplemented as described above and with 10% FBS and 1% AB-serum in a T75 tissue culture flask (21). BLCL was developed from the same donor using EBV transformation of B cells from PBMC, following standard protocols.

### Immunocytochemistry

DU145 cells were grown on coverslips and incubated for 72h after 0Gy or 12Gy radiation. They were fixed with acetone/methanol 1:1 (vol/vol) and labeled with

FITC-conjugated Hsp70 antibody (Enzo Life Sciences, Farmingdale, NY). The slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and visualized next day using an Axiovert 40 fluorescence microscope (Zeiss, Jena, Germany).

### **Flow cytometry**

Cells ( $1-2 \times 10^5$ /sample) in 100  $\mu$ l flow cytometry buffer (PBS, 1 mM EDTA and 2% FBS) were labeled with fluorochrome-conjugated 5T4, CRT, HMGB1 (R&D Systems), Hsp70 (Enzo Life Sciences), TLR-4, HLA-DR, CD86 and CD83 (E-Bioscience) antibodies and incubated on ice for 40 min. For intracellular labelling the cells were fixed and permeabilized with E-Biosciences Fix/Perm reagents before antibodies were added for 40 min incubation at room temperature. For cytokine flow cytometry, T cells were fixed and permeabilized as above, and CD3, CD8 and IFN $\gamma$  antibodies were added together to the cells for 40 min. Flow cytometry was carried out using a FACSCanto flow cytometer with FACSDiva software (Becton Dickinson Biosciences, Franklin Lakes, NJ).

### **Inhibition of TRIF, MyD88, HMGB1 and Hsp70**

The MyD88 and TRIF inhibitory peptides correspond to the sequence of the BB-loop of MyD88 (RDVLPQT) and TRIF (FCEEVQVPGRGELH), respectively, and serve as decoys by binding to the TIR domains and interfering with TLR-adaptor interactions (22, 23). The peptides are linked to protein transduction sequences, which render the peptides cell permeable. The control peptides consist of the protein transduction sequence alone. DC were pre-treated with 20  $\mu$ M MyD88 inhibitory or control peptide or 10  $\mu$ M TRIF inhibitory or control peptide, respectively, for 6h before adding DC to the irradiated or non-irradiated DU145 cells as described in the method for the cross-presentation assay.

Glycyrrhizin acid, an inhibitor for HMGB1, was added at 50  $\mu$ M at the time of irradiation, while VER155008, an inhibitor for Hsp70, at 5  $\mu$ M to 0Gy and 12Gy irradiated DU145 cells at 0h, 24h and 48h of the 72h incubation, respectively, as described in the method for the cross-presentation assay.

### **Antigen cross-presentation**

DU145 cells were set up into two 96 well U-bottom plates at  $5 \times 10^3$  cells/well. After an overnight incubation, one plate was irradiated with 12Gy prior to incubation for 72h at 37°C in 5% CO $_2$ . DC were then added to the wells with tumor cells at a 1:1 ratio. The co-cultured cells were re-suspended and incubated for 48h after which 5T4 specific T cells were added at a 1:1:5 (tumor cell:DC:T cell) ratio. Golgi Plug (0.5 $\mu$ l/500ml) and Golgi Stop (0.35 $\mu$ l/500ml) (both from Sigma) were added to the wells 1h later and the cultures were incubated overnight. Cytokine flow cytometry was carried out to determine the percentage of CD8 $^+$  T cells producing IFN $\gamma$  in response to the antigen presented by DC.

### **T cell proliferation in response to cross-presented antigen**

DU145 cells were plated out in triplicates in two 24 well plates at  $1 \times 10^5$  cells/well in 1.5 ml. One plate was irradiated with 12Gy prior to incubation for 72h. DC at  $1 \times 10^5$  cells/well in 0.5 ml were added per well to the tumor cells. After 4h, CFSE labelled T cells ( $5 \times 10^5$  cells/well) were added to the DC:tumor cell co-culture and the plates were incubated at an angle for 48h after which they were laid flat. After 5 days of tumor cell:DC:T cell incubation, the cells were re-suspended and surface stained with CD3 and CD8-antibodies for flow cytometry analysis of CFSE dilution.

### **Single nucleotide polymorphism analysis**

SNP analysis was carried out by the Department of Medical Genetics (Cardiff and Vale NHS Trust, University Hospital of Wales, Cardiff). The method comprises of DNA amplification from blood or established BLCL by PCR followed by pyrosequencing. The SNP analysis was optimised for the Asp299Gly sequence of the TLR4 polymorphism. Out of 67 samples tested, 4 donors were found to carry the Asp299Gly SNP.

### **Statistical analysis**

Statistical analysis was carried out by applying Student's t-test, paired t-test and ANOVA with Tukey's post-hoc test (GraphPad InStat 3.06). Significant differences are marked as \* $p < 0.05$ , \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

## Results

### **Irradiation induces immunologically relevant changes in tumor cells**

Ionizing radiation causes DNA damage in individual cells and tissue damage in the tumor environment, the result of which maybe a pro-inflammatory immune reaction. To establish the optimum minimal radiation dose and incubation time which cause significant changes in DU145 PCa cells in vitro, dose escalation and time-kinetics experiments were carried out. Cell cycle changes were measured by flow cytometry. There was a highly significant arrest in the G<sub>2</sub>/M phase in the irradiated cells, detectable at 24h post-radiation and remaining stable at 48h. There was a small but significant radiation dose-dependent increase at 48h in the proportion of cells in sub-G<sub>0</sub> phase representing apoptotic cells with fragmented DNA (Fig. 1A). The type of cell death detected with Annexin/Propidium iodide (PI) labeling was mainly late apoptotic/necrotic and the proportion of cells with early apoptotic markers remained low throughout the incubation period (Fig. 1B). Other irradiation-associated, immunologically relevant changes were also observed. Surface MHC Class-I expression was not altered by radiation (not shown) but the cellular content of the target antigen 5T4 was significantly elevated following 12Gy radiation (Fig. 1C). The total cellular HMGB1 content was also measured by flow cytometry and a significant increase was observed, with early time kinetics (Fig. 1D). Hsp70 expression was strongly upregulated in the cytosol and on the cell surface by irradiation (Fig. 1E) as imaged at 72h post-irradiation. Multicolor flow cytometry analysis confirmed that upon irradiation the proportion of surface Hsp70 positive cells increased more than two-fold. CRT surface expression also increased more than 10-fold. Most CRT expressing cells were also positive for surface Hsp70 (Fig. 1F middle vs. right panel). These results demonstrate that radiation induces dramatic changes of numerous immunologically relevant parameters in tumor cells.

### **Dendritic cell activation and tumor antigen cross-presentation following uptake of irradiated prostate cancer cells**

Next we studied whether irradiated tumor cells are taken up by monocyte derived DC and whether this event can phenotypically activate DC and trigger their antigen presenting function. Tumor cells were irradiated with 12Gy and labelled with CFSE 72h later, when DC were added at 1:1 ratio. Phagocytosis was measured 24h later by determining the proportion of HLA-DR<sup>+</sup>CFSE<sup>+</sup> cells. Although some tumor cell uptake (<5%) occurred when tumor cells were not irradiated (Fig. 2A i), the proportion of phagocytic DC increased significantly when encountering irradiated DU145 tumor cells (Fig. 2A ii and iii). DC phenotype following phagocytosis of irradiated or non-irradiated tumor cells was also studied 24h after DC-tumor cell co-culture. CD86 and HLA-DR expression was significantly increased on DC that have encountered irradiated tumor cells compared to those that have been exposed to non-irradiated tumor cells. The increase in CD83 expression was not significantly different in this donor (Fig. 2B), however, in repeated experiments with DC from other donors the difference was significant, leaving CD83 upregulation inconclusive.

In order to maximise irradiation-induced changes in tumor cells and tumor cell-uptake associated changes in DC before testing tumor antigen cross-presentation by the activation of 5T4-specific T cells, cross-presentation experiments were optimised and the method is described in detail in the Materials and Methods. Antigen cross-presentation from HLA-A2 negative tumor cells by HLA-A2<sup>+</sup> DC was measured by the proliferation and intracellular cytokine production of a HLA-A2-

restricted 5T4-peptide-specific T cell line (21). The T cell line can recognize naturally processed 5T4, as these T cells can kill 5T4<sup>+</sup> HLA-A2<sup>+</sup> tumor cells directly (21). Significant T cell proliferation was triggered by DC which were exposed to non-irradiated DU145 cells, however T cell proliferation increased significantly compared to this, when DC co-cultured with irradiated DU145 cells were used to stimulate T cells (Fig. 2C). In contrast, when IFN $\gamma$  production by these T cells was measured, DC cultured either with tumor cells that received no irradiation or necrotic tumor cells generated by repeated freeze-thawing, induced no IFN $\gamma$  production (Fig. 2D). DC co-cultured with irradiated tumor cells were efficient in inducing IFN $\gamma$  production by these T cells. The experiments demonstrate that DC are able to present naturally-expressed tumor antigens to T cells and that this process is significantly enhanced following tumor cell irradiation.

### **The TLR4-MyD88 pathway contributes to antigen cross-presentation**

Tumor antigen cross-presentation from tumor cells treated with chemotherapy, especially with anthracyclines, has been studied more extensively than that from irradiated tumor cells. However, it has been suggested that in both cases antigen cross-presentation depends on the TLR4-HMGB interaction and consequently patients with TLR4 polymorphism are unable to mount immune responses to tumor antigens (19). In order to study the role of TLR4-HMGB signalling in antigen cross-presentation from irradiated tumor cells, we used an HMGB1 inhibitor (24) and measured the effect on antigen cross-presentation. The inhibitor was applied to tumor cells 1h before irradiation. A significant decrease in T cell IFN $\gamma$  production was observed (Fig. 3Ai), however HMGB1 inhibition did not affect CD86 expression on DC after their co-culture with tumor cells (Fig 3Aii). As HMGB1 can bind to multiple receptors, such as TLR2 and TLR4, we also inhibited the MyD88/TRIF signalling pathway by applying inhibitory peptides and compared T cell stimulation results to those obtained with the membrane permeable control peptides. The optimum concentrations of the inhibitory peptides were established in LPS-induced TNF $\alpha$  production experiments (not shown). Based on the results, both peptides were used at 25 mM in the cross-presentation blocking experiments. While neither TRIF nor the MyD88 inhibitory peptide had any effect on their own (not shown), when applied together, they resulted in a small but significant inhibitory effect on antigen cross-presentation (Fig 3B).

### **TLR4 polymorphism does not impact on antigen cross-presentation from irradiated tumor cells**

In order to elucidate whether TLR4 SNP would have a detrimental effect in PCa patients receiving radiotherapy, due to impaired antigen cross-presentation (19) as shown with anthracycline treated tumor cells, we conducted a series of experiments with DC generated from monocytes of donors with the normal (Asp299) TLR4 allele (n=5) or with the allele carrying the Asp299Gly SNP (n=4). All donors were HLA-A2<sup>+</sup>. The general characterisation of monocytes and DC revealed that TLR4 expression levels were identical in both groups (Fig. 4Ai, ii) and LPS-activated TNF $\alpha$  production was also comparable in both group of donors (Fig 4B). We also established that phenotypic maturation of DC (CD86, HLA-DR and CD83), carrying the SNP allele, following co-culture with irradiated tumor cells was unimpaired (Fig. 4B). However, the most relevant question was whether antigen cross-presentation by DC was affected in donors carrying the SNP allele. The results in Fig 4C show that cross-presentation of a naturally expressed tumor antigen from irradiated tumor cells

was similarly efficient by DC with the Asp299 or the Gly299 TLR4 allele, respectively.

Taken together, our results indicate that the TRIF/MyD88 signalling pathway and HMGB1 contribute to tumor antigen cross-presentation; however, we found no evidence that TLR4 polymorphism impairs antigen cross-presentation from irradiated tumor cells.

### **Hsp70 inhibition has no effect on Hsp70 upregulation but blocks antigen cross-presentation**

As shown in Fig. 1E and F, Hsp70 is significantly upregulated in irradiated tumor cells. As the contribution of heat shock proteins to antigen cross-presentation in other systems has been well demonstrated, we carried out experiments to establish whether Hsp70 plays an important role in cross-presentation of irradiated tumor cells. To test this we used the novel small molecule inhibitor, VER155008, which inhibits the activity of both the induced and the constitutive form of Hsp70 via binding to the ATPase pocket and allosterically preventing substrate binding (25, 26). VER155008 treatment of tumor cells has been shown to inhibit tumor cell growth and induce tumor cell death. We applied this inhibitor to tumor cells before irradiation and for the 72h culture preceding their co-culture with DC. Tumor cell numbers after 72h were only 18% lower when VER155008 was applied to irradiated tumor cells, presumably because tumor cell proliferation was already slowed down by the radiation. However, as expected, it inhibited the growth of unirradiated tumor cells by ~70% (Fig. 5A). Similarly, VER155008 significantly increased cell death of unirradiated DU145 cells (Fig. 5B). However, the inhibitor did not affect Hsp70 cell surface upregulation on irradiated tumor cells (Fig 5C). After establishing that VER155008 delivers the expected effects to otherwise un-manipulated tumor cells, we studied the effect on the ability of irradiated tumor cells to upregulate CD86 on DC (Fig. 5C) following a 24h co-culture of DC and DU145 cells. We found that CD86 upregulation failed on DC when they encountered VER155008-treated irradiated tumor cells (Fig. 5D). Finally, we applied VER155008 in the cross-presentation model described earlier. VER155008 significantly decreased both the background level cross-presentation of tumor antigen from non-irradiated tumor cells and the enhanced level of cross-presentation observed from irradiated tumor cells (Fig 5E). This experiment provides strong evidence that Hsp70 plays an important role in tumor antigen cross-presentation following irradiation.

## Discussion

Antigen cross-presentation has been indicated as an important mechanism for generating CD8<sup>+</sup> T cell responses against solid tumors which do not migrate into lymph nodes or viruses which do not infect professional antigen presenting cells. While chemotherapy-induced antigen cross-presentation has been studied extensively, there is a paucity of information about ionizing radiation-mediated antigen cross-presentation. The abscopal effect, observed in patients undergoing radiation therapy, has been demonstrated to be immune mediated and is likely to involve antigen cross-presentation from irradiated tumors (4, 5). Further studies in this field would aid better understanding of how radiation therapy could be made more successful.

Our study addresses the mechanistic details of antigen cross-presentation from irradiated human tumor cells. We established a model using a tumor-specific T cell line as a detector of cross-presentation of a naturally expressed tumor antigen from irradiated, HLA-mismatched PCa tumor cells. The radiation dose (12 Gy) used in these experiments is in line with the latest technical developments of radiation therapy in prostate cancer and other malignancies. High dose brachytherapy and intensity modulated radiotherapy offer treatments with fewer fractions but higher doses, delivered more precisely to the cancer (27). The cellular effect of radiation is complex, resulting in growth arrest, senescence and different types of cell death. We observed cell cycle arrest at the G2/M phase, as reported by others (28), and a gradual increase of cell death with time following radiation. Cell death was predominantly of late apoptotic/necrotic type. The p53 gene is mutated in DU145 cells, which may impact on radiation-mediated repair response and apoptosis (29). As p53 mutations are frequent in PCa (30), our observations are likely to be representative of the physiological behaviour of the majority of prostate cancer cells.

Radiation-induced upregulation of CRT from the endoplasmic reticulum to the cell surface is one of the typical stress responses with an important immunological impact, such as the facilitation of phagocytosis (31). The results show a partial contribution by the TLR signalling pathway and HMGB1 to antigen cross-presentation. HMGB1 is both a nuclear factor and a secreted protein. In the nucleus, it acts as an architectural chromatin-binding factor that influences DNA tertiary structure (32). When released from dying cells, it functions as a pro-inflammatory cytokine (33). However, its effects are pleiotropic and they depend not only on its redox status but also on the particular receptor it binds, such as RAGE, TLR2 or TLR4. Glycyrrhizin, which binds directly to HMGB1 and inhibits its chemokine function and autophagy induction (34, 35) among other potential effects, was proved inhibitory in the cross-presentation model, but did not inhibit DC activation. This indicates that HMGB1's role in antigen cross-presentation does not depend on its cytokine-like function.

HMGB1 has been shown to associate with TLR4 (36). The Asp299Gly SNP of TLR4 causes structural changes of the TLR4 extracellular domain, with a potential impact on LPS binding (37), while cross-presentation was implied to be affected as well, similar to that observed in TLR4<sup>-/-</sup> knockout mice (19). However, LPS-induced cytokine production is not affected by this TLR4 SNP, even when the SNP is present in a homozygous form (38). Our experiments confirmed that LPS-induced TNF $\alpha$ -production is not affected by the Asp299Gly SNP in DC but, unlike in the anthracycline-induced cross-presentation model (19), we observed no impact by the SNP on antigen cross-presentation either. Our DC were heterozygous for the SNP allele thus functionally not comparable to the TLR4<sup>-/-</sup> mouse DC. However, there is a

discrepancy between the DC results by Apetoh et al. (19) and that observed by us; this maybe due to differences between tumour cell responses to chemotherapy vs. radiation, or that TLR4 SNP has an impact on tumor responses via mechanisms other than antigen cross-presentation.

Removing the supernatant from irradiated DU145 cells before co-culturing them with DC reduced T cell activation but had no effect on DC maturation (not shown), indicating that both cell-associated and soluble factors are needed for efficient antigen cross-presentation from irradiated tumour cells. Another damage associated molecule group, upregulated by irradiation and released into the extracellular space, is heat shock proteins. We studied Hsp70 in detail in this paper as it has an important and well-documented function of chaperoning antigen when released from stressed or necrotic cells (39). Hsp70 levels are variable in PCa but generally higher in cancer tissue than in non-malignant prostate tissue (40). Irradiation induces upregulation of Hsp70 gene expression in PCa cells as a means of protection from radiation (41). We observed increased cytosolic and surface Hsp70 levels upon irradiation in DU145 cells. The molecular chaperone activity of Hsp70 is conferred by two functional domains: a peptide-binding and an ATP-binding domain. One domain reciprocally affects the other, and when polypeptides bind to the peptide-binding domain, ATP is hydrolysed to ADP but when ATP binds, the polypeptides dissociate (42). As ATP levels usually drop in irradiated cells, protein binding by ADP-associated Hsp70 is likely to become a relatively stable association, linking stress-response with chaperoning function. VER155008, a novel and highly specific Hsp70 family inhibitor, functions as an ATP mimetic and binds to the ATPase pocket of Hsp70 (43), thereby inhibiting the activity of Hsp70. Thus, in our model, its effect in inhibiting antigen cross-presentation can be explained by the inability of Hsp70 to remain associated with polypeptides.

Hsp70 can bind to TLR2/TLR4, CD91 or CD40 and to scavenger receptors such as SREC-I and LOX-1. As Hsp70 can bind directly to TLR4 as one of its potential receptors and upregulate HMGB1 (44) in DC, there is a possibility for cross-talk between Hsp70 and the HMGB/TLR4 pathways. On the other hand, SREC-I and LOX-1 were each responsible for about 50% of antigen cross-presentation in a murine system (17). Hsp70 uptake and the subsequent intracellular events in DC are subjects of ongoing studies in our laboratory.

Despite the growing list of players for the “ideal” antigen cross-presentation, the plasticity of the process has also been demonstrated, as e.g. highly polarized (type-1) DC can efficiently prime T cells even when co-cultured with apoptotic cells (45). Furthermore, DC can acquire antigen from live cells for antigen cross-presentation both in tumor and viral settings (46-48). In the latter, while apoptosis of infected fibroblasts is inhibited by the virus, Hsp70 expression is significantly upregulated by the infection (49). These examples illustrate that if any key player of the antigen-cross-presentation process is overexpressed or hyper-activated, it can generate a shortcut leading to antigen cross-presentation even if not all the elements, as discussed earlier, are present.

Taken together, our study provides strong evidence that pre-existing tumor antigen-specific T cells can be re-activated as a consequence of high dose radiation. We also demonstrate the crucial role that Hsp70 plays in antigen cross-presentation from irradiated tumor cells. These observations have practical implications in the design of future immuno-radiotherapy combinations.

### Acknowledgements

We thank Dr Mario Labeta (Cardiff University) for helpful discussions, Drs Rachel Butler and Rana Hussein (Cardiff and Vale NHS Trust) for the TLR4 SNP analysis, Ms Lynda Churchill for technical help and all our blood donors at Velindre Cancer Centre.

### References

1. Tabi, Z., Spary, L. K., Coleman, S., Clayton, A., Mason, M. D., and Staffurth, J. Resistance of CD45RA- T cells to apoptosis and functional impairment, and activation of tumor-antigen specific T cells during radiation therapy of prostate cancer. *J Immunol*, *185*: 1330-1339, 2010.
2. Dewan, M. Z., Galloway, A. E., Kawashima, N., Dewyngaert, J. K., Babb, J. S., Formenti, S. C., et al. Fractionated but not single-dose radiotherapy induces an immune-mediated abscopal effect when combined with anti-CTLA-4 antibody. *Clin Cancer Res*, *15*: 5379-5388, 2009.
3. Hodge, J. W., Sharp, H. J., and Gameiro, S. R. Abscopal regression of antigen disparate tumors by antigen cascade after systemic tumor vaccination in combination with local tumor radiation. *Cancer Biother Radiopharm*, *27*: 12-22, 2012.
4. Golden, E. B., Demaria, S., Schiff, P. B., Chachoua, A., and Formenti, S. C. An abscopal response to radiation and ipilimumab in a patient with metastatic non-small cell lung cancer. *Cancer Immunol Res*, *1*: 365-372, 2013.
5. Postow, M. A., Callahan, M. K., Barker, C. A., Yamada, Y., Yuan, J., Kitano, S., et al. Immunologic correlates of the abscopal effect in a patient with melanoma. *N Engl J Med*, *366*: 925-931, 2012.
6. Schmidtner, J., Distel, L. V., Ott, O. J., Nkenke, E., Sprung, C. N., Fietkau, R., et al. Hyperthermia and irradiation of head and neck squamous cancer cells causes migratory profile changes of tumour infiltrating lymphocytes. *Int J Hyperthermia*, *25*: 347-354, 2009.
7. Suwa, T., Saio, M., Umemura, N., Yamashita, T., Toida, M., Shibata, T., et al. Preoperative radiotherapy contributes to induction of proliferative activity of CD8+ tumor-infiltrating T-cells in oral squamous cell carcinoma. *Oncol Rep*, *15*: 757-763, 2006.
8. Tabachnyk, M., Distel, L. V., Buttner, M., Grabenbauer, G. G., Nkenke, E., Fietkau, R., et al. Radiochemotherapy induces a favourable tumour infiltrating inflammatory cell profile in head and neck cancer. *Oral Oncol*, *48*: 594-601, 2012.
9. McDonnell, A. M., Currie, A. J., Brown, M., Kania, K., Wylie, B., Cleaver, A., et al. Tumor cells, rather than dendritic cells, deliver antigen to the lymph node for cross-presentation. *Oncoimmunology*, *1*: 840-846, 2012.
10. Schrama, D., thor Straten, P., Fischer, W. H., McLellan, A. D., Brocker, E. B., Reisfeld, R. A., et al. Targeting of lymphotoxin-alpha to the tumor elicits an efficient immune response associated with induction of peripheral lymphoid-like tissue. *Immunity*, *14*: 111-121, 2001.
11. Chen, L., Fabian, K. L., Taylor, J. L., and Storkus, W. J. Therapeutic use of dendritic cells to promote the extranodal priming of anti-tumor immunity. *Front Immunol*, *4*: 388, 2013.

12. Garg, A. D., Martin, S., Golab, J., and Agostinis, P. Danger signalling during cancer cell death: origins, plasticity and regulation. *Cell Death Differ*, *21*: 26-38, 2014.
13. Lauber, K., Ernst, A., Orth, M., Herrmann, M., and Belka, C. Dying cell clearance and its impact on the outcome of tumor radiotherapy. *Front Oncol*, *2*: 116, 2012.
14. Dunn, G. P., Koebel, C. M., and Schreiber, R. D. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol*, *6*: 836-848, 2006.
15. Gameiro, S. R., Jammeh, M. L., Wattenberg, M. M., Tsang, K. Y., Ferrone, S., and Hodge, J. W. Radiation-induced immunogenic modulation of tumor enhances antigen processing and calreticulin exposure, resulting in enhanced T-cell killing. *Oncotarget*, 2013.
16. Park, S. H., Lee, S. J., Chung, H. Y., Kim, T. H., Cho, C. K., Yoo, S. Y., et al. Inducible heat-shock protein 70 is involved in the radioadaptive response. *Radiat Res*, *153*: 318-326, 2000.
17. Murshid, A., Gong, J., and Calderwood, S. K. The role of heat shock proteins in antigen cross presentation. *Front Immunol*, *3*: 63, 2012.
18. Southall, P. J., Boxer, G. M., Bagshawe, K. D., Hole, N., Bromley, M., and Stern, P. L. Immunohistological distribution of 5T4 antigen in normal and malignant tissues. *Br J Cancer*, *61*: 89-95, 1990.
19. Apetoh, L., Ghiringhelli, F., Tesniere, A., Obeid, M., Ortiz, C., Criollo, A., et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med*, *13*: 1050-1059, 2007.
20. Miller, Y. I., Choi, S. H., Wiesner, P., and Bae, Y. S. The SYK side of TLR4: signalling mechanisms in response to LPS and minimally oxidized LDL. *Br J Pharmacol*, *167*: 990-999, 2012.
21. Al-Taei, S., Salimu, J., Lester, J. F., Linnane, S., Goonewardena, M., Harrop, R., et al. Overexpression and potential targeting of the oncofoetal antigen 5T4 in malignant pleural mesothelioma. *Lung Cancer*, *77*: 312-318, 2012.
22. Loiarro, M., Sette, C., Gallo, G., Ciacci, A., Fanto, N., Mastroianni, D., et al. Peptide-mediated interference of TIR domain dimerization in MyD88 inhibits interleukin-1-dependent activation of NF- $\kappa$ B. *J Biol Chem*, *280*: 15809-15814, 2005.
23. Toshchakov, V. U., Basu, S., Fenton, M. J., and Vogel, S. N. Differential involvement of BB loops of toll-IL-1 resistance (TIR) domain-containing adapter proteins in TLR4- versus TLR2-mediated signal transduction. *J Immunol*, *175*: 494-500, 2005.
24. Mollica, L., De Marchis, F., Spitaleri, A., Dallacosta, C., Pennacchini, D., Zamai, M., et al. Glycyrrhizin binds to high-mobility group box 1 protein and inhibits its cytokine activities. *Chem Biol*, *14*: 431-441, 2007.
25. Massey, A. J., Williamson, D. S., Browne, H., Murray, J. B., Dokurno, P., Shaw, T., et al. A novel, small molecule inhibitor of Hsc70/Hsp70 potentiates Hsp90 inhibitor induced apoptosis in HCT116 colon carcinoma cells. *Cancer Chemother Pharmacol*, *66*: 535-545, 2010.
26. Schlecht, R., Scholz, S. R., Dahmen, H., Wegener, A., Sirrenberg, C., Musil, D., et al. Functional analysis of Hsp70 inhibitors. *PLoS One*, *8*: e78443, 2013.
27. Zaorsky, N. G., Harrison, A. S., Trabulsi, E. J., Gomella, L. G., Showalter, T. N., Hurwitz, M. D., et al. Evolution of advanced technologies in prostate cancer radiotherapy. *Nat Rev Urol*, *10*: 565-579, 2013.

28. Janicke, R. U., Engels, I. H., Dunkern, T., Kaina, B., Schulze-Osthoff, K., and Porter, A. G. Ionizing radiation but not anticancer drugs causes cell cycle arrest and failure to activate the mitochondrial death pathway in MCF-7 breast carcinoma cells. *Oncogene*, *20*: 5043-5053, 2001.
29. Lehmann, B. D., McCubrey, J. A., Jefferson, H. S., Paine, M. S., Chappell, W. H., and Terrian, D. M. A dominant role for p53-dependent cellular senescence in radiosensitization of human prostate cancer cells. *Cell Cycle*, *6*: 595-605, 2007.
30. Ritter, M. A., Gilchrist, K. W., Voytovich, M., Chappell, R. J., and Verhoven, B. M. The role of p53 in radiation therapy outcomes for favorable-to-intermediate-risk prostate cancer. *Int J Radiat Oncol Biol Phys*, *53*: 574-580, 2002.
31. Obeid, M. ERP57 membrane translocation dictates the immunogenicity of tumor cell death by controlling the membrane translocation of calreticulin. *J Immunol*, *181*: 2533-2543, 2008.
32. Scaffidi, P., Misteli, T., and Bianchi, E. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature*, *418*: 191-195, 2002.
33. Magna, M. and Pisetsky, D. S. The Role of HMGB1 in the Pathogenesis of Inflammatory and Autoimmune Diseases. *Mol Med*, 2014.
34. Mollica, L., De Marchis, F., Spitaleri, A., Dallacosta, C., Pennacchini, D., Zamai, M., et al. Glycyrrhizin binds to High-Mobility Group Box 1 protein and inhibits Its cytokine activities. *Chem Biol*, *14*: 431-441, 2007.
35. Livesey, K. M., Tang, D., Zeh, H. J., and Lotze, M. T. Autophagy inhibition in combination cancer treatment. *Curr Opin Investig Drugs*, *10*: 1269-1279, 2009.
36. Park, J. S., Gamboni-Robertson, F., He, Q., Svetkauskaite, D., Kim, J. Y., Strassheim, D., et al. High mobility group box 1 protein interacts with multiple Toll-like receptors. *Am J Physiol Cell Physiol*, *290*: C917-924, 2006.
37. Ohto, U., Yamakawa, N., Akashi-Takamura, S., Miyake, K., and Shimizu, T. Structural analyses of human Toll-like receptor 4 polymorphisms D299G and T399I. *J Biol Chem*, *287*: 40611-40617, 2012.
38. van der Graaf, C., Kullberg, B. J., Joosten, L., Verver-Jansen, T., Jacobs, L., Van der Meer, J. W., et al. Functional consequences of the Asp299Gly Toll-like receptor-4 polymorphism. *Cytokine*, *30*: 264-268, 2005.
39. Basu, S., Binder, R. J., Suto, R., Anderson, K. M., and Srivastava, P. K. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. *Int Immunol*, *12*: 1539-1546, 2000.
40. Cornford, P. A., Dodson, A. R., Parsons, K. F., Desmond, A. D., Woolfenden, A., Fordham, M., et al. Heat shock protein expression independently predicts clinical outcome in prostate cancer. *Cancer Res*, *60*: 7099-7105, 2000.
41. Gibbons, N. B., Watson, R. W., Coffey, R. N., Brady, H. P., and Fitzpatrick, J. M. Heat-shock proteins inhibit induction of prostate cancer cell apoptosis. *Prostate*, *45*: 58-65, 2000.
42. Massey, A. J. ATPases as drug targets: insights from heat shock proteins 70 and 90. *J Med Chem*, *53*: 7280-7286, 2010.
43. Massey, A., Williamson, D., Browne, H., Murray, J., Dokurno, P., Shaw, T., et al. A novel, small molecule inhibitor of Hsc70/Hsp70 potentiates Hsp90 inhibitor induced apoptosis in HCT116 colon carcinoma cells. *Cancer Chemother Pharmacol*, *66*: 535-545, 2010.

44. Gong, W., Wang, Z. Y., Chen, G. X., Liu, Y. Q., Gu, X. Y., and Liu, W. W. Invasion potential of H22 hepatocarcinoma cells is increased by HMGB1-induced tumor NF-kappaB signaling via initiation of HSP70. *Oncol Rep*, *30*: 1249-1256, 2013.
45. Wieckowski, E., Chatta, G. S., Mailliard, R. M., Gooding, W., Palucka, K., Banchereau, J., et al. Type-1 polarized dendritic cells loaded with apoptotic prostate cancer cells are potent inducers of CD8(+) T cells against prostate cancer cells and defined prostate cancer-specific epitopes. *Prostate*, *71*: 125-133, 2011.
46. Harshyne, L. A., Watkins, S. C., Gambotto, A., and Barratt-Boyes, S. M. Dendritic cells acquire antigens from live cells for cross-presentation to CTL. *J Immunol*, *166*: 3717-3723, 2001.
47. Matheoud, D., Baey, C., Vimeux, L., Tempez, A., Valente, M., Louche, P., et al. Dendritic cells crosspresent antigens from live B16 cells more efficiently than from apoptotic cells and protect from melanoma in a therapeutic model. *PLoS One*, *6*: e19104, 2011.
48. Tabi, Z., Moutaftsi, M., and Borysiewicz, L. K. Human cytomegalovirus pp65- and immediate early 1 antigen-specific HLA class I-restricted cytotoxic T cell responses induced by cross-presentation of viral antigens. *J Immunol*, *166*: 5695-5703, 2001.
49. Santomenna, L. D. and Colberg-Poley, A. M. Induction of cellular hsp70 expression by human cytomegalovirus. *J Virol*, *64*: 2033-2040, 1990.

## Figure Legends

**Figure 1.** Radiation causes cell cycle arrest, cell death and changes to key immunologically relevant molecules in prostate cancer cells. A, Cell cycle analysis of DU145 cells irradiated with increasing dose (x-axis) and incubated for 4, 24 or 48h as indicated above the graphs. Means±SEM of cells from triplicate cultures in different cell cycle phases are shown as indicated. B, Percentage of DU145 cells labelled with Annexin and Propidium iodide (PI), respectively, following 12Gy radiation and cultured up to 72h (x-axis) is shown from triplicates (mean+SEM). C, Medium fluorescence intensity (mfi) of 5T4 expression in fixed and permeabilized DU145 cells 48h following irradiation from triplicate cultures (Mean+SEM). D, Mfi of HMGB1 in DU145 cells (total staining in fixed and permeabilized cells) at different times after 12 Gy irradiation (x-axis) from triplicate cultures (Mean+SEM). E, Hsp70-FITC (green) and DAPI (blue) labelled DU145 cells 72h after 0Gy (left) or 12Gy (right) irradiation. 63x magnification (Axiovert-40, Zeiss). F, Representative dot plots of DU145 cells 72h after 0Gy or 12Gy radiation showing CRT (x-axis) and Hsp70 (y-axis) co-expression or isotype control (first panel). The numbers show Hsp70 single positive (upper left) or Hsp70+CRT+ double positive cells (right quadrant). All experiments were repeated at least three times.

**Figure 2.** Irradiated tumor cells are taken up by DC and trigger DC maturation and antigen cross-presentation. A, Representative dot plot showing uptake of CFSE-pre-labelled DU145 cells (x-axis) after 0Gy (i) or 12Gy (ii) irradiation by DC. The latter were identified by HLA-DR staining. Phagocytic DC are cells in the upper right quadrant. iii. Summary of results from triplicates, mean+SEM are shown. B, flow cytometry analysis of DC markers after 24h co-culture with 0Gy or 12Gy irradiated DU145 cells, as indicated. Mean+SEM of mfi from triplicate cultures are shown. C, Proliferation of CFSE-labeled 5T4-specific T cells 5 days after stimulation by autologous DC co-cultured with DU145 cells, as indicated. Mean+SEM of CFSE mfi from triplicate cultures are shown. D, 5T4-specific T cells were stimulated overnight with DC co-cultured with DU145 cells as shown (FT=freeze-thawed). IFN $\gamma$ <sup>+</sup> T cells were defined by flow cytometry using intracellular cytokine staining. Mean+SEM of % IFN $\gamma$ <sup>+</sup> T cells from triplicate cultures are shown. All experiments were repeated at least three times.

**Figure 3.** The effect of HMGB1/TLR4 pathway inhibitors on DC maturation and antigen cross-presentation. A, DC were co-cultured with 0Gy or 12Gy treated DU145 cells in the presence or absence of 50  $\mu$ M HMGB1 inhibitor (Glycyrrhizin) as indicated for 72h. 5T4-specific T cell stimulation as described for the cross-presentation assay (i) and CD86 upregulation on DC (ii) were analyzed by flow cytometry. The columns show mean+SEM of results from triplicate cultures. B, DC were cultured as in A, but in the presence of 25  $\mu$ g/ml MyD88 and 25  $\mu$ g/ml TRIF inhibitory peptides together (inhibitory) or of the same concentration of control peptide. Mean+SEM of % IFN $\gamma$ <sup>+</sup> T cells from triplicate cultures are shown. The experiments were repeated 2-3 times.

**Figure 4.** TLR4 polymorphism does not affect cross-presentation. A, i, TLR4 expression on monocytes from 5 donors with the Asp299 TLR4 allele (299A) and 4 donors with the Gly299 (299G) allele are shown; ii, as in i, expression is shown on

day 5 DC. iii, day 5 DC were stimulated with LPS and TNF $\alpha$  production measured by flow cytometry. Symbols represent % positive cells from individual donors. The boxes show the 25% and 75% percentiles of the combined data, the lines in the boxes show the median values. B, DC phenotyping from the donors as in A. Each line represent an individual donor. C, Stimulation of 5T4-specific T cells with DC, from donors as in A, loaded with 0Gy or 12Gy irradiated DU145 cells. Symbols represent means of triplicate results, each line represents an individual donor.

**Figure 5.** Hsp70 inhibitor VER155008 prevents DC maturation and antigen cross-presentation. A-C, The effects of VER155008 on DU145 cells during 72h post-irradiation culture. Mean+SEM of results from triplicate samples are shown. A, the effect on cell numbers expressed as % of cell numbers in the absence of the inhibitor; B, Annexin<sup>+</sup>PI<sup>+</sup> cell frequencies as determined by flow cytometry; C, surface expression of Hsp70. D-E, The effect of VER155008-treated or untreated DU145 cells on DC; D, CD86 expression following 24h coculture of DU145 cells with DC; E, stimulation of 5T4-specific T cells in a cross-presentation experiment. The experiments were repeated 2-3 times.

Figure 1

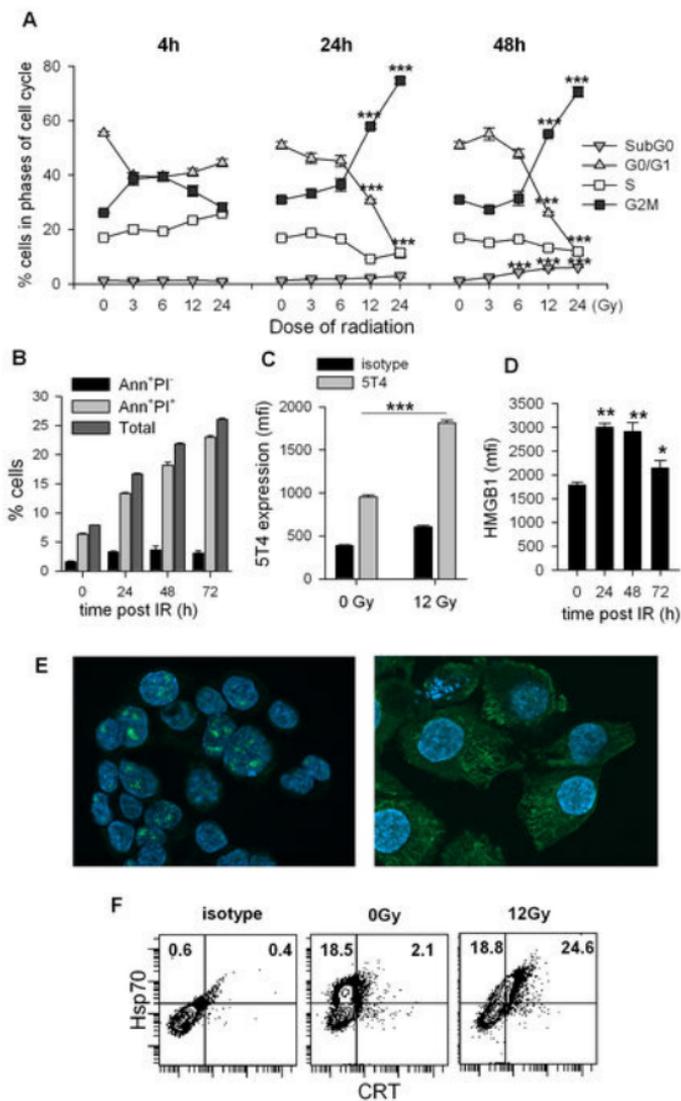


Figure 2

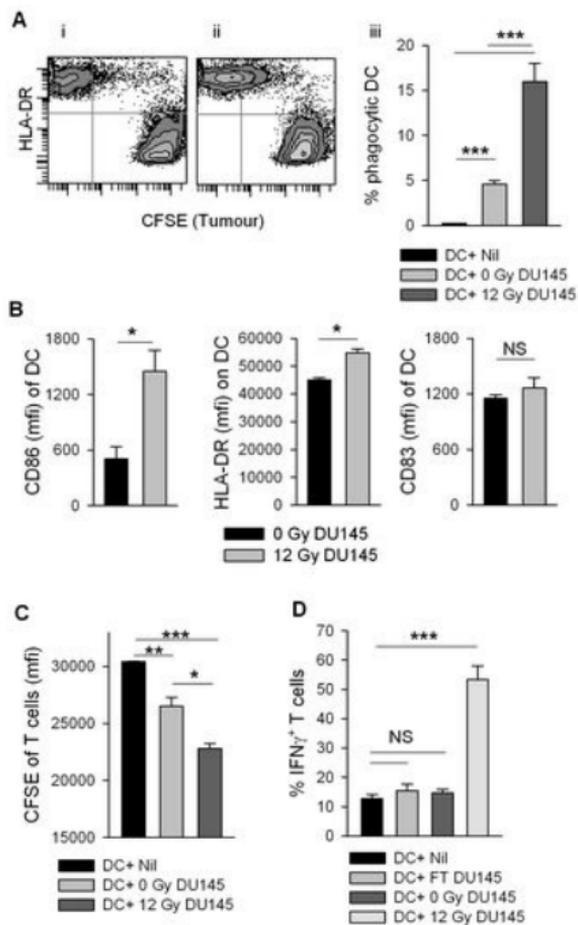


Figure 3

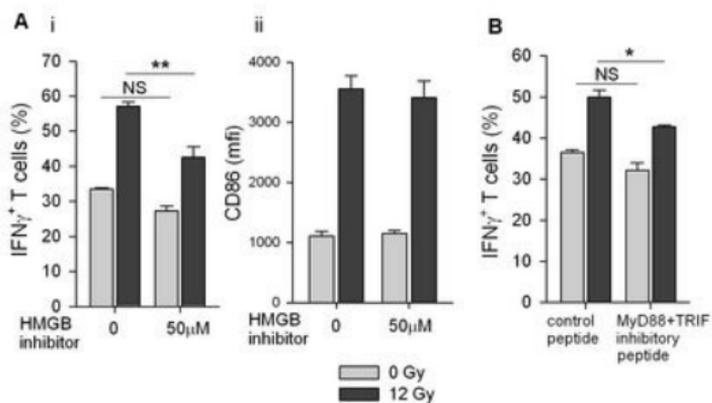


Figure 4

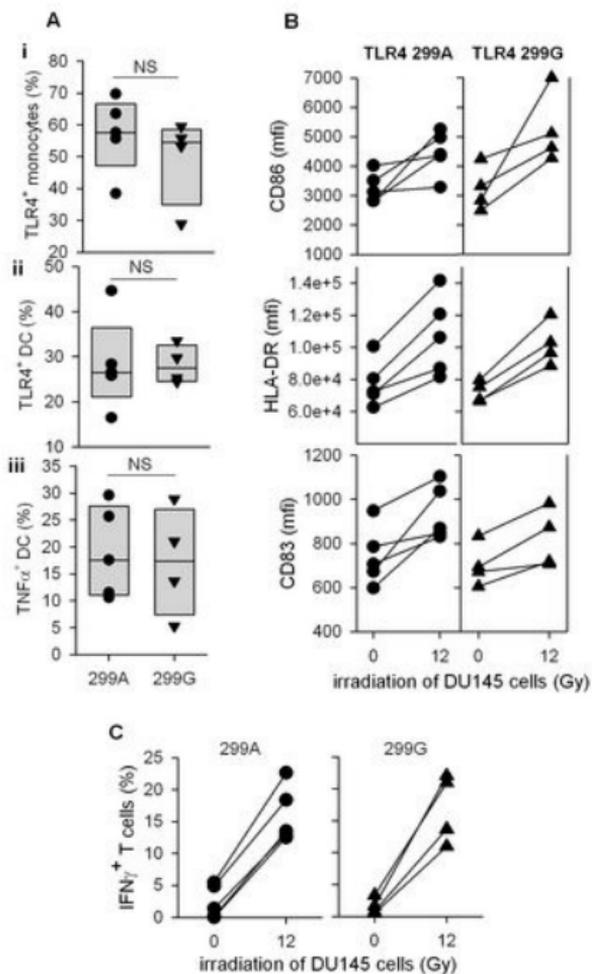


Figure 5

