Cross-presentation of the oncofoetal tumor antigen 5T4 from irradiated prostate cancer cells - a key role for heat shock protein 70 and receptor CD91

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Abstract

Immune responses contribute to the success of radiation therapy of solid tumors; however, the mechanism of triggering CD8⁺ T-cell responses is poorly understood. Antigen cross-presentation from tumor cells by dendritic cells (DC) is a likely dominant mechanism to achieve CD8⁺ T-cell stimulation. We established a cross-presentation model in which DCs present a naturally expressed oncofoetal tumor antigen (5T4) from irradiated DU145 prostate cancer cells to 5T4-specific T cells. The aim was to establish which immunogenic signals are important in radiation-induced cross-presentation. Radiation (12 Gy) caused G₂/M cell-cycle arrest and cell death, increased cellular 5T4 levels, high-mobility protein group-B1 (HMGB1) release and surface calreticulin and heat-shock protein-70 (Hsp70) expression in DU145 cells. DCs phagocytosed irradiated tumor cells efficiently, followed by upregulation of CD86 on phagocytic DCs. CD8⁺ 5T4-specific T cells, stimulated with these DCs, proliferated and produced IFNγ. Inhibition of HMGB1 or the TRIF/MyD88 pathway only had a partial effect on T-cell stimulation. Unlike previous reports, we found no evidence that DCs carrying Asp299Gly toll-like receptor-4 (TLR4) single nucleotide polymorphism had impaired ability to cross-present tumor antigen. However, pre-treatment of tumor cells with Hsp70 inhibitors resulted in a highly significant and robust prevention of antigen cross-presentation and CD86 upregulation on DCs co-cultured with irradiated tumor cells. Blocking the Hsp70 receptor CD91 also abolished cross-presentation. Together, results from our study demonstrate that radiation induces immunologically relevant changes in tumor cells, which can trigger CD8⁺ 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 not yet clear what are the predictors of complete responses. RT in PCa has been shown to be associated with increased frequencies of tumor antigen-specific T cells (1). The abscopal effect of radiation (tumor regression at a distant site following localized radiation) has been shown to be immune-mediated not only in mouse tumor models (2, 3) but also in patients with metastatic melanoma and lung adenocarcinoma (4, 5). Furthermore, CD8+ T-cell infiltration in the irradiated tumor tissue serves as a prognostic factor (4-7), indicating that radiation can switch the immunosuppressive tumor milieu to a pro-immune environment.

For solid tumors, tumor antigen-specific CD8+ T-cell responses can be induced either by tumor cells entering lymph nodes (8) or dendritic cells (DC) cross-presenting tumor antigens either in lymph nodes or ectopic lymphoid tissues present in some tumors (9, 10). Efficient cross-presentation requires tumor cell damage or cell death and is associated with translocation or release of 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. The release of chemoattractant molecules and HMGB1, representing DAMPs, also has been observed. 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 (11). Our study focuses on
ionizing radiation, which is known to cause primarily DNA damage, cell-cycle arrest and cellular-damage responses. These changes can trigger either DNA-repair or cellular senescence and also apoptotic, necrotic or necroptotic cell death. The early release of IFNα/β by irradiated tumor cells can polarize antigen-presenting cells and aid their cross-presenting function (13). High-dose (10-100Gy) in vitro radiation of 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 (14). These typical ICD markers may facilitate phagocytosis of damaged/dead cells and provide maturation signals for DCs (12).

The aim of our study was to determine the relative importance of immunogenic signals in radiation-mediated tumour antigen cross-presentation. As antigen cross-presentation studies often use artificially overexpressed antigens, which may provide false positive results, we established a model focusing on a naturally occurring oncofoetal antigen, 5T4, which is expressed in most solid tumors (15). This cross-presentation model enabled us to study the effect of irradiated tumor cells on DC phenotypic and functional maturation, while the use of specific inhibitors revealed the main players of the cross-presentation process. We show here that in radiation-induced tumor antigen cross-presentation the toll-like receptor 4 (TLR4) pathway is not the major mechanism and the Asp299Gly TLR4 single nucleotide polymorphism (SNP) is not associated with any impairment of the process. Instead, we found that Hsp70 is crucially important both in activating DCs and triggering CD8+ T-cell responses to DCs co-cultured with irradiated tumor cells. Our results highlight the plasticity of tumor antigen cross-presentation and demonstrate the important immunologic role of Hsp70 following tumor radiation.
Materials and Methods

Media and reagents

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

Tumor cells and treatment

DU145 PCa cells were obtained from ECACC and maintained in culture with regular passaging for less than 6 months. Authentication was carried out by the supplier using cytogenetic, isoenzymatic and DNA profile analysis. The HLA type of DU145 cells is 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). Irradiation was carried out using a $^{137}$Cs-source (with dosimetry quality assurance) at a rate of 0.627Gy/min. Oxaliplatin (Sigma) was used at 20µM.

Donors, dendritic cell preparation

Ethical approval was granted and informed consent was obtained from healthy volunteers. HLA Class-I typing was carried out as above. Peripheral blood mononuclear cells (PBMC) from venous blood, collected in EDTA vacutainers, were
Antigen cross-presentation from irradiated tumor cells prepared by density gradient centrifugation. CD14+ monocytes were isolated by negative selection using the EasySep Human Monocyte Enrichment Kit without CD16 Depletion (Stem Cell Technologies, France). Average purity of CD14+ cells was 70-80%. Cells were incubated at 5x10^6 cells/well in 6-well trays in 5ml/well of 10% FBS-RPMI plus 500ng/ml human recombinant GM-CSF (ProSpec, Israel) and 500U/ml IL4 (Gentaur, Belgium) for 5-6 days.

T-cell and B-cell lines

A CD8+ T-cell line was developed from a HLA-A2+ healthy donor by repeated stimulation of non-adherent PBMCs with autologous DCs loaded with 2µg/ml 5T4\textsubscript{17-25} peptide (RLARLALVL; ProImmune), as described (16). T cells (1–2×10^6) were expanded with a mixture of 5x10^6 peptide-pulsed autologous B lymphoblastoid cells (BLCL) irradiated with 40Gy; 5x10^7 allogeneic PBMCs from 2-3 donors, irradiated with 30Gy; 50U/ml IL2 and 1µl/ml OKT3 hybridoma supernatant in 50ml RPMI, supplemented as above, and with 10% FBS and 1% AB-serum (16).

Immunocytochemistry

DU145 cells were seeded on coverslips and left untreated or were irradiated with 12Gy. After 72h cells were fixed with a 1:1 (v:v) mixture of ice cold acetone:methanol for 5 min. After drying, cells were blocked in 1% BSA/PBS for 1h then stained with an anti-Hsp70 antibody (Enzo Life Sciences), and a goat anti-mouse Alexa-488 secondary (Life Technologies). Nuclei were stained with DAPI. Images were gathered on a Zeiss Observer Z.1 microscope, fitted with an Apotome 2 module for structured illumination, using a 63x/1.4 numerical aperture oil immersion
objective, and an Axiocam 506 monochrome camera system. Representative images from Z axis sections were overlaid to generate maximum projection images.

Flow cytometry

Cells were labelled in flow cytometry buffer (PBS, 1mM EDTA and 2% FBS) with fluorochrome-conjugated 5T4, CRT, HMGB1 (R&D Systems), Hsp70 (Enzo Life Sciences), CD91 (BD Pharmingen), TLR4, HLA-DR, CD86 and CD83 (E-Bioscience) antibodies or unconjugated SREC-I (R&D Systems) followed by a goat anti-mouse AlexaFluor-488 (Invitrogen) antibody and incubated on ice for 40min. For intracellular labelling, the cells were fixed and permeabilized with E-Biosciences Fix/Perm reagents before antibodies were added for 40min at room temperature. For cytokine flow cytometry, T cells were fixed and permeabilized as above and CD3, CD8 and IFNγ antibodies were added together for 40min. Flow cytometry was carried out using a FACSCanto flow cytometer with FACSDiva software (BD Bioscience, Franklin Lakes, NJ).

For cell-cycle analysis, Guava Cell Cycle Reagent (Millipore) was used; cell death was assessed using the Annexin-V/Propidium Iodide (PI) Apoptosis Detection Kit (BD Bioscience).

For phagocytosis assays, DU145 cells at 72h post-irradiation were labelled with 5µM CFSE (E-Bioscience) and were co-cultured with DCs (5x10^4 cells/well, 1:1 ratio) in a 96-well U-bottom plate for 24h at 37°C. After incubation, the cells were surface labelled with HLA-DR or CD86 antibodies.

Western blotting

Cell pellets were resuspended in 1ml cold RIPA Lysis Buffer (Santa Cruz) with freshly added protease and phosphatase inhibitors. Cells were incubated on ice
for 30min, vortexing every 10min, followed by centrifugation at 14,000g for 15min.

Protein estimation from the supernatant was carried out using the BCA assay.

5 μg protein was loaded and separated on NuPage 4-12% Bis-Tris gels under reducing conditions and transferred onto PVDF membranes using the iBlot Gel Transfer Stack System (Life Technologies). The membrane was blocked with 5% non-fat dry milk and probed with a sheep polyclonal IgG 5T4 antibody (R&D Systems, AF4975) or a mouse monoclonal IgG1 anti-Hsp70 antibody (C92F3A-5) and with a mouse monoclonal antibody for GAPDH (0411) (both from Santa Cruz) for 1h at room temperature followed by horseradish-peroxidase-conjugated secondary antibody (Santa Cruz). Bands were developed by ECL on film (GE Healthcare, UK). Relative density of the bands exposed was calculated using the Image J Software.

**HMGB1 ELISA**

Supernatants from 0Gy- or 12Gy-irradiated DU145 cells grown in T25 flasks (5x10^5 input cell number) were collected 0h, 24h, 48h and 72h post-radiation and tested using a HMGB1 ELISA-kit (IBL International) according to the manufacturer’s protocol.

**Inhibition of TRIF, MyD88, HMGB1, Hsp70 and its receptors**

The MyD88 and TRIF inhibitory peptides which correspond to the sequence of the BB-loop of MyD88 (RDVLPGT) and TRIF (FCEEFQVPGRGELH), respectively, serve as decoys by binding to the TIR domains and interfering with TLR-adaptor interactions (17,18). The control peptides consist of the protein transduction sequence alone, which renders the peptides cell-permeable. DCs were pre-treated with 20μM MyD88 or 25μM TRIF inhibitory or control peptide,
respectively, for 6h before LPS stimulation (100ng/ml) or with 25µM of both when adding DCs to DU145 cells for the cross-presentation assay.

Glycyrrhizin, an HMGB1-inhibitor, was added at 50µM at the time of irradiation, while VER155008, an Hsp70-inhibitor, at 5µM to 0Gy- and 12Gy-irradiated DU145 cells at 0h, 24h and 48h of the 72h incubation, respectively. PES was added to DU145 cells at 20µM at the time of irradiation. Hsp70 receptor blocking was carried out by treating DCs with the SREC-I-specific purified goat IgG polyclonal antibody (R&D Systems) or CD91 mouse IgG1 monoclonal antibody (Thermo Scientific) or relevant isotypes (R&D Systems) at 1µg/ml for 1h before adding DCs to DU145 cells.

**Antigen cross-presentation**

DU145 cells were set up in two 96-well U-bottom plates at 5x10^3 cells/well. One plate was irradiated with 12Gy prior to incubation for 72h. DCs were then added to the wells at a 1:1 DU145:DC ratio. After 48h, 5T4-specific T cells were added at a 1:1:5 (DU145:DC:T cell) ratio. Golgi Plug (0.5µl/500µl) and Golgi Stop (0.35µl/500µl) (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 IFNγ^+CD8^+ T cells.

**T-cell proliferation**

DU145 cells were plated in two 24-well plates at 1x10^5 cells/well in 1.5ml. One plate was irradiated with 12Gy and 10^5 DCs in 0.5 ml was added to the wells. After 4h, CSFE-labelled T cells (5x10^5 cells/well) were added and incubated for 5 days. Flow cytometry analysis of CFSE dilution in CD3^+CD8^+ cells was carried out.
SNP analysis

SNP analysis was performed by the Department of Medical Genetics (Cardiff and Vale NHS Trust, University Hospital of Wales, Cardiff). DNA amplification was carried out from blood or established BLCLs by PCR followed by pyrosequencing optimized 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). Statistical significant differences are marked as *p<0.05, **p<0.01; ***p<0.001.
Results

Irradiation induces immunologically relevant changes in tumor cells

To establish the optimum minimal radiation causing significant changes in DU145 cells in vitro, dose escalation and time-kinetics experiments were performed. A significant proportion of irradiated cells were arrested in the G2/M phase, detectable first at 24h post-radiation. There was a small but significant radiation dose-dependent increase at 48h in the proportion of cells in sub-G0 phase representing apoptotic cells with fragmented DNA (Fig.1A). The type of cell death, detected with Annexin/PI-labeling, was mainly late apoptotic/necrotic and the proportion of cells with early apoptotic markers remained low throughout the 72h incubation (Fig.1B). Other, radiation-associated, immunologically relevant changes were also observed. The total cellular HMGB1 content increased with early time kinetics (Fig.1Ci), while significant amounts of HMGB1 were released from the cells, detectable from 48h post-radiation by ELISA (Fig.1Cii). 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.1D i,ii) as detected by flow cytometry and confirmed by western blotting (Fig.1E).

Irradiation of tumor cells induces Hsp70 cytoplasmic translocation and surface expression

Radiation resulted in the significant upregulation of surface Hsp70 on DU145 cells (Fig.2A). Multicolor flow cytometry analysis confirmed that upon irradiation in a large proportion of cells with cell surface Hsp70 expression CRT was also translocated to the cell surface; this double positive subset was not observed without irradiation (Fig.2B right vs. middle plot). Immunocytochemistry confirmed that while
Hsp70 expression was predominantly nuclear in untreated DU145 cells, 72h after irradiation cytosolic and cell surface expression became dominant (Fig.2C and Suppl.Fig.1). While a slight increase in Hsp70 content was observed 3h after irradiation in DU145 cells by western blotting (Fig.2D), it was not as significant as that observed 2h after 42°C heat treatment; by 72h post-irradiation no increase was detectable. This suggests that Hsp70’s cellular localization may be important in antigen cross-presentation.

Dendritic-cell activation and tumor antigen cross-presentation following uptake of DU145 cells

Next we studied whether irradiated tumor cells are taken up by DCs and if they activate DCs and trigger their antigen-presenting function. Tumor cells were labelled with CFSE 72h post-radiation and added to DCs at a 1:1 ratio. Phagocytosis was measured 24h later by determining the proportion of HLA-DR⁺CFSE⁺ cells. Although some uptake of non-irradiated tumor cells was observed (Fig.3Ai), the proportion of phagocytic DCs increased significantly upon encountering irradiated DU145 tumor cells (Fig.3Aii and iii). DC phenotype following phagocytosis of irradiated or non-irradiated tumor cells was also studied 24h after DC:tumor cell co-culture. CD86 expression was significantly elevated on DC cultured with irradiated, but not with non-irradiated, tumor cells (Fig.3B). Furthermore, CD86 expression was significantly higher on those DCs that phagocyted tumor cells i.e. that were positive for CFSE (Fig.3C, cells in Q2 of Fig 3Aii) compared to those in Q1.

5T4 antigen cross-presentation from DU145 tumor cells by HLA-A2⁺ DCs was measured by assessing proliferation and intracellular cytokine production of 5T4-peptide-specific T cells (16). While some T-cell activation was triggered by DCs
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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 crucially depends on the TLR4-HMGB1 interaction and consequently patients with TLR4 polymorphism are unable to mount immune responses to tumor antigens (19). In order to study TLR4-HMGB1 signaling in antigen cross-presentation from irradiated tumor cells, we applied Glycyrrhizin (GA), an HMGB1-inhibitor (20), to tumor cells before irradiation. HMGB1 inhibition resulted in a small decrease in CD86 expression on DCs after their co-culture with tumor cells (Fig.4Ai), and also a small but significant decrease in T cell IFNγ-production (Fig.4Aii). HMGB1 can bind to multiple receptors, such as TLR2 and TLR4, so next we targeted the MyD88/TRIF signaling pathway with inhibitory
peptides. We established that 20-25 μM of the inhibitory peptides significantly reduced LPS-induced TNFα production by DCs compared to that of control peptides (Fig.4B). While neither TRIF nor the MyD88 inhibitory peptide alone (25μM each) had any effect on T-cell stimulation (not shown), when applied together, they resulted in a small inhibitory effect (Fig.4C), indicating the importance of pathways other than TLRs in antigen cross-presentation.

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

In order to elucidate whether TLR4 SNP results in impaired antigen cross-presentation, as it has been shown with oxaliplatin-treated tumor cells (19), we conducted a series of experiments with DCs generated from monocytes of donors carrying the normal (Asp299) (n=5) or the polymorphic Gly299 (n=4) TLR4 allele. All donors were HLA-A2+. The general characterization of monocytes and DCs revealed that TLR4 expression levels (Fig.5Ai, ii) and LPS-induced TNFα production were comparable between the two groups (Fig.5Aiii). Phenotypic maturation of DCs (CD86, HLA-DR and CD83), carrying the SNP allele, following co-culture with irradiated tumor cells was also unimpaired (Fig.5B). Most importantly, cross-presentation of 5T4 from irradiated tumor cells was similarly efficient by DCs carrying the Asp299 or the Gly299 TLR4 allele, respectively (Fig.5C). In order to see whether TLR4 SNP was impacting on chemotherapy-induced but not radiation-induced tumor antigen cross-presentation, T-cell responses were also studied with oxaliplatin-treated tumor cells. T-cell responses were generally weaker than those induced by DCs co-cultured with irradiated tumor cells, but significantly elevated responses were observed in one out of three normal and two out of three TLR4 SNP
donors (Fig.5C). Despite the low number of donors, the experiments clearly show that donors with TLR4 SNP are able to cross-present tumor antigen either from irradiated or chemotherapy-treated tumor cells.

**Hsp70 inhibition blocks antigen cross-presentation**

The contribution of heat shock proteins to antigen cross-presentation has been demonstrated in several models; we performed experiments to establish whether Hsp70 plays a role in cross-presentation of irradiated tumor cells. To test this, first we applied to tumor cells before irradiation the small molecule inhibitor VER155008, which inhibits the activity of both the induced and constitutive forms of Hsp70 (21,22). Tumor cell numbers after 72h were only slightly lower when VER155008 was applied to irradiated cells, presumably because cell proliferation was already slowed down by the radiation. However, as expected, the treatment inhibited the growth of non-irradiated tumor cells by ~70% (Fig.6A). Similarly, VER155008 significantly increased cell death of non-irradiated but not irradiated DU145 cells (Fig.6B). The inhibitor did not impair Hsp70 cell surface expression on irradiated tumor cells (Fig.6C). After establishing that VER155008 delivers the expected effects to untreated tumor cells, we studied its effect on the ability of irradiated tumor cells to upregulate CD86 on DCs. CD86 upregulation was partially inhibited (Fig.6D) when DCs encountered irradiated tumor cells pre-treated with VER155008. Finally, we applied VER155008 in the cross-presentation model to test its effect on T-cell activation. The inhibitor significantly decreased both the background cross-presentation of tumor antigen from non-irradiated tumor cells and the enhanced level of cross-presentation observed from irradiated tumor cells (Fig.6E), as detected by decreased T cell IFNγ-production. The results were confirmed with DCs derived from
two donors. In order to test that the inhibition of cross-presentation with VER155008 was not due to an off-target effect, we applied PES, another Hsp70-inhibitor. PES binds only to stress-induced but not constitutive Hsp70 (23). Interestingly, PES only inhibited T-cell stimulation induced by irradiated tumor cell-loaded DCs but not by the addition of non-irradiated tumor cells (Fig.6E). These experiments indicate a crucial role for radiation-induced Hsp70 in tumor antigen cross-presentation.

Irradiated tumor cell-derived Hsp70 signals mainly via CD91 on DCs

In order to further elucidate the importance of Hsp70 in the cross-presentation model, we tested the expression of potential receptors CD91 and SREC-I on day 5 DCs. Significant surface expression of both receptors was observed (Fig.7A). When neutralizing antibodies against these receptors were applied in the cross-presentation experiments, T-cell activation was completely inhibited in the presence of the anti-CD91- but not the SREC-I-specific neutralizing antibody (Fig.7B). These results demonstrate that CD91-mediated effects in DCs, such as tumor cell-derived Hsp70 binding, are necessary for efficient antigen cross-presentation from irradiated tumor cells.
Discussion

Antigen cross-presentation has been indicated as an important mechanism for generating CD8+ 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.

We studied the relative importance of immunogenic signals in 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 by DCs. The radiation dose (12 Gy) applied to tumor cells 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. The cellular effect of radiation is complex, resulting in growth arrest, senescence and different types of cell death. We observed cell-cycle arrest in the G2/M phase, as reported by others (24), 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 (25). As p53 mutations are frequent in PCa, our observations
are likely to be representative of the physiologic 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 immunologic impact, such as the facilitation of phagocytosis (26). The results show a partial contribution by the TLR signaling 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. When released from dying cells, it functions as a pro-inflammatory cytokine (27). 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 (20) among other potential effects, was proved inhibitory in the cross-presentation model. HMGB1 has been shown to associate with TLR4 (28). The Asp299Gly SNP of TLR4 causes structural changes of the TLR4 extracellular domain, with a potential impact on LPS binding (29). Cross-presentation has been implied to be affected by this SNP, similar to that observed in TLR4\textsuperscript{-/-} knockout mice (19). However, LPS-induced cytokine production is not affected by the TLR4 SNP even when present in a homozygous form (30). Our results are in agreement with this, as we observed no influence of Asp299Gly TLR4 SNP on LPS-induced TNF\ensuremath{\alpha}\textsuperscript{-} production in DCs. However, we also found no effect by the TLR4 SNP on radiation-induced antigen cross-presentation. Furthermore, contrary to the observation by others (19) we also did not find any evidence of impaired antigen cross-presentation from oxaliplatin-treated tumor cells by DCs carrying the TLR4 SNP. The possible
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explanation behind this discrepancy may lay in donor variation as the previously published observation (19) was based on results from a single donor with TLR4 SNP.

Heat shock proteins represent another group of damage-associated molecules, upregulated by irradiation and released into the extracellular space from dying cells or secreted from live cells (31). In PCa, Hsp70 has been shown to be protective, as its silencing enhanced tumor cell sensitivity to radiation (32). Hsp70 has well-described immunologic roles as well, as tumor tissue-derived Hsp70 has been shown to be protective against tumor challenge in mice (33). We observed a predominantly nuclear expression of Hsp70 in untreated DU145 cells, while in irradiated cells nuclear expression seemed lower and cytosolic and cell-surface expression increased significantly. This translocation observed at 72h post-irradiation may have been associated with enhanced repair activity in the irradiated cells. While Hsp70 inhibition with VER155008, a specific Hsp70-family inhibitor (21) did not influence tumor cell growth and cell death following radiation, it significantly inhibited the growth of non-irradiated tumour cells. VER155008 also did not influence Hsp70 cell surface expression on irradiated DU145 cells; however it partially inhibited the ability of irradiated DU145 cells to activate CD86 upregulation on DCs. The reason behind this observation is not clear. The inhibition of antigen cross-presentation was complete when VER155008 was added either to irradiated or control DU145 cells. Another Hsp70 family inhibitor, PES (23) only affected the enhanced T-cell response observed with irradiated but not that with untreated tumor cells.

Hsp70 can bind to TLR2 or TLR4, CD91, CD40 or to scavenger receptors such as SREC-I and LOX-1. Hsp70 binding to TLR4 can upregulate HMGB1 in DCs (34), providing cross-talk between the heat shock and the HMGB/TLR4 pathways. On the other hand, SREC-I and LOX-1 were shown to be both responsible for antigen
cross-presentation in a murine system (35). In order to determine which receptor is important in this model, DC phenotyping and antibody-blocking experiments were carried out. The results agree with previous works (36,37), showing that CD91 is the dominant receptor for Hsp70 during the cross-presentation process.

We have not addressed directly whether Hsp70’s role in this model lies mainly in antigen-chaperoning (33,38,39) or enhancing autophagy (40) or triggering DC maturation (41,42) with subsequent increase in T-cell stimulation. All of these known functions can be important and they likely play a synergistic role. Our model provides an opportunity to study the fine details of heat shock protein-mediated chaperoning of a naturally expressed antigen in a human tumor model. Such an analysis would represent an interesting follow-up to the work presented here.

Despite the long list of players necessary for “optimal” antigen cross-presentation, the plasticity of the process has also been demonstrated, as e.g. highly polarized (type-1) DCs can efficiently prime T cells even when co-cultured with apoptotic cells (43). Furthermore, DCs can acquire antigen from live cells for antigen cross-presentation both in tumor and viral settings (44-46). In the latter, while apoptosis is inhibited by the virus, Hsp70 expression is significantly upregulated (47). 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, results from our study provide strong evidence that pre-existing tumor antigen-specific T cells can be re-activated as a consequence of radiation of tumor cells. We also demonstrate that Hsp70 plays a crucial role in antigen cross-presentation from irradiated tumor cells. These observations have practical implications for the design of future immuno-radiotherapy combinations.
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References

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Figure Legends

Figure 1. Radiation causes cell-cycle arrest, cell death and changes to immunologically relevant molecules in DU145 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. B, Percentage of DU145 cells undergoing different types of cell death, as indicated, following 12Gy radiation and cultures up to 72h (x-axis). Ci, HMGB1 expression in fixed and permeabilized DU145 cells at different times after 12 Gy irradiation (x-axis) shown as mean fluorescence intensity (mfi); ii, soluble HMGB1, as detected by ELISA in the supernatant of DU145 cells as in (i). Di, 5T4 expression in fixed and permeabilized DU145 cells 48h post-irradiation; Dii, Representative FACS histograms. A-Di: Means+SEM of results from triplicate samples. E, Western blotting of 5T4 antigen in DU145 cell lysate with or without irradiation. Raw data (left panel), adjusted density (right panel). All experiments were repeated 2-3 times.

Figure 2. Hsp70 translocation in irradiated tumour cells. A, Hsp70 surface expression was measured by flow cytometry on DU145 cells 24h or 72h post-irradiation. Means+SEM of Hsp70 mfi (minus isotype control mfi values) from triplicate samples are shown. B, Representative dot plots of DU145 cells 72h after irradiation, showing CRT (x-axis) and Hsp70 (y-axis) expression or isotype control (first panel). The numbers represent Hsp70 single positive (upper left) or Hsp70+CRT+ double positive cells (upper right). C, Hsp70-FITC (green) and DAPI (blue)-labelled DU145 cells 72h after 0Gy (left) or 12Gy (right) irradiation. 63x magnification (Axiovert-40, Zeiss). The white arrows indicate surface Hsp70 expression. D, Western blotting of Hsp70 antigen in DU145 lysate, treatments are: (1) positive control: 42°C heat-treatment for...
2h followed by 1h incubation at 37°C; (2) 12Gy radiation followed by 72h incubation; (3) 12Gy radiation followed by 3h incubation; (4) no treatment. Blots are shown on left panel, adjusted density on the right.

**Figure 3.** DC maturation and antigen cross-presentation by irradiated tumor cells. A, Representative dot plot showing uptake of CFSE-pre-labelled DU145 cells (x-axis) after 0Gy (i) or 12Gy (ii) irradiation by DCs (HLA-DR+ cells). Phagocytic DCs are in the upper right quadrant (Q2). iii, summary of results from five donors; each symbol represents the mean of % DCs in Q2 from triplicate samples per donor. B, Flow cytometry analysis of CD86 expression after 24h co-culture of DCs without (NIL) or with 0Gy- or 12Gy-irradiated DU145 cells. Mean+SEM of CD86 mfi from triplicate cultures are shown. C, Flow cytometry of CD86 expression, analysed on DCs gated as Q1 or Q2 DCs, respectively, after DCs co-culture with DU145 cells. Di, Proliferation of CFSE-labeled 5T4-specific T cells 5 days after stimulation by autologous DCs co-cultured with DU145 cells, as indicated. Mean+SEM of CFSE (mfi) of T cells from triplicate cultures are shown; ii, representative histograms of CFSE dilution in T cells. The numbers represent the % of T cells that proliferated. E, 5T4-specific T cells were stimulated overnight with DCs (from six donors), co-cultured with DU145 cells. Each symbol represents the mean of % IFNγ+ T cells from triplicates from an individual donor. Fi, 5T4 antigen cross-presentation is inhibited by HLA-Class I blocking antibody; ii, 5T4+ (DU145, PC3, M38) but not 5T4− (LNCaP) tumor cells stimulate T cells in the cross-presentation assay. Means+SEM of % IFNγ+ T cells from triplicates are shown.

**Figure 4.** Partial effect of HMGB1/TLR4 pathway-inhibitors on DC maturation and antigen cross-presentation. A, DCs were co-cultured with 0Gy- or 12Gy-treated
DU145 cells in the presence or absence of 50μM glycyrrhizin (GA) for 72h. CD86 upregulation on DCs (i) and 5T4-specific T-cell stimulation (ii) were analyzed by flow cytometry. The columns show means+SEM of results from triplicate cultures. B, DCs were treated with LPS in the presence of inhibitory peptides targeting MyD88 (20μM) or TRIF (25μM). Control peptides (cell-permeable domain of the inhibitory peptide) were used at the same concentrations. Means+SEM of % TNFα-producing DCs are shown, as detected by cytokine flow cytometry. C, DCs were cultured in a cross-presentation assay in the presence of MyD88 and TRIF inhibitory peptides together or with control peptides (25μM each). Mean+SEM of % IFNγ+ T cells from triplicate cultures are shown. The experiments were repeated 2-3 times.

**Figure 5.** TLR4 polymorphism does not affect tumor antigen cross-presentation. Ai, TLR4 expression on monocytes from 5 donors with the Asp299 (299A) and 4 donors with the polymorphic Gly299 (299G) allele; ii, TLR4 expression on day 5 DCs. iii, day 5 DCs were stimulated with LPS and TNFα production was 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 represent the medians. B, DC phenotyping from donors as in A. DCs were co-cultured with 0Gy- or 12Gy-irradiated DU145 cells. Each line represents an individual donor. C, Stimulation of 5T4-specific T cells with DCs, derived from 3 donors in each group (as in A), loaded with untreated or 12Gy-irradiated or oxaliplatin-treated DU145 cells, respectively. Means+SEM of % IFNγ+ T cells are shown from triplicate samples.

**Figure 6.** Hsp70 inhibition abolishes antigen cross-presentation. A, The effect of VER155008 on DU145 cell numbers after 72h culture. B, Different types of cell death
as detected by Annexin/PI staining in the absence or presence of VER155008. C, Surface expression of Hsp70 (gray) vs. isotype (black) in the absence or presence of VER155008: (i) summary from triplicates; (ii) representative histograms. D, The effect of VER155008-treated or untreated DU145 cells on CD86 expression of DCs following a 24h co-culture: (i) representative histograms; (ii) summary from triplicates. E, Stimulation of 5T4-specific T cells in a cross-presentation experiment with DCs loaded with VER155008 or PES-treated or untreated DU145 cells. This experiment was carried out with DCs derived from two donors. A-E, Means+SEM of results from triplicate samples are shown.

Figure 7. Hsp70 receptor expression on DCs and the effect of receptor-blocking on antigen cross-presentation. A, Representative data of CD91 or SREC-I expression on the surface of day 5 DCs (i) compared to isotype control. Numbers in histograms represent % positive DCs. (ii) Means+SEM of mean fluorescence intensity (mfi) of antibody binding by DCs, as indicated, from triplicate samples. B, T-cell stimulation in a cross-presentation experiment in the presence of CD91 or SREC-I neutralizing (N) antibodies or isotype controls (Iso), respectively. Means+SEM of % IFNγ+ T cells are shown from triplicate samples.
Figure 2

A

B

C

D

M = Marker
1 = 2h 42°C
2 = 72h post IR
3 = 3h post IR
4 = Nil
Figure 5

A i

\[
\begin{array}{c}
\text{TLR4}^+ \text{monocytes (\%)} \\
\text{TLR4}^+ \text{DC (\%)} \\
\text{TNF-\alpha}^+ \text{DC (\%)} \\
\end{array}
\]

299A 299G

\[
\begin{array}{c}
\text{NS} \\
\text{NS} \\
\text{NS} \\
\end{array}
\]

B

\[
\begin{array}{c}
\text{CD86 (mfi)} \\
\text{HLA-DR (mfi)} \\
\text{CD83 (mfi)} \\
\end{array}
\]

299A 299G

\[
\begin{array}{c}
\text{\*} \\
\text{\*} \\
\text{\*} \\
\end{array}
\]

irradiation of DU145 cells (Gy)

C

\[
\begin{array}{c}
\text{IFN-\gamma}^+ \text{T cells (\%)} \\
\end{array}
\]

Donor: 1 2 3

299A 299G

\[
\begin{array}{c}
\text{\*} \\
\text{\*} \\
\text{\*} \\
\text{\*} \\
\text{\*} \\
\text{\*} \\
\text{\*} \\
\text{\*} \\
\text{\*} \\
\end{array}
\]

Untreated 12Gy Oxaliplatin

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Figure 6

A

**

B

NS

C i

NS

D i

no inhib | VER

DC

isotype

DC

0 Gy

DC

12 Gy

DC

0 Gy + VER

DC

12 Gy + VER

CD86

% CD86

or isotype Ab + DC

C ii

0 Gy

0 Gy + V

12 Gy

12 Gy + V

Hsp70

Hsp70 surface expression

D ii

% CD86 or isotype Ab + DC

E

**

**

NS

**

***

***

**

No inhibitor

VER

PES

% IFNγ+ T cells

Donor A

Donor B

DC+ Nil

0 Gy DU145

12 Gy DU145

DC+ Nil

0 Gy DU145

12 Gy DU145
Cross-presentation of the oncofoetal tumor antigen 5T4 from irradiated prostate cancer cells - a key role for heat shock protein 70 and receptor CD91

Josephine Salimu, Lisa Kate Spary, Saly Al-Taei, et al.

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