TUMOUR ANTIGEN CROSS-PRESENTATION FROM IRRADIATED TUMOUR CELLS AND THE ROLE OF TLR4 POLYMORPHISM

PhD dissertation

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Publications

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Manuscripts under review

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Abstracts

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Presentations

Presentations to learned Societies: International

Salimu J, Spary LK, Al-Taei S, Tabi Z., (03/2013) **Cross-presentation of tumor antigens from irradiated prostate cancer cells by dendritic cells.** Keystone Symposium: Understanding Dendritic Cell Biology to Advance Disease Therapies, (03/2013) Colorado, USA. Poster Presentation

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Summary

Immune responses contribute to the success of radiation therapy of solid tumours; however, the mechanism of triggering $CD8^+$ T cell responses is poorly understood. Antigen cross-presentation from tumour cells by dendritic cells (DC) is a likely dominant mechanism to achieve $CD8^+$ T cell stimulation. We established a cross-presentation model in prostate cancer in which DC present a naturally expressed oncofetal tumour antigen (5T4) from irradiated DU145 tumour 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 tumour cells lead to efficient phagocytosis of tumour cells and upregulation of CD86 and HLA-DR on DC. $CD8^+$ 5T4-specific T cells, stimulated with these DC, proliferated and produced IFNγ. 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, I found no functional evidence that DC with Asp299Gly toll-like receptor-4 (TLR4) single nucleotide polymorphism had impaired ability to cross-present tumour antigen. However, I observed a highly significant and robust prevention of antigen cross-presentation when tumour cells were pretreated with the novel Hsp70 inhibitor, VER 155008. The inhibitor also prevented CD86 upregulation on DC co-cultured with irradiated tumour cells. Together, the results in this thesis demonstrate that radiation induces immunologically relevant changes in tumour cells, which can trigger $CD8^+$ T cell responses via a predominantly Hsp70-dependent antigen cross-presentation process.

Abbreviations

ADCC	Antibody Dependent Cell-Mediated Cytotoxicity
ADT	Androgen Deprivation Therapy
APC	Antigen Presenting Cells
ATP	Adenosine Triphosphate
BLCL	B Lymphoblastoid Cell Lines
CFSE	Carboxyfluorescein Succinimidyl Ester
CRT	Calreticulin
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte Antigen – 4
DAMPs	Damage Associated Molecular Patterns
DC	Dendritic Cells
DMSO	Dimethyl Sulphoxide
EBRT	External Beam Radiotherapy
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
F-Actin	Filamentous Actin
FADD	Fas-associated Death Domain
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GRP	Glucose Regulated Protein
HD	Healthy Donor
HDR	High Dose Rate
HLA	Human Leukocyte Antigen
HMGB1	High-Mobility Group Protein B1
HNSCC	Head and Neck Squamous Cell Carcinoma
HSP	Heat Shock Protein
IL	Interleukin
IR	Ionising Radiation
IRF3	Interferon Regulatory Factor 3
LDR	Low Dose Rate
LPS	Lipopolysaccharide
MCA	Methycholanthrene
mCRPC	Metastatic Castrate Resistant Prostate Cancer
MFI	Mean Fluorescent Intensity
MGUS	Monoclonal Gammopathy of Undetermined Significance
MHC	Major Histocompatibility Complex
MM	Multiple Myloma
MyD88	Myeloid Differentiation Primary Response Protein 88
NK	Natural Killer
NLRP3	NLR Family, Pyrin Domain Containing-3
NOD	Nucleotide-binding Oligomerization Domain

PAP	Prostatic Acid Phosphatase
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCa	Prostate Cancer
PD-1	Programmed Death-1
PD-L1	Programmed Death-Ligand 1
PDGF	Platelet Derived Growth Factor
PRR	Pattern Recognition Receptor
PSA	Prostate Specific Antigen
PSMA	Prostate Specific Membrane Antigen
NF-κB	Nuclear Factor κ B
RAG-2	Recombination Activation Genes-2
RAGE	Receptor for Advanced Glycation Endproducts
RER	Rough Endoplasmic Reticulum
RIPK-1	Receptor Interaction Kinase-1
RLR	RIG-I Like Receptor
ROS	Reactive Oxygen Species
RT	Radiation Therapy
SREC-I	Scavenger Receptor Expressed by Endothelial Cell-i
SNP	Single Nucleotide Polymorphism
TAA	Tumour Associated Antigen
TAP	Transporter Associated with Antigen Processing
TGF-β	Transforming Growth Factor-Beta
TILs	Tumour-Infiltrating Lymphocytes
TIM-3	T-cell Ig and Mucin Containing Domain-3
TIR	Toll IL-1 Resistance
TLR	Toll Like Receptor
TNF-α	Tumour Necrosis Factor-Alpha
TRAM	TRIF-Related Adaptor Molecule
TRIF	Toll/IL-1R Domain Adaptor Inducing Interferon
VEGF	Vascular Endothelial Growth Factor

I. Introduction. The Role of the Immune System in Protection Against Tumours

Immunosurveillance Theory

In 1909 Paul Ehrlich proposed the notion that cancer occurs spontaneously and that the immune system is able to both recognize and protect against it (Ehrlich, 1909). However, this theory could not be experimentally tested because so little was known at the time about the molecular and cellular basis of immunity. Years later, based on the increasing number of observations in mouse models, Lewis Thomas and McFarlane Burnet proposed a theory of "immune surveillance". This theory suggests that effector T cells of the immune system actively patrol the body to identify and eradicate nascent malignancies (Thomas, 1982, Burnet, 1970).

At the time, such work was considered controversial given the evidence that appeared to disapprove the immunosurveillance hypothesis. Athymic nude mice did not have increased susceptibility to tumours induced by 3-methycholanthrene. However, it is now known that Natural Killer (NK) cells are present and functional in nude mice (Shouval et al., 1983). Since then, gene-targeted mice, specific immune system activators and blocking monoclonal antibodies specific for immunologic components have helped to substantiate the immunosurveillance theory.

The important question is how cells of the immunosurveillance network distinguish nascent transformed cells or established tumour cells from normal cells. A role for the immune system in the prevention of tumours is to specifically identify and eliminate tumour cells based on the expression of tumour-associated antigens (TAA) or molecules induced by cellular stress (Swann and Smyth, 2007). Cancer cells express antigens that differentiate them from their non-transformed counterparts. These TAA are often products of mutated cellular genes, over-expressed or aberrantly expressed normal genes or genes encoding viral proteins (Criscitiello, 2012). During adaptive immune responses, tumour cells expressing TAA are eliminated by tumour-specific T cells that recognise the peptide-Major

Histocompatibility Complex (pMHC) complexes in which the peptide components are encoded by e.g. mutant DNA sequences (Nakachi et al., 2004). On the other hand, overexpression of stress-inducible proteins such as NKG2D ligands (MICA, MICB, ULBPs) is required for tumour recognition by the innate immune system (Vesely et al., 2011).

Evidence of immunosurveillance

The first piece of evidence for immunosurveillance came from a study of mice deficient in the recombination activation genes (RAG-2), which are completely deficient in antigen-specific immune cells, such as T, B, and NKT cells due to an inability to rearrange lymphocyte antigen receptors. When RAG-$2^{-/-}$ and wild type mice were subcutaneously injected with chemical carcinogen methycholanthrene (MCA) and monitored for tumour development, RAG-2 knockout mice developed tumours earlier than wild type mice. Thus, T, B and NKT cells are essential to suppress the development of chemically induced tumours (Shankaran et al., 2001).

NK cells are important in cancer immunosurveillance as NK deficient mice were found to have significantly greater death rates with spontaneous malignant tumours late in life (Haliotis et al., 1985). C57BL/6 mice depleted of both NK and NKT cells using the NK1.1 mAb were two to three times more susceptible to MCA-induced tumour formation than wild-type controls (Smyth et al., 2001).

Perforin is a key component of cytolytic granules, which mediate $CD8^+$ T cell and NK cell cytotoxicity. Perforin controlled tumour growth in wild type C57BL/6 mice compared to perforin-deficient mice when tumour elimination was dependent on NK cells (Street et al., 2001). Additionally, perforin-deficient mice were also 1000-fold more susceptible to transplanted lymphomas compared with immunocompetent mice when tumour rejection was controlled by $CD8^+$ T cells (Smyth et al., 2000). This demonstrates that lymphocyte-mediated cytotoxicity induced by perforin plays an important role in promoting host resistance to tumours.

The role of cytokines in immunosurveillance is important as they contribute to the tumour elimination by immune cells. Antibody neutralisation of IFNγ or the genetic

deficiency of IFNγ or the IFNγ receptor have consistently shown to result in an increase in chemically induced carcinogenesis and spontaneous tumour development (Dighe et al., 1994, Kaplan et al., 1998). IFNγ has demonstrated antitumor effects by inhibiting tumour proliferation (Kominsky et al., 1998).

Cancer immunosurveillance in humans

A number of clinical observations have provided evidence supporting the notion of cancer immunosurveillance. Firstly, immunocompromised individuals with congenital or acquired immunodeficiencies or immunosuppressed transplant recipients have a heightened risk of malignancy. Most of the cancers that do develop during states of immunodeficiency are cancers related to viral infections such as human herpes virus 8 (Kaposi sarcoma), Epstein-Barr virus (various lymphomas) and Human Papillomavirus (cervical cancer) (Boshoff and Weiss, 2002). However, increased frequencies of numerous solid non-haematological cancers without known viral aetiology have also been observed in immunocompromised individuals (Sampaio et al., 2012). For example, there is evidence of increased incidences of solid cancers in AIDS patients such as a 3.5-fold elevated risk of lung cancer, independent of smoking, compared to the wider population (Chaturvedi et al., 2007, Kirk et al., 2007). The lung cancer risk of patients undergoing organ transplantation is approximately 20 to 25 times that of the general population in the USA, with an incidence of 0.28% to 4.1% in patients after heart and lung transplants (Bellil and Edelman, 2006). In another study, assessment of over 5000 Nordic renal transplant recipients between 1964 and 1982, showed increased standardized cancer incidence ratios for colon, lung, bladder, kidney, ureter, and endocrine tumours compared to the general population (Birkeland et al., 1995).

Human tumours often contain immune cells referred to as tumour-infiltrating lymphocytes (TILs). The association between favourable patient prognosis and TILs was first observed in patients with melanoma (Clark et al., 1989, Clemente et al., 1996), where it was reported that patients with high levels of $CD8^+$ T cell infiltration survive longer than those whose tumours contain low numbers of lymphocytes. The presence of TILs, and in some studies $CD8^+$ T cells, has now been shown to be a favourable independent predictor of survival for many tumours including ovarian

cancer (Zhang et al., 2003), colorectal cancer (Baier et al., 1998), urothelial cancer (Sharma et al., 2007) and cervical cancer (Piersma et al., 2007).

Antibody and T cell responses against TAA such as the cancer-testis antigen NY-ESO in cancer patients compared with healthy individuals provide evidence that the immune system can recognize malignant cells (Jäger et al., 1999, Jäger et al., 2000). This may be due to overabundance of antigen or its enhanced presentation to generate immunogenicity in the malignant setting. Paraneoplastic autoimmune syndrome is caused by activation of antitumor immune responses specific for self-antigens expressed on tumour cells. For example, neurological paraneoplastic syndromes are characterised by both high titres of antibodies and lymphocytes reactive to antigens shared between tumour and neural tissue (Posner, 2003). A paraneoplastic immune response can precede tumour diagnosis by a number of years, indicating that antitumor responses might be primed even by undetectable microscopic tumours at pre-clinical stages of development (Mathew et al., 2006).

Epidemiologic studies found childhood infections might lower the risk for cancer in adulthood. Sera samples from patients with mumps induced parotitis and healthy controls were obtained, and anti-MUC-1 antibodies as well as antigen levels of the ovarian cancer antigen CA-125 and MUC-1 were analysed. The level of anti-MUC-1 antibodies was significantly higher in mumps cases compared to controls. Free circulating levels of CA-125, but not MUC-1, were also higher in mumps cases. Meta-analysis addressing the association showed a 19% decrease in risk of ovarian cancer associated with a history of mumps-induced parotitis. The suggestion is that mumps-induced parotitis may lead to the expression and immune recognition of normal or aberrant MUC-1 and creates effective immune memory against the MUC-1 antigen which may provide protection against ovarian cancer (Cramer et al., 2010).

The spontaneous recognition and destruction of human cancers by cells of the adaptive immune system substantiates the occurrence of cancer immunosurveillance in humans. However, tumours do still develop in the presence of a functioning immune system. The concept of cancer immunoediting explains how tumour can arise in seemingly immunocompetent hosts, despite the multitude of immune effector functions in place to protect against carcinogenesis.

Cancer immunoediting theory

Cancer immunoediting emphasizes the dual roles of immunity in protecting the host from tumour development whilst also promoting tumour growth (Dunn et al., 2002). The theory of immunoediting is composed of 3 phases: elimination, equilibrium, and escape (Figure 1.1). The elimination phase of cancer immunoediting is the same process described in the initial theory of immunosurveillance whereby the immune cells locate, recognize, and destroy transformed cells and prevent the development of malignancy (Dunn et al., 2002).

In the equilibrium phase, the host immune system and any tumour cells that have survived the elimination phase enter into a dynamic equilibrium phase, where lymphocytes and cytokines exert potent effects sufficient to prevent any tumour expansion but not enough to completely eliminate all the tumours. The survival of the remaining tumour cells is favoured by numerous genetic instabilities and immunoselection making them resistant to immune mediated killing. This process could take place over many years (Prestwich et al., 2008). The existence of a vigorous T cell immune response to pre-malignant monoclonal gammopathy of undetermined significance (MGUS) cells that eventually progress to multiple myeloma (MM) is consistent with the equilibrium phase. At this disease stage, the immune system controls but does not eliminate the MGUS cells that eventually evolve and progress to malignancy (Dhodapkar et al., 2003, Swann and Smyth, 2007).

In breast cancer patients, successful treatment of primary tumour and subsequent relapse, at least 10 years later, of patients remaining disease free despite evidence of micrometastatic disease is suggestive of tumour dormancy (Karrison et al., 1999). Reported cases in which a donated organ transmitted tumours to the recipient is also suggestive of tumour dormancy in the donor (Myron et al., 2002). It is possible that tumour development was being controlled by the immune system of the immunocompetent donor and that transplantation of the organ into an immunosuppressed host allowed tumour outgrowth.

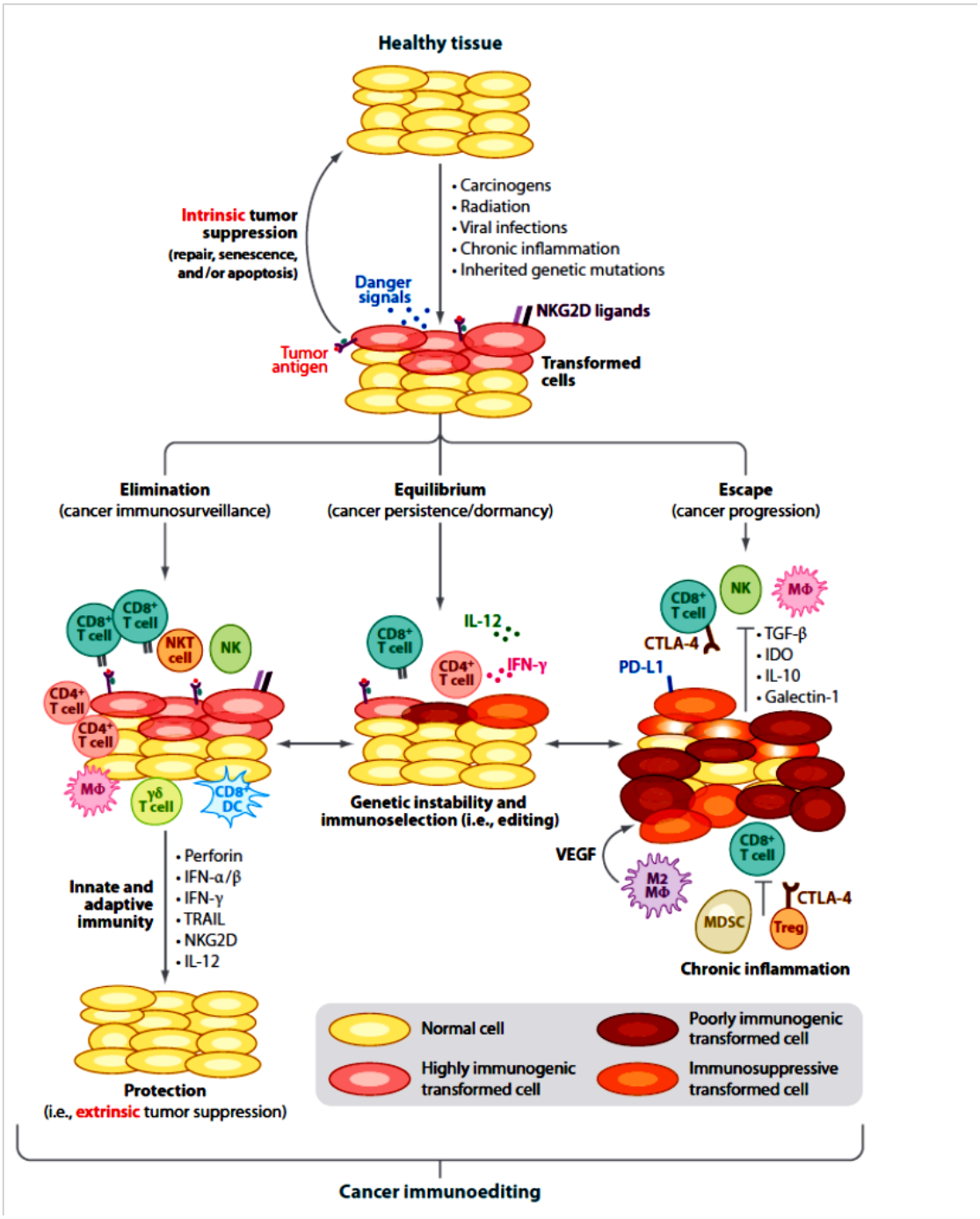


Figure 1.1: Cancer immunosurveillance and immunoediting. In cancer immunosurveillance, transformed cells escaping intrinsic tumour suppression mechanisms are subjected to extrinsic tumour suppression mechanisms that detect and eliminate developing tumours. Cancer immunoediting is composed of 3 phases: 1) Elimination of cancer cells (representing the classical concept of cancer immunosurveillance); 2) Equilibrium, a phase of tumour dormancy where tumour cells and immune cells reach a state that keeps tumour expansion in check. This phase may select for the survival of tumour cells with new mutations and favour resistance to immune control. 3) Escape, the balance between immunological control of the tumour and tumour progression tips in favour of tumour growth even in the presence of an antitumor immune response (Vesely et al., 2011).

The escape phase represents the failure of the immune system to either eliminate or control transformed cells, allowing them to become malignant. Tumour cells can evade the immune system by a host of different strategies that entail reduced immunogenicity, resistance to killing by immune effector cells or subversion of the immune responses (Zitvogel et al., 2006). Tumour cells are able to prevent T cell recognition of TAAs via the downregulation of MHC-molecules (Bai et al., 2003). In some cases, tumour cells are unable to produce the intracellular machinery that facilitates antigen processing and presentation (i.e. TAP1 and TAP2). Genomic instability of the tumour cells may result in the loss of TAA, creating antigen loss variants that are no longer detectable by the antigen-specific T cells (Vesely et al., 2011).

Resistance to immune mediated killing is accomplished by altering major mechanisms that mediate immune cytotoxicity. These alterations include impaired binding of perforin to the tumour cell surface which provides resistance to perforin mediated killing (Lehmann et al., 2000), downregulation or mutation of the cell death inducer receptor (FAS) in tumours which affects the binding of the cell death inducer ligand Fas-ligand (FasL) on T cells (Real et al., 2001), or mutations in the TNF-related apoptosis-inducing ligand receptors in tumours (Shin et al., 2001). Tumours can also evade effector lymphocytes by upregulating expression of antiapoptotic molecules such as FLIP and BCL-XL (Kataoka et al., 1998, Hinz et al., 2000) or expressing inhibitory cell surface molecules that induce cytotoxic T cell apoptosis such as programmed death-ligand 1 (PD-L1) (Dong et al., 2002) and FasL (Li et al., 2002).

Tumour cells also secrete factors to directly subvert the function of both innate and adaptive immune cells. Antitumor immunity can be subverted at an early stage by tumour-derived factors that inhibit dendritic cell (DC) function. In response to danger or cellular stress, DC are stimulated to mature, migrate and carry tumour antigens to lymph nodes to alert the adaptive immune system to the presence of transformed cells. To inhibit this initial priming event, tumour cells secrete sterol metabolites to suppress the expression of CCR7 on the DC, thereby disrupting DC migration to the lymph nodes (Villablanca et al., 2010). Many tumours produce

vascular endothelial growth factor (VEGF), which is critical for tumour angiogenesis, but also inhibits the ability of DC to stimulate T cells (Mimura et al., 2007). TGF-β secretion by tumour cells leads to inhibition of DC activation as well as direct inhibition of T cell and NK cell function (Wrzesinski et al., 2007). IL-10 present within tumours can suppress DC function and skew T cell responses towards a Th2-type immune response that is less effective against malignant cells (Itakura et al., 2011, Corinti et al., 2001, Aruga et al., 1997). Stromal cells in the tumour microenvironment can skew DC differentiation and function towards an immunosuppressive phenotype with elevated PD-L1 expression (Spary et al., 2014)

A variety of immunosuppressive leukocytes can suppress immune function. The production of GM-CSF, IL-1β, VEGF, and prostaglandin E_2 (PGE_2) by tumour cells leads to the expansion of myeloid-derived suppressor cells (MDSC) and their accumulation within the tumour. MDSC are a heterogeneous group of myeloid progenitor cells and immature myeloid cells that can inhibit lymphocyte function by a number of mechanisms (Gabrilovich and Nagaraj, 2009). The production of TGF-β by MDSC induces anergy of NK cells (Li et al., 2009a). MDSC inhibit T cell activation by depleting or sequestering amino acids arginine and cysteine (Srivastava et al., 2010) as well as directly disrupting the binding of specific pMHC complexes to $CD8^+$ T cells (Nagaraj et al., 2007). The development of regulatory T cells (Tregs) is induced by MDSC (Huang et al., 2006).

Tregs are critical mediators of peripheral tolerance under physiological settings but are often recruited to the tumour site where they suppress antitumor immunity. They inhibit $CD8^+$ T cell function in a number of ways, including IL-10 and TGF-β production, cytotoxic T lymphocyte antigen-4 (CTLA-4) and PD-L1 expression, and IL-2 consumption (Terabe and Berzofsky, 2004). Furthermore, TGF-β production by tumour cells can convert effector T cells into Tregs, that in turn suppress other effector T cells, which infiltrate the tumour (Sakaguchi et al., 2009).

Cytokines, produced at the tumour site, such as IL-4, IL-13 and IL-10 induce M2 macrophages. M2 macrophages can inhibit antitumor immunity through the production of TGF-β and IL-10 and can promote stromal development and

angiogenesis through secretion of platelet-derived growth factor (PDGF) (Sica et al., 2008).

The complexity of cancer immunobiology

According to the immunoediting hypothesis, tumour cell selection favours not only cells that can evade the immune system, but also tumour cells that may support a tumour-promoting immune response. Whereas full activation of adaptive immune cells in response to the tumours might result in eradication of malignant cells, chronic activation of various types of innate immune cells in or around pre-malignant tissue sometimes promotes tumour development (de Visser et al., 2006).

Innate immune cells, such as DC, NK cells, macrophages, neutrophils, basophils, eosinophils and mast cells, are the first line of defence against foreign pathogens. DC, macrophages and mast cells serve as sentinel cells that are found in tissues and continuously monitor their microenvironment for signs of distress. When tissue homeostasis is perturbed, sentinel macrophages and mast cells immediately release soluble mediators such as cytokines, chemokines, matrix remodelling proteases, and reactive oxygen species (ROS), as well as biochemical mediators such as histamine that induce mobilization and infiltration of additional leukocytes into damaged tissues, a process known as inflammation (de Visser et al., 2006). However, chronic inflammation can promote tumour development, with the innate cells providing proliferation and angiogenic signals. Malignant tissues that contain infiltrates of some innate cell types, such as macrophages in human breast carcinoma and mast cells in lung adenocarcinoma and melanoma, tend to be associated with an unfavourable clinical prognosis (Leek et al., 1996, Leek et al., 1999, Imada et al., 2000, Ribatti et al., 2003). Moreover, population based studies reveal that individuals who are prone to chronic inflammatory diseases have an increased risk of cancer development (Balkwill et al., 2005). In addition, over 15% of all human cancers are believed to be caused by infectious conditions (Pagano et al., 2004), some of which indirectly promote carcinogenesis through induction of chronic inflammatory states (Balkwill and Mantovani, 2001).

In contrast, infiltration of NK cells in human gastric or colorectal carcinoma is associated with a favourable prognosis (Ishigami et al., 2000, Coca et al., 1997). Therefore, the innate immune system can play a key role in initiating a protective antitumor immune response but can also inhibit it. Cancer immunoediting and tumour-promoting inflammation might not be mutually exclusive processes, but rather potentially overlapping immune responses. Both MyD88 and IL-1β have been shown to promote tumourigenesis in a number of primary carcinogen models (Swann et al., 2008, Krelin et al., 2007), but MyD88 and IL-1β are also critical to the development of antitumor immunity against established tumours through recognition of dying tumour cells undergoing immunogenic cell death (Apetoh et al., 2007b, Ghiringhelli et al., 2009). Furthermore, while TNF-α is important for tumour apoptosis and the priming, proliferation and recruitment of T cells (Calzascia et al., 2007), it can also mediate cancer development (Szlosarek and Balkwill, 2003). The various mechanisms by which TNF-α promotes cancer growth, invasion, and metastasis include acting as a growth factor in certain tumour types by increasing concentrations of positive cell-cycle regulators (and decreasing levels of CDK inhibitors) and components of growth-factor-receptor signalling pathways such as RAS or c-MYC (Gaiotti et al., 2000). TNF-α also induces chemoresistance in several cancers (Maeda et al., 1994) and mediates androgen independence in prostate cancer (Mizkami et al., 2000).

Given the complexity of cancer immunoediting, the identification of key immune molecules and cells important for the elimination of nascent transformed cells may provide opportunities to harness specific aspects of immunity to induce tumour regression. The inhibition of tumour escape mechanisms may also render tumour cells visible for immune recognition, enabling immune mediated destruction, which is achieved by some traditional cancer treatments such as radiotherapy and chemotherapy.

Immune cells involved in antitumor responses

Dendritic Cells (DC)

DC are members of the innate immune system and function as key players during the induction phase of adaptive immune responses. For an anticancer immune response

to lead to effective killing of tumour cells, a series of events must be initiated and allowed to proceed. In the first step, tumour cells expressing TAA are captured by DC for processing; secondly, DC present the captured antigen on MHC molecules to T cells leading to the third step involving priming and activation of effector T cell responses against the tumour specific antigen.

DC are a set of antigen presenting cells (APC) present in lymph nodes, spleen and at low levels in blood that are particularly effective at stimulating T cells. They are one of the key features of the innate immune system as they have the ability to rapidly recognize pathogen and tissue injury and have the ability to signal the presence of danger to cells of the adaptive immune system. DC are unique APC as they are the only ones that are able to induce primary immune responses by priming naïve T cells thus permitting establishment of immunological memory (Banchereau et al., 2000).

The origin and subsets of human DC

DC originate from $CD34^+$ hematopoietic stem cells within the bone marrow and circulate through the blood and lymphoid organs. In human blood, plasmacytoid DC and myeloid DC represent two major DC subsets derived from different developmental pathways. In steady state, they can be distinguished based on morphology, surface markers and gene expression profiles. Plasmacytoid DC have a plasma cell-like morphology, are negative for CD11c and CD1a and express relatively low levels of HLA-DR. They are phenotypically distinguished by the presence CD123, CD303 (BDCA-2) and CD304 (BDCA-4) (Chan et al., 2012). Plasmacytoid DC have a strong capacity to produce Type 1 interferon after viral exposure but primarily mediate regulatory rather than stimulatory T cell immune responses in a cancer setting (Wei et al., 2005).

In contrast, myeloid DC are classically characterised by the high expression of CD11c, CD1a, and HLA-DR with the distinguishing morphology of protruding dendrites (Chan et al., 2012). $CD11c^+$ blood DC are divided according to the specific expression of CD1c (BDCA-1) and CD141 (BDCA3). $CD14^+$ peripheral blood monocytes obtained from peripheral blood mononuclear cells (PBMC) and cultured with granulocyte monocyte-colony stimulating factor (GM-CSF) and IL-4 *in vitro* differentiate into myeloid DC (Sallusto and Lanzavecchia, 1994). Myeloid DC can

produce pro-inflammatory cytokines such as IL-12, can prime naïve T cells and activate T cell responses. They are also able to cross-present tumour antigens to antigen-specific T cells. Myeloid DC are widely used for human *in vitro* immunological studies (Mittag et al., 2011, Palucka and Banchereau, 2013) and in cancer vaccines (Guardino et al., 2006, Rosenblatt et al., 2011).

DC maturation

Newly generated myeloid DC home to tissues where they reside as immature cells. Immature DC (iDC) are characterised by high levels of antigen capture and processing but low T cell stimulatory capacity with low expression of co-stimulatory molecules (CD40, CD80 and CD86) and are negative for the DC maturation marker CD83. DC are recruited by chemokines such as CCL2, CCL3 and RANTES to the site of tissue damage or infection upon local inflammation. iDC efficiently capture cells or pathogens at the site using several ways such as phagocytosis, macropinocytosis and endocytosis.

DC express numerous pattern-recognition receptors (PRRs), which permit sensing and transmission of danger signals to adaptive immune cells. PRRs include C-type lectins, Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD-like) receptors, as well as RIG-I like receptors (RLR). These receptors allow DC to sense pathogens, apoptotic and necrotic cells and stressed cell products. Activation of PRRs induces phenotypic changes in DC, specifically the upregulation of the CD83 maturation marker, and CCR7, increased expression of co-stimulatory molecules CD40, CD80 and CD86, and redistribution of MHC molecules from intracellular endocytic compartments to the DC surface (Aguilera et al., 2011, Banchereau et al., 2000). The maturation processes also include the loss of endocytic and phagocytic receptors and downregulation of CD14 on the cell surfaces. PRR activation on DC also leads to the secretion of IL-6, IL-10, TNF-α and IL-12 (Shen et al., 2008). These activated DC play an important role in the bystander activation of other DC, NK, NKT and $CD8^+$ T cells, which secrete IFNγ and other cytokines that aid in tumour and microbe eradication (Rossi and Young, 2005).

DC migration towards secondary lymphoid organs

The ability of DC to migrate from antigen encounter to the sites of T cell priming is fundamental to their capacity to induce primary immune responses. Soon after activation, maturing DC undergo a rapid switch in the expression of chemokines. DC start downregulating the expression of the inflammatory chemokine receptors upon activation, resulting in an unresponsiveness of the maturing DC to inflammatory cytokines such as CCL2 and RANTES. At the same time, the expression of the lymphoid chemokine receptors CXCR4 and CCR7 are strongly upregulated, enabling maturing DC to respond to the lymphoid chemokines CXCL12, CCL21 and CCL19, which are expressed in lymphoid organs (Ricart et al., 2011, Vecchi et al., 1999, Sallusto et al., 2000).

Antigen processing and presentation

During the migration toward secondary lymphoid organs, DC switch from an antigen capturing to an antigen presenting mode allowing them to induce T cell responses. T cells only recognize antigen that has been processed and presented on MHC molecules. Antigen processing is the conversion of native proteins into MHC-associated peptides. MHC molecules play a role in the determination of adaptive immune responses, as the particular set of MHC molecules expressed influences the repertoire of antigens to which that $CD4^+$ and $CD8^+$ T cells can respond (Doherty and Zinkernagel, 1975, Kaye et al., 1989).

Antigen processing and presentation to $CD4^+$ T cells

The role of $CD4^+$ T cells in antitumor responses is to predominantly provide help during priming of naive $CD8^+$ T cell to achieve full activation and effector function of tumour-specific $CD8^+$ T cells. However, they also express both Th1 and Th2 cytokines required for maximal systemic antitumor immunity and recruitment of other immune cells. $CD4^+$ T cells recognize peptides bound to MHC class II molecules expressed on APC such as macrophages, DC and B cells (Germain, 1994).

Structure of MHC class II molecule

MHC Class II molecules have two nonidentical glycoprotein chains, a 33kDa α chain and a 28kDa β chain associated by non-covalent interactions. Each chain in the class II molecule contains two external domains: α1 and α2 domains in one chain and β1 and β2 domains in the other (Brown et al., 1993). MHC class II molecules interact

with peptides derived from endocytic degradation of exogenous antigens. Peptides recovered from MHC class II peptide complexes generally contain 13-18 amino acid residues. The peptide-binding cleft in MHC class II molecules is open at both ends allowing longer peptides to extend beyond the ends (Rudensky et al., 1991, Hunt et al., 1992).

The MHC class II exogenous antigen presentation pathway

APC can internalise exogenous antigen by phagocytosis, endocytosis, or both. Once an antigen is internalized, it is degraded into peptides within compartments of the endocytic processing pathway. The endocytic pathway appears to involve three increasingly acidic compartments: early endosomes (pH 6.0-6.5); late endosomes, or endolysosomes (pH 5.0-6.0); and lysosomes (pH 4.5-5.0) (Clague M.J, 1998). Within the compartments of the endocytic pathway, antigen is degraded into oligopepetides of about 13 to 18 residues, which bind to MHC class II molecules and are thus protected from further proteolysis. The invariant chain interacts with the peptide-binding cleft of the class II molecules, preventing any endogenously derived peptides from binding to the cleft. The invariant chain is also involved in the folding of the class II α and β chains, their exit from the rough endoplasmic reticulum (RER), and the subsequent routing of the class II molecules to the endocytic processing pathway from the trans-golgi network. As the proteolytic activity increases in each successive compartment, the invariant chain is gradually degraded leaving a short fragment of the invariant chain termed CLIP bound to the MHC class II molecule. CLIP physically occupies the peptide-binding groove of the class II MHC molecule, preventing any premature binding of the antigenic peptide. A non-classical MHC class II molecule called HLA-DM is required to catalyze the exchange of CLIP with antigenic peptides (Kropshofer et al., 1999). Once a peptide has bound, the peptide-class II complex is transported to the plasma membrane, where the neutral pH appears to enable the complex to assume a compact, stable form (Nielsen et al., 2010, Blum et al., 2013).

Antigen processing and presentation to $CD8^+$ T cells

MHC class I molecules are expressed on all nucleated cells. Given that nonhematopoietic tumour cells express MHC class I molecules, required for $CD8^+$ T cell recognition, but do not express MHC class II molecules required for $CD4^+$ T cell recognition, predominant tumour recognition and killing occurs by $CD8^+$ T cell. The

generation of $CD8^+$ T cell responses occurs in two phases, both of which involve the process of antigen presentation. In the first phase, APC such as DC gather antigens present in tissues, as described above, and then present them to naive $CD8^+$ T cells in the draining lymph nodes in ways that stimulate their maturation into effector T cells. In the second phase, these effector T cells seek out and eliminate the infected or abnormal cells expressing the appropriate antigens.

Structure of MHC class I molecules

MHC class I molecules have a heavy 45kDa glycoprotein chain associated non-covalently with the small (12kDa) β_2 microglobulin molecule. The α chain of MHC class I molecules is organized into three external domains (α1, α2, α3) (Madden et al., 1992). MHC class I molecules interact with peptides derived from cytosolic degradation of endogenously synthesized proteins. The peptides that bind MHC class I molecules are eight to ten amino acid long and contain specific amino acids (motifs) in key positions that are essential for binding to a particular MHC molecule. This peptide length is most compatible with the closed-ended peptide binding cleft of the class I molecules (Madden et al., 1991).

The MHC class I endogenous antigen presentation pathway

$CD8^+$ T cells seeking out and eliminating infected and abnormal cells use the endogenous antigen presentation pathway. Intracellular proteins are degraded into short peptides by cytosolic protease complexes called proteasomes. Peptides generated in the cytosol by the proteasome are translocated by the transporter protein called transporter associated with antigen processing (TAP) into the RER by a process that requires the hydrolysis of ATP. The optimal peptide length of 9 amino acids for MHC class I binding is achieved by trimming with aminopeptidases present in the ER such as ERAP. The α chain and β_2–microglobulin components of the MHC class I molecule are synthesized on polysomes along the RER. Within the RER membrane, a newly synthesized class I α chain associates with calnexin, until the β_2 microglobulin binds to the α chain. Binding to β_2 microglobulin releases calnexin and allows binding to the chaperonin calreticulin and to tapasin, which is associated with TAP. This association promotes binding of an antigenic peptide, which stabilizes the class I molecule-peptide complex, allowing its release from the RER

and transit to the cell surface via the Golgi complex (Rock et al., 2010, Blum et al., 2013).

The cross-presentation pathway of exogenous antigens

Naive antigen-specific $CD8^+$ T cells cannot directly eliminate tumour cells. To become effector T cells, naive $CD8^+$ T cells need first to be activated by professional APC such as DC (Bousso and Robey, 2003). $CD8^+$ T cells are primarily generated within the lymph nodes and tumour antigens are only present within the lymph node if the tumour cells migrate there (Ochsenbein et al., 2001). Therefore, in the majority of cases, DC acquire tumour antigens in the tumour tissue, and migrate to lymph nodes where they prime naive $CD8^+$ T cells by presenting antigens on MHC class I molecules, by a mechanism known as cross-presentation (Rock et al., 2010). Following uptake, exogenous antigens are internalized into specialized organelles that are termed phagosomes for particulate/cell-associate antigens, or endosomes for soluble protein antigen (McDonnell et al., 2010). There are two best-characterized mechanisms by which peptides for cross-presentation are generated from protein.

The phagosome-cytosol pathway is one of the major cross-presentation mechanisms and involves transfer of the internalized protein from phagosomes to the cytosol. The transferred antigen is then degraded by proteasomes and the resulting peptides are transported to newly synthesized MHC class I molecules by TAP. Hence, similar to direct presentation, this pathway is proteasome- and TAP-dependent (Shen and Rock, 2006). However, the mechanism allowing transfer of proteins into the cytosol is unclear.

The second mechanism of cross-presentation is the vacuolar pathway. This pathway is TAP independent and insensitive to proteasome inhibitors thus it is clearly different from the phagosome-to-cytosol pathway. The generation of cross-presented peptides in the vacuolar pathway is inhibited by cysteine protease inhibitors such as leupeptin; therefore it is suggested that exogenous proteins are degraded into peptides by lysosomal proteases within the lumen of the phagosome or endosome (Rock et al., 2010). These peptides are then loaded onto recycling MHC class I

molecules by peptide exchange. It may be dependent on the type of antigen and the mechanism of uptake that decides the internal route to cross-presentation.

Factors influencing cross-presentation

Although numerous studies have suggested that the capacity to cross-present exogenous antigen may be restricted to a specialized DC subset such as CD1c and CD141 DC, it seems that a cross-presentation program can be initiated in most if not all DC subsets (Nierkens et al., 2013). Factors emerging as important for the modulation of cross-presentation activity in DC are the type and source of antigen, presence of DC immunogenic/stimulatory factors and endocytic/signalling receptors. Potential mechanisms for transfer of tumour antigens to DC for cross-presentation include (Melief, 2008):

- Phagocytosis of cell associated antigens (Albert et al., 1998, Fonseca and Dranoff, 2008),
- Pinocytosis/endocytosis of soluble antigen (Norbury et al., 2004),
- Capture of soluble antigens bound to heat shock proteins (Binder et al., 2007, Giodini and Cresswell, 2008),
- Transfer of small antigenic protein fragments through gap-junctions (Neijssen et al., 2005),
- Capture of antigen-carrying exosomes (Zeelenberg et al., 2008),
- Nibbling of live tumour cell membrane (Harshyne et al., 2001),
- Cross-dressing whereby DC acquire peptide-MHC complexes from contact with necrotic cells (Dolan et al., 2006).

Cell-associated antigens, especially from dead cells, are cross-presented more efficiently than soluble proteins to generate $CD8^+$ T cell responses (Albert et al., 1998). Therefore, while dead cells can generate immune responses, the immunological outcome fundamentally depends on the type of cell death. DC efficiently take up a variety of apoptotic and necrotic tumour cells. However, only exposure to the latter induces DC maturation. Apoptotic cells can suppress the transcription of pro-inflammatory cytokine genes, promote the secretion of anti-inflammatory cytokines by phagocytes and can cause DC to cross-present apoptotic cell-derived antigen in a matter that promotes immunological tolerance (Stuart et al., 2002, Rock and Kono, 2008). This event is associated with the release of anti-

inflammatory mediators like TGF-β or PGE_2 and recruitment of Tregs in order to avoid local inflammation (Tesniere et al., 2007, Lauber et al., 2012, Golden et al., 2012). In contrast, necrotic cell death, which is often passive, leads to the exposure of damage-associated molecular patterns (DAMP) and consequent activation of inflammatory and immune effectors (Sauter et al., 2000). Autophagy also has a role in antigen cross-presentation and T cell cross-priming with cell-associated antigen. Autophagy was required for efficient antigen cross-presentation of OVA-expressing HEK-293T cells or gp100-expressing melanoma cells both *in vitro* and *in vivo* (Albert and Joubert, 2012, Li et al., 2009b, Li et al., 2008). This suggests that cell death modality determines how dead cells are degraded and antigens contained in them are presented.

Immunogenic cell death (ICD) signals

In 1994 Polly Matzinger proposed the 'danger theory', which states that the immune system can distinguish between dangerous and innocuous endogenous signals (Matzinger, 1994). It became evident that dying, stressed or injured cells release or expose molecules on their surface that can function as either adjuvant or danger signals for the innate immune system These signals were later called DAMPs (Garg et al., 2010). Some DAMPs are released (such ATP and high mobility group protein B1 (HMGB1)) or become exposed on the outer leaflet of the plasma membrane (such as calreticulin (CRT) and heat shock protein 70 (Hsp70)). Most of these DAMPs have no immunological functions within the cells until they are secreted into the extracellular space or exposed on the plasma membrane. Table 1.1 has an overview of DAMPs associated with various types of cell death and their immunodulatory function.

Despite the growing list of players contributing to the "ideal" antigen cross-presentation setting, the plasticity of the process has also been demonstrated, for example, highly polarized (type-1) DC can efficiently prime T cells even when co-cultured with apoptotic cells (Wieckowski et al., 2010). Furthermore, DC can acquire antigen from live cells for antigen cross-presentation both in tumour and viral settings (Harshyne et al., 2001, Matheoud et al., 2011, Tabi et al., 2001). In the latter, while apoptosis of infected fibroblasts is inhibited by the virus, Hsp70 expression is significantly upregulated by the infection (Santomenna and Colberg-Poley, 1990).

DAMPs	Receptor	Type of cell death (and mode of emergence)	Immunomodulatory Functions	Refs
ATP	P2Y2 and P2X7	Primary necrosis (passively released) immunogenic apoptosis, cell death accompanied by autophagy	Can act as a 'find me' signal, causes NLRP3-inflammasome-based IL-1β production from DC and mediates mitoxantrone- and oxaliplatin- induced antitumor immunity	(Garg et al., 2012b), (Ghiringhelli et al., 2009), (Michaud et al., 2011), (Elliott et al., 2009)
CRT	CD91	Immunogenic apoptosis (either pre-apoptotic or early or mid apoptotic surface exposure)	A potent 'eat me' signal and mediator of tumour immunogenicity crucial for antitumor immunity.	(Obeid et al., 2007b), (Gardai et al., 2005)
F-actin	DNGR1	Accidental necrosis and secondary necrosis	Helps in recognition of necrotic cells by $CD8\alpha^{+}$ dendritic cells	(Ahrens et al., 2012)
Hsp70, Hsp90, Hsp60, Hsp72, GRP78 and GP96	CD91, TLR2, TLR4, SREC-I and Stabilin-1	Necrosis (passively released) and immunogenic apoptosis (either pre-apoptotic or early or mid-apoptotic surface exposure)	Can attract monocytes and neutrophils. Can cause NK cell activation and DC maturation. Surface-exposed HSP90 can mediate T cell-based antitumor immunity.	(Garg et al., 2012a), (Basu et al., 2000), (Vega et al., 2008)
HMGB1	TLR2, TLR4, RAGE and TIM3	Primary necrosis and secondary necrosis, (passively released). Cell death accompanied by autophagy (early or mid apoptotic active secretion)	Can act as a strong cytokine and attract various immune cells. Can cause DC maturation. Immunostimulatory activity of HMGB1 might be inactivated during apoptosis	(Apetoh et al., 2007a), (Scaffidi et al., 2002), (Thorburn et al., 2008), (Chiba et al., 2012)

Table 1.1: An overview of DAMPs associated with various types of cell death and their immunomodulatory functions (adapted from Krysko et al., 2012). ATP, adenosine triphosphate; CRT, calreticulin; DAMPs, damage-associated molecular patterns; DC, dendritic cells; DNGR1, dendritic cell NK lectin; F-actin, filamentous actin; GRP, glucose regulated protein; HMGB1, high-mobility group protein b1; HSP, heat shock protein; NLRP3, NRL family pyrin domain containing 3; RAGE, receptor for advanced glycation end products; SREC-I, scavenger receptor class F member 1; TIM3, T-cell Ig and mucin containing domain 3; TLR; toll like receptor.

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.

TLR4

TLRs are a family of receptors with a main role in binding a wide array of pathogens and bridge the gap between the innate and the adaptive immune system. TLRs consist of three domains: an exterior region that contains many leucine-rich repeats (LRRs), a membrane-spanning domain and an interior domain called the TIR domain. The ligand-binding site of the TLR is found among the LRRs whilst the TIR domain interacts with the signalling machinery of immune response stimulation. There are at least 10 TLRs expressed in humans. Their cytoplasmic domains are highly homologous, but because of differences in the extracellular domain structure they recognize diverse microbes differently (Kutikhin, 2010). TLR4 is one of the most investigated TLR and has been shown to be essential for tumour antigen cross-presentation in mouse models (Apetoh et al., 2007b).

The gene encoding for TLR4 is located on chromosome 9q32-q33, contains 4 exons and is expressed on lymphocytes, monocytes, macrophages and DC. TLR4 binds microbial ligands such as lipopolysaccharide (LPS), respiratory syncytial virus (RSV) fusion protein and the component of *Cryptococcus neoformans*, glucuronoxylomannan (Kutikhin, 2010). TLR4 also binds various endogenous ligands, including Hsp60, Hsp70 and gp96, β-defensin and HMGB1. Upon association with the ligands, TLR4 transduces signals through two pathways involving distinct adaptors, Toll/IL-1R (TIR) domain containing adaptor inducing interferon (TRIF) and myeloid differentiation primary response protein 88 (MyD88) (Figure 1.2). The MyD88 adapter-like protein (MAL) mediates the MyD88 pathway. Initiation of the MyD88-dependent pathway activation leads to the activation of the nuclear factor κB (NF-κB) and AP-1 and the transcription of pro-inflammatory genes. The TRIF-related adapter molecule (TRAM) mediates the TRIF-dependent pathway. Initiation of the TRIF pathway leads to the activation of interferon regulatory factor 3 (IRF3), and the expression of IFN-β and IFN-inducible genes (Ferwerda et al., 2008).

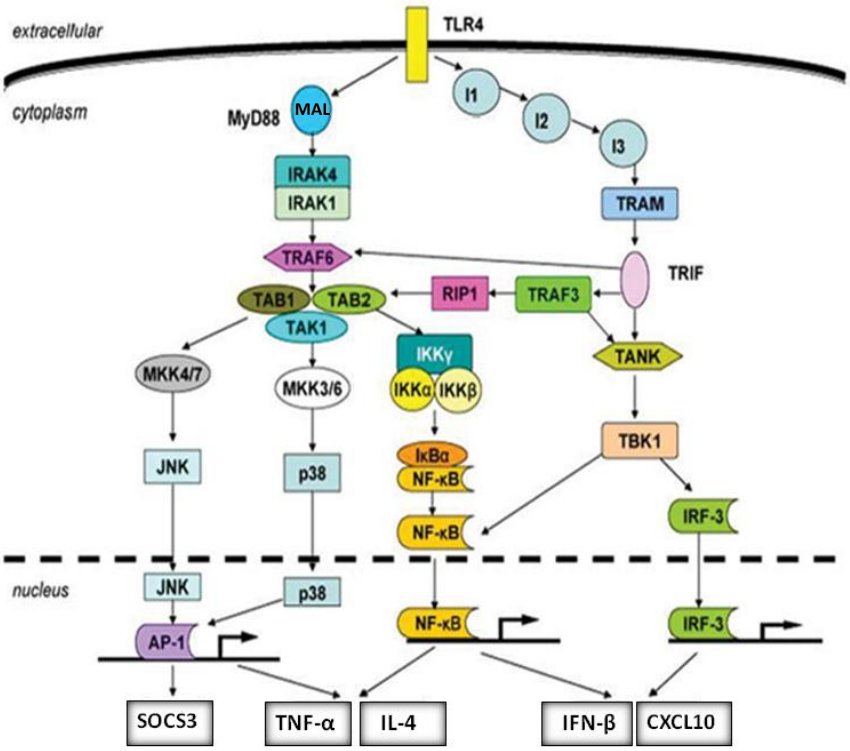


Figure 1.2 TLR4 activates the MyD88-dependent and the TRIF-dependent pathways. MAL and TRAM are required for the activation of MyD88- and TRIF dependent pathways, respectively, MyD88 recruits IRAK4 and TRAF6 upon ligand stimulation. TRAF6 activates TAK1/TAB1/TAB2/TAB3 complex via K63-linked ubiquination (Ub). Activated TAK1 complex then activates the IKK complex consisting of IKKα, IKKβ and IKKγ/NEMO, which catalyze IκBs (P). IκBs are destroyed by the proteasome pathway, allowing NF-κB to translocate into nuclei. TAK1 simultaneously activates the MAP kinase pathway, which results in phosphorylation (P) and activation of AP-1. NF-κB and AP-1 control inflammatory responses by inducing pro-inflammatory cytokines. TLR4 also recruits TRAM and TRIF, which interacts with TBK1. TBK1 together with IKKi mediates phosphorylation of IRF3 (P). Phosphorylated IRF3 is dimerized and translocated into nucleus to bind DNA. TRIF also interacts with TRAF6 and RIP1, which mediate NF-κB activation. Activation of IRF3, NF-κB and AP-1 is required for induction of type I IFN, particularly IFN-β. (Adapted from (Selvarajoo, 2013))

TLR4 in antigen cross-presentation

The role of TLR4 in efficient cross-presentation has been demonstrated in $TLR4^{-/-}$ mouse DC which failed to cross-present antigen from irradiated dying tumour cells to T cells *in vitro* (Apetoh et al., 2007b). Within $TLR4^{-/-}$ mouse DC, antigenic particles were more rapidly destroyed via the lysosomal pathway than in wild type DC. In the same study, HMGB1 was detected in the supernatant of dying cells. Binding of HMGB1 to TLR4 was previously shown using fluorescence resonance energy transfer analyses and immunoprecipitation (Park et al., 2005) and recently knockdown or neutralization of HMGB1 was also carried out. Consequently, protection against tumours was lost and inhibition of tumour antigen presentation and lack of T cell priming were observed *in vivo*. Therefore, the research by Apetoh et al (2007) demonstrated that both the release of HMGB1 by dying tumour cells and the TLR4-MyD88 signalling pathway are required for the immune response against tumours and also for the efficacy of anticancer chemotherapy and radiotherapy in mice (Apetoh et al., 2007b).

TLR4 single nucleotide polymorphism (SNP)

Two cosegregating missense SNPs have been identified in the *TLR4* gene at minor allele frequencies between 8 and 10% in Caucasian populations, which result, respectively, in aspartic acid to glycine substitution at position 299 (Asp299Gly) and threonine to isoleucine substitution at position 399 (Thr399Ile) in the receptor protein. These SNPs are situated within the extracellular domain of TLR4 and are associated with impaired ligand-receptor binding (Apetoh et al., 2007b), alteration in the dimerization of the TLR4/MD2 complex (Yamakawa et al., 2013) and/or interference with the recruitment of TLR4 adaptors, MyD88 and TRIF (Figueroa et al., 2012). The Asp299Gly SNP removes a potential negative charge and increases rotational freedom about the peptide bond, while the Thr399Ile SNP increases the overall steric bulk in the extracellular domain, possibly preventing ligand/cofactor (MD2) docking (Rallabhandi et al., 2006). This results in the reduced capacity for individuals to mount immune responses against TLR4 ligands.

Activation of NFκB leads to the release of inflammatory cytokines, chemokines and co-stimulatory molecules. The inflammatory mediators can exert various atherogenic

effects involving the expression of adhesion molecules on endothelial cells, proliferation of smooth muscle cells, activation of immune cells and activation of the acute phase response (Kiechl et al., 2002). A study by Kiechl et al (2002), showed that compared with the carriers of the wild-type TLR4, subjects with the Asp299Gly mutated allele had lower levels of some inflammatory cytokines, acute phase reactants, soluble adhesion molecules, and other mediators of inflammation such as IL-6, soluble vascular cell adhesion molecule-1 (sVCAM), and Neopterin. Most importantly, these individuals had a reduced risk of atherosclerosis. The reason for this has been suggested to be the reduced inflammatory mediators responsible for exerting atherogenic effects (Kiechl et al., 2002).

Additionally, carriers of the Asp299Gly allele appeared to be more susceptible to bacterial infections compared to those carrying the wild type (Kiechl et al., 2002). The TLR4 Asp299Gly allele was found exclusively in patients with septic shock. Patients with septic shock with the TLR4 Asp299Gly/Thr399Ile alleles had a higher prevalence of gram-negative infections (Lorenz et al., 2002). The lower levels of cytokine production in individuals with the polymorphisms may subsequently increase their susceptibility to bacterial infection, as they are unable to clear the invading microorganisms.

The functional consequence of the Asp299Gly SNP in a tumour antigen cross-presentation setting has only been demonstrated by Apetoh et al (2007). DC from individuals bearing the mutation had a severely impaired capacity to cross-present MART-1 antigen derived from dying melanoma cells to a MART-1 specific CTL clone compared to individuals with the Asp299 allele. It was suggested in the study that impaired cross-presentation might be due to defective binding of HMGB1 to the mutated TLR4 allele (Apetoh et al., 2007b).

However, contradicting studies regarding the functional effects of the TLR4 polymorphism have also been reported. Tulic et al (2007) demonstrated that impaired responses to RSV and LPS were associated with reduced NF-κB signalling post-TLR4 engagement, reduced IFN, IL-8, IL-10 IL-12p35, IL-18 and CCL8 release and the absence of acute phase TNF-α in the group with TLR4 Asp299Gly or Thr399Ile SNPs (Tulic et al., 2007). Conversely, Dourville et al (2010) found that the TLR4

SNPs did not influence immune responses evoked by LPS and RSV infection as measured by the intermediate phenotype of pro-inflammatory and anti-inflammatory cytokines (Douville et al., 2010). The diversity in the findings may be due to differences in the experimental systems. While Tulic et al. (2007) preactivated PBMC with IFNγ before stimulating the cells with the TLR4 ligands, Douville et al., (2010) did not. This may change the phenotype of PBMC and hence affect the results.

Although the studies above illustrate the effects of Asp299Gly and/or Thr399Ile TLR4 SNPs, there are numerous other studies that found no association between the mutations and cellular immune responses (Allen et al., 2003, Read et al., 2001, Feterowski et al., 2003). In one study, the consequences of the Asp299Gly polymorphism were investigated after stimulation of mononuclear cells with LPS, the non-LPS TLR4 microbial stimuli *Aspergillus fumigates* and *Cryptococcus neoformans*, and the endogenous TLR4 ligand Hsp60. No differences in either the production of the pro-inflammatory cytokine TNF-α or the anti-inflammatory cytokine IL-10 were observed between volunteers with the wild-type allele, volunteers heterozygous for the Asp299Gly allele and one volunteer homozygous for the Asp299Gly variant (van der Graaf et al., 2005b).

Discrepancies in the results may be attributed to the genotypic differences observed between TLR4 haplotypes. Many experimental and clinical studies observed no functional differences between individuals with the cosegregating Asp299Gly/Thr399Ile haplotype and those with the wild type TLR4 allele (Calvano et al., 2006, Erridge et al., 2003, Schippers et al., 2004, Ferwerda et al., 2008). In an *in vitro* experiment using whole blood, Ferweda et al (2007) found that individuals with the Asp299Gly allele alone, a genotype often found within the African population, had an altered cytokine profile in response to LPS whilst individuals with both Asp299Gly and Thr399Ile alleles did not (Ferwerda et al., 2008). This was however, the only study that observed an increase in pro-inflammatory cytokines therefore contradicting the other studies. In the only study that investigated the role of all TLR4 haplotypes on the susceptibility to septic shock, the presence of the Asp299Gly alone, but not that of the Asp299Gly/Thr399Ile haplotype, was associated with an increased mortality to septic shock (Lorenz et al., 2002).

Investigations to determine the haplotype functionally relevant have also been carried out in transfected cells. Transfection of THP-1 cells demonstrated that the Asp299Gly mutation (but not the Thr399Ile mutation) interrupts TLR4-mediated LPS signalling measured by NF-κB activity (Arbour et al., 2000). In another study, cells transfected with Asp299Gly TLR4 exhibited impaired LPS-induced phosphorylation of p38 and TANK-binding kinase 1 (TBK1), activation of NF-kB and IRF3, and induction of IL-8 and IFN-β mRNA, whereas the Thr399Ile TLR4 did not cause statistically significant changes. However, the use of transfection to investigate the phenotypic effect of certain mutations meets with several complications. Transfections represent a stripped-down model performed in altered cell lines. As a result, transfected cells may behave differently from cells expressing these mutations naturally. Furthermore, transfections only represent the homozygous state. Most studies of TLR4 haplotypes include heterozygous state, whereas the homozygous state of these polymorphisms is rare. Therefore, determination of the association between the Asp299Gly/Thr399Ile haplotype and the phenotype, and its impact on the susceptibility to Gram-negative infection or diseases (based on transfection experiments), is difficult and prone to artifacts (Ferwerda et al., 2008).

The impact of TLR4 polymorphism on the outcomes of cancer treatment

Apetoh et al (2007) investigated the clinical relevance of the TLR4 Asp299Gly SNP in the response to anticancer treatments. A retrospective cohort study of 280 breast cancer patients presenting with lymph node involvement, treated with local radiation and anthracycline-based chemotherapy, was designed. No significant differences for all classical prognostic factors between normal individuals and patients bearing the mutated TLR4 allele were observed (Apetoh et al., 2007b). However, metastasis-free survival was significantly decreased in women carrying the variant allele of TLR4. Forty percent of women with the mutated allele relapsed in five years compared to twenty-six percent with the normal allele. Therefore, patients with breast cancer who carry a TLR4 polymorphic allele relapse more quickly after radiotherapy and chemotherapy than those carrying the normal TLR4 allele (Apetoh et al., 2007b).

Bergmann et al (2011) also looked at the impact of the Asp299Gly SNP on the clinical outcome of adjuvant systemic therapy with chemotherapy and radiotherapy after surgery in a large cohort of head and neck squamous cell carcinoma (HNSCC) patients. Genotype analysis was done using DNA from tissue samples of 188 patients with HNSCC. Ten percent of patients carried the TLR4 Asp299Gly allele. Patients with the heterozygous genotype TLR4 Asp299Gly had a significantly reduced disease-free and overall survival. These associations seem to be attributable to relatively poor therapy-response among HNSCC patients carrying the Asp299Gly variant receiving adjuvant treatment (Bergmann et al., 2011). Conclusively, this study demonstrates that TLR4 Asp299Gly allele may serve as a marker for prognosis of head and neck patients with adjuvant systemic therapy, particularly chemotherapy, and might indicate therapy resistance (Bergmann et al., 2011).

The relevance of immune responses in prostate cancer

Prostate cancer

PCa is the most common male malignancy in the western world. Cases have tripled over the past few years and this is largely attributable to earlier detection and screening following the introduction of prostate-specific antigen (PSA) testing into routine clinical practice in the late 1980s (Quon et al., 2011, Challapalli et al., 2012). Three-quarters of PCa cases are diagnosed in men aged over 65 years and as life expectancy increases, the prevalence will also increase in a more aged population (Cancer Research UK, 2013). At presentation approximately 60% of patients have localised, 30% locally advanced and 10% metastatic disease (Tabi et al., 2011). Based on the PSA levels, histopathological grading and clinical staging, PCa is classified as low-, intermediate- and high-risk for disease recurrence. The risk status often plays a major role in deciding further therapy.

Radiation therapy (RT) can be used as part of curative therapy for both localised and locally advanced disease but has no proven role in the metastatic setting. There are four major treatment approaches for localised PCa, active surveillance, radical prostatectomy, external beam radiotherapy (EBRT) and low-dose rate (LDR) brachytherapy. Traditional EBRT treatment requires patients to receive repeated

doses of approximately 2 Gy/fraction for a total of 50-70 Gy to control PCa (Challapalli et al., 2012). Considerable advances in EBRT technology over the last decade have led to the development of three-dimensional conformal radiotherapy (3D-CRT) and intensity-modulated radiotherapy (IMRT), which closely match and modulate the high-dose volume to the tumour target while reducing the radiation to dose-limiting normal tissues (Challapalli et al., 2012). Hypofractionation using EBRT delivery systems (treatment in approximately 4 weeks) has also improved PCa treatment (Khoo and Dearnaley, 2008). LDR brachytherapy, which uses multiple permanently planted radioactive seeds, can be used to deliver a very high radiation dose to a highly targeted volume in a single treatment with equivalent outcomes to EBRT and surgery (Challapalli et al., 2012). As part of the treatment for locally advanced PCa, high dose rate (HDR) brachytherapy, which uses a single high-intensity radiation source that is temporarily inserted into multiple positions in the prostate, can be used as a single agent or in combination with androgen deprivation therapy (ADT) and/or RT (Hoskin, 2008). ADT consists of lowering the levels of testosterone, the male hormone that fuels hormone-dependent tumour growth.

Immunotherapy in prostate cancer

PCa is an immunogenic cancer, as evidenced by a positive correlation between the frequency of $CD8^+$ tumour-infiltrating T cells and PSA recurrence-free survival (Karja et al., 2005). PCa is also an ideal model for cancer immunotherapies based on the ready demonstration of humoral and cellular immunity to a range of cancer antigens as well as often slow progression (Schweizer and Drake, 2014). The majority of work has gone into developing immune-based therapies that are either antigen-specific (i.e. cancer vaccines and antibody-based therapies) or monoclonal antibodies that function as immune checkpoint inhibitors.

Unlike CD28 which is stimulatory, interaction between CTLA-4 and PD-1 and their respective ligands results in inhibitory signals which effectively turn off T cell function. The molecules that mediate thes negative interactions have collectively been termed immune checkpoints. The human anti-CTLA-4 monoclonal antibody ipilimumab is the only Food and Drug Administration (FDA) approved immune checkpoint inhibitor (Schweizer and Drake, 2014). Preclinical data support the use of

ipilimumab in PCa, not only as a monotherapy but also as a means to augment the immune response elicited by other therapies (i.e. cancer vaccines, RT). In the clinical trial with or without RT in patients with metastatic castrate-resistant prostate cancer (mCRPC), the major PSA response of ≥50% PSA decline rate among those receiving the maximum dose of ipilimumab was reported at 8/50 (16%) patients; four in the radiation group and four in the monotherapy group (Slovin et al., 2013).

Cancer vaccines work through eliciting an antigen-specific response leading to activation of effector T cells and tumour eradication. Given that both normal and cancerous prostate cells express unique antigens such as PSA and prostatic acid phosphatase (PAP), there are several attractive potential targets for vaccines (Schweizer and Drake, 2014). Different vaccination approaches for treating PCa have been evaluated in both the pre-clinical and clinical settings. The most well known vaccine strategy is Sipuleucel-T, which was FDA approved in 2010 for the treatment of asymptomatic or mildly symptomatic CRPC. Sipuleucel-T is an ex vivo generated autologous DC-based vaccine designed to target PAP. It is also the only cancer vaccine ever shown to improve overall survival in the phase III setting (Kantoff et al., 2010).

Another immunotherapy strategy showing promise in clinical testing is PROSTVAC-VF, a viral-based vaccine engineered to express PSA along with three co-stimulatory molecules (CD80, ICAM-1 and LFA-3) (Madan et al., 2009). A randomized phase II trial involving 125 patients with mCRPC showed an 8.5-month improvement in median overall survival and a larger phase III trial is currently underway (Schweizer and Drake, 2014, Kaufman et al., 2004).

In regard to passive immunotherapies for PCa, there have been no major successes thus far. The humanized monoclonal antibody, J591, that targets the prostate specific membrane antigen (PSMA) has gone through perhaps the most clinical testing. While it was found to elicit a dose-dependent antibody-dependent cell-mediated cytotoxicity (ADCC) effect, it did not produce a robust antitumor effect in patients with mCRPC (Morris et al., 2005).

Link of immune responses and radiotherapy in prostate cancer

RT is the medical use of ionising radiation (IR). IR has been harnessed for over a century to treat cancer largely on the rationale that rapidly proliferating cancer cells are more sensitive than normal cells to DNA damage induced by radiation. Cellular DNA damage induced by IR leads to the activation of a DNA damage response-signalling cascade. Depending on the extent of damage, this leads to transient or permanent cell cycle arrest, and/or cell death, respectively (Formenti and Demaria, 2009, Lauber et al., 2012).

Current understanding of cell death mechanisms demonstrate that the fate of dying cells can be classified according to phenomenological and ultrastructural changes. Based on the morphologies of dying cells, three types of cell death have been recognized. These are Type I, or apoptotic cell death; Type II, or autophagic cell death; and Type III or necrotic cell death. Cell death can either be programmed, were it is controlled by molecular processes that not only dictate the morphology but also the fate of the cell; or passive, a consequence of damage so extensive that the cell cannot survive (Tesniere et al., 2007, Green, 2011). However, IR not only exerts a local cytotoxic effect but also has the ability to augment a host's anti-cancer immune response. Radiation-induced forms of cell death trigger the production of stress or danger signals that mobilize the innate and adaptive immune system to deal with the damage and tissue repair with the goal of maintaining the integrity of the tissue and body. Some of these signals can be highly immunogenic, stimulating the clearance of tumours by innate immune cells, and improving DC activation and antigen processing, thereby switching on antitumor T cells responses. As a result, radiation-induced cell death provides an important link between innate and adaptive immunity.

Apoptosis is a programmed process of cell death and it is a constantly occurring mechanism in living organisms, which is essential for normal development, tissue homeostasis and numerous other physiological processes. Morphologically, it is characterised by cellular shrinkage, chromatin condensation, nuclear fragmentation, and membrane blebbing (Green, 2011). IR primarily regulates apoptosis via the mitochondrial intrinsic death pathway that involves permeabilization of the mitochondrial outer membrane and the release of various proteins including

cytochrome *c*, and apoptosome formation. However, IR can also induce apoptosis via the extrinsic pathway through a death receptor mediated caspase activation process (Lauber et al., 2012, Golden et al., 2012). Upregulation of death receptors during apoptosis plays an important role in the elicitation of antitumor T cell responses. Radiation induced upregulation of FAS on tumour cells sensitises them to antigen-specific $CD8^+$ T cells killing via the FAS/FAS ligand pathway and promotes more effective antitumor responses (Chakraborty et al., 2003). Furthermore, exposure of CRT on the membrane of pre-apoptotic and apoptotic cells following radiation, enhances phagocytosis of dying tumour cells by DC and induces a protective antitumor immune response (Obeid et al., 2007a, Gardai et al., 2005).

Autophagy, another type of programmed cell death is a major intracellular pathway for the degradation and recycling of proteins, ribosomes and entire organelles. It is characterized by membrane blebbing, partial chromatin condensation and autophagic vacuoles in the cytoplasm. In the context of response of cancer cells to RT, autophagy leads to either cell survival or cell death (Zois and Koukourakis, 2009). Autophagy is a slow process leading to the degradation of intracellular organelles after sequestration in double-membrane vacuoles (Tesniere et al., 2007). There are three major stages in the autophagy pathway. The initiation stage is the *de novo* formation of an isolation membrane (also called phagophore); the second stage is elongation during which the isolation membrane expands and damaged organelles or cytosolic materials are captured; and the third stage involves the formation of autophagosomes, which is followed by rapid transition into autolysosomes upon fusion with lysosomes, and targets the captured materials for degradation (Li et al., 2008). Various preclinical models have revealed that autophagy is activated in irradiated tumour cells (Ito et al., 2005, Apel et al., 2008, Chen et al., 2011). Autophagy induced by IR is immunogenic as it contributes to the release of cell death-associated danger signals that trigger antitumor host immune responses. Autophagic cell death following radiation causes the release of ATP from dying tumour cells which in turn is required for attracting immune cells including DC into the tumour tissue (Ko et al., 2014).

IR induces necrosis when applied at high doses. Necrosis is morphologically characterized by swelling of the mitochondrial, cytoplasmic and nucleic swelling,

leading to the rupture of the plasma membrane, and the release of swollen and damaged organelles (Tesniere et al., 2007). It can be caused by excessive damage and/or catastrophic energy loss and thus can be a passive process. Recent evidence has shown, however, that necrosis can be a result of regulated processes and therefore, programmed (Green, 2011). Programmed necrotic cell death is known as necroptosis and is characterized by the cellular morphology of necrosis but with a signalling that more closely resembles apoptosis. Necroptosis occurs via the activation of two kinases: receptor interaction kinase-1 (RIPK-1) and RIPK-3. In both apoptosis and necrosis, the intracellular death domain recruits the adaptor protein, Fas-associated death domain (FADD). Therefore, in both forms of cell death, the deciding factor of whether a cell commits apoptosis or necrosis depends on the FADD-associated activities of caspase-8 (for apoptosis) and RIPK-1 (for programmed necrosis/necroptosis). Necroptosis is induced in cells that are caspase-8 deficient or inhibited. However, if caspase-8 is intact and active, it can cleave RIPK-1, thereby turning off necroptosis and alter the balance of cell death in favour of apoptosis (Golden et al., 2012, Lauber et al., 2012, Green, 2011). Necrotic cell death is considered immunogenic as it often causes the release of pro-inflammatory cytokines and danger signals such as HMGB1. The release of HMGB1 by irradiated tumour cells has been demonstrated in some studies (Apetoh et al., 2007a, Suzuki et al., 2012). However, further studies are required to clarify the role of necroptosis in IR-induced cell death and the subsequent spillage of immune stimulating "danger signals".

Radiation of different tumour cells induces the expression of pro-inflammatory cytokines, such as IL-1β , TNF-α and type 1 interferons (Formenti and Demaria, 2013, Hallahan et al., 1989, Burnette et al., 2011) and the chemokine CXCL16, which promotes recruitment of effector $CD8^+$ and T-helper 1 $CD4^+$ T cells, (Matsumura et al., 2008). In addition, tumour cells that receive sublethal doses of radiation undergo phenotypic changes that enhance their susceptibility to immune effectors (Garnett et al., 2004). This indicates that radiation can switch the immunosuppressive tumour milieu to a pro-immune environment.

IR can reduce tumour growth outside the field of radiation, known as the abscopal effect. The abscopal effect of radiation has been shown to be immune-mediated as

demonstrated not only in mouse tumour models (Dewan et al., 2009, Hodge et al., 2012) but also in patients with metastatic melanoma and lung adenocarcinoma (Golden et al., 2013, Postow et al., 2012). The first direct evidence to implicate the immune system was reported by Demaria et al., (2004) who showed that an abscopal effect could be elicited in mice harbouring two 67NR breast cancer cell line tumours when radiation was applied to one of the tumours in conjunction with Flt3 ligand (Flt3-L), a DC growth factor (Demaria et al., 2004). The study found that RT alone led to growth delay exclusively of the irradiated 67NR tumour, as expected. However, growth of the non-irradiated tumour was also impaired by the combination of RT and Flt3-L. As a control, Flt3-L had no effect without RT. Importantly, the abscopal effect was shown to be tumour specific, because growth of a non-irradiated A20 lymphoma in the same mice containing a treated 67NR tumour was not affected. Moreover, no growth delay of non-irradiated 67NR tumours was observed when T cell deficient (nude) mice were treated with RT plus Flt3-L (Demaria et al., 2004).

Immune responses are important for PCa radiotherapy as illustrated by Tabi et al., (2010). PBMC were collected from PCa patients with locally advanced tumour before, during and after hypofractionated RT and analysed for T cell phenotype and function. The study observed significantly more loss of naïve and early memory compared with more differentiated T cells during RT. More importantly, TAA-specific antitumor T cell responses were detectable after but not before or during RT (Tabi et al., 2010). Similar findings were observed by others (Nesslinger et al., 2007) indicating that RT induces or amplifies antigen-specific immune responses in PCa patients. Thus, immunological mechanisms may contribute to clinical outcomes after RT alone or in combination with hormone therapy.

HYPOTHESIS AND AIM

We hypothesize that (a) ionising radiation treatment of PCa cells triggers antitumor T cell responses via cross-presentation of tumour antigens and (b) this process is influenced by TLR4 Asp299Gly SNP on DC.

In order to investigate this, the aims of the thesis are to:

1. Determine the effects of IR on the DU145 PCa cell line, positive for the 5T4 oncofetal glycoprotein.
2. Investigate the ability of irradiated tumour cells to induce DC activation.
3. Determine the ability of the irradiated tumour cells to induce 5T4-specific T cell responses via cross-presentation.
4. Study the influence of TLR4 Asp299Gly SNP on tumour antigen cross-presentation.

II. Materials and Methods

DONORS

Ethical approval for the project was obtained from the South Wales Ethics Committee and informed consent to provide peripheral blood samples was obtained from both healthy donors and prostate cancer patients. HLA Class I and II typing was determined by low resolution PCR using sequence specific primers. The Welsh Blood Transfusion Service, Cardiff, UK, provided this service.

TISSUE CULTURE MEDIA

RPMI (RPMI 1640, Lonza, Belgium) was supplemented with low endotoxin fetal bovine serum (FBS, PAA Laboratories, Austria) and/or human AB serum (Sigma, UK), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 2 mM L-glutamine (Gibco), 25 mM Hepes buffer (Sigma, UK) and 1 mM sodium pyruvate (Sigma, UK). This will be referred to as supplemented RPM1 (sRPMI).

CELL LINES

Cells were maintained at 37^{o}C in an atmosphere of 5% CO_2 in a humidified CO_2 incubator. Mycoplasma test was carried out regularly using a MycoAlert Mycoplasma Detection Kit (Lonza). All tissue culture work was carried out in a class II biosafety cabinet. Authentication of cell lines was carried out by the supplier using cytogenetic isoenzymatic and DNA profile analysis. Cell lines in our lab are not used beyond 30 passages, however, if they were, further validation would be required.

DU145 is a prostate cancer cell line derived from a metastatic brain and obtained from ATCC. Tumour cells were grown in sRPMI 10% FBS.

LNCaP is a human prostate adenocarcinoma cell line derived from a metastatic left supraclavicular lymph node and obtained from ATCC. Tumour cells were grown in sRPMI 10% FBS.

B lymphoblastoid cell lines (BLCL) were prepared according to the standard method described below by infecting PBMC with EBV-containing B95.8 cell supernatant and PHA (Louie and King 1991). BLCL were grown in sRPMI 10% FBS.

T2 cells are a hybrid cell line derived from a B cell and a T cell lymphoblastoid line. T2 cells are HLA-A2 positive and TAP-negative (Salter 1985).

They were grown in sRPMI 10% FBS and were used as target cells in peptide-specific cytotoxicity assays.

Mesothelioma cell lines were generated by long term culture of explanted tumour biopsies and maintained by regular passaging in sRPMI 5% FBS.

ISOLATION, GENERATION AND CULTURE OF IMMUNE CELLS

Peripheral blood mononuclear cells (PBMC)

Venous blood samples of healthy donors from EDTA-vacutainers were subjected to density gradient centrifugation on Histopaque (Sigma, UK) and PBMC were isolated from the buffy coat. The PBMC were either used fresh or kept cryopreserved.

T cell isolation

The EasySep™ Human T Cell Enrichment Kit (Stem Cell Technologies) was used to isolate T cells from fresh or cryopreserved PBMC by negative selection according to the manufacturer's protocol. Unwanted cells were targeted for removal with Tetrameric Antibody Complexes recognizing CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, glycophorin A and dextran-coated magnetic particles. The labelled cells were separated using an EasySep magnet without the use of columns and non-labelled cells were poured off into a new tube. The recommended buffer used for this protocol was Phosphate Buffered Saline (PBS) (Lonza), + 2% FBS with 1 mM EDTA (Sigma).

DC generation

Two methods were used to generate monocyte-derive DC.

1. PBMC, setup in a 6 well tray at $1.5x10^7$ cells/well in 5 ml 1% sRPMI were incubated for 2 h at 37^oC in order for cells to adhere. After gentle resuspension, non-adherent cells were removed, cryopreserved (as detailed later) and stored in liquid nitrogen. Adherent cells left on the plate were then grown in 5 ml/well 10% sRPMI in the presence of 500 ng/ml GM-CSF (Prospec-Tany Technogene Ltd., Israel) and 500 U/ml IL-4 (Peprotech, UK) for 5-6 days.

2. $CD14^+CD16^+$ monocytes were isolated from fresh or previously frozen PBMC by negative selection using the EasySep Human Monocyte Enrichment Kit without CD16 Depletion (Stem Cell Technologies) according to the manufacturer's protocol. Unwanted cells were targeted for removal with Tetrameric Antibody Complexes recognizing CD2, CD3, CD19, CD20, CD56, CD66b, CD123, glycophorin A and dextran-coated magnetic particles. The cocktail also contains an antibody to human Fc receptor to minimize nonspecific binding. The labelled cells were separated using an EasySep magnet without the use of columns and the non-labelled cells were poured off into a new tube. The isolated cells were incubated at $5x10^6$ cells/well in a 6 well tray and grown in 5 ml/well 10% sRPMI in the presence of 500 ng/ml GM-CSF and 500 U/ml IL-4 for 5-6 days. Approximately $1\text{-}2x10^7$ cells were recovered with an average purity of 70-80% $CD14^+$ cells.

Generation of a 5T4 peptide-specific T-cell line (RLAR-T cells)

A $CD8^+$ T-cell line was developed from a HLA-$A2^+$ healthy donor by stimulation of non-adherent PBMC with autologous monocyte-derived DC, generated from adherent PBMC as described above. DC were loaded with 2 µg/ml $5T4_{17\text{-}25}$ peptide (RLARLALVL; 90.4% purity; ProImmune, UK) (Shingler et al., 2008) and treated simultaneously with 5 ng/ml LPS for 1 h. DC were washed and plated out in 24-well trays at 2×10^5 cells/well with 4×10^6 non-adherent PBMC in 2 ml. Cultures were supplemented with 1000 U/ml IL-6 and 5 ng/ml IL-12 (Fonteneau et al., 2001) and grown for eight days. Peptide-specific stimulation was repeated weekly with peptide-loaded autologous DC as above, supplemented with 5 ng/ml IL-7 and 10 U/ml IL-2 (Fonteneau et al., 2001). Seven days after the fourth stimulation, a T cell specificity assay was carried out. The peptide-specific T cells were enriched using a kit specific for IFN-γ producing T cells (Miltenyi Biotech, UK). Separated T cells ($1–5\times10^5$) were expanded using a mixture of 5×10^6 peptide-pulsed autologous BLCL irradiated with 40 Gy, 5×10^7 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 sRPMI 10% FBS and 1% AB-serum (Sigma) in a T75 flask (Al-Taei et al., 2012). The flask was placed at an angle for the first 3-4 days. Half the media was replaced at day four with fresh media containing fresh cytokines

but no OKT3. CD8+ T-cell separation was carried out after seven days using the EasySep CD8+ T-cell enrichment kit (StemCell Technologies, UK). T cell assays were carried out after 7–9 days in expansion.

GENERAL METHODS

Passaging of adherent cells

To passage cells, media was first removed and cell monolayers dissociated by the addition of 2 ml Trypsin/EDTA solution (Lonza). Trypsin is a proteolytic enzyme that causes detachment of cells from the growth surface by cleaving cell-cell and cell-matrix adhesions whilst EDTA acts to chelate $Ca2^{+}$ and $Mg2^{+}$ ions present in FBS that otherwise act as trypsin inhibitors. After addition of the trypsin/EDTA solution, the flasks were returned to the incubator for 5-10 min until the cells had detached. Trypsin/EDTA was neutralised with 6-8 ml sRPMI 10% FBS and the cells were pelleted by centrifugation for 3 min at 1300 RPM. The cell pellet was gently re-suspended in 10 ml of the appropriate medium and gently mixed to ensure no cell clumps were evident. For cell maintenance, 1 ml of this suspension, (1/10 of cell suspension volume), was diluted in sRPMI 10% FBS and used to seed a T75 flask which was then cultured as normal. Prior to setting up cells for an experiment, the re-suspended cells were counted using the methods below to enable seeding at an appropriate cell density. The remaining cells were cryopreserved and stored.

Cryopreservation and Storage

Freezing media for storing cells in the liquid N_2 contained sRPMI supplemented with 20% FBS and 10% dimethyl sulphoxide (DMSO, Sigma). The freezing media was always prepared fresh on the day of use and kept on ice. Cells were counted, and then centrifuged at 1300 RPM for 3 min. After this, the supernatant was removed and cells re-suspended in the freezing media to yield $2x10^{7}$ cells/ml for PBMC or 1-$5x10^{6}$ cells/ml for cell lines, DC and T cells. 1 ml aliquots of cell suspension were then transferred to pre-chilled cryogenic vials. These were placed into CoolCell alcohol-free cell freezing containers (Biocision) to ensure controlled cooling at -1^{o}C/min in a -80^{o}C freezer. The vials were transferred to liquid nitrogen for long-term storage within 2-3 days.

Recovery of cryopreserved cells

To recover cryopreserved cells, the vials were placed into a 37^{o}C water bath until the liquid is partially thawed. sRPMI with 10% FBS (1 ml) was added dropwise to the partially thawed vial, re-suspended and then all the cells were transferred dropwise into a 15 ml falcon tube with pre-warmed (37^{o}C) 10 ml sRPMI 10% FBS. The cell suspension was centrifuged at 1300 RPM for 3 min; the working media was discarded and the pellet re-suspended in 5 ml media for counting. In general, a recovery of >70% viable cells was achieved; if less than that, the cells were not used.

Evaluation of cell number and viability

The number and viability of cells was determined by using a Neaubauer haemocytometer in conjunction with trypan blue exclusion assay or by using the ViaCount Assay (Millipore).

a) Trypan blue is membrane-impermeable and so can only penetrate dead cells thus viable cells remained unstained. A 1:10 dilution of cell suspension in 0.1% trypan blue was carried out and the cells were counted in a quadrant of known volume. The number of cells were determined by the following formula:

   Mean number of cells per quadrant x dilution factor x 10^4 = number of cells/ml

b) The Guava ViaCount Assay is an alternative to trypan blue exclusion for determining absolute cell count and viability. The assay differentially stains viable and non-viable cells based on their permeability to the DNA-binding dyes in the ViaCount Reagent. A uniform cell suspension was prepared for counting. The cell samples were stained by mixing the cells with Guava ViaCount Reagent in a small tube. Following this, the cells were incubated for 5 min and the data were acquired on the Millipore Guava® EasyCyteTM8 flow cytometer. Accurate cell counting on the Guava system occurs at a concentration range of $1x10^4$-$5x10^5$ cells/ml in the stained sample. Therefore, if an approximate concentration of the original cell suspension was known, then the dilution guide table below was used as a reference to determine the optimum dilution factor:

Conc. of Original Cell Suspension	Dilution Factor	Cell Suspension Volume	ViaCount Reagent Volume	Conc.of Diluted Cells
$1x10^5$ to $1x10^6$ cells/ml	10	50 µl	450 µl	$<1x10^5$ cells/ml
$1x10^6$ up to $1x10^7$ cells/ml	20	20 µl	380 µl	$<5x10^5$ cells/ml
$>1x10^7$ cells/ml	40*	20 µl	780 µl	$>2.5x10^5$ cells/ml

*Further dilution may be necessary for highly concentrated cell suspensions.

If the concentration was not known, stained samples were prepared by mixing cells with ViaCount Reagent at a 20-fold dilution. Further dilutions were carried if the total cell number/ml value remained high.

Flow cytometry

Anti-human antibodies together with appropriate isotype controls used for flow cytometric analysis are described in the tables below (Table 1 and 2). Unstained cells were used as a negative control in some experiments. The correct dilutions of the antibodies to give efficient staining were established by titration of the antibodies. All cell staining was performed in 5 ml sterile non-pyrogenic FACS tubes. A FACSCanto, 6-colour cytometer with BD FACS-DIVA software version 6.1.2 (BD Bioscience) was used for all experiments unless otherwise stated.

Intracellular staining

Cells were fixed with 100 µl fixation buffer (eBiosciences) for 15 min at room temperature, washed with PBS, and permeabilized with 100 µl permeabilization buffer (eBiosciences) for 40 min at room temperature. The respective antibodies were added at the same time as the permeabilization solution in the dark.

Surface staining

Non-specific binding was reduced by firstly incubating cells with 100 µl FACS buffer: PBS, 2% FBS and 5 mM EDTA for 10 min on ice. The cells were then stained with antibody or isotype for 40 min on ice in the dark. To remove unbound antibodies, cells were washed in 2 ml FACS buffer.

Intracellular Cytokine Staining (ICCS)

One hour after stimulation of DC or T cells, 1 µl/ml Golgi Plug (BD Biosciences) and 0.7 µl/ml Golgi Stop (BD Biosciences) were added for either 6 h or 12 h further incubation at 37^{o}C. Golgi Plug contains brefeldin A while Golgi Stop contains monensin both of which block intracellular protein transport processes in lymphoid cells resulting in the accumulation of cytokines and/or proteins in the Golgi complex. Optimisation experiments in the laboratory revealed that the combined use of the two reagents results in increased sensitivity of the assays. The cells were washed with 2 ml PBS after the total incubation time and then fixed and permeabilized as above for intracellular staining with cytokine specific antibodies.

Gating strategy

Tumour cells

When assessing tumour cells for surface expression of immunogenic signals, 5T4 and HLA-ABC, a gating strategy was used. Firstly, in order to distinguish between live and dead populations, cells were stained with 7AAD, a solution with a strong affinity for DNA (Figure 2.1a). Cells with a compromised membrane readily stain for 7AAD and therefore have higher fluorescence intensity than cells with intact cell membranes. Dead cells (7AAD^{+}) were gated out by gating on the 7AAD^{-} cells (Figure 2.1a). DU145 cells were gated from 7AAD^{-} cells based on the forward scatter height (FSC-H) and side scatter height (SSC-H) pattern (Figure 2.1B and C).

Dendritic cells

To analyse DC co-cultured with tumour cells, gating for the DC population was firstly based on the typical FSC-H and SSC-H pattern. DC were distinguished from tumour cells by staining with HLA-DR. Since tumour cells do not express HLA-DR, DC were identified by gating on the HLA-DR^{+} population. Phagocytosis and DC activation was assessed from the HLA-DR^{+} population.

T cells

When the size of a single cell passing through the FSC-H correlates with the forward scatter area (FSC-A), cells appear as a diagonal display on a FSC-H versus FSC-A dot plot. However, when cells clump together, this correlation is lost and as a result

doublets can be discriminated from singlets. In the gating strategy using T cells, doublets were firstly excluded by placing the gate on the singlets (Figure 2.3A). The lymphocyte population was then gated from the singlets based on the FSC-A and SSC-A pattern (Figure 2.3B). T cells were identified by gating on the $CD3^+$ lymphocytes (Figure 2.3C) and $CD8^+$ T cells were gated from the $CD3^+$ T cell population (Figure 2.3D). The percentage of $CD8^+$ T cells expressing IFNγ (Figure 2.3F) was determined by setting quadrants using unstimutaled APC (Figure 2.3E).

5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE)

CFSE is widely used for cell tracking and proliferation studies. CFSE readily crosses intact cell membranes. Once inside the cells, intracellular esterases cleave the acetate groups to yield the fluorescent carboxyfluorescein molecule. The succinimidyl ester group reacts with primary amines, cross-linking the dye to intracellular proteins. The stable incorporation of the intracellular fluorescent dye CFSE into cells provides a powerful tool to monitor cell migration, and to quantify cell division, because of the sequential decrease in fluorescent labelling in daughter cells (Parish et al., 2001).

Single cell suspension was washed twice with PBS to remove any serum. The cells were then re-suspended at $5\text{-}10x10^6$ cells/ml with PBS. A final CFSE concentration of 5 μM was added to the cells, mixed immediately and incubated for 10 min at room temperature in the dark. The labelling was stopped by adding 4-5 volumes of cold complete sRPMI media and incubated for 5 min on ice. Following this, the cells were washed three times with sRPMI media and then used in experiments as desired.

Statistics

All statistical analysis was performed with Graphpad Prism 4 or 6 software. A student T-Test was performed when comparing 2 unpaired groups. One-way ANOVA was utilised when studying more than 2 unpaired groups. Mean and standard deviation (SD) are stated throughout the result section.

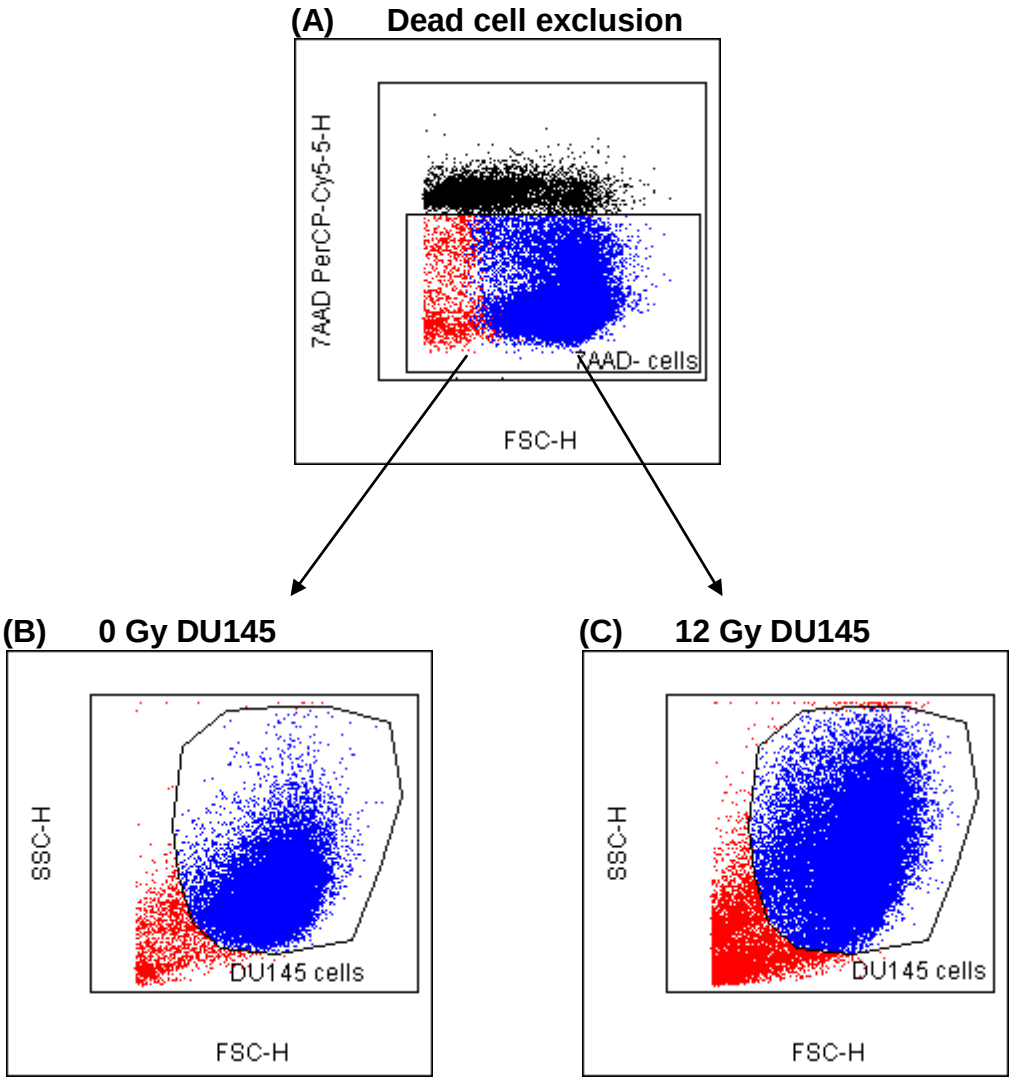


Figure 2.1: Example of gating strategy for assessing surface expression of immunogenic signals, 5T4, and MHC molecules. After detachment, cells were stained with CRT, Hsp70, 5T4 or HLA-ABC antibodies for 40 min and subsequently with 7AAD for 5 min before flow cytometry analysis. (A) Dead cells were gated out by gating on the 7AAD- cells. DU145 cells treated with (B) 0 Gy or (C) 12 Gy IR represented by the blue population were gated from 7AAD- cells based on the FSC and SSC dot plot pattern. The red population is possibly debris.

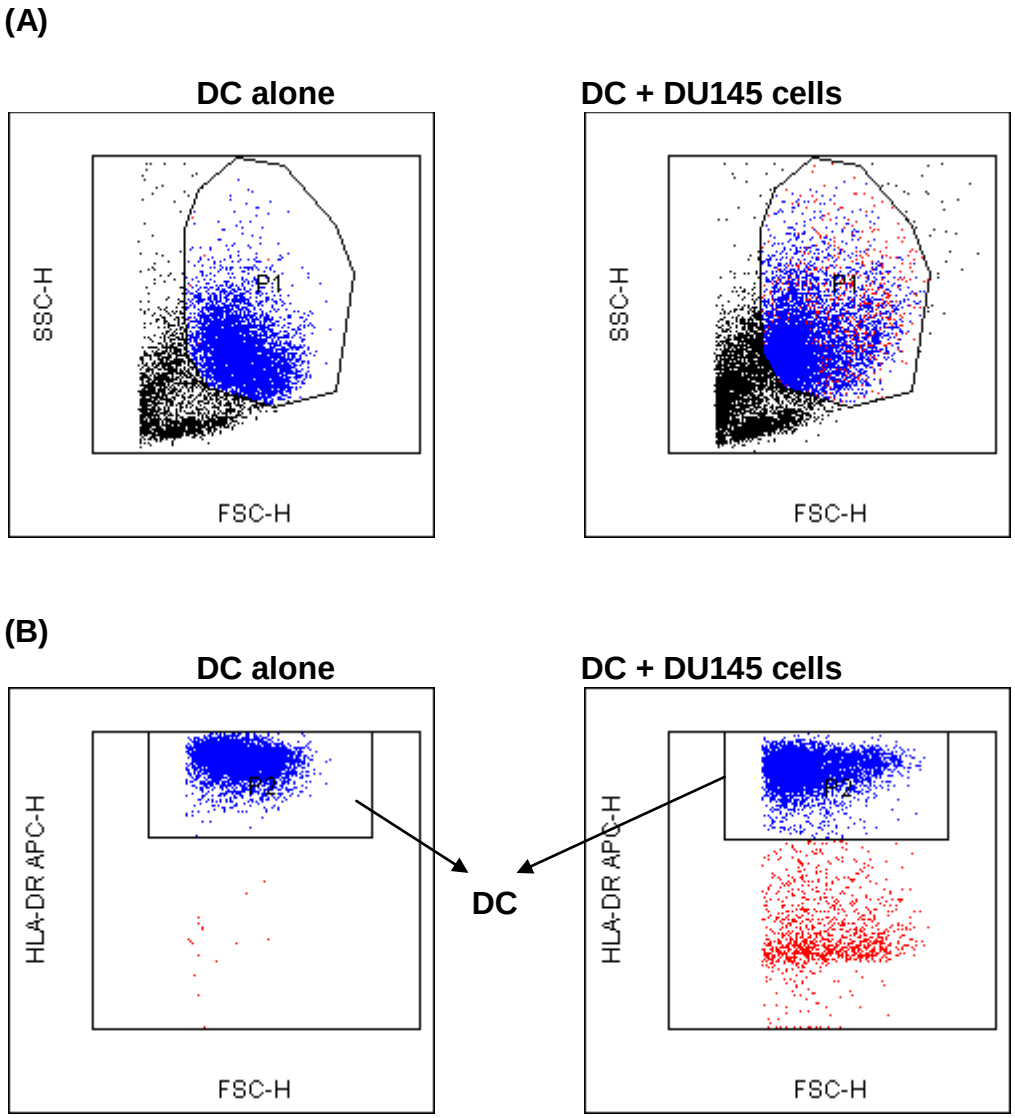


Figure 2.2: Example of gating strategy when carrying out DC phagocytosis or activation assays. (A) Gating of the DC population was based on the typical FSC-H and SSC-H pattern. (B) Tumour cells (red events) were gated out by gating on HLA-DR^{+} identified as DC (blue events).

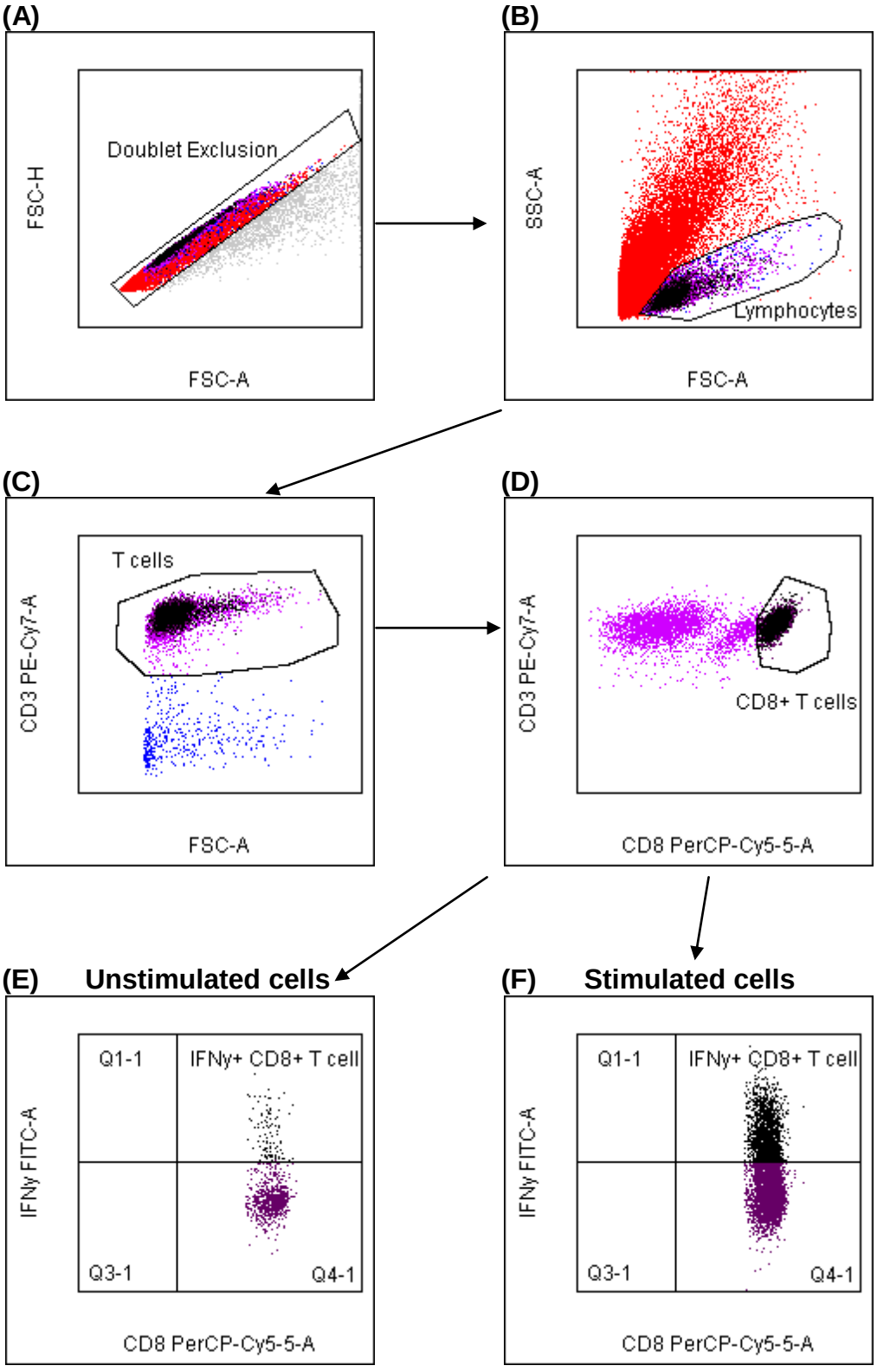


Figure 2.3: Example of gating strategy for assessing the frequency of $CD8^+$ T cells producing IFNγ. Following an overnight ICCS assay, T cells were collected for flow cytometry analysis. Cells were stained with anti-CD3-PE-Cy7, anti-CD8-PE-Cy5 and anti-IFNγ-FITC. (A) Doublets were gated out by gating on the single cells. (B) Lymphocytes were defined according to their FSC and SSC. (C) T cells were identified by gating on $CD3^+$ lymphocytes and (D) $CD8^+$ T cells were gating from the T cells. (E) Quadrants in dot plots were set using unstimulated T cells to assess (F) the percentage of $CD8^+$ T cells producing IFNγ.

Production of Soluble Peptide-MHC Class I (pMHCI)

Soluble biotinylated MHC class I monomers were produced in Professor Linda Wooldrige's laboratory according to their established methods (Glick et al., 2002). Briefly, HLA-A2 heavy chain and β_2m inclusion body preparations were denatured separately in 8 M urea buffer (Sigma) and mixed at a 1:1 molar ratio. pMHCI was refolded in 2-mercaptoethylamine/cystamine (Sigma) redox buffer with added synthentic peptide. HLA-A2 heavy chains were refolded with the $5T4_{17-25}$ peptide. Following buffer exchange into 10 mM Tris, pH 8.1, refolded monomer was purified by anion exchange. Purified monomers were biotinylated as previously described (O'Callaghan et al., 1999) using *d*-biotin (Sigma) and BirA enzyme. Excess biotin was removed by gel filtration.

Tetramerization and Flow Cytometry

Biotinylated pMHCI monomers were conjugated by addition of streptavidin-R-phycoerythrin (PE) (Invitrogen) at a pMHCI:streptavidin molar ratio of 4:1 to produce tetrameric pMHCI complexes. Once prepared, tetramers were stored in the dark at 4 °C. 1×10^5 5T4 specific T cells in 20 µl of PBS were stained with 1 µg of $5T4_{17-25}$ specific/HLA-A2 PE-tetramer for 15 min at 37 °C, stained with CD3 and CD8 antibodies, incubated on ice for 40 min before being washed twice in PBS, and then analyzed using the FACS Canto flow cytometer.

TABLE 1

Antibody	Fluorochrome	Clone	Species and Isotype	Company	Final Concentration
PRIMARY ANTIBODY					
5T4	Alexa Fluor 488	H8	Mouse IgG1	Oxford Biomedica	1 µg/test
Annexin V	FITC	-	-	BD Pharmingen	2.5 µl/test
Calreticulin	PE	326203	Mouse IgG2b	R&D Systems	0.25 µg/test (10 µl/test)
CD14	APC-eFluor 780	61D3	Mouse IgG1	EBioscience	0.25 µg/test (2.5 µl/test)
CD3	PE-Cy7	UCHT1	Mouse IgG1	EBioscience	0.25 µg/test (2.5 µl/test)
CD8	PE-Cy5	RPA-T8	Mouse IgG1	EBioscience	0.0625 µg/test (2.5 µl/test)
CD8	FITC	RPA-T8	Mouse IgG1	EBioscience	0.5 µg/test 2.5 µl/test
CD83	PerCP-eFluor 710	HB15e	Mouse IgG1	EBioscience	0.125 µg/test (2.5 µl/test)
CD86	PE	IT2.2	Mouse IgG2b	EBioscience	0.25 µg/test (2.5 µl/test)
CD107a	PE-Cy5	eBioH4A3	Mouse IgG1	EBioscience	0.25 µg/test (2.5 µg/test)
CD209	FITC	eB-h209	Rat IgG2a	EBioscience	0.25 µg/test (2.5 µl/test)
CD284 (TLR4)	Alexa Fluor 488	HTA125	Mouse IgG2a	EBioscience	0.5 µg/test (2.5 µl/test)
HLA-A2	FITC	BB7.2	Mouse IgG2b	BD Pharmingen	1 µg/test (2 µl/test)
HLA-ABC	PE-Cy5	W6/32	Mouse IgG2a	EBioscience	10 µl/test
HLA-DR	APC	LN3	Mouse IgG2b	EBioscience	0.0038 µg/test (2.5 µl/test)
HMGB1	PE	115603	Mouse IgG2b	R&D Systems	0.25 µg/test (10 µl/test)
Hsp70	FITC	C92F3A-5	Mouse IgG1	Enzo Life Sciences	1 µg/test (1 µl/test)
IFNγ	FITC	4S.B3	Mouse IgG1	EBioscience	0.75 µg/test (1.5 µl/test)
MIP-1β	PE	24006	Mouse IgG2b	R &D Systems	10 µl/test
TNF-α	APC	MAb11	Mouse IgG1	EBioscience	0.5 µg/test (2.5 µl/test)

TABLE 2

Isotype Antibody	Fluorochrome	Clone	Species and Isotype	Company	Final Concentration
ISOTYPES					
Mouse IgG1 Isotype Control	Alexa Fluor 488	P3.6.2.8.1	Mouse IgG1	EBioscience	1µg/ml
Mouse IgG1 Isotype Control	FITC	P3	Mouse IgG1	EBioscience	1µg/test (20µl/test)
Mouse IgG2a Isotype Control	Alexa Fluor 488	eBM2a	Mouse IgG2a	EBioscience	0.5µg/test (1µl/test)
Mouse IgG2b Isotype Control	PE	133303	Mouse IgG2b	R&D Systems	0.25µg/test (10µl/test)

ANALYSIS OF TUMOUR CELL DEATH

Tumour cell death was induced by ionising irradiation (IR) with a ^{137}Cs-source at a dose rate of 0.63 Gy/min. The effects of IR were assessed by cell cycle analysis, Annexin-V/PI apoptosis assay and by imaging using the Incucyte Kinetic Imaging System. For all cell death analysis experiments, tumour cells were set up and irradiated in T25 flasks unless otherwise stated. Floating cells were collected from the flasks before trypsinisation of attached cells and included in the analysis since cell detachment is a classical feature of cell death.

Cell cycle analysis

The cell cycle describes the process of the replication and division of chromosomes within the nucleus, which occurs prior to cell division. One of the ways of finding out the potential effects of cancer treatment regimes is to measure changes in cell cycle kinetics under varying conditions. The effect of IR on the tumour cells was assessed using the Guava Cell Cycle Reagent (Millipore). The Guava Cell Cycle Reagent contains the nuclear DNA stain, Propidium Iodide (PI). Resting cells (G0/G1) contain two copies of each chromosome. Cycling cells synthesize chromosomal DNA (S phase), which results in increased fluorescence intensity. When all chromosomal DNA has doubled (G2/M phase), cells fluoresce with twice the intensity of the initial population. Cells irradiated with varying doses and/or incubated for different periods after radiation were trypsinized, washed and transferred into FACS tubes. In order to fix the cells, 200 µl ice-cold 70% methanol was added drop-wise into the tubes while vortexing on medium speed. The cell preparation was refrigerated for 24 h. The methanol-fixed cells were then re-suspended in 1 ml PBS, vortexed, and then incubated for 1 min at room temperature, before centrifuging at 1300 RPM, for 3 min. The supernatant was discarded and the cells re-suspended in 200 µl of Guava Cell Cycle Reagent. After 30 min incubation in the dark, all samples were transferred to 1.5 ml microcentrifuge tubes and analysed on the Millipore Guava® EasyCyteTM8 flow cytometer. Based on the data attained from this cell cycle analysis, 12 Gy was chosen as the IR dose to be used in further experiments and for the cross-presentation model.

Annexin-V/PI apoptosis assay

Annexin-V is used to quantitatively determine the percentage of cells undergoing apoptosis. Annexin-V is a Ca^{++} dependent phospholipid-binding protein that has a high affinity for the membrane phospholipid phosphatidylserine (PS). PS is translocated from the inner to the outer leaflet of the plasma membrane during the early stages of apoptosis, thereby exposing PS to the external cellular environment for Annexin-V to bind. PI is a DNA-binding vital dye, which is excluded by viable cells with intact membranes but is permeable through membranes of damaged or dead cells.

The cells were transferred into FACS tubes, washed twice with cold PBS and then re-suspended in 100 µl 1X Binding Buffer (BD). A master mix of equal volumes of FITC Annexin V and PI was prepared and 5 µl was added to each tube. The cells were gently vortexed and incubated for 15 min in the dark at room temperature. After incubation, 400 µl of 1X Binding Buffer was added to each tube and the cells were analysed with 2 h by flow cytometry.

Incucyte Kinetic Imaging System

The IncuCyte system (Essen BioScience) allows the microscope to be placed inside the incubator while the control of the system and access to the images and data are enabled from a computer on the local network. This imaging method provides an efficient way of documenting and understanding cellular growth, behaviour, and morphology. Irradiated and non-irradiated tumour cells were setup in 6 well plates and grown in the incubator with the system set to capture images and record the percentage of confluency after every 4 h for 3 days.

Immunocytochemistry

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

HMGB1 ELISA

DU145 cells setup in eight T25 flasks were incubated overnight before four of the flasks were treated with 12 Gy irradiation. Both irradiated and non-irradiated cells were incubated for an extra 0, 24, 48 or 72 h. Supernatant from the cells were collected after each time point, centrifuged at 1 300 RPM for 3 min and filtered to remove debris. The supernatant was then kept at -20^{o}C until all the time points were collected. HMGB1 levels in the supernatant were determined using a HMGB1 ELISA kit (IBL International) according to the manufacturer's protocol. Since HMGB1 can be measured in FBS in the cell culture supernatant, a control of media alone was used to determine background levels.

HMGB1 western blotting

Supernatants from irradiated DU145 cells were collected and HMGB1 was immunoprecipitated using Protein G Dynabeads (Life technologies). Broad-spectrum protein markers (Biorad, UK), HMGB1 protein (R and D Systems) and immunoprecipitated supernatants were separated on a 12% polyacrylamide gel (30% acrylamide/bis-acrylamide; Sigma) by SDS-PAGE under reducing conditions, transferred to HybondTM-P PVDF membrane (GE Healthcare, UK), blocked in PBS/Tween 20 (Sigma) with 5% nonfat dry milk for 2 h and probed with a monoclonal HMGB1 antibody (Santa Cruz) overnight. Horseradish-peroxidase-conjugated secondary antibody was used (Santa Cruz). Bands were developed by ECL+ on film (GE Healthcare, UK). The arbitrary units of labelling intensity were calculated using Image J software.

DC ASSAYS

DC phagocytosis of tumour cells

DU145 cells were set up into two T25 flasks and cells were allowed to adhere overnight. One of the flasks was irradiated with 12 Gy IR while the other left untreated and both flasks were further incubated for 72 h. Following this, the cells were collected and trypsinized before being labelled with CFSE using the protocol outlined above. CFSE-labelled DU145 cells were counted and re-suspended in their original media. Tumour cells ($5x10^4$ cells/well) were co-cultured with DC ($5x10^4$ cells/well) in a 96 well U-bottom plate and incubated for 24 h at 37^{o}C. After

incubation, the cells were collected from the wells, washed and surface stained with HLA-DR.

During FACS analysis, DC and DU145 tumour cells were distinguished from each other on the basis that DC were HLA-$DR^{+}CFSE^{-}$ while tumour cells were HLA-$DR^{-}CFSE^{+}$. Phagocytosis of tumour cells by DC was analysed by observing the population of HLA-$DR^{+}CFSE^{+}$ cells.

Cytokine ELISA

DC were co-cultured with tumour cells at a 1:1 ratio either in a 96 well U-bottom or a 24 well plate. The cells were incubated for 48 h. The supernatant from each well was collected and spun at 1300 RPM for 3 min to remove debris. The supernatant was then transferred into microcentrifugal tubes and stored at -80^{o}C prior to assessing the cytokines released by DC. Quantitation of IL-12, IL-10 and IL-6 was performed using Standard ELISA Development Kits (PeproTech) with a Europium-based detection method. After removal of the biotinylated detection antibody, 1 µg/ml Europium-labelled Streptavidin-conjugate (Perkin Elmer), diluted in DELFIA Assay Buffer, was added to each well and incubated at room temperature for 45 min. Following washing, 100 µl DELFIA Enhancement Solution (Perkin Elmer) was added to each well and incubated for 5 min at room temperature. Bound europium was measured by time-resolved fluorescence on a Wallac Victor2 1420 plate reader (Perkin Elmer).

Cytokine Array

DC were co-cultured with irradiated or non-irradiated tumour cells, respectively, at a 1:1 ratio in a 24 well plate. The cells were gently re-suspended and incubated for 48 h. The supernatant from each well was collected and spun at 1300 RPM for 3 min to remove debris. After which, the supernatant was transferred into microcentrifugal tubes and stored at -80^{o}C prior to analysis. The cytokine profile of DC was analysed using the Proteome Profiler Human Cytokine Array Panel A kit (R&D Systems) according to the manufacturer's protocol.

Inhibition of TLR4 and its pathways in DC determined by LPS stimulation

DC were treated for 1 h with different concentrations of VIPER peptide (Lysakova-Devine et al., 2010), TRIF or MyD88-inhibitory peptides or control peptides (Toshchakov et al., 2005, Loiarro et al., 2005) before stimulation with 10 ng/ml or 100 ng/ml LPS, respectively, for 1 h. GolgiPlug and GolgiStop were added and DC were incubated for a further 5 h. An ICCS was conducted to determine TNF-α positive cells.

T cell functional experiments

To determine if 5T4-specific T cells recognise and proliferate in response to 5T4 antigen on tumour cells, a ^{3}H-thymidine incorporation assay was carried out. To ensure that the tumour cells would not proliferate and thus incorporate the ^{3}H-thymidine, they were irradiated with 30 Gy. Irradiated tumour cells ($1x10^{4}$) and increasing numbers of T-cells, at Stimulator: Responder (S: R) ratios ranging from 80:1 to 2.5:1, were plated in triplicates in a 96-well tray. Control wells contained tumour cells or T cells only. Following incubation for three days, 0.5 µCi/well ^{3}H-thymidine (GE Healthcare, Waukesha, WI) was added to each well for 8 h and the plates were frozen at -20^{o}C. Cells after thawing were harvested onto filtermats (PerkinElmer) and counted on a Wallac 1450 MicroBeta-TriLux 3 Detector (PerkinElmer).

^{51}Cr-release assay

Peptide-pulsed or unpulsed T2 cells or tumour cells were labelled with ^{51}Cr. Target cells $3x10^{3}$/100µl/well and increasing numbers of T cells in 100 µl, at Effector:Target (E:T) ratios ranging from 80:1 to 2.5:1, were plated in triplicates in a 96-well tray. Control wells contained target cells only with 100 µl media or 100 µl 5% Triton-X100, to determine spontaneous and maximum release, respectively. Supernatant (50 µl/well) was collected from each well 4 h later and ^{51}Cr-release was measured on a Wallac 1450 MicroBeta-TriLux 3 Detector. The percent specific lysis from each well was calculated as [(experimental release - spontaneous release) / (maximum release - spontaneous release)] x 100.

T cell proliferation in response to cross-presented antigen

The rate of RLAR-T cell proliferation was measured to determine T cell activation by DC cross-presenting 5T4 antigen. DU145 cells ($1x10^5$ cells/well in 1.5 ml) were plated out in triplicates in two 24 well plates. One of the plates was irradiated with 12 Gy while the other left untreated and both plates were incubated for 72 h. DC at $1x10^5$ cells/well in 0.5 ml were added per well to the tumour cells. Four hours later, CSFE labelled T cells ($5x10^5$ cells/well) were added to the DC:tumour cell co-culture and the plate incubated at an angle for 48 h after which it was laid flat. After 5 days of tumour cell:DC:T cell incubation, the cells were re-suspended, collected into 5 ml FACS tubes and surface stained with CD3 and CD8 for flow cytometry analysis of CFSE dilution.

IFNγ-production in response to cross-presented antigen

DU145 cells were set up into two 96 well U-bottom plates at $5x10^3$ cells/well in 200 µl. After an overnight incubation, one of the plates was irradiated with 12 Gy and both plates were further incubated for 72 h. DC were then added to the wells containing tumour cells at a 1:1 ratio. The co-cultured cells were gently re-suspended and incubated for 48 h after which RLAR-T cells were added at a 1:1:5 (Tumour cell:DC:T cell) ratio. Golgi Plug (0.25 µl/well) and Golgi Stop (0.175 µl/well) were added to the wells an hour later and the cultures were incubated overnight. An ICCS flow cytometry analysis was carried out to determine the percentage of $CD8^+$ T cells producing IFNγ in response to the antigen presented by the DC.

Inhibitors and blocking antibodies

In order to determine if TLR4 or its pathways and receptors affect tumour Ag cross-presentation, DC were pretreated with inhibitory peptides before being co-cultured with the tumour cells.

VIPER (viral inhibitory peptide of TLR4) KYSFKLILAEY-9R (Severn Biotech) has been reported as a TLR4 inhibitor that consists of an 11 amino acid inhibitory sequence (underlined). It was derived from the A46 vaccinia virus protein and linked to a 9R (RRRRRRRRR) homopolymer delivery sequence (Lysakova-Devine et al., 2010). It is thought that the TLR4 inhibitor binds to the TIR domains of the receptor

and adaptor proteins, thereby inhibiting TLR4 signalling by interfering with TLR4-Mal and TLR4-TRAM (Lysakova-Devine et al., 2010).

The MyD88 and TRIF inhibitory peptides correspond to the sequence of the BB-loop of MyD88 (RDVLPGT) (Proimmune) and TRIF (FCEEFQVPGRGELH) (Invivogen), respectively, and serve as decoys by binding to the TIR domains and interfering with TLR-adaptor interactions (Loiarro et al., 2005, Toshchakov et al., 2005). The peptides are linked to protein transduction sequences, which render the peptides cell permeable (RQIKIWFQNRRMKWKK) (Derossi et al., 1994). The control peptides consist of the protein transduction sequence alone. DC were pre-treated with 20 µM MyD88 inhibitory or control peptide or 10 µM 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 (Sigma), an inhibitor for HMGB1, was added at 50 µM at the time of irradiation, while VER155008 from Tocris Bioscience (R and D Systems), an inhibitor for Hsp70, at 5 µM to 0 Gy and 12 Gy irradiated DU145 cells at 0 h, 24 h and 48 h of the 72 h incubation, respectively, as described in the method for the cross-presentation assay.

TLR4 nucleotide sequencing for the Asp299Gly SNP

Pyrosequencing

SNP analysis was carried out in Dr Rachel Butler's laboratory in the department of Medical Genetics (Cardiff and Vale NHS Trust, University Hospital of Wales). 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 known TLR4 polymorphism and 68 samples were tested.

TaqMan Predesigned SNP Genotyping Assay

The TaqMan SNP Genotyping Assays use TaqMan 5´-nuclease chemistry for amplifying and detecting specific TLR4 SNP **(SNP ID: rs4986790)** (Applied Biosystems) in purified genomic DNA samples. The assay was carried out on 10 samples. In order to extract genomic DNA from PBMC or BLCL samples, the cells were resuspended in 3 ml Nucleic Lysis Buffer (1 M Tris-HCL pH8, 1.5 M NaCL,

0.5 M EDTA in dH_2O), 600 µl Proteinase K (2 mg/ml in 1% SDS and 2 mM EDTA) and 200 µl 10% SDS and incubated overnight at 37^oC on a rotor. After which, 1 ml 6 M NaCL was added to each sample and the samples were shaken for 15 seconds before spinning at 1300 RPM for 25 min. The supernatants were transferred into clean 15 ml falcon tubes, 8 ml 100% ethanol was added to the supernatant and supernatant+ethanol solution was mixed by inverting the tubes several times. After the extracted DNA was collected from the solution and ethanol had evaporated, the DNA samples were transferred into 100 µl dH_2O. The genomic DNA was quantitated using the Nanodrop spectrophotometer. Genomic DNA (20 ng/per well) was added to the reaction mix for a total of 20 µl/per well. All the components were purchased from Applied Biosystems.

Preparation for reaction mix:

Component	**Volume per well**
2X TaqMan Genotyping Master Mix	10 µl
40X Genotyping Assay Mix	0.5 µl
Nuclease-Free Water	5.5 µl
Genomic DNA (20 ng/ml)	4 µl

III. Development and Characterisation of a Tumour Antigen Cross-Presentation Model from Irradiated Tumour Cells

INTRODUCTION

The development of antitumor $CD8^+$ T cell responses relies on DC to acquire tumour antigens from tumour cells and present the processed antigen on MHC class I molecules via the process of cross-presentation to $CD8^+$ T cells. Cross-presentation is thought to occur through one of two main pathways. The endosome-to-cytosol pathway involves transport of exogenous antigens from endosomal vesicles into the cytosol, where they are trimmed and processed by the proteasome and subsequently loaded on MHC class I molecules in the endoplasmic reticulum, similar to endogenous antigens. In the second, proteasome-independent cytosol-independent pathway, DC use endosomal proteases to process and load captured antigens directly onto MHC class I molecules in endosomal compartments (Rock et al., 2010). Antigen cross-presentation should not be confused with cross-priming, which requires additional signals in order to generate primary T cell responses.

Many tumours do not express TAA at a sufficiently high amount to cause significant cross-presentation. Moreover, most growing tumours do not cause robust DC activation to create a pro-inflammatory environment. These obstacles have been overcome in some studies by using 1) tumour cells infected with non-replicating viral vectors encoding viral or TAA proteins (Russo et al., 2000, Zhou et al., 2003); 2) tumour cells made apoptotic after infection with oncolytic viruses (Moehler et al., 2005, Donnelly et al., 2013); 3) inducing cell death using chemotherapeutic agents (Apetoh et al., 2007b). However, very few *in vitro* studies carried out with human cells have evaluated antitumor specific T cell responses when DC cross-present natural cell-associated TAA from irradiated tumour cells. Radiation increases the expression of some TAA (Sharma et al., 2011) and creates a pro-inflammatory tumour microenvironment as highlighted in the introduction. T cell responses after the cross-presentation of antigen from irradiated tumour cells have only been

documented using melanoma antigens, such as Melan A/MART-1 (Barrio et al., 2012) and gp100 (von Euw et al., 2007).

In this study the 5T4 oncofetal glycoprotein, which is normally expressed in the placenta and rarely in fully developed healthy tissues, was the target antigen. It is highly expressed in a range of human carcinomas, including PCa (Southall et al., 1990). 5T4 has been detected in the majority of primary PCa tissues studied (Amato and Stepankiw, 2012). 5T4 expression is not evident in normal prostate tissues but is present at low levels in benign prostatic hyperplasia (BPH). 5T4 has been linked to altering cell adhesion, motility and morphology and is associated with aggressive metastasis (Carsberg et al., 1996). In order to study T cell responses generated via cross-presentation of 5T4 antigen, an antigen-specific T cell line was required; the readout in the cross-presentation model would then be T cell proliferation and IFNγ production generated by DC in a MHC Class I-restricted manner.

In this work, monocyte-derived DC were used to take up and cross-present tumour antigen, mainly because it would have been impractical to carry out the experiments with the subset of blood DC which are highly specialised to cross-present antigen (Bachem et al., 2010, Segura et al., 2013). It has been demonstrated that monocyte-derived DC are able to cross-present exogenous antigen (Albert et al., 1998, von Euw et al., 2007). As a generally accepted, universal method for tumour antigen cross-presentation does not exist, a series of optimisation experiments need to be carried out to determine the most efficient setup in this model.

Question

Does irradiation of tumour cells generate antitumor T cell responses by cross-presentation of 5T4 antigen via DC to antigen-specific T cells?

Specific aims

1. Characterise PCa cell lines for the expression of 5T4 and HLA-A2.
2. Develop a 5T4 specific HLA-$A2^+$ $CD8^+$ T cell line.
3. Characterise the T cell line for the ability to recognise naturally expressed 5T4 antigen.
4. Developing an antigen cross-presentation model with irradiated PCa cells where the readout is T cell activation.

RESULTS

Characterisation of PCa cell lines

To determine which of the PCa cell lines expressed 5T4 antigen, DU145 and LNCaP lines were examined by flow cytometry for 5T4 expression using a 5T4 antibody conjugated with Alexa Fluor 488 in the laboratory by Dr Saly Al-Taei (the antibody was a gift from Oxford Biomedica) (Figure 3.1). 5T4 was expressed at a significant level on DU145 but not LNCaP cells compared to the isotype control. Tumour cells can downregulate the expression of MHC class I molecules as a mechanism to escape recognition and destruction by cytotoxic $CD8^+$ T cells. However, DC can acquire cellular antigens, regardless of the MHC expression on these cells, for cross-presentation. The T cell line that was generated in the lab was HLA-$A2^+$ and it was desirable to use an HLA-$A2^-$ tumour cell line, so direct recognition of the tumour antigen by the T cell line can be excluded. Surface staining for MHC class I expression on DU145 and LNCaP cells was carried out and analysed by flow cytometry. While both cell lines express MHC class I, the expression on LNCaP cells was slightly lower compared to that expressed on DU145 cells. The PCR data obtained from the Welsh Blood Transfusion Services revealed that DU145 cells encode for: **HLA-A03, HLA-A33, HLA-B50 and HLA-B57** while LNCaP cells encode for: **HLA-A01, HLA-A02, HLA-B37 and HLA-B08**. Based on 5T4 positivity and the lack of HLA-A2 expression, DU145 cells were used in the cross-presentation model.

Specificity and function of the 5T4 specific HLA-$A2^+$ $CD8^+$ T cell line

The precursor frequency of naive $CD8^+$ T cells that recognize a specific antigen presented by MHC class I molecules is low and is estimated to be approximately 1:200 000 (Blattman et al., 2002). These small numbers of antigen-specific $CD8^+$ T cells need to undergo significant expansion in order to eradicate tumour cells and this requires activation by DC (Arens and Schoenberger, 2010). To determine the ability of 5T4 antigen from irradiated PCa cells to induce 5T4-specific T cell responses via cross-presentation, I generated a 5T4 specific $CD8^+$ T cell line (RLAR-T cells). The

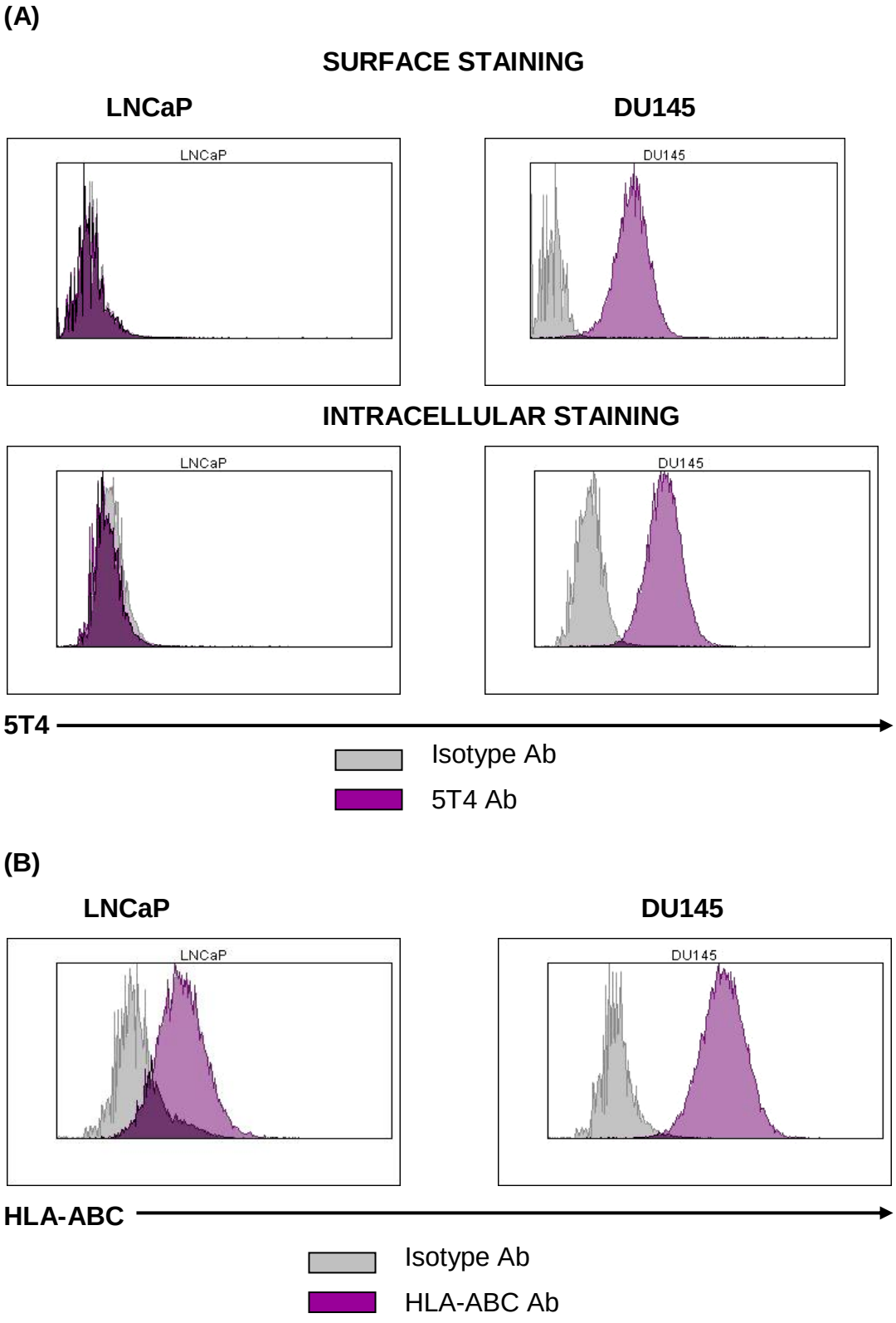


Figure 3.1: 5T4 and MHC-Class I expression on PCa cell lines. (A) Representative histograms of surface or intracellular 5T4 expression on PCa cells as indicated. Cells were labelled with either IgG1-Alexa488 isotype (grey) or 5T4-Alexa Fluor 488 antibody (purple). (B) Representative histograms of the tumour cells (as indicated above the figures) labelled with HLA-ABC PE-Cy5 Ab (purple) or IgG2a PE-Cy5 isotype control (grey) antibodies.

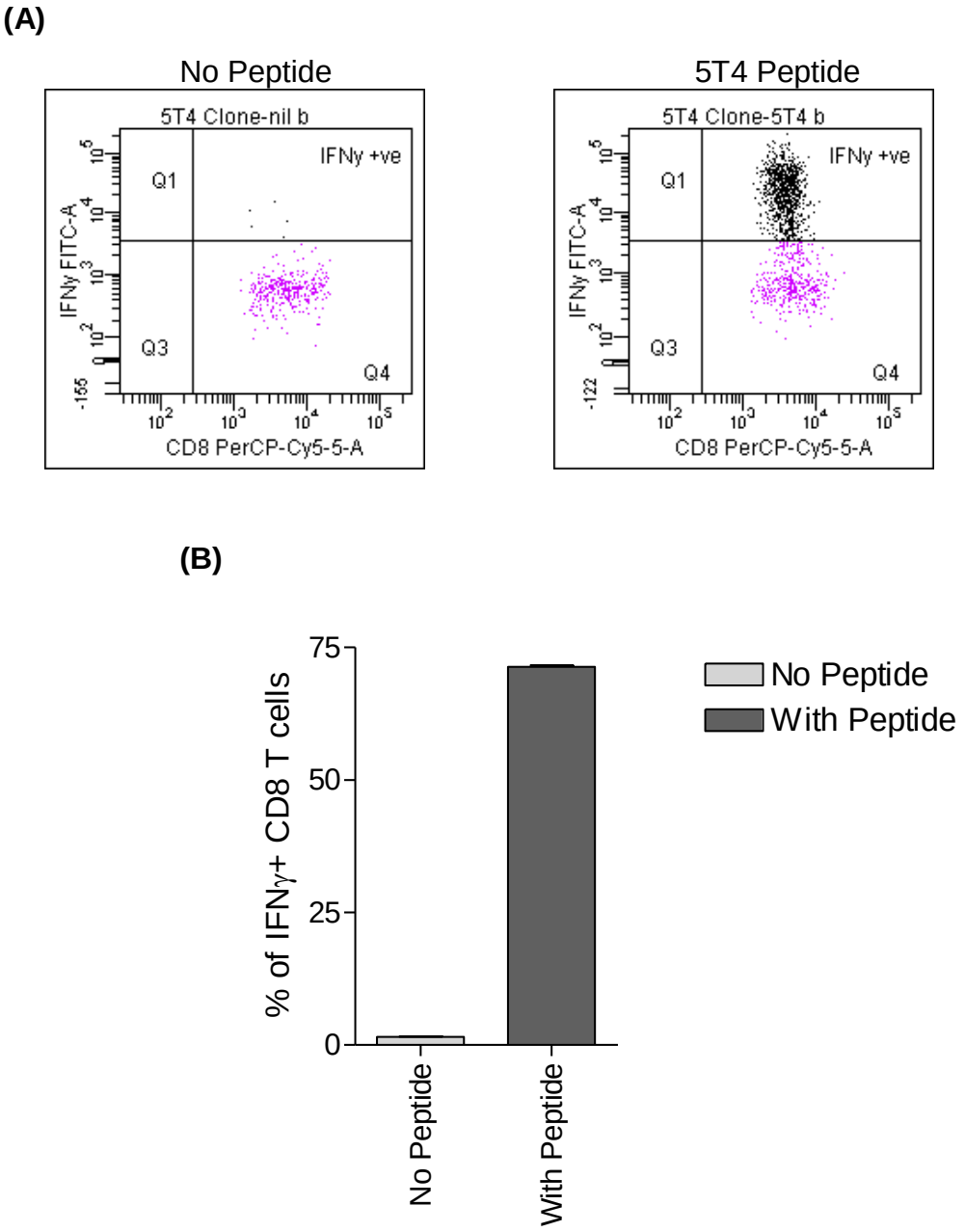


Figure 3.2: Characterisation of the RLAR-T cells. (A) Representative dot plots of the $CD8^+$ RLAR-T cell responses to the $5T4_{17\text{-}25}$ peptide. (B) Mean and SD percentage of 5T4-specific IFNγ positive T cells from duplicates shown with or without peptide. One of >10 experiments is shown.

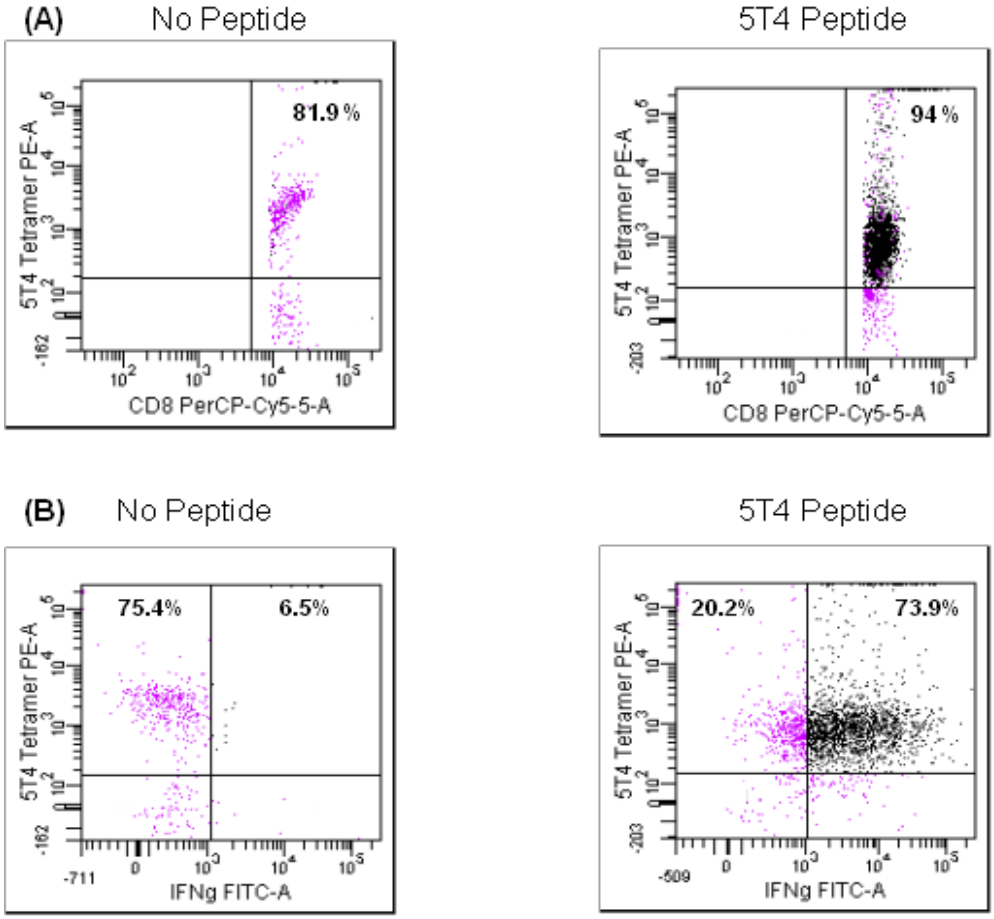


Figure 3.3: Assessment of T cell IFNγ production by tetramer positive RLAR-T cells. (A) Representative dot plot showing the percentage of $CD8^+$ T cells positive for the tetramer without or with peptide stimulation. (B) Representative dot plot showing the percentage of tetramer positive cells responding to no peptide or 5T4 peptide stimulation. The number represent the mean of triplicate samples.

RLAR-T cells were developed from a HLA-$A2^+$ healthy donor by stimulation of non-adherent PBMC with autologous monocyte-derived DC, generated from adherent PBMC (Al-Taei et al., 2012), and loaded with 2 µg/ml $5T4_{17-25}$ peptide (RLARLALVL) (Shingler et al., 2008) as described in the Methods. After CD8 enrichment, a peptide specificity assay was carried out on the RLAR-T cells. Approximately 71% of the $CD8^+$ T cells responded to the $5T4_{17-25}$ peptide as evaluated by the percentage of IFNγ-producing T cells (Figure 3.2). To determine the percentage of antigen-specific T cells responding to the $5T4_{17-25}$ peptide, the RLAR-T cells were labelled with a PE-conjugated $5T4_{17-25}$ specific/HLA-A2 tetramer I prepared in the laboratory of Dr Linda Wooldridge (Figure 3.3). While 94% of the $CD8^+$ T cells were labelled by the tetramer (Figure 3.3A), about 73.9% of the tetramer positive $CD8^+$ T cells produced IFNγ upon stimulation overnight with the $5T4_{17-25}$ peptide (Figure 3.3B).

The RLAR-T cells were also tested for the ability to kill HLA-$A2^+$ T2 cells with or without pulsing them with the 5T4 peptide (Figure 3.4A). In order to determine if the T cells also recognise naturally processed 5T4 on tumour cells in an HLA-A2-restricted manner, several tumour cell lines with known 5T4 expression were also tested as targets (Figure 3.4B): $5T4^+A2^+$ M15 (mesothelioma) cells; $5T4^+A2^-$ M38 (mesothelioma) cells; $5T4^-A2^+$ LNCaP (PCa) cells and $5T4^+A2^-$ DU145 cells. Due to the lack of available PCa cell lines, mesothelioma cell lines were used in these experiments as they are being studied in an independent project in the laboratory.

The RLAR-T cells killed $5T4_{17-25}$ peptide-pulsed T2 cells efficiently at any E:T ratios (2.5:1-80:1) studied, with low background lysis of peptide-unpulsed T2 cells (Figure 3.4A). However, none of the tumour cells were killed even at the highest (80:1) E:T ratio (Figure 3.4B). The RLAR-T cells were then tested for their ability to kill a larger selection of HLA-$A2^+$ (M15, M18, M24, M34, M36, M40) and HLA-$A2^-$ (M38) tumour cell lines, all expressing 5T4 (Figure 3.5A). Four of the six $A2^+$ cell lines (M18, M24, M34, M36) were killed at a range of 16-27% specific lysis at 20:1 E:T ratio in a 5h CTL assay. The lysis of these cells was significantly higher than that of the HLA-$A2^-$ cell line, M38 (Figure 3.5B). As in the previous experiment, the M15 cells were not killed, indicating that although the RLAR-T cells can recognise

naturally processed 5T4, some tumour cells are resistant to T cell killing or recognition by T cells is impaired due to 5T4 being differentially expressed.

In order to find out if the RLAR-T cells can recognise 5T4 on M15 tumour cells in spite of their inability to kill these cells, a stimulation assay with irradiated (30 Gy) M15 and negative control LNCaP cells was set up (Figure 3.6). The RLAR-T cells recognized native 5T4 antigen on M15 but not on LNCaP cells in a dose dependent manner. However, T cell proliferation was enhanced in the presence of the peptide. This confirms that the RLAR-T cells can recognise naturally processed 5T4, but shows that some tumour cells can be poor stimulators or targets of T cells. These findings have been published (Al-Taei et al., 2012).

To determine if the 5T4 antigen can be cross-presented, a model was developed and $5T4^-$ LNCaP cells were used as an antigen negative control, while DC incubated without any tumour cells were used to calculate background stimulation of T cells. Since T cell activation results in a marked expansion of T cells, cross-presentation of the 5T4 antigen by DC was first evaluated by assessing the proliferation of CFSE labelled RLAR-T cells (Figure 3.7). DC were co-cultured with DU145 or LNCaP cells in a 24 well plate for 4 h before the CFSE labelled RLAR-T cells were added. T cell proliferation was measured after 5 days. Analysis of MFI by flow cytometry was carried out to determine the dilution of the CFSE label in the gated $CD8^+$ T cell population. DC cross-presenting antigen from DU145 cells induced the proliferation of the RLAR-T cells above the background levels induced by DC alone. However, cross-presentation of antigen from LNCaP cells did not occur as T cell proliferation was even lower than the background (Figure 3.7).

Antigen-specific $CD8^+$ T cells can mobilize two main effector mechanisms: production of cytokines and chemokines and cytolysis of target cells. To determine antitumor T cell responses, 5T4 specific T cell activation with antigen, cross-presented by DC, was assessed by evaluating the percentage of IFNγ positive $CD8^+$ T cells. Optimisation was carried out to determine the most efficient cell culture conditions. As culture of these cells in flat bottomed trays did not result in antigen cross-presentation measured by IFNγ, the cell number and vessels were optimised

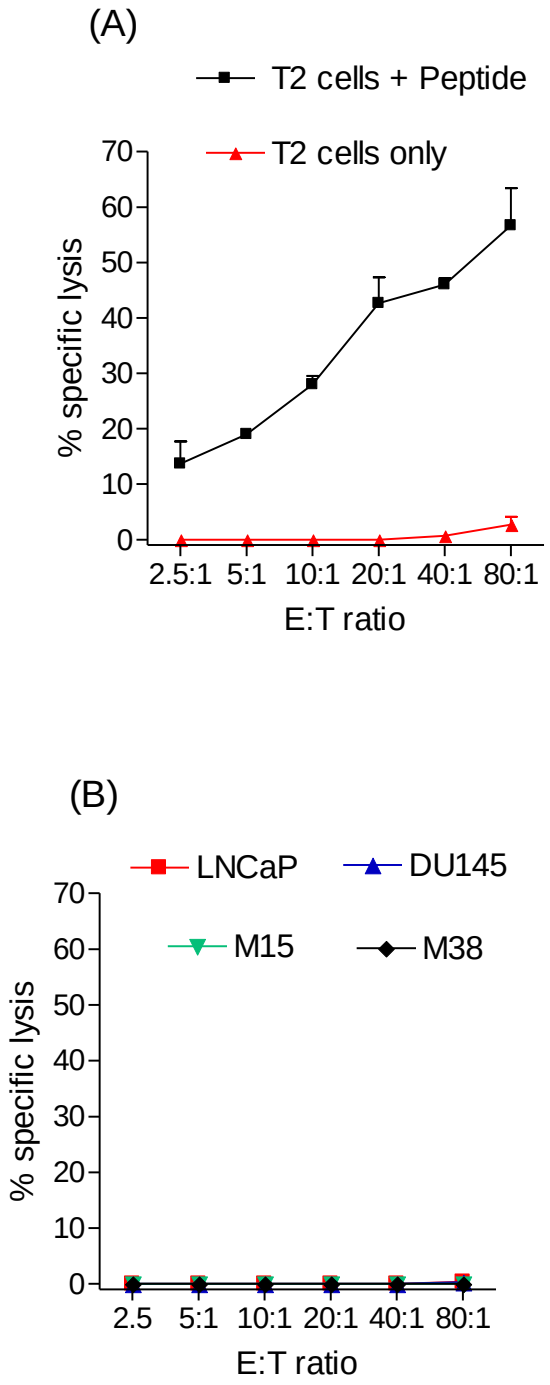


Figure 3.4: Cytotoxic activity of the RLAR-T cells. Targets: (A) $5T4_{17-25}$ peptide-pulsed *(black)* and unpulsed T2 cells *(red)* and (B) Tumour cells – LNCaP, DU145, M15 and M38. Mean and SD percent of specific lysis at different effector:target (E:T) ratios from triplicate samples are shown.

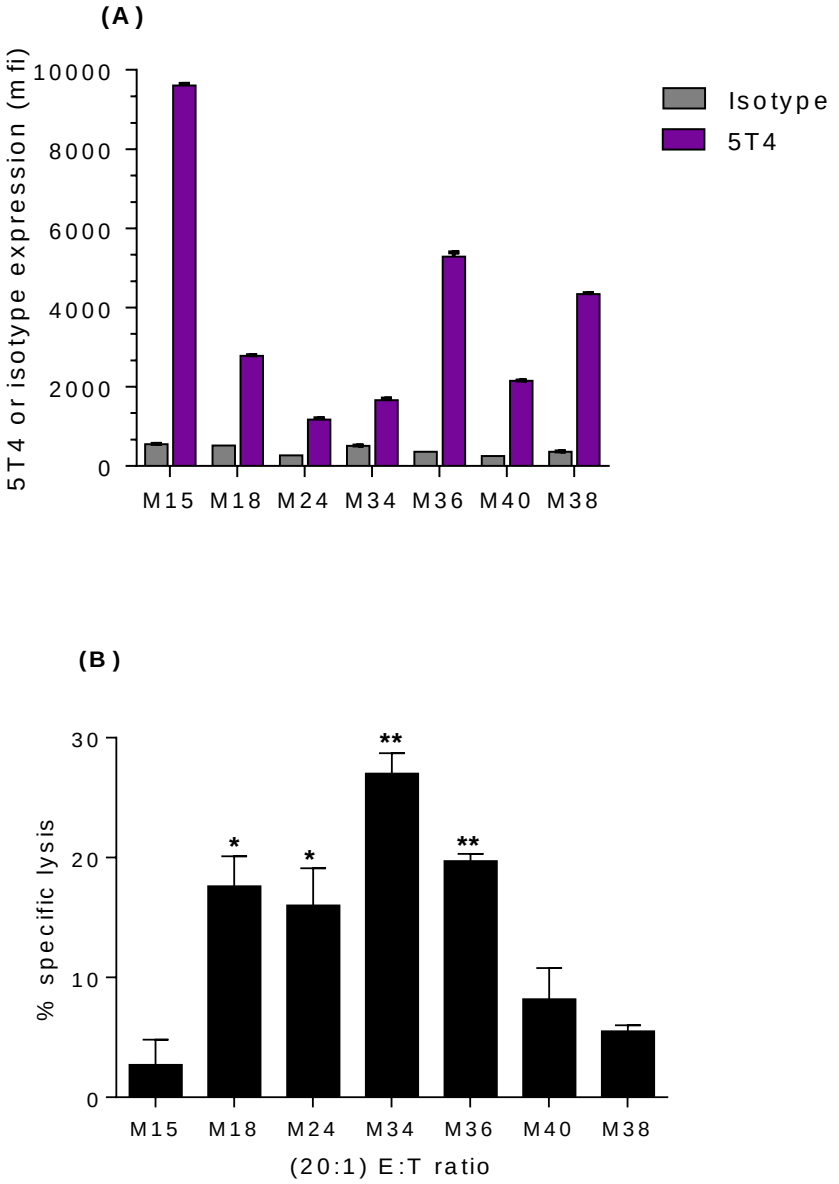


Figure 3.5: 5T4-dependent mesothelioma cell killing by the RLAR-T cells. (A) 5T4 surface expression on the target cells *(mesothelioma tumour cells)* of the CTL assay. Mean and SD of MFI are shown, calculated from two independent experiments. (B) HLA-A2^{+} mesothelioma cell lines *(M15 - M40)* and an HLA-A2^{-} cell line *(M38)* were pulsed with ^{51}Cr and served as targets of T-cell killing by the RLAR-T cells in a 5 h ^{51}Cr-release assay at 20:1 E:T ratio. The bars represent the means and SD of percentage specific lysis from triplicate samples. *(*p < 0.05; **p < 0.01; Student's t-test).*

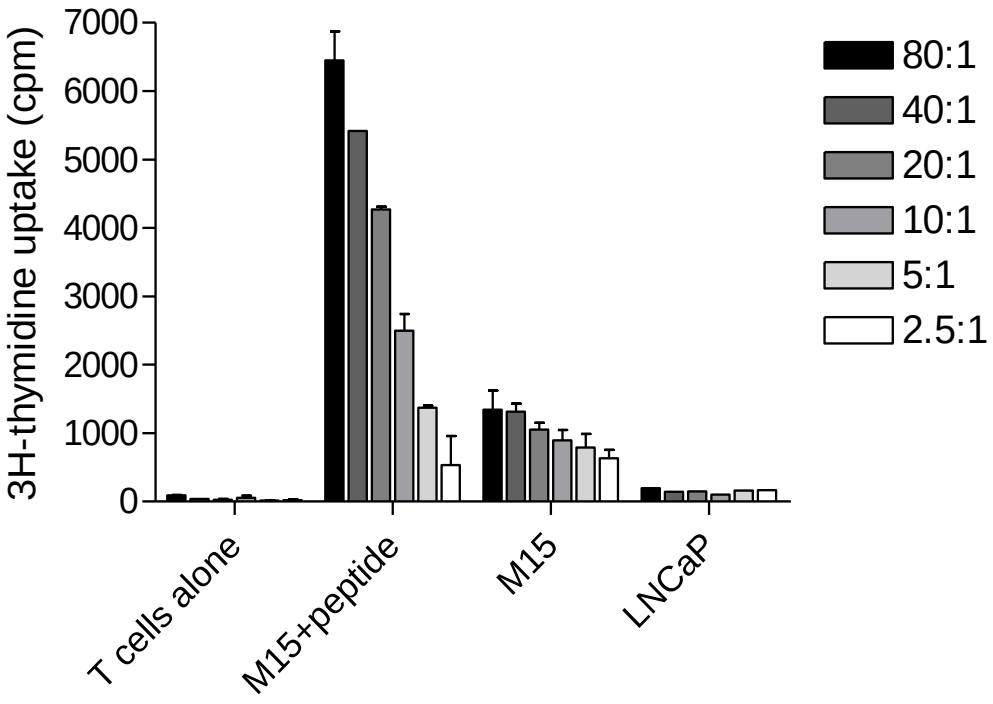


Figure 3.6: Proliferation of the RLAR-T cells stimulated by tumour cells. The RLAR-T cells were cultured either alone or with $5T4_{17-25}$ peptide + M15, M15 or LNCaP cells at different tumour:T cell ratios. The bars represent the mean and SD for ^{3}H-thymidine uptake (count/min, cpm) by RLAR-T cells in response to the tumour cells or tumour+peptide from triplicate samples.

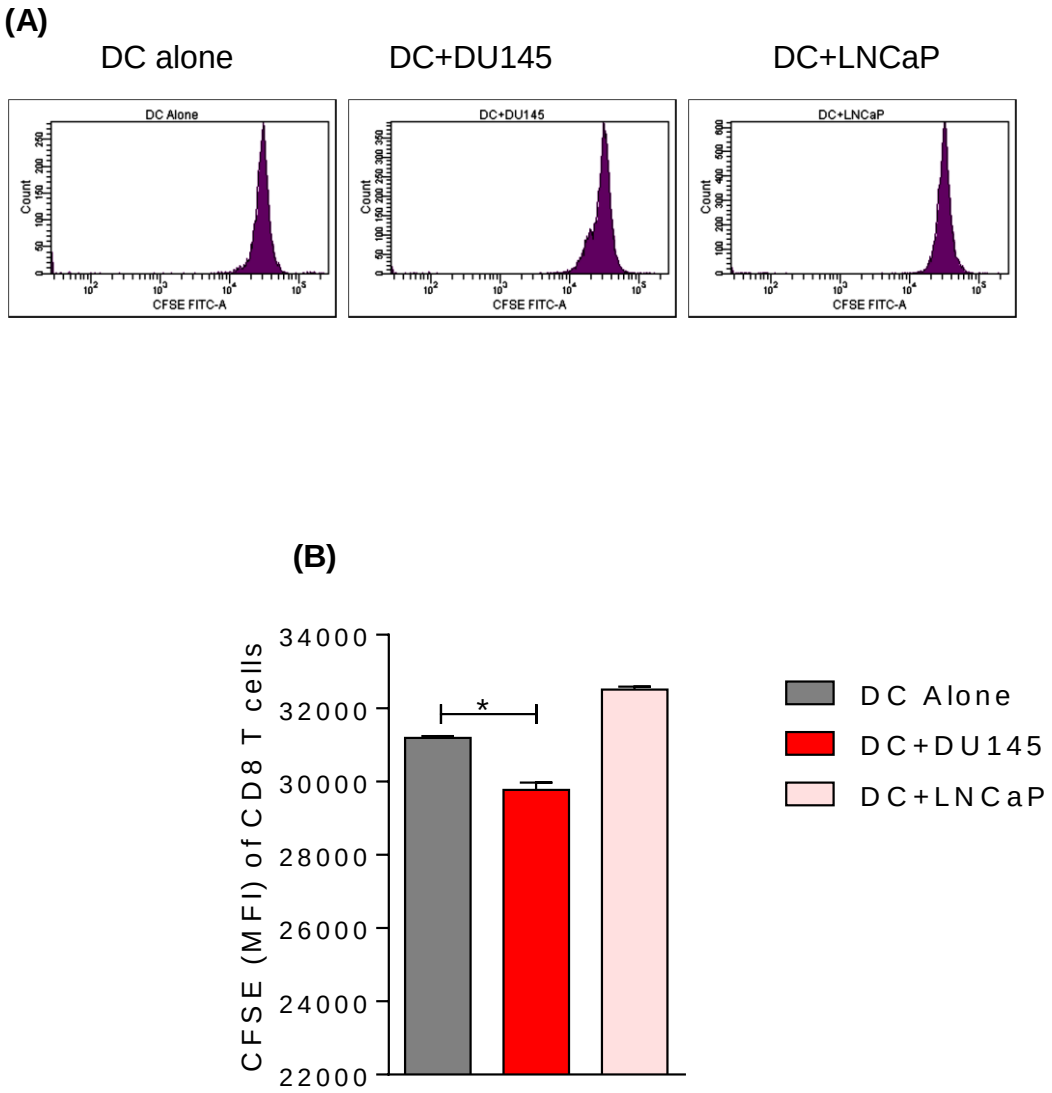


Figure 3.7: Proliferation of the CFSE labelled RLAR-T cells; cross-presentation from non-irradiated tumour cells. (A) Representative histograms showing CFSE dilution from the labelled RLAR-T cells in response to either DC alone or DC in the presence of LNCaP or DU145 tumour cells which were not irradiated. (B) The bars represent the mean and SD for the MFI of CFSE from triplicate samples. *(*p < 0.05; Student's t-test).* Representative of two experiments

(not shown) and it was concluded that U-bottom trays were needed to detect cross-presentation by T cell IFNγ production. DC were co-cultured with DU145 or LNCaP cells in a 96 well plate for 48 h before the RLAR-T cells were added for 6 h in the presence of Golgi blockers. DU145 and LNCaP cells were also cultured with RLAR-T cells in the absence of DC to ensure that the tumour cells were not inducing any T cell stimulation. IFNγ production by $CD3^+CD8^+$ gated population was analysed by flow cytometry. Similar to the proliferation data, DC co-cultured with DU145 cells resulted in a small but significant stimulation of IFNγ production by the RLAR-T cells above the background induced by DC alone (Figure 3.8). DC co-cultured with LNCaP cells inhibited this background cytokine production by the RLAR-T cells. The T cell proliferation and IFNγ production data demonstrate that 5T4 specific antitumor T cells responses can be generated against 5T4 antigen cross-presented by DC from non-irradiated tumour cells.

Next, the ability of irradiated DU145 cells to induce enhanced T cell activation by DC was investigated by carrying out a T cell proliferation assay with CFSE labelled RLAR-T cells (Figure 3.9). To assess effector T cell responses, in addition to measuring IFNγ production (Figure 3.10A), MIP-1β production (Figure 3.10B and C) and T cell degranulation via CD107a mobilization (Figure 3.11) were also measured by flow cytometry in a 6 h assay.

Cross-presentation of antigen from non-irradiated DU145 cells induced significant T cell proliferation similar to data shown earlier. However, cross-presentation of the 5T4 antigen was greatly increased as detected by T cell proliferation when DC cross-presented antigens from irradiated cells compared to non-irradiated ones as highlighted by further dilution of the CSFE and decrease in MFI (Figure 3.9).

Although DC, cross-presenting antigen from both irradiated and non-irradiated DU145 cells stimulated T cell proliferation, irradiated tumour cells induced approximately twice as many RLAR-T cells to produce IFNγ and MIP-1β compared to that by non-irradiated tumour cells (Figure 3.10). Upon cross-presentation by DC with antigen from irradiated DU145 cells, 40% of the gated $CD8^+$ T cells produced MIP-1β compared to 28% by DC with non-irradiated cells and 20% by DC alone (Figure 3.10B). In a similar manner, 15% of the gated $CD8^+$ T cells were double

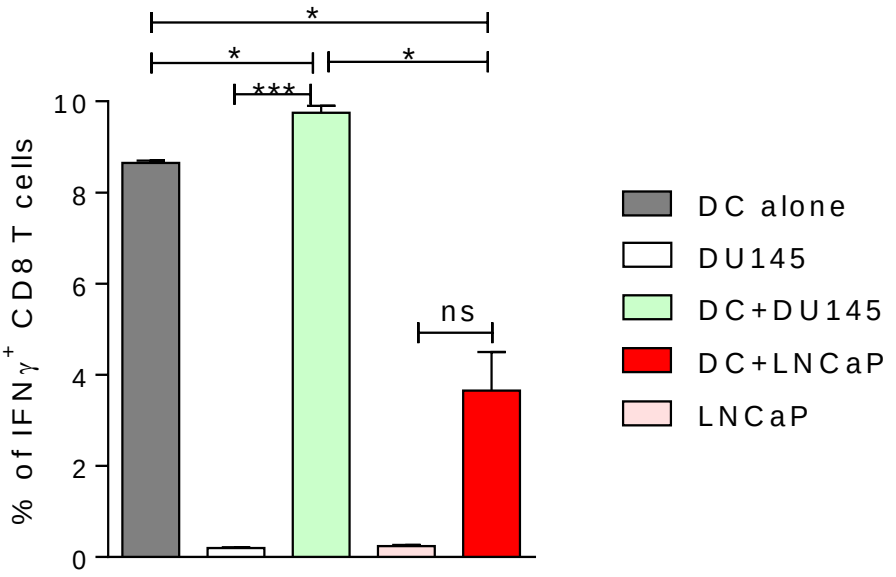


Figure 3.8: RLAR-T cell stimulation by DC co-cultured with non-irradiated LNCaP or DU145 cells. Mean and SD for the percentage of IFNγ positive $CD8^+$ T cells from triplicate samples. *(*p < 0.05; ***p<0.001 Student's t-test).* Representative of two experiments.

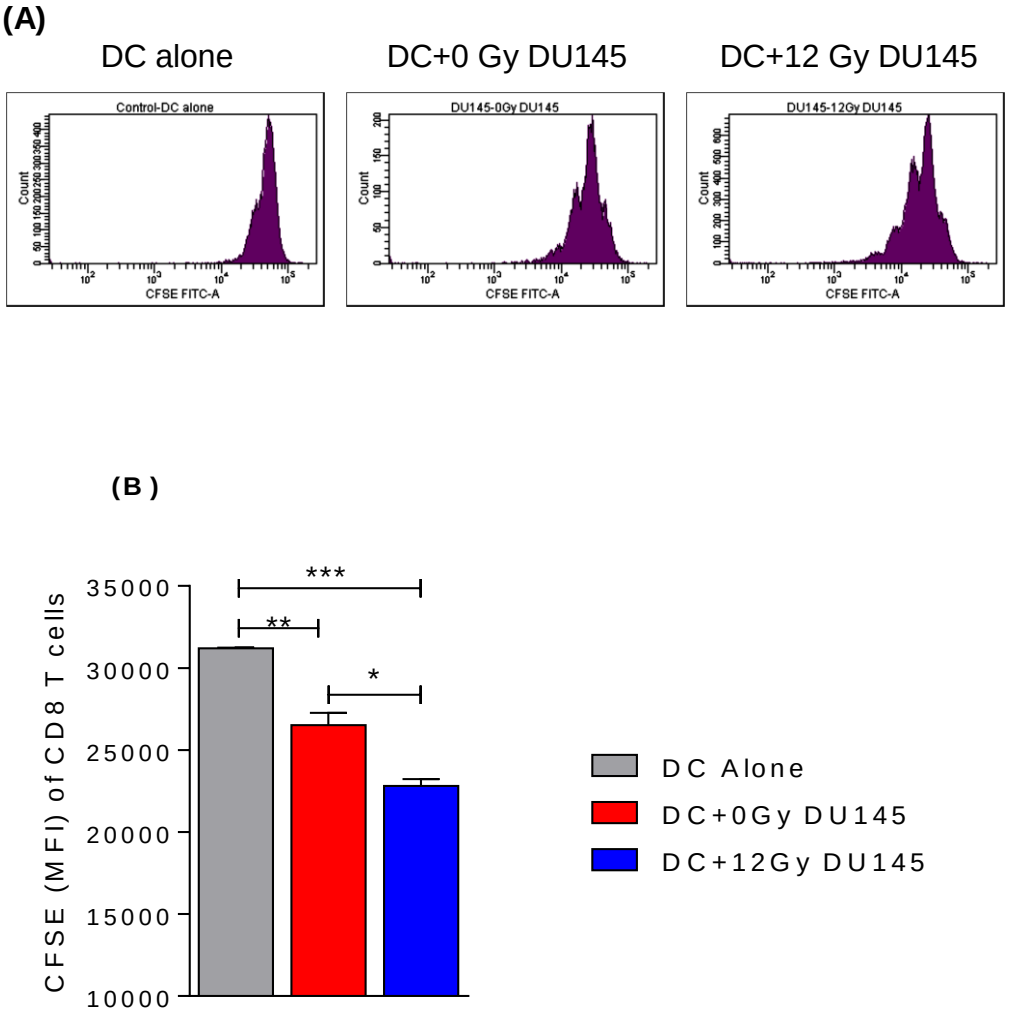


Figure 3.9: Proliferation of RLAR T cells; cross-presentation from irradiated tumour cells. (A) Representative histograms showing CFSE dilution by RLAR T cells in response to either DC alone or DC in the presence of 0 Gy or 12 Gy treated DU145 tumour cells. (B) The bars represent the mean and SD for the MFI of CFSE of the RLAR-T cells in response to either DC alone or DC in the presence of 0 Gy or 12 Gy treated DU145 tumour cells from triplicate samples. *(*p < 0.05; **p<0.01; ***p < 0.001; Student's t-test).* Representative of two experiments.

positive for MIP-1β and IFNγ in response to DC with irradiated cells compared to 7.5% by DC with non-irradiated cells and 5.5% by DC alone (Figure 3.10C). CD107a is a marker of cytotoxic T cell degranulation and its mobilization correlates well with cytotoxic activity of $CD8^+$ T cells. Yet again, cross-presentation of antigen from irradiated cells caused twice as many $CD8^+$ T cells to mobilize CD107a to the cell surface and a large proportion of these cells T cells produced IFNγ (Figure 3.11A and B).

Using live (non-irradiated), necrotic (through repeated freeze thawing) and apoptotic/necrotic (12 Gy irradiated) cells, a cross-presentation experiment was carried out to determine if any form of cell death was sufficient to induce antitumor T cell responses or if IR was superior in generating 5T4 specific T cell responses. The freeze-thawed fraction was prepared by placing trypsinized DU145 cells in a -20^{o}C freezer until completely frozen and then thawing them at room temperature. This was repeated three times. Cross-presentation of the 5T4 antigen was not enhanced above background by freeze thawing the cells (Figure 3.12), indicating that the availability of the 5T4 antigen on the tumour cells alone is not sufficient for the induction of antigen cross-presentation.

In order to prove that T cell responses being observed are due to cross-presentation, the cytosolic and vacuolar cross-presentation pathways were blocked. Events within the cytosolic pathway were inhibited using lactacystin to prevent antigen degradation by proteasomes and brefeldin A to prevent loading of peptides on MHC class I molecules and transport to the cell surface through the Golgi apparatus. The inhibitors were used separately or in combination. Within the endosomal (vacuolar) pathway, aspartic proteases such as cathepsin D and E were inhibited using Pepstatin A while cysteine proteases such as cathepsin B, L and S were inhibited using Leupeptin. These inhibitors were also used either separately or in combination. Firstly, to ensure that the inhibitors were not toxic to DC, DC were pretreated with a combination of either cytosolic or endosomal pathway inhibitors before being pulsed with the $5T4_{17\text{-}25}$ peptide for T cell stimulation (Figure 3.13). Significant differences were not observed between the control group (no inhibitor) and those with the inhibitors when the percentage of IFNγ producing $CD8^+$ T cells was assessed. This demonstrated that the inhibitors did not affect DC viability or function (Figure 3.13).

In the cross-presentation experiment, DC were pretreated for 1 h with the respective inhibitors or controls before co-culturing them with irradiated or non-irradiated DU145 cells (Figure 3.14). Cross-presentation of the 5T4 antigen to RLAR-T cells was completely inhibited by blocking events of the cytosolic pathway either separately or in combination. However, inhibition of the proteases involved in the vacuolar pathway did not affect cross-presentation (Figure 3.14). This suggests that 5T4 antigen is processed by proteasomes and transported on newly synthesised MHC class I molecules out of the ER to the cell surface through Golgi apparatus.

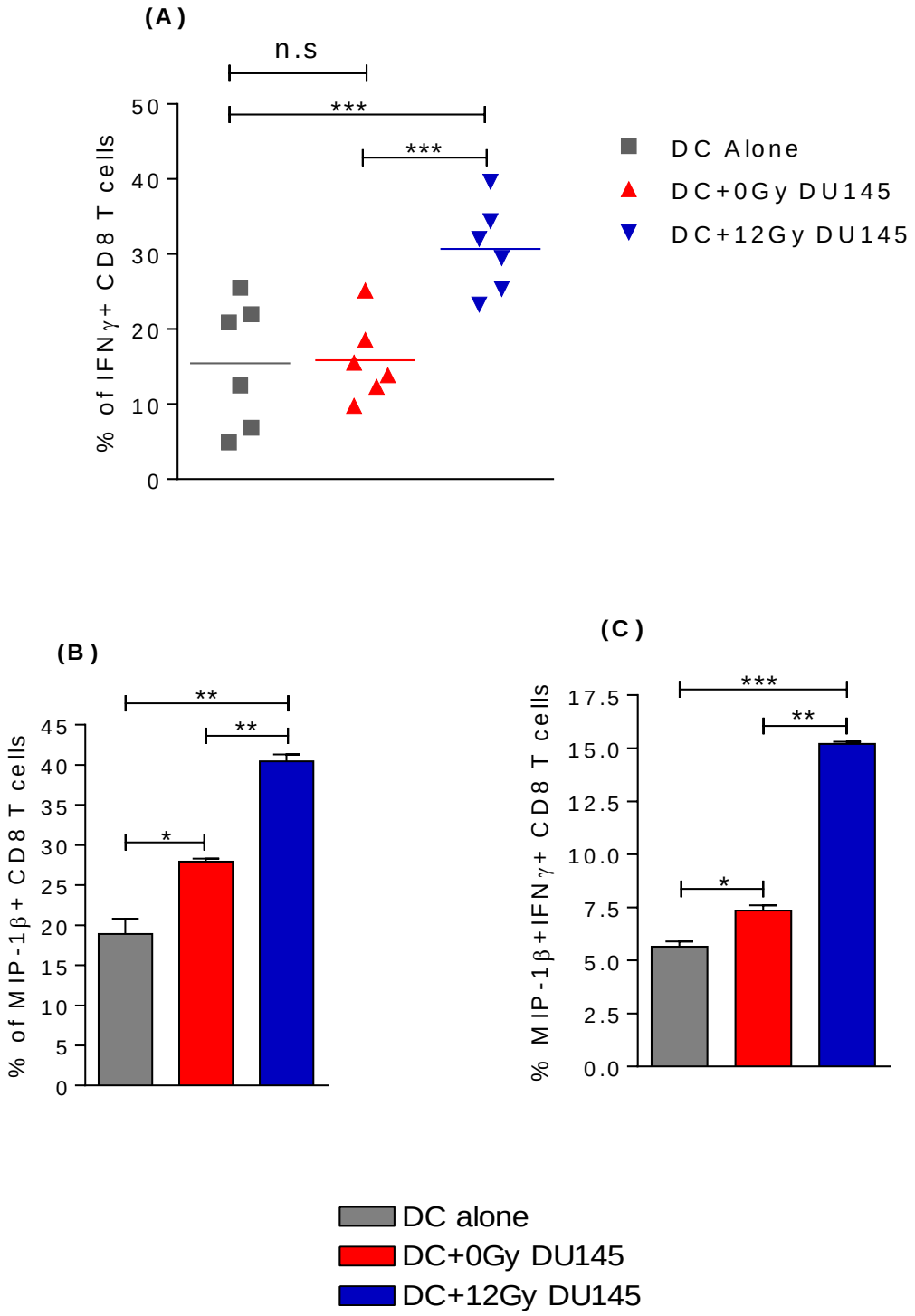


Figure 3.10: Stimulation of RLAR-T cells after cross-presentation of irradiated and non-irradiated DU145 cells by DC measured by IFNγ and MIP-1β production. (A) IFNγ positive $CD8^+$ T cells. Summary of results from 6 donors. Each symbol represents a different donor and is a mean of triplicate samples. The lines represent the mean of IFNγ production in each group. Mean and SD for the percentage of (B) MIP-1β positive or (C) MIP-1β and IFNγ double positive $CD8^+$ T cells from triplicate samples are also shown. *(*p < 0.05;**p <0.01; ***p < 0.001; Student's t-test).*

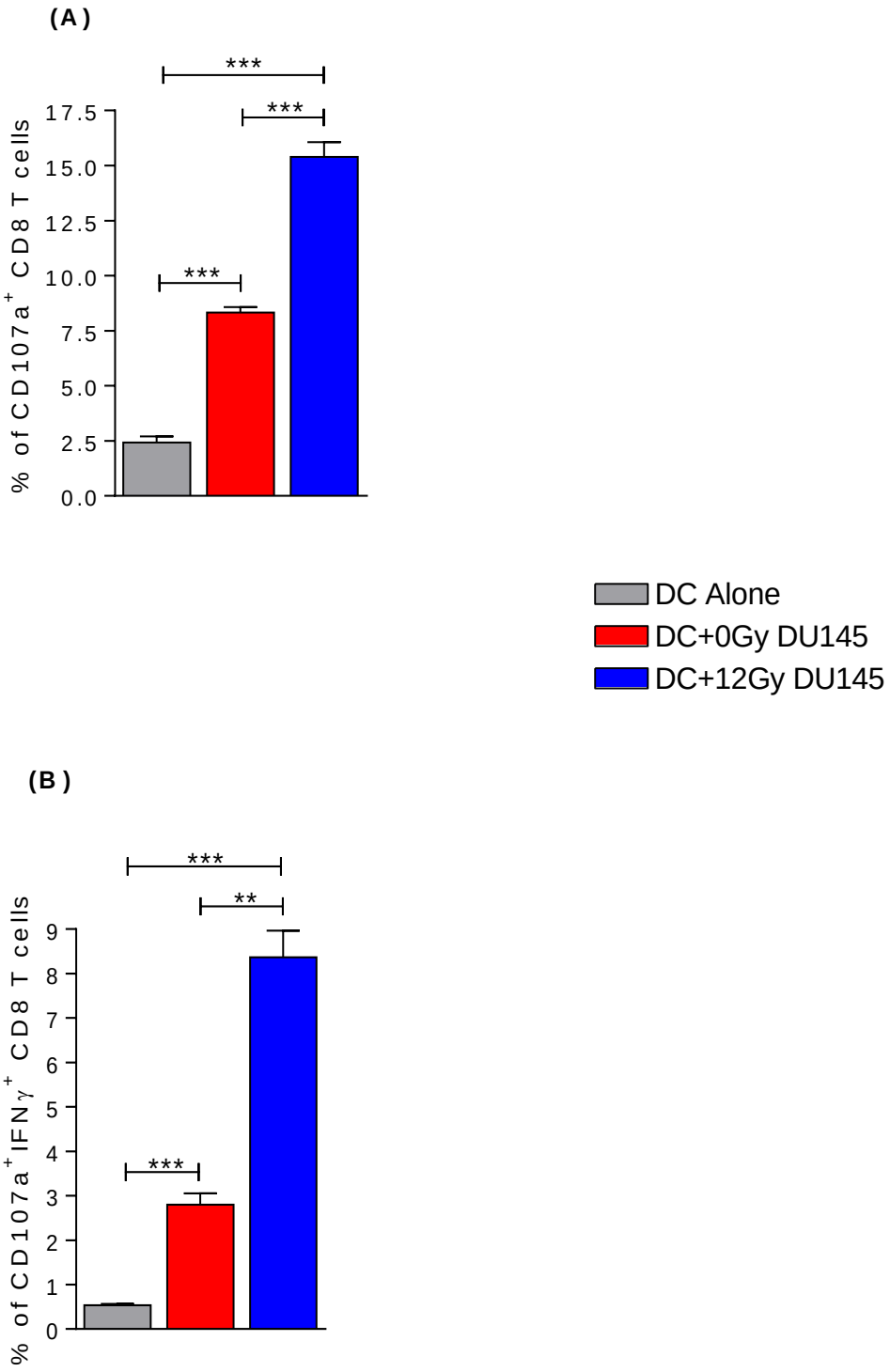


Figure 3.11: Stimulation of RLAR-T cells after cross-presentation of irradiated and non-irradiated DU145 cells by DC measured by CD107a mobilisation. Mean and SD for the percentage of (A) CD107a positive or (B) CD107a and IFNγ double positive $CD8^+$ T cells from triplicate samples. *(**p<0.01; ***p < 0.001 Student's t-test)*

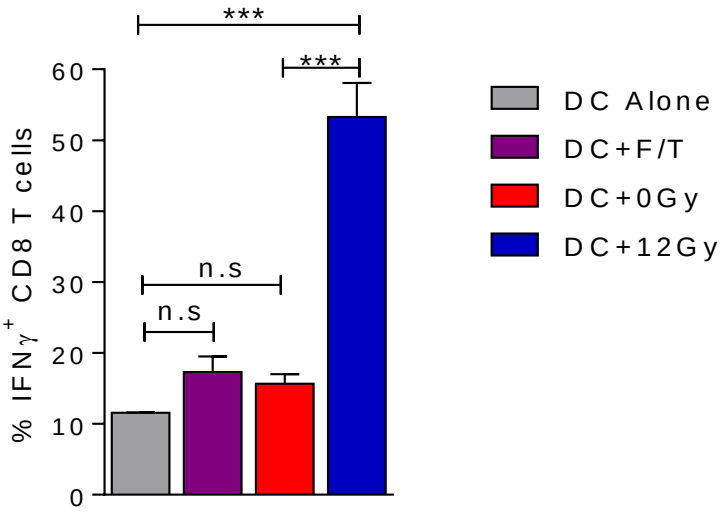


Figure 3.12: Stimulation of RLAR-T cells after the cross-presentation of freeze thawed (F/T), irradiated or non-irradiated DU145 cells by DC. Mean and SD for the percentage of IFNγ positive CD8 T cells from triplicate samples. (****p < 0.001; Student's t-test)*

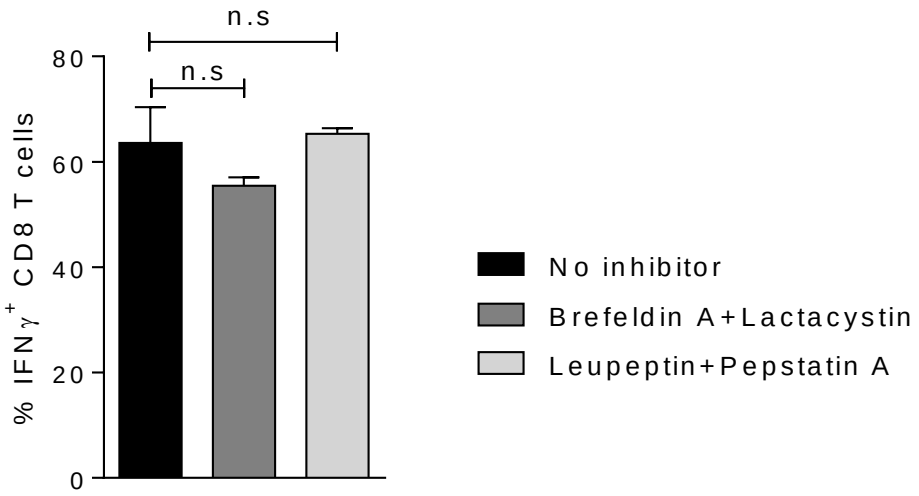


Figure 3.13: Stimulation of RLAR-T cells by peptide-pulsed DC treated with cytosolic or endosomal pathway inhibitors. Mean and SD for the percentage of IFNγ positive $CD8^+$ T cells from triplicate samples. (*n.s = not significant; Student's t-test)*

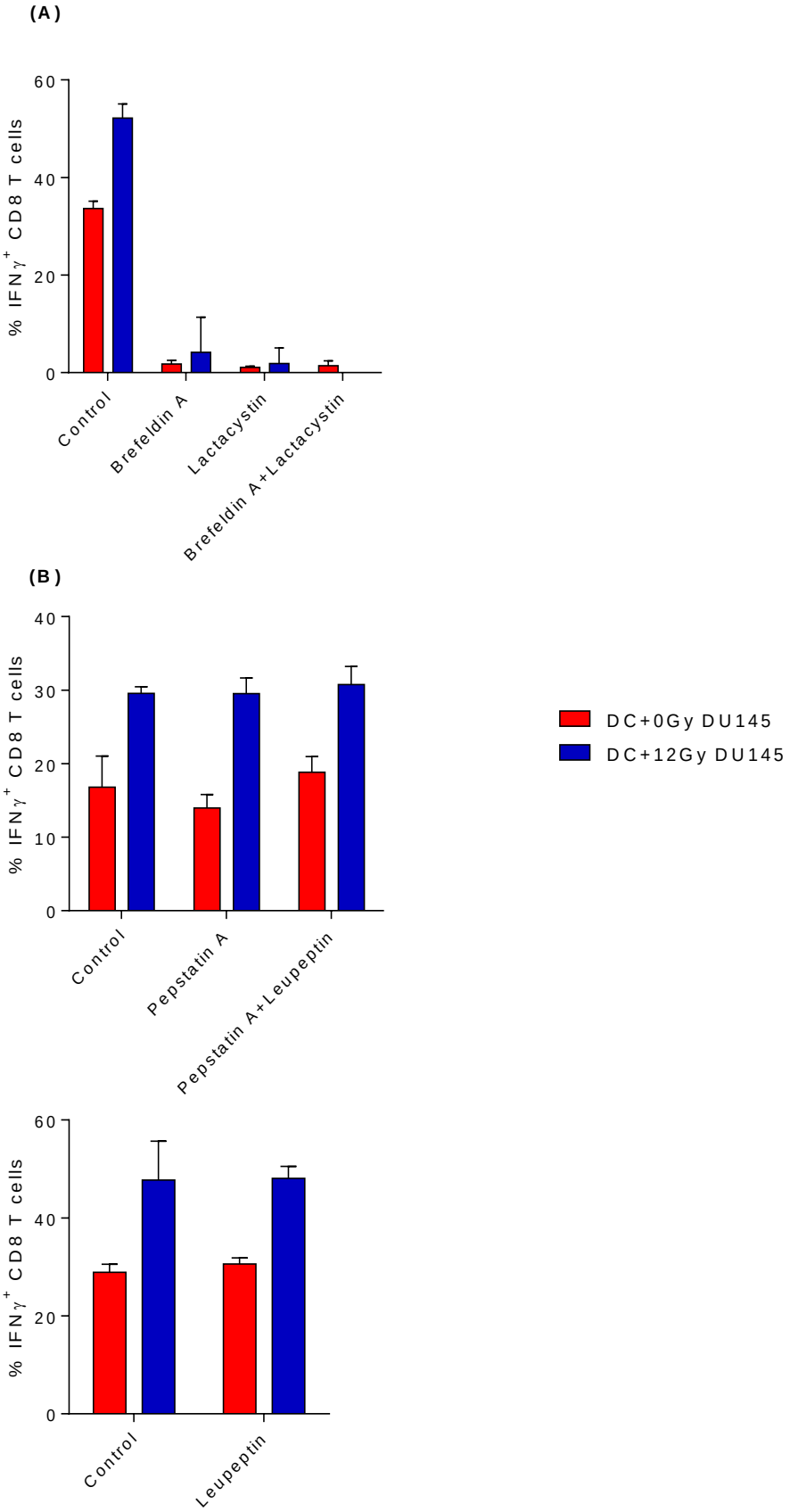


Figure 3.14: Stimulation of RLAR-T cells by cross-presentation with DC treated with (A) cytosolic or (B) endosomal pathway inhibitors. Mean and SD for the percentage of IFNγ positive CD8 T cells from triplicate samples.

DISCUSSION

The question in this chapter was whether irradiation of tumour cells triggers antitumor T cell responses generated by cross-presentation of the 5T4 tumour antigen to antigen-specific T cells. In order to do this, I identified a 5T4 expressing PCa cell line (DU145) and generated a T cell line, which was then characterized for its ability to specifically recognize naturally processed 5T4 antigen. Using these cell lines, I developed an antigen cross-presentation model with irradiated PCa cells and monocyte-derived DC, where the readout was T cell activation.

Of the two PCa cell lines tested, high levels of 5T4 were detected in DU145 but not LNCaP cells, while HLA-A2 was only expressed on LNCaP but not DU145 cells. DU145 cells were used in the subsequent cross-presentation experiments and a 5T4 specific HLA-$A2^+$ $CD8^+$ T cell line (RLAR-T cells) was developed using the HLA-A2 binding peptide $5T4_{17-25}$ (RLARLALVL) (Shingler et al., 2008). The specificity and cytotoxic capabilities of the RLAR-T cells against $5T4_{17-25}$ peptide-pulsed target cells were studied using IFNγ intracellular staining and a standard ^{51}Cr release assay, respectively, to verify the functionality of the RLAR-T cells before use in the cross-presentation experiments. Almost 71% of the RLAR-T cells produced IFNγ and were also cytolytic to peptide-pulsed target cells. Cytotoxic killing of the mesothelioma cells by the RLAR-T cells and proliferation of the T cells confirmed the ability of the RLAR-T cells to recognise the naturally expressed 5T4 peptides in association with HLA-A2 on tumour cells. Other studies have also demonstrated the cytotoxic activity of $CD8^+$ T cells specific for the $5T4_{17-25}$ peptide against $5T4^+$HLA-$A2^+$ renal cell carcinoma in an *in vitro* assay (Tykodi et al., 2012). However, to our knowledge, the antitumor potential of 5T4 specific $CD8^+$ T cells against 5T4 antigen cross-presented from 5T4 positive PCa tumour cells has not been reported.

The RLAR-T cells were then characterised for their ability to specifically recognise 5T4 antigen cross-presented from the tumour cells. In our experimental setting, recognition of the 5T4 antigen by RLAR-T cells was assessed by measuring T cell proliferation and production of IFNγ in response to tumour cells alone or tumour cells co-cultured with DC. T cells cultured with tumour cells alone were not stimulated to either proliferate or produce IFNγ; confirming the ^{51}Cr data

demonstrating that the RLAR-T cells cannot recognise 5T4 antigen directly on DU145 or LNCaP cells. However, $5T4^+$ DU145 but not $5T4^-$ LNCaP cells co-cultured with DC caused a small but significant stimulation of proliferation and IFNγ production by RLAR-T cells. This indicates that DC are required for the recognition of 5T4 antigen on DU145 cells by RLAR-T cells and that cross-presentation occurs at a low level from non-irradiated tumour cells. This is similar to findings by Matheoud et al., (2010) which demonstrated cross-priming and cross-presentation of OVA from live (non-irradiated) cells to naïve OT-1 specific $CD8^+$ T cell (Matheoud et al., 2010).

Comparison of irradiated and non-irradiated DU145 cells co-cultured with DC revealed that antigen from irradiated tumour cells significantly enhances anti-5T4 T cell responses either measured as proliferation or production of IFNγ, MIP-1β or CD107a. These data support the findings shown in other studies that antigen from irradiated tumour cells is cross-presented by DC and generate antigen-specific T cell responses (von Euw et al., 2007, Barrio et al., 2012) but contradicts other studies that did not find that irradiation increases antigen cross-presentation (Matheoud et al., 2010, Matheoud et al., 2011). One of the noticeable differences that might be contributing to the contradicting data within the studies is the experimental setup. Whilst our study, as well as the studies of von Euw et al., (2007) and Barrio et al., (2012) co-cultured tumour cells, DC and T cells together during a cross-presentation experiment, Matheoud et al., (2010 and 2011) purified DC using positive selection after uptake of tumour cells, and then co-cultured the purified DC with T cells. In the latter setting, the T cells may not be exposed to the immunostimulatory cytokines released by activated DC (IL-1β and IL12) (Ghiringhelli et al., 2009, Morelli et al., 2001) and/or irradiated tumour cells (TNF-α and IFN-β) (Hallahan et al., 1989, Burnette et al., 2011). Furthermore, the radiation dose, used by Matheoud, of 100 Gy is much higher than that we used (12 Gy) and generated >70% apoptotic cells while in our model apoptotic tumour cell frequencies remained low. Another reason why we were able to detect cross-presentation may lie in the carefully optimised model where the timing and cell-to-cell contact was probably superior to that of Matheoud et al., (2010 and 2011). They co-cultured DC and tumour cells for 16 h instead of 48 h as carried out in our experiments. Cross-presentation of Mart-1 to specific $CD8^+$ T cells at different times (3 - 48 h) revealed that antigen-specific T cells were

stimulated more efficiently after longer periods of antigen processing as IFNγ production increased with the co-culture time (Barrio et al., 2012).

To determine if similar results can be attained with any form of cell death or cell death induced by irradiation was necessary, a cross-presentation assay was carried out using freeze-thawed DU145 cells. The level of T cell stimulation by antigen from the necrotic (freeze-thawed) fraction was similar to that achieved by non-irradiated cells. It has been suggested that apoptotic or programmed cell death is immunologically silent while necrotic cell death is not. Apoptotic cell death is physiological and normal and, as such, poses no danger, while necrotic cell death releases danger signals upon rupturing, hence, immunogenic. However, very few studies have shown tumour lysates prepared from freeze-thawed cells to be immunogenic and to elicit potent antitumor responses (Chiang et al., 2011). My study as well as work by others clearly demonstrates that only irradiated and not freeze-thawed necrotic tumour cells induce potent antitumor immune responses (Scheffer et al., 2003, Buckwalter and Srivastava, 2013).

Cross-presentation of antigen can occur via two distinct mechanisms, as discussed in the introduction. To assess if 5T4 derived from DU145 cells is delivered from endosomes to cytosol and require proteasome degradation for presentation, lactacystin was used as it blocks the catalytic activity of the β-subunits of the proteasome (Craiu et al., 1997). Other studies have shown that cross-presentation by human DC of exogenous viral antigens derived from infected cells could be blocked by the inhibition of the proteasome (Arrode et al., 2000, Fonteneau et al., 2003). In our study, cross-presentation of tumour antigens is likely to occur via the cytosolic pathway, because we found treating DC with the proteasome inhibitor completely diminished their ability to stimulate IFNγ production by the RLAR-T cells.

Newly synthesized MHC class I molecules loaded with peptides are transported from the ER to the cell surface through the Golgi apparatus for presentation. In the presence of brefeldin A, MHC class I complexes are withheld in the ER (Yewdell et al., 1999). However, a modified phagosome-to-cytosol model has been proposed, which involves proteasomal processing and the return of antigenic peptides to the phagosome by TAP. This does not require newly synthesized MHC class I molecules

in the ER and bypasses the Golgi apparatus so brefeldin A does not block cross-presentation (Houde et al., 2003, Mant et al., 2012). Cross-presentation of the 5T4 antigen to the RLAR-T cells by DC treated with brefeldin A was completely inhibited in our experimental setting, therefore demonstrating that processed 5T4 antigen is transported on newly synthesized MHC class I molecules out of the ER to the cell surface through the Golgi apparatus.

In the endosomal pathway, antigens enter the endocytic pathway and progressively pass through increasing acidic compartments. In a late acid endosomal compartment, the antigens undergo proteolysis; this requires either the aspartic (cathepsin D) or cysteine (cathepsin B and S) proteases to process/degrade antigen (Riese and Chapman, 2000). Inhibition of aspartic proteases using pepstatin A or cysteine proteases using leupeptin did not affect the cross-presentation of 5T4 antigen. This demonstrates that 5T4 antigen processing in our cross-presentation model occurs in the cytosolic compartment and not the endosomal compartment of the DC.

In conclusion, the RLAR-T cells I generated can recognise cross-presented 5T4 antigen in an antigen-specific manner as they responded to $5T4^{+}$HLA-$A2^{-}$ DU145 but not $5T4^{-}$HLA-$A2^{+}$ LNCaP cells co-cultured with DC. Antitumor T cell responses are several fold enhanced when DC cross-present antigen from irradiated DU145 cells compared to that from non-irradiated cells. Antigen cross-presentation is likely to proceed via the cytosolic pathway. T cell responses are dependent on the effects induced by ionising radiation and not only on the presence of dead cells, as shown by tumour cells rendered necrotic by freeze-thawing. Therefore, in the next chapter I will analyse what is causing the immunogenicity of irradiated cells.

IV. Investigating the Immunogenicity of Irradiated Tumour Cells

INTRODUCTION

Cell succumbing to immunogenic cell death induced by chemotherapeutic agents or IR undergo specific changes in their surface characteristics and release pro-immunogenic factors. This stimulates DC to efficiently take up tumour cells, process them and cross-present tumour antigens to $CD8^+$ T cells, thus eliciting antitumor specific responses (Kepp et al., 2011). Since in the previous chapter, IR was shown to induce significant antigen-specific antitumor T cell responses by DC from PCa tumour cells, this chapter will focus on examining what is driving the immunogenicity of irradiated cells. In addition, I will study if IR-induced cell death activates DC.

Question

What are the immunogenic changes ionising radiation causes in tumour cells?

Specific aims

1. Investigate cell cycle and proliferation changes in DU145 cells induced by IR.
2. Examine the type of cell death in DU145 cells induced by IR.
3. Investigate if immunogenic signals are translocated or upregulated in DU145 cells following IR treatment.
4. Determine whether irradiation of DU145 cells enhances their phagocytosis by DC.
5. Determine the ability of the irradiated DU145 cells to induce DC activation.
6. Examine the cytokine and chemokine profile of DC in response to irradiated and non-irradiated DU145 cells.

RESULTS

In vitro IR alters tumour cell morphology and proliferation

In order to investigate the growth of irradiated DU145 cells, the Incucyte Kinetic Imaging System was used. DU145 cells were either irradiated with 12 Gy or left untreated before placing the plate in the imaging system for 72 h (Figure 4.1 and 4.3). During this incubation period, the system monitored cell morphology and growth by capturing images and recording the percentage of confluence every 4 h.

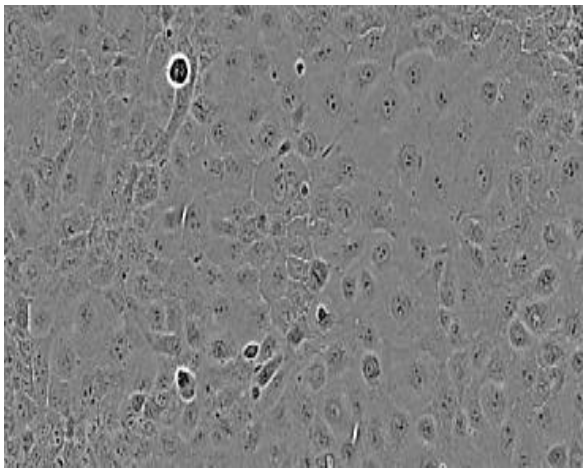
The images revealed fundamental morphological differences between the irradiated and non-irradiated cells. Cell senescence and breakdown of the monolayer; debris, disintegration of the membrane, cell swelling, as well as membrane damage (signs of necrosis) and also cell membrane blebbing and apoptotic bodies (Figure 4.1B), were evident in the irradiated cultures. The use of flow cytometry analysis also showed how irradiation causes morphological changes in the irradiated cells as they became enlarged and more granular, as illustrated by the forward scatter versus side scatter dot plot (Figure 4.2). Cell growth slowed down 18 h after IR to approximately 67% confluency and to less than 80% at 72 h. Non-irradiated cells continued to grow until they reached 98% confluence (Figure 4.3) at approximately 42 h. The differences in confluence reflect those shown by the images (Figure 4.1).

IR causes cell cycle arrest and necrotic cell death

IR induces damages to the cellular DNA, which leads to the activation of a DNA damage response signalling cascade. Depending on the extent of damage, this may result in transient or permanent cell cycle arrest, and/or cell death, respectively (Lauber et al., 2012). To investigate the radio-sensitivity of DU145 cells, the cell line was irradiated with increasing IR doses (0 Gy, 3 Gy, 6 Gy, 12 Gy and 24 Gy) and incubated for different periods post-irradiation. Cell cycle analysis was carried out using the Guava easyCyte flow cytometer.

Accumulation of cells arrested at the G2/M phase, (Figure 4.4), became detectable after the cells had been incubated for 24 h after IR with ≥12-24 Gy. IR also caused

(A) Non-Irradiated DU145 cells



(B) 12 Gy Irradiated DU145 cells

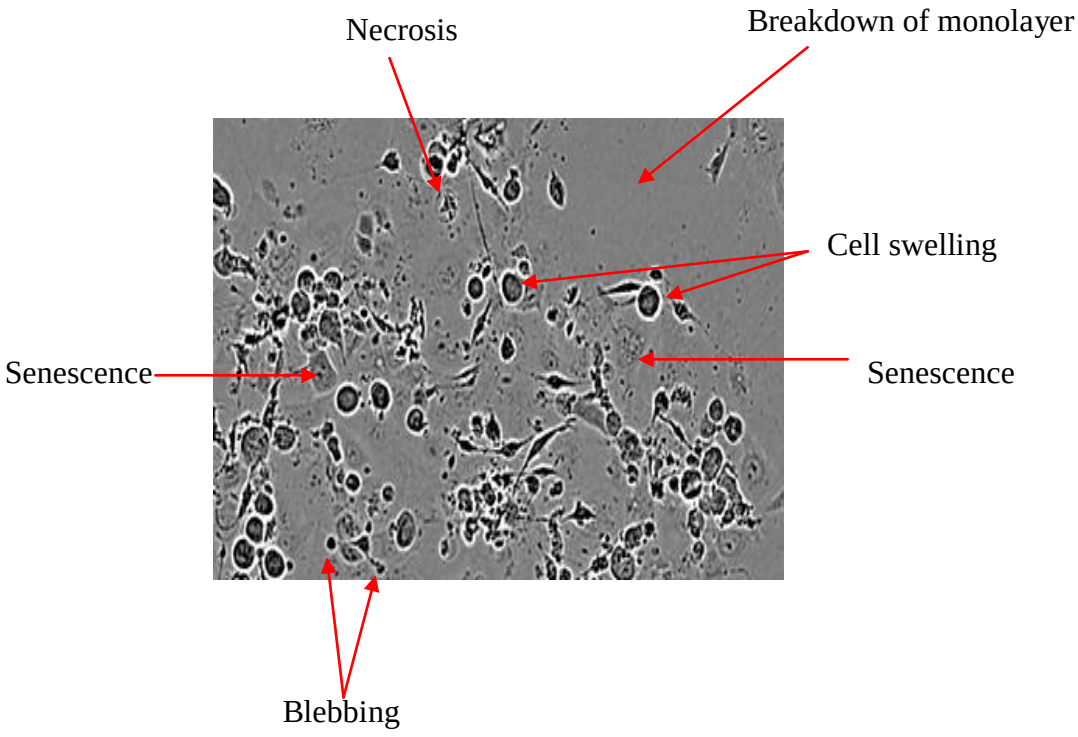


Figure 4.1: Images of DU145 cells using the Incucyte Kinetic Imaging System. Images of (A) Non-irradiated and (B) irradiated DU145 cells taken 72 h after 12 Gy IR. Red arrows indicate the morphological features only observed in the irradiated cultures.

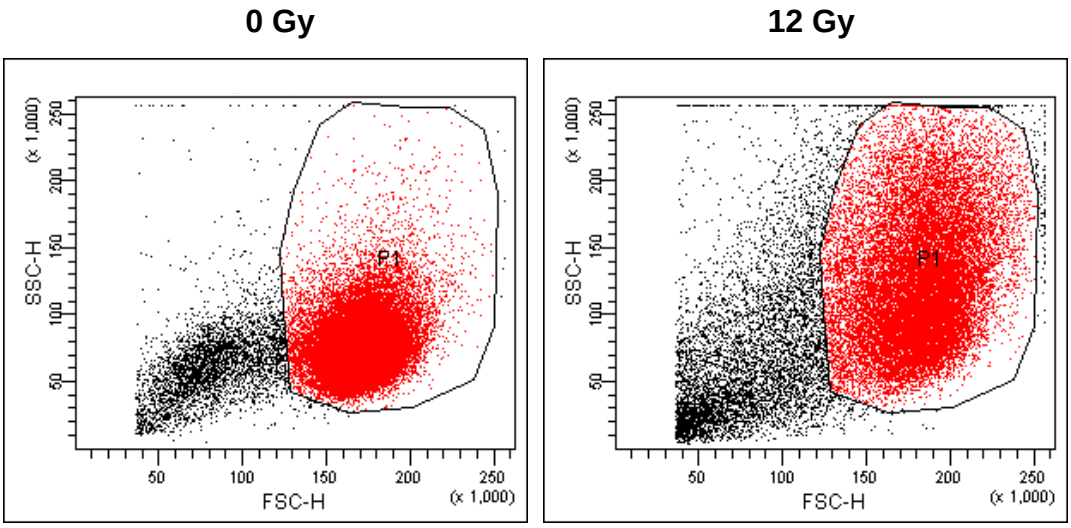


Figure 4.2: Increased scatter parameters displayed by irradiated DU145 cells. Non-irradiated and irradiated 12 Gy DU145 cells analysed by flow cytometry 72 h after IR. P1 gate (Red) illustrates the change in cell size while the black events represent increased debris compared to non-irradiated cells.

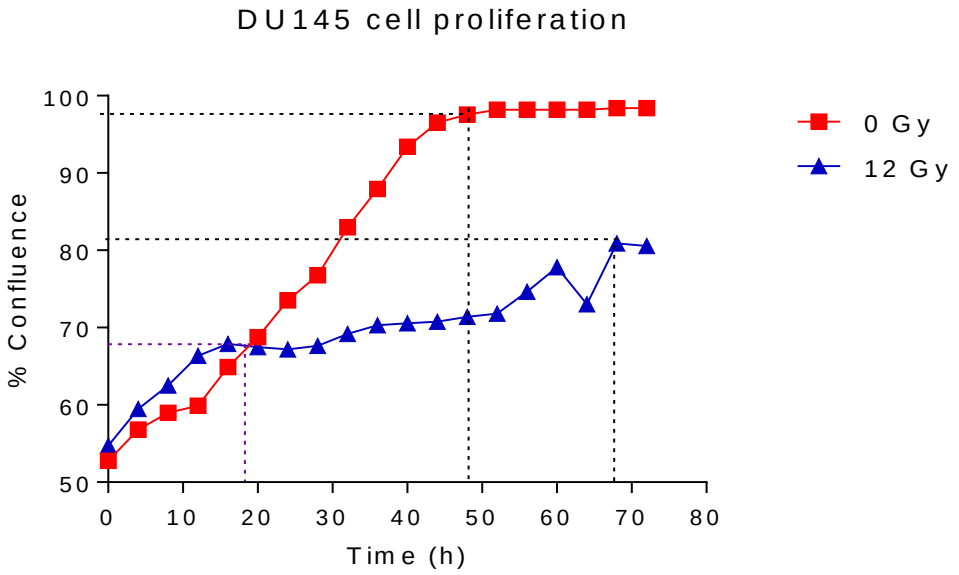


Figure 4.3: Growth kinetics of non-irradiated (red) versus irradiated (blue) DU145 cells. Graph shows the mean of metrics calculated across multiple regions of a cell culture plate captured every 4 h for 3 days by the Incucyte Kinetic Imaging System.

DNA fragmentation detectable by the increased proportion of cells in the Sub-G0 fraction (Figure 4.4). IR effects such as ER stress and ROS accumulation may occur immediately but the detection method being used is optimum for detecting events only after 24 h. 12 Gy was chosen as the IR dose to be used in further experiments and for the cross-presentation model.

An Annexin-V/PI assay was carried out to investigate more closely the extent and the type of cell death induced by IR. DU145 cells were irradiated with 12 Gy and incubated for 24 h, 48 h and 72 h (Figure 4.5A and B). Non-irradiated cells only had 6.3% total cell death. The relatively high percentage of cell death detected in the non-irradiated group might be due to lack of adhesion. 24 h post-IR, the percentage of cell death doubled to 13.3% and a gradual increase was observed after 48 h (18.2%) and 72 h (23%). While longer incubation periods allowed the detection of the increased proportion of late apoptotic/necrotic cells, the proportion of early apoptotic cells remained constant throughout the entire period (Figure 4.5A and B).

The effect of IR on tumour antigen and MHC Class I expression

Radiation can lead to alterations in the tumour cells, including de novo synthesis of particular proteins such as tumour antigens and the upregulation of MHC class I expression (Sharma et al., 2011). In the previous section, we established that 12 Gy had significant effects on cell cycle and survival of DU145 cells measured at 72 h post IR. Therefore, these conditions were used to determine if IR alters the expression of 5T4 and MHC class I molecules on the DU145 cells, which would affect their immunological behaviour. To determine the total expression of 5T4 antigen, cells were fixed and permeabilized with eBioscience buffers.

Surface and total 5T4 expression was higher on irradiated DU145 cells compared to non-irradiated cells (Figure 4.6A). However, MHC class I expression was not increased following IR treatment (Figure 4.6B). This suggests that IR increases the amount of antigen that can be processed by DC upon uptake of tumour cells.

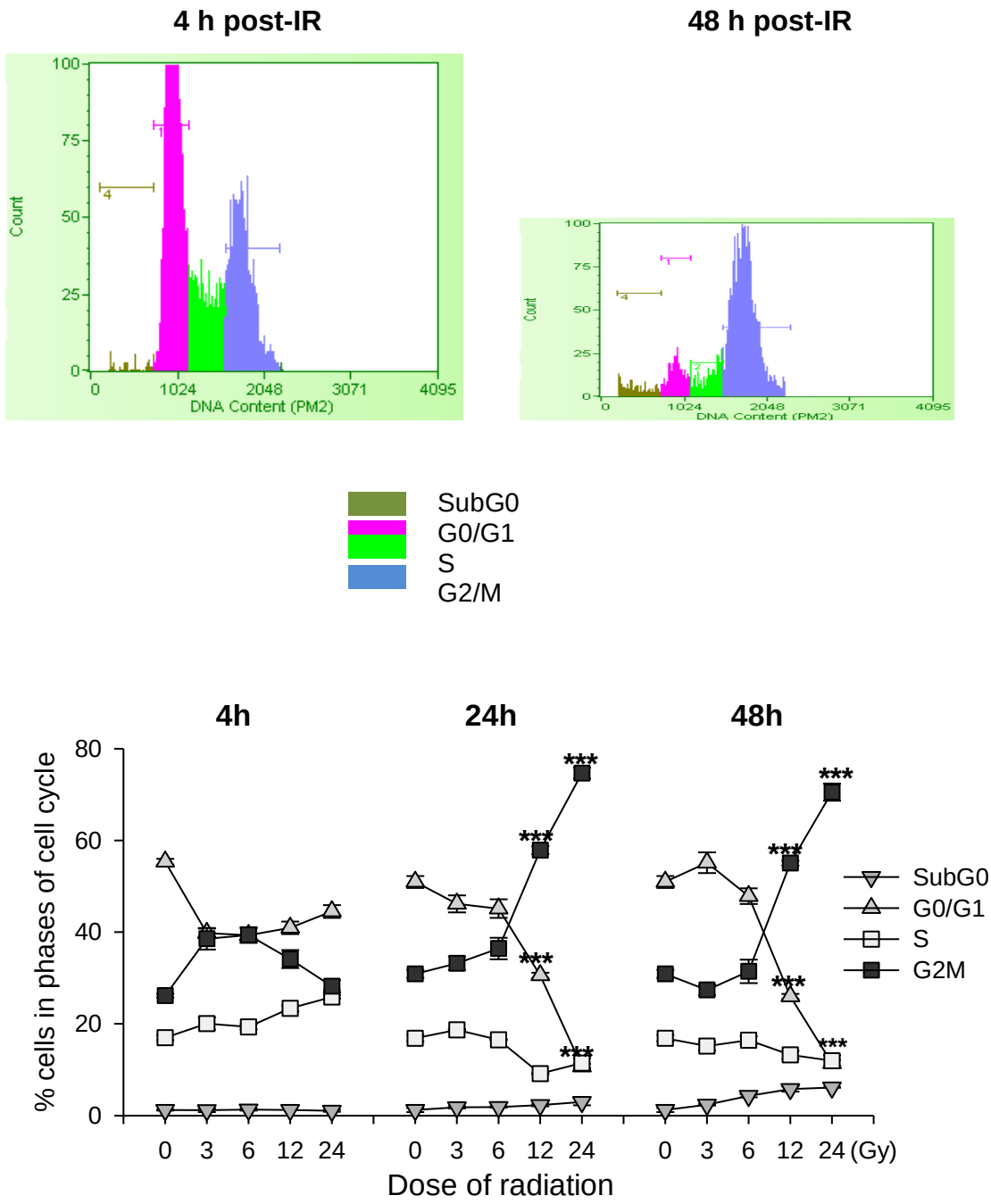


Figure 4.4: Cell cycle analysis of irradiated cells. (A) Representative cell cycle graphs. (B) DU145 cells were irradiated with increasing doses of IR (x-axis) and incubated for 4 h, 24 h and 48 h. Means were calculated from triplicate samples.

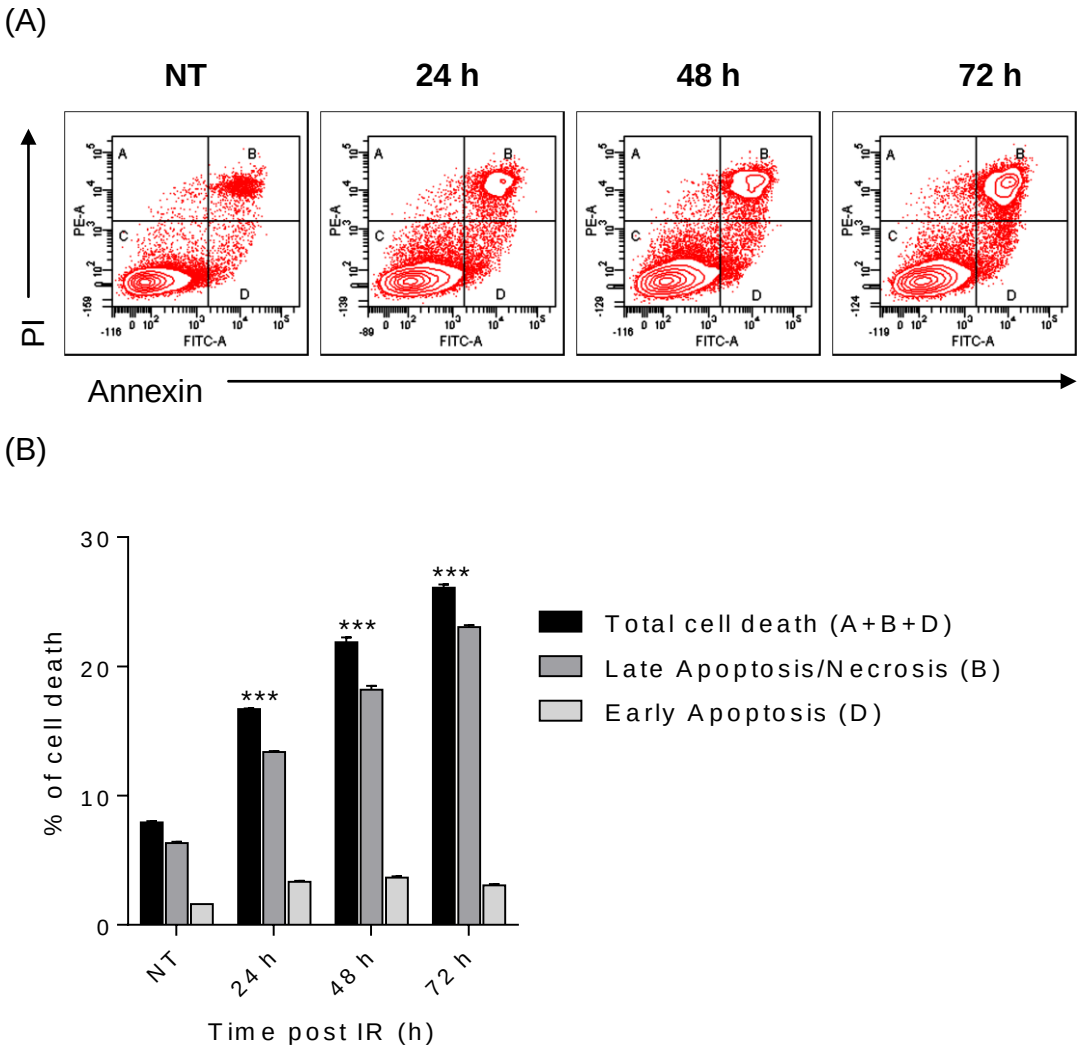


Figure 4.5: Analysis of DU145 cell death after irradiation. (A) Representative figure of the population of cells in different stages of cell death as labelled on the dot plot. (B) Summary of triplicate samples Mean and SD of duplicate samples are shown. NT = non-irradiated

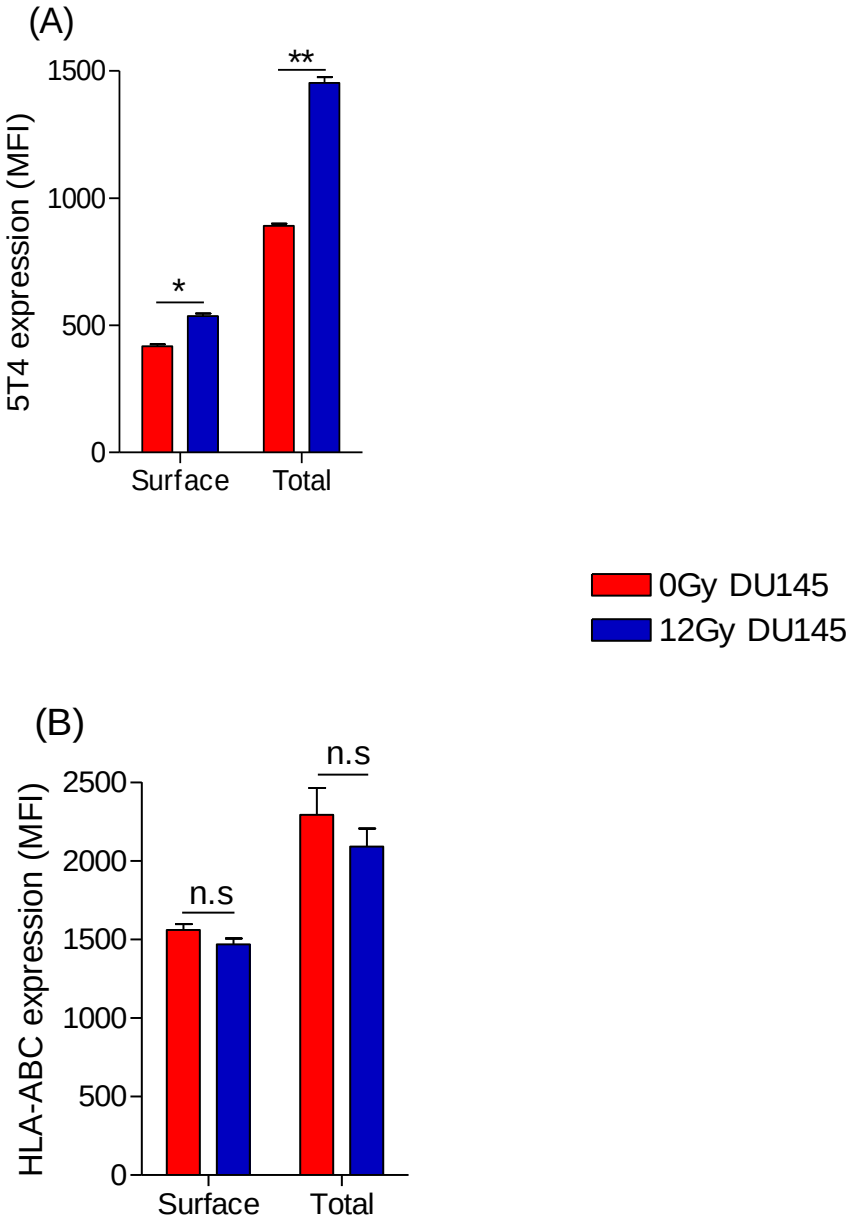


Figure 4.6: IR-induced effect on (A) 5T4 and (B) MHC class I expression. (A) Mean and SD of MFI for surface and intracellular (A) 5T4 or (B) MHC class I expression from triplicate samples after background from isotype has been subtracted.

IR induces the exposure or release of immunogenic signals

Chemotherapy- or irradiation-induced cell death results in the translocation of immunologically relevant molecules from within the cell to the plasma membrane or release in the extracellular space (Zitvogel et al., 2010). The translocation of CRT, Hsp70 and HMGB1 were examined in irradiated DU145 cells. The intracellular form of CRT is important for protein folding and assembly and maintaining ER calcium homeostasis. It also contributes to the quality control of MHC class I assembly, and has multiple roles in the MHC class I pathway. Although CRT is normally ER-resident, it can also be found on the surface of living and dying cancer cells. Upon induction of cell death, surface CRT increases as cells become more damaged and is redistributed into patches. Cell surface CRT is an "eat me" signal that mediates phagocytic uptake and immunogenicity of dying cells (Gardai et al., 2005, Raghavan et al., 2012).

It has been reported that surface exposure of CRT correlates with the downregulation and redistribution of CD47, a "don't eat me" signal (Gardai et al., 2005). CD47 binds to SIRPα, which prevents viable cells from mistakenly being ingested, hence contributing to appropriate dying cell recognition (Kepp et al., 2011). To assess if CD47 is downregulated on dying tumour cell while CRT is upregulated, irradiated and non-irradiated DU145 cells were surface stained with an APC conjugated CD47 and PE conjugated CRT antibody or respective isotypes and analysed by flow cytometry. Upon irradiation, the proportion of surface CRT positive cells increased more than 8-fold. About a third of CRT positive cells did not express CD47 (Figure 4.7).

Intracellular Hsp70 plays an essential role as molecular chaperone by assisting the correct folding of nascent and stress-accumulated misfolded proteins and preventing their aggregation. Hsp70 levels are high in tumour cells compared to normal cells due to their increased requirement for molecular chaperones needed to stabilize the abundant mutant and over-expressed oncoproteins found in cancer and act as a powerful antiapoptotic protein in the cytosol (Schmitt et al., 2007, Joly et al., 2010). In response to stress such as IR, Hsp70 levels within the cell increase and the protein can be translocated to the plasma membrane for cell surface exposure or even

released into the extracellular space. Extracellular exposure/release in response to radiation is considered as an immunogenic signal (Schildkopf et al., 2011).

Immunocytochemistry was carried out to examine the effects of IR on cellular and surface expression of Hsp70 on DU145 cells (Figure 4.8A). The surface expression on the tumour cells was also confirmed by flow cytometry 24-72 h after irradiation with 12 Gy (Figure 4.8B). A correlation between Hsp70 and CRT was also assessed by flow cytometry (Figure 4.8C). Radiation of DU145 cells induced the translocation of Hsp70 from the nucleus to the cytoplasm and cell surface (Figure 4.8A). Hsp70 surface expression was significantly elevated by 24 h after irradiation compared to the non-irradiated controls as measured by flow cytometry and further increased by 72 h (Figure 4.8B). Upon irradiation, the proportion of surface Hsp70 positive cells increased more than two-fold and CRT surface expression increased more than 10-fold. Most CRT expressing cells were also positive for surface Hsp70 (Figure 4.8C).

HMGB1 is passively released by dying tumour cells in response to anthracyline or IR treatment. HMGB1 secretion into the supernatant of dying cells is associated with the reduction of its nuclear expression (Apetoh et al., 2007a). Therefore, in order to determine if radiation of DU145 cells causes changes in cellular HMGB1 levels in our experimental setting, intracellular HMGB1 was analysed by flow cytometry. DU145 cells were irradiated with 12 Gy and incubated for 24 h, 48 h, and 72 h before analysis. After a significant initial increase at 24 h, there was a gradual decrease of cellular HMGB1 content with time (Figure 4.9A). In order to determine if the decrease in cellular HMGB1 levels was due its release into the extracellular space, an ELISA assay was carried out from the supernatant collected from the cells used in Figure 4.9A. Since HMGB1 can be measured in FBS in the cell culture supernatant, a control of media alone was used to determine background levels. When supernatants from irradiated and non-irradiated DU145 cells were assessed, HMGB1 concentrations above background levels were only detected 48 and 72 h after IR treatment. The concentration of HMGB1 at these later time points were significantly higher in the irradiated group compared to the non-irradiated (Figure 4.9B).

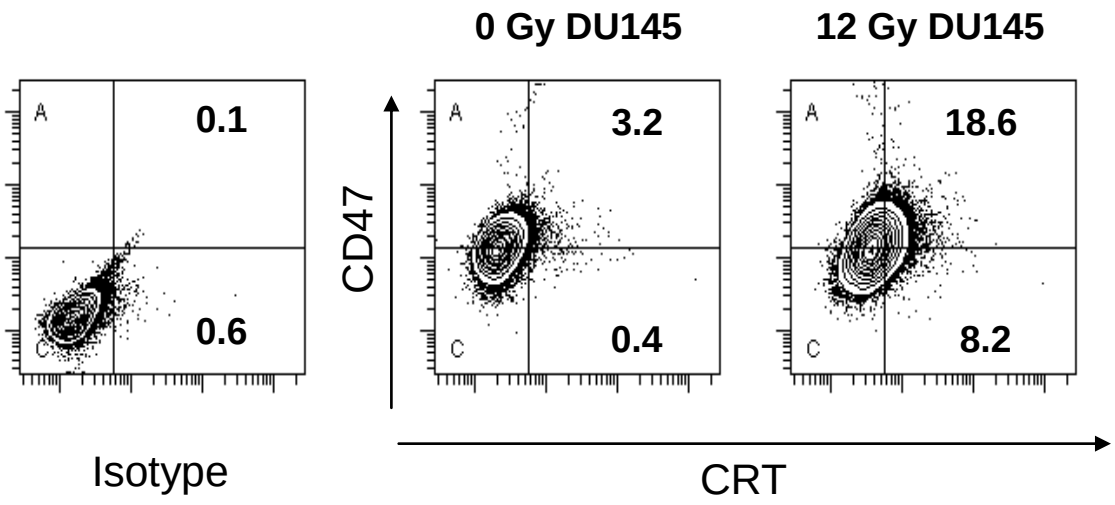


Figure 4.7: IR-induced effects on surface CRT and CD47 expression. 0 Gy and 12 Gy treated DU145 cells were analysed by flow cytometry 72 h after IR. Representative dot plots; the numbers represent means of triplicate samples.

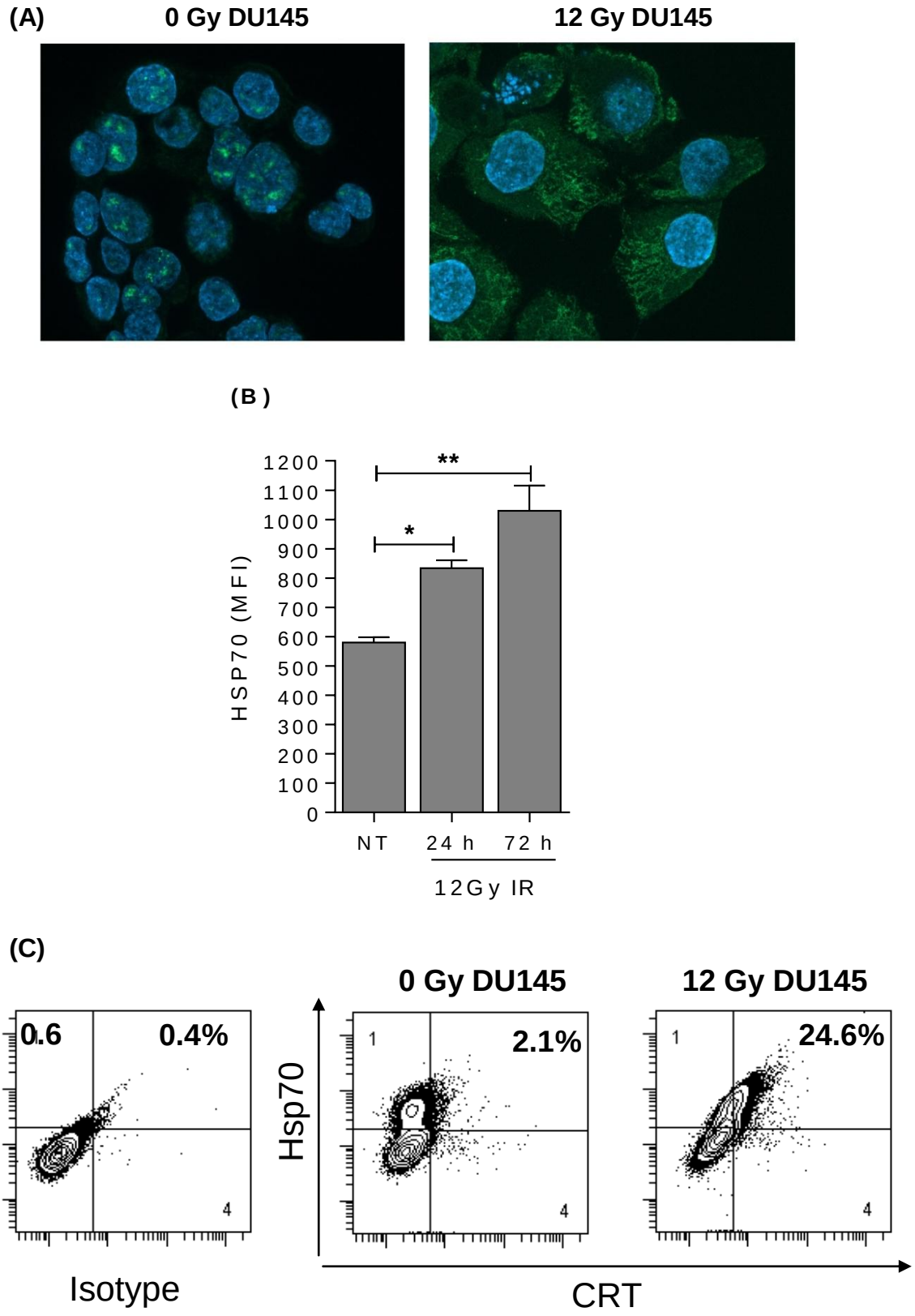


Figure 4.8: IR-induced translocation of Hsp70 to the cytosol and surface. (A) Immunoflouresence staining of nuclear and cytoplasmic Hsp70 in DU145 cells 72 h post-IR. (B) Surface staining of Hsp70 24 h and 72 h post-IR. Mean and SD of triplicate samples for MFI of Hsp70 after background of the Isotype has been subtracted. *(*p < 0.05;**p <0.01; Student's t-test).* (C) IR induced exposure of surface Hsp70 and CRT on DU145 72 h after IR. Representative dot plots; the numbers represent means of triplicate samples.

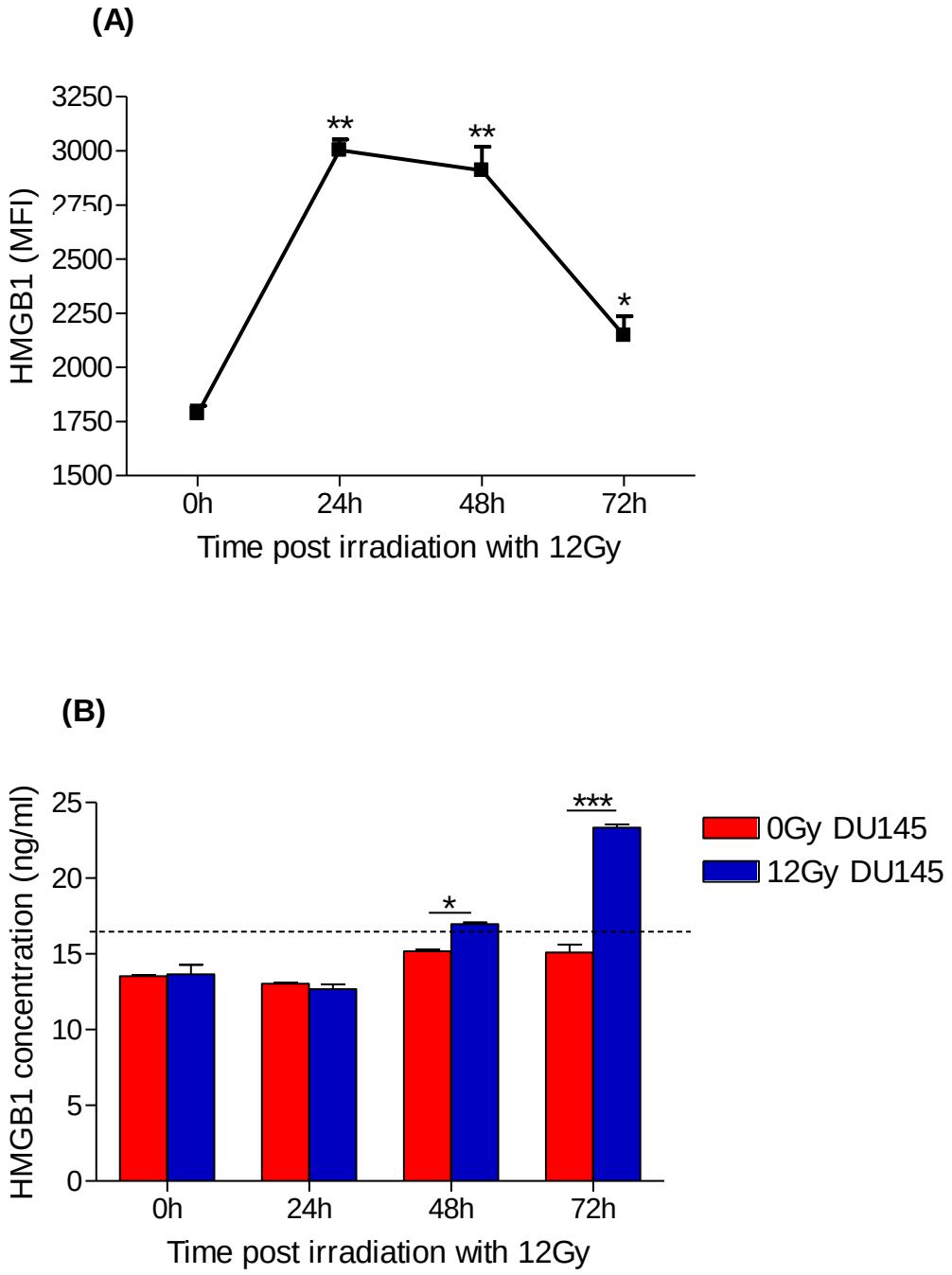


Figure 4.9: Cellular and extracellular levels of HMGB1 after different incubation periods post irradiation with 12 Gy. (A) Intracellular HMGB1 staining of irradiated DU145 cells. (B) Extracellular HMGB1 detected from cell supernatant. Mean and SD of triplicate samples showing the MFI of HMGB1 after background of the Isotype has been subtracted. The dotted line represents the levels detected from media alone. *(*p < 0.05; **p < 0.01; *** p < 0.001; Student's t test)*

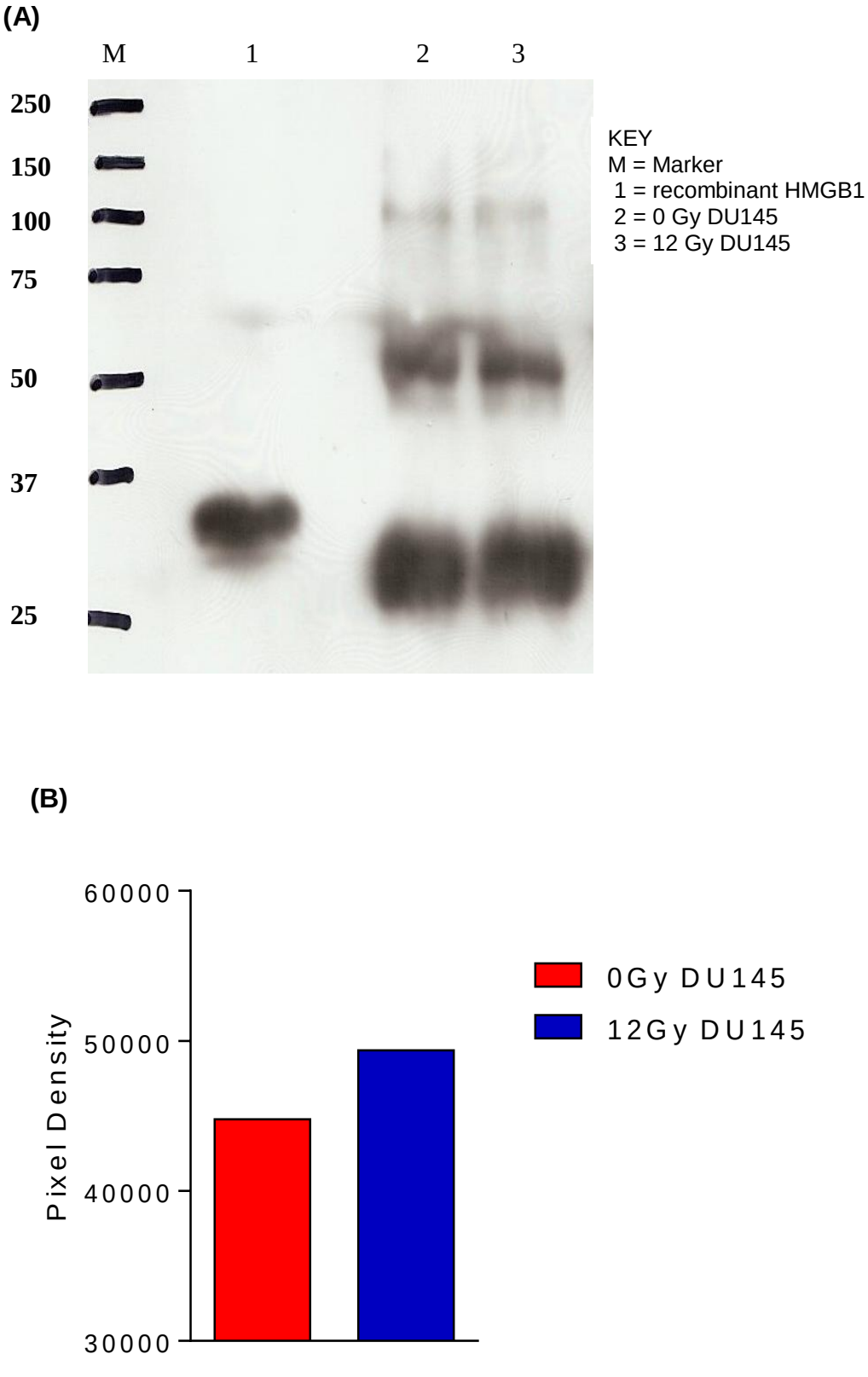


Figure 4.10: Detection of HMGB1 in the supernatant of DU145 cells following irradiation. (A) Western blot of immunoprecipitated proteins. Samples listed above were probed with mouse anti-HMGB1 primary antibody and goat anti-mouse HPR-conjugated secondary antibody (1:5000) (B) Pixel densitometry of the western blotting bands for 0 Gy and 12 Gy irradiated DU145 cells.

Levels of released HMGB1 were also analysed by western blotting. The tumour supernatants were concentrated using immunoprecipitation for enhanced detection. The band detected at approximately 50-60 kDa was albumin as the media contained 0.5% FBS. The band at approximately 30-36 kDa was HMGB1 (Figure 4.10A). Quantification of the bands sizes was carried out using imageJ (Figure 4.10A and B). Pixel densities calculated using imageJ confirmed that supernatants from irradiated DU145 cells had more HMGB1 compared to the non-irradiated supernatant (Figure 4.10B).

Phagocytosis of tumour cells

Dying cells undergo specific surface changes that signal professional APC such as DC to bind and engulf them. Among these markers is the surface exposure of PS as well as the translocation of CRT to the cell surface. Both PS and CRT were highly expressed on the surface of irradiated DU145 cells in our experiments. To investigate if DC preferentially take up irradiated tumour cells versus non-irradiated cells, a phagocytosis experiment was carried out. DC were co-cultured overnight with CFSE labelled irradiated and non-irradiated DU145 cells. To identify DC in the DC:Tumour cell co-culture, DC were labelled with a HLA-DR antibody since DU145 cells are negative for HLA-DR. Engulfment of CFSE labelled tumour cells by DC was determined by flow cytometry, by assessing the percentage of HLA-DR and CFSE double positive cells. Although uptake of non-irradiated tumour cells was observed, approximately 30% more double positive DC were detected after co-culture of irradiated DU145 cells with DC (Figure 4.11), indicating that radiation induces cellular changes in tumour cells which enhance their phagocytic uptake.

DC activation: maturation and cytokine release following uptake of tumour cells

Nearly all processes mediated by DC depend on their differentiation and maturation state. These processes involve migration to peripheral lymphoid organs as well as expression of MHC molecules, co-stimulatory molecules, and cytokines resulting in T cell stimulation. Thus, IR-induced changes in the state of DC maturation and

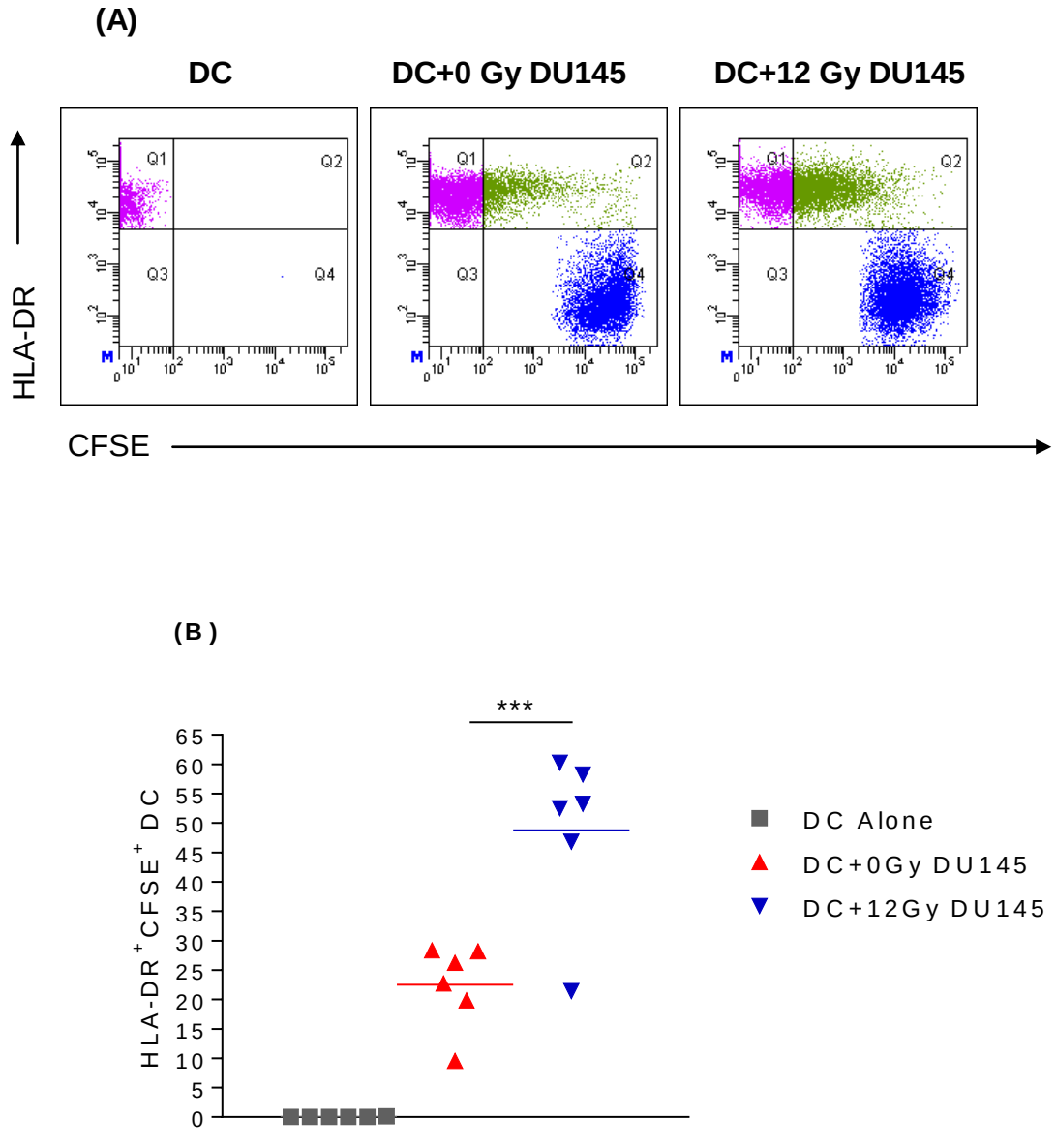


Figure 4.11: Phagocytosis of DU145 cells by DC. (A) Dot plots with cells labelled with HLA-DR antibody *(y-axis)* representing DC and CFSE *(x-axis)* representing DU145 cells. Double positive population represents DC with phagocytosed tumour cells. (B) HLA-DR and CFSE double positive DC, following uptake of non-irradiated DU145 cell (red) versus irradiated DU145 cells (blue). Summary of results from 6 donors. Each symbol represents a different donor and is a mean of triplicate samples. The lines represent the mean of DC in each group.

activation would affect cellular immunity. The ability of the tumour cells to mature DC was assessed by co-culturing irradiated or non-irradiated DU145 cells with DC at 1:1 ratio for 48 h before phenotypic analysis. The phenotype of immature and mature DC was characterised by detecting the expression of cell surface molecules HLA-DR, CD83, CD86 and CCR7 by flow cytometry (Figure 4.12). Immature DC express high to medium levels of surface HLA-DR, relatively low levels of co-stimulatory molecules (CD86), and are negative for CD83 and CCR7. DC co-cultured with irradiated tumours displayed significant upregulation of HLA-DR, CD86, CCR7 and CD83 compared to those co-cultured with non-irradiated tumour cells (Figure 4.12).

As shown above, when tumour cells undergo IR-induced cell death, they translocate immunogenic signals to the cell surface. Release of danger signals such as Hsp70, HMGB1 and ATP into the extracellular space by irradiated tumour cells has also been reported (Garg et al., 2012a, Apetoh et al., 2007a, Ko et al., 2014). To determine whether signals from the two cellular compartments work together or independently, to induce activation and improve tumour antigen cross-presentation, the original supernatant (IR/non-IR conditioned supernatant) from irradiated and non-irradiated tumour cells was removed and replaced with fresh media before DC were added. The conditioned supernatant was transferred into new wells and DC were added to these. DC activation was assessed by the upregulation of CD86 molecules (Figure 4.13A). Irradiated tumour cells were able to activate DC in the absence of the IR conditioned supernatant. However, the conditioned supernatant alone from irradiated cells was not able to activate DC. Similar to the results obtained above, upregulation of the co-stimulatory molecule was not observed when DC were cultured with non-irradiated cells in the presence or absence of non-IR conditioned media or with non-IR conditioned supernatant alone (Figure 4.13A). This suggests that only signals on the surface of irradiated tumour cells are able to activate DC.

Conversely, DC cross-presentation of antigens from irradiated DU145 cells in the absence of IR conditioned supernatant moderately reduced T cell stimulation compared to when DC were co-cultured with irradiated cells in the presence of conditioned supernatant (Figure 4.13B). Cross-presentation of antigens from non-

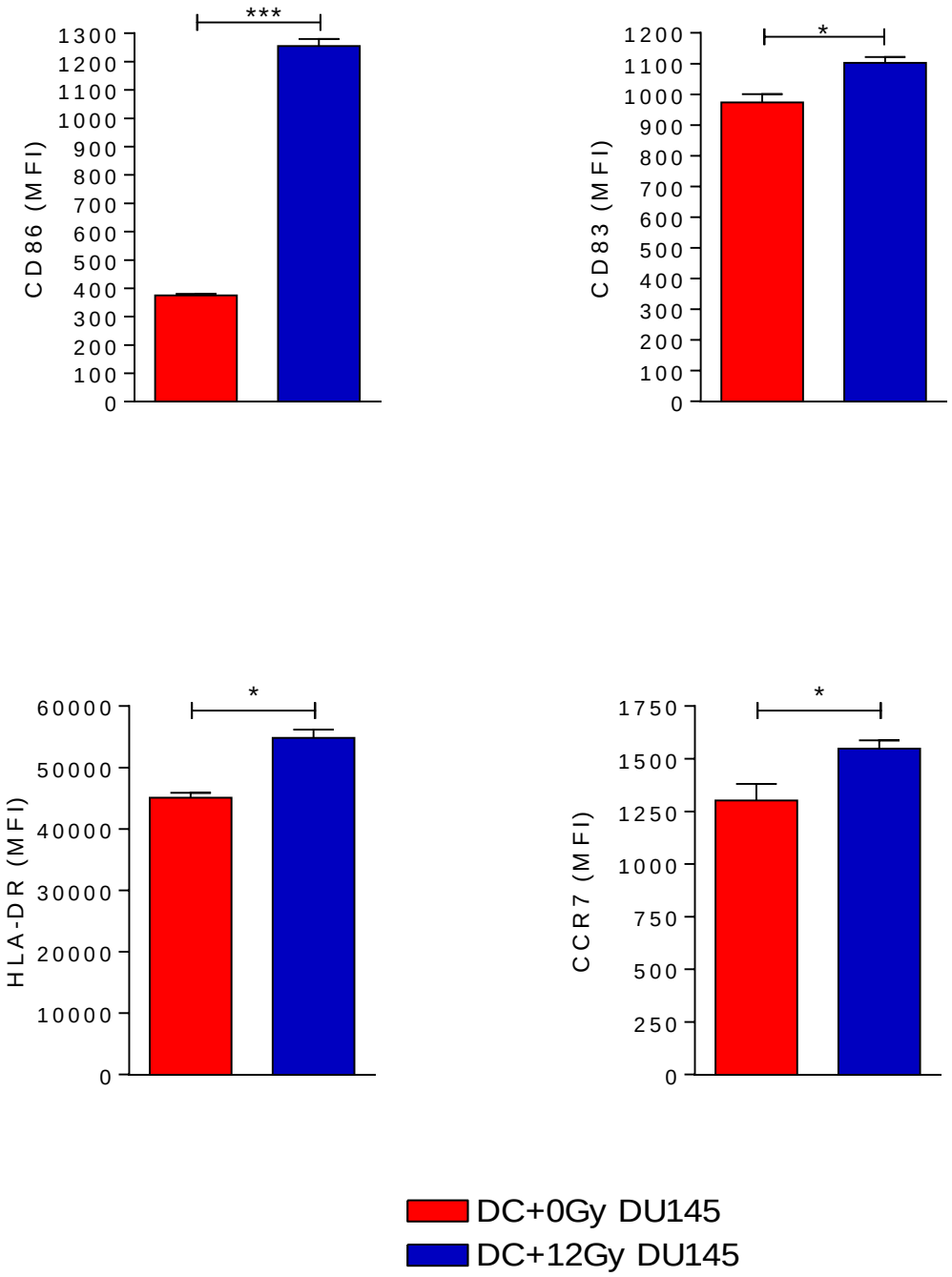


Figure 4.12: DC maturation by non-irradiated and irradiated DU145 cells. Mean and SD of MFI for CD86, CD83, HLA-DR and CCR7 for triplicate samples are shown. A representative of three repeated experiments from different donors.

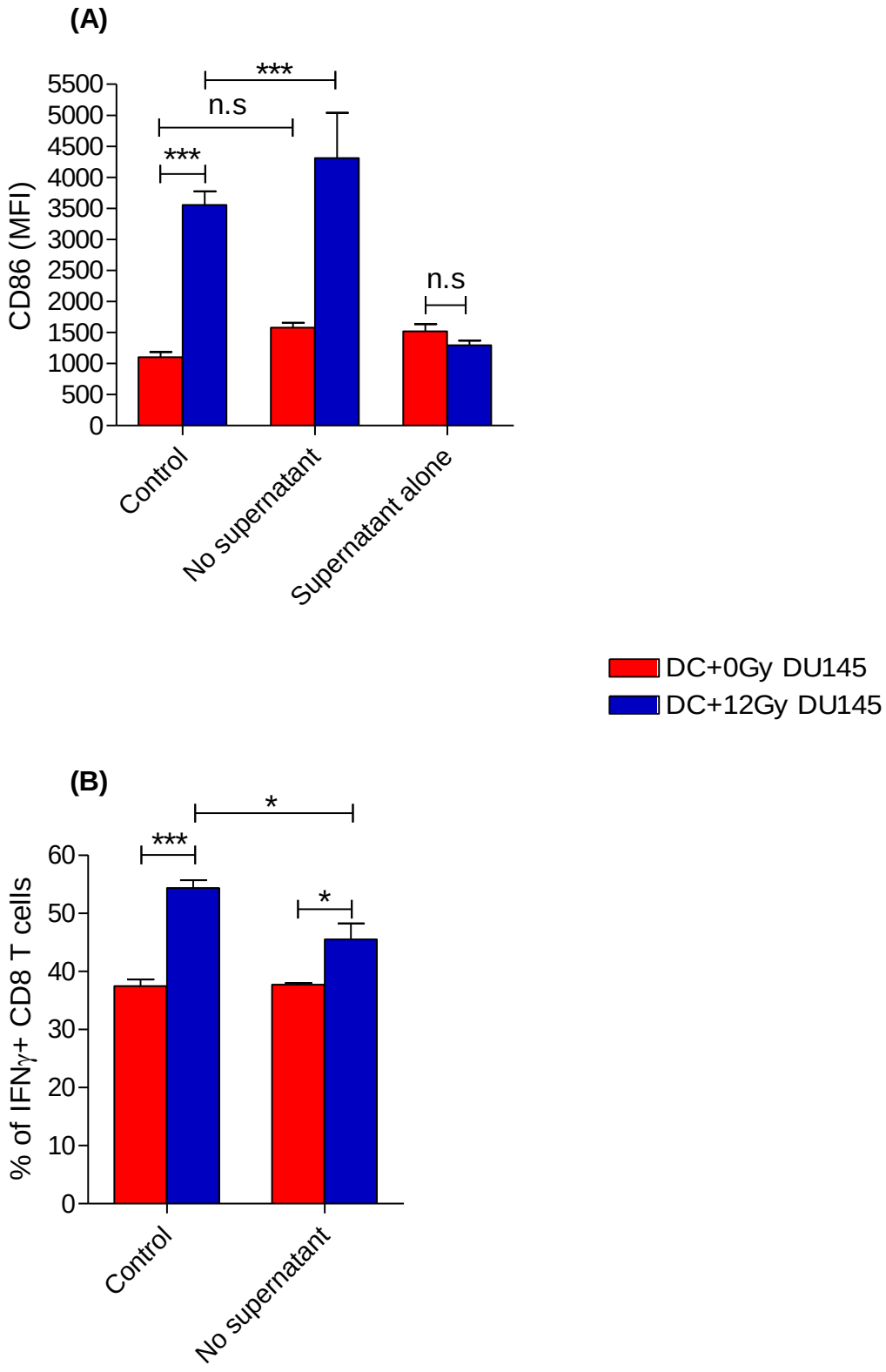


Figure 4.13: Effects of soluble factors vs. cellular elements of DU145 cells on (A) DC activation and (B) 5T4 specific T cell responses. (A) Mean and SD of CD86 (MFI) expression on DC from triplicate samples are shown. (B) Mean and SD of percentage of IFNγ positive $CD8^+$ T cells from triplicates samples. *(*p < 0.5; **p < 0.01; ***p < 0.001; Student's t test).*

irradiated DU145 was not affected by the presence or absence of non-IR conditioned media. This demonstrates that the signals on the irradiated tumour cells and not those released into the extracellular space are important for DC activation while the latter partially improves antigen cross-presentation. Not only the dying cell itself, but also soluble factors, released by dying cells contribute to the immunological outcome in a cross-presentation setting (Figure 4.13B).

Production of certain cytokines during the DC maturation process can influence DC to induce either anti- or pro-inflammatory immune responses. A standard ELISA protocol was used to assess IL-6, IL-12 and IL-10 produced by DC in response to the irradiated and non-irradiated DU145 cells. DC produced significantly more IL-6 in response to irradiated DU145 cells compared to the non-irradiated cells (Figure 4.14A). IL-12 release was only detected from DC that had taken up irradiated cells (Figure 4.14B) while IL-10 produced by DC cultured with irradiated versus non-irradiated cells was not significantly different (Figure 4.14C). The events provide evidence that irradiated tumour cells are able to activate DC, upregulate co-stimulatory molecules and produce pro-inflammatory cytokines required for T cell stimulation during antigen cross-presentation.

Cytokine and Chemokine Array

After establishing that IR causes a pro-inflammatory cytokine shift, a protein profiler array was carried out to investigate which other cytokines or chemokines are produced by DC in response to irradiated tumour cells. Supernatants were collected from 48 h co-cultures of DC with irradiated or non-irradiated DU145 cells. Supernatants from DC alone or irradiated DU145 cells alone were used as controls (Figure 4.15). The Protein Profiler Array revealed that DC downregulated CCL2 secretion by 56%, but upregulated CXCL10 secretion by 58% following uptake of irradiated tumour cells compared to that of non-irradiated tumour cells (Figure 4.15 and 4.16). As CCL2 is a Th2-type, while CXCL10 is a Th1-type chemokine, the switch in their ratio is a further confirmation of irradiation inducing pro-inflammatory changes in DC.

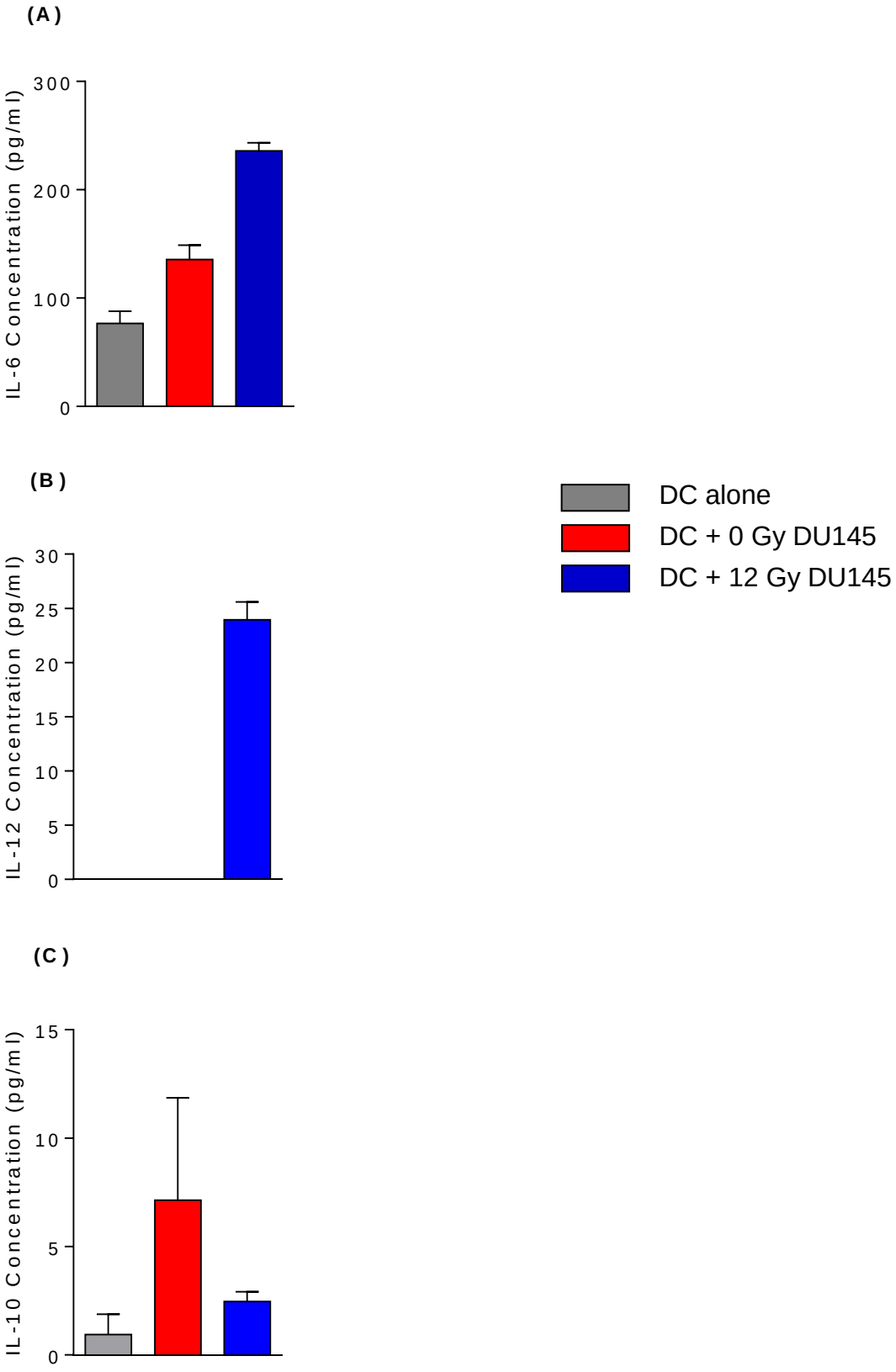


Figure 4.14: Secretion of IL-6, IL-12 and IL-10 by DC. Supernatants from DC co-cultured with irradiated and non-irradiated DU145 cells for 48 h were analysed by ELISA. Mean and SD for IL-6, IL-12 or IL-10 concentration of triplicate samples are shown.

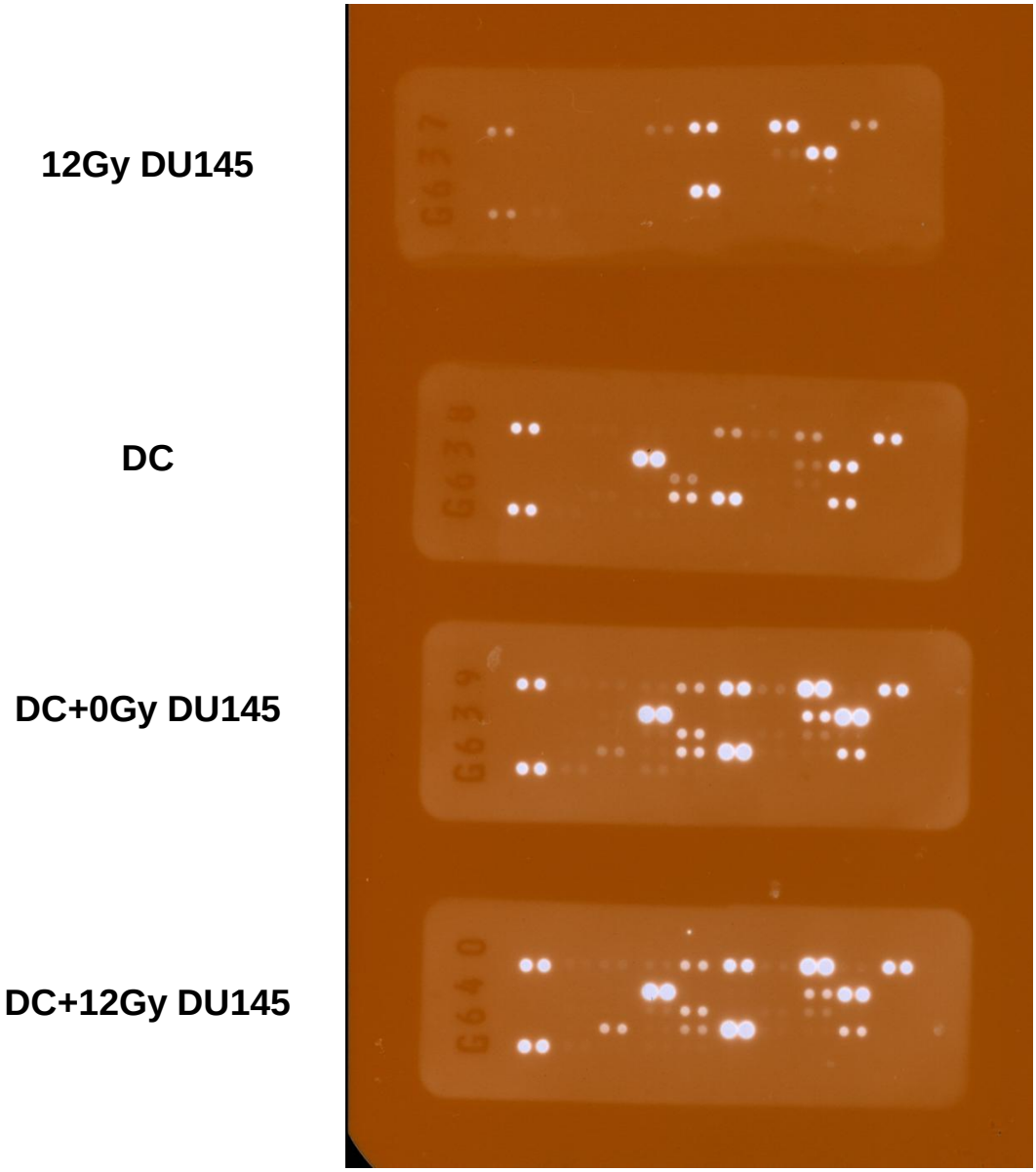


Figure 4.15A: Proteome Profiler Human Cytokine Array- Scanned images of the membranes used in the array for each group as shown above.

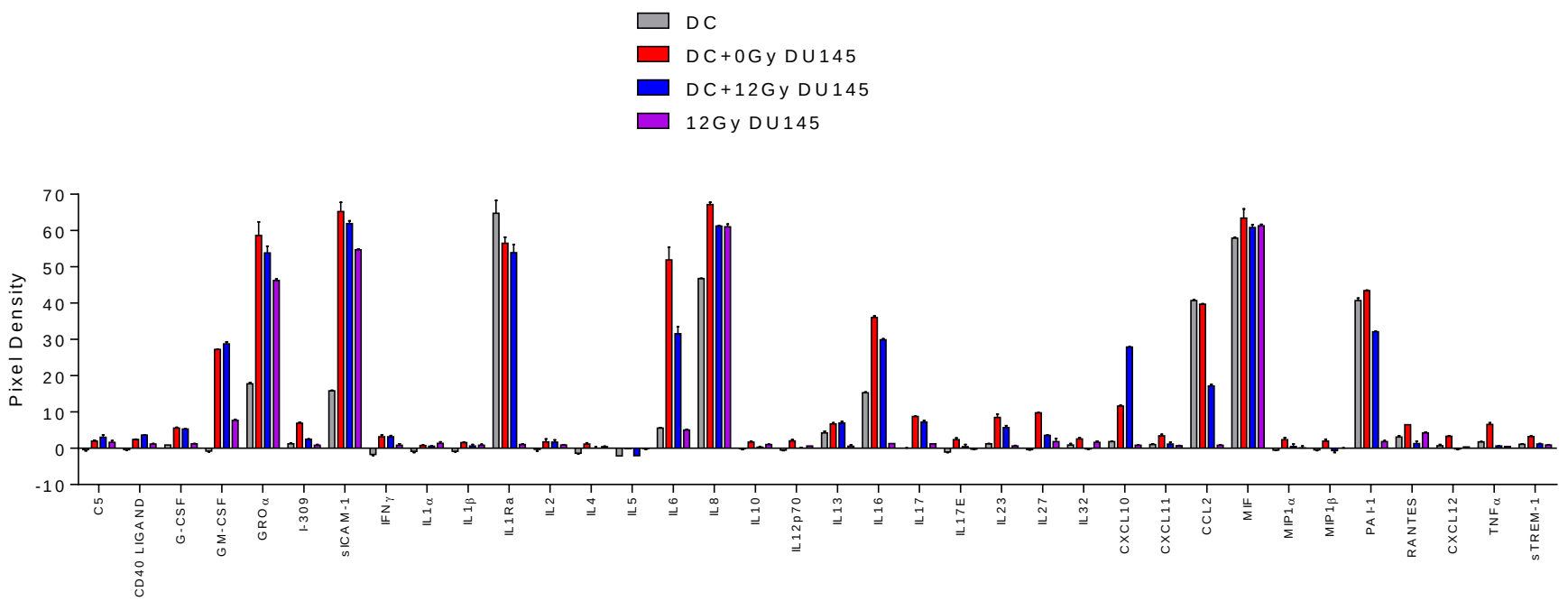


Figure 4.15B: Cytokine and chemokine responses by DC following uptake of irradiated and non-irradiated DU145 cells. Densitometry of proteins was carried using ImageJ software and data was normalized by subtracting the averages of the negative control from the test samples. Mean and SD of pixel densities from duplicate samples are shown.

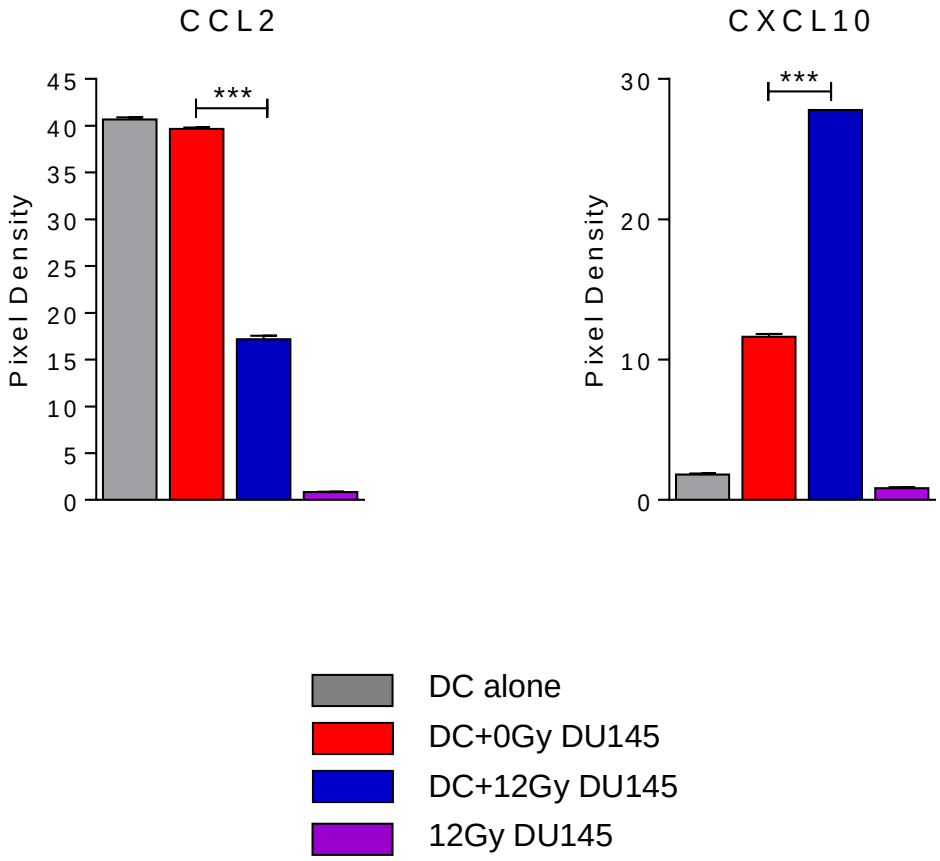


Figure 4.16: Significant differences in cytokine and chemokine production by DC following the uptake of irradiated and non-irradiated DU145 cells. Densitometry as on Figure 4.15B from the same experiment. Mean and SD of pixel densities from duplicate samples are shown.

DISCUSSION

The question in this chapter was whether IR causes immunogenic changes in tumour cells. In order to answer this, the effects of IR on DU145 cells in the context of cell cycle arrest, proliferation, type of cell death and translocation of immunogenic signals were examined. Furthermore, the activation status of DC upon uptake of irradiated or non-irradiated DU145 cells was investigated.

The DNA damage induced by IR initiates signals that can ultimately activate either temporary checkpoints that permit time for genetic repair or irreversible growth arrest that results in cell death (necrosis or apoptosis) (Pawlik and Keyomarsi, 2004). In mammalian cells, progression through the cell cycle can be halted at G1 and/or G2 in response to IR. The cell-cycle DNA damage checkpoints occur late in G1, which prevents entry to S phase, and late in G2, which prevents entry to mitosis (DiPaola, 2002). Irradiating DU145 cells caused cell cycle arrest at the G2/M phase in a radiation dose dependent manner. Our findings are in agreement with the work by others (Xu et al., 2002, Miyata et al., 2001, Aquilina et al., 1999) which showed late G2/M accumulation in irradiated cells.

Many of the common solid tumours take a long time to respond to cytotoxic treatments such as radiotherapy or chemotherapy. The rate usually correlates with the turnover of cells within the tumour, and this reflects the mechanism of mitotic catastrophe. Mitotic catastrophe is a form of cell stress, which occurs as a result of failed mitosis. In this mechanism, treated cells remain viable until they enter the cell cycle, either initially or at some later point, when the accumulated genetic damage makes the cell non-viable (Shinomiya, 2001, Garcia-Lora et al., 2003). Based on the growth curve of irradiated DU145 cells, altered proliferation was not detectable for the first 18 h after IR treatment. The occurrence of G2/M arrest, which prevents damaged cells from proceeding to mitosis for cell division, probably contributed to the halt in proliferation observed in tumour cells. Images of irradiated cultures taken by the Incucyte system showed among other signs, cell senescence, a form of irreversible growth arrest. The hallmarks of cell senescence include enlarged and flattened cellular morphology and increased granularity. This correlates with some of the data obtained from the flow cytometry analysis where irradiated DU145 cells

were larger and more granular compared to non-irradiated cells. Numerous signs of cell death were also observed in the images.

Prolonged incubation after IR treatment allowed the detection of increased cell death using the Annexin-V/PI staining protocol. At 72 h, I observed higher levels of late-apoptotic/necrotic cell death than apoptotic death. IR-induced necrotic tumour cell death provides an abundant cellular source of tumour antigen for uptake and presentation by APC. Although not in a cross-presentation experiment, a study has demonstrated that irradiation of a NY-ESO negative breast cancer cell line induced de novo synthesis and upregulation of NY-ESO on the tumour cells. Consequently, NY-ESO $CD8^+$ T were able to recognize and respond to the breast cancer cell line in an antigen-specific manner (Sharma et al., 2011). In a cross-presentation setting, antigen from tumour cells taken up by DC is processed and presented to $CD8^+$ T cells in the context of MHC class I molecules. Therefore, it seems likely that the increased expression of 5T4 on DU145 cells in response to radiation provides DC with more tumour antigen to process and present to T cells compared to the antigen from non-irradiated cells. This sequentially would enhance the stimulation of $CD8^+$ T cells.

HMGB1 concentrations above background levels (media alone) were only detected in the supernatant from irradiated DU145 cells suggesting passive release by dying tumour cells in response to IR. This confirms the work by Apetoh et al. (2007a and b). In order to detect HMGB1 by western blotting, immunoprecipitation of the supernatant was carried out. Although HMGB1 was detected in the supernatants of both irradiated and non-irradiated cells after immunoprecipitation, there was more in the supernatant from irradiated DU145 cells. While the positive control of the rhHMGB1 was observed at approximately 30-36 kDa, the bands from the DU145 cell supernatants were observed at approximately 26-33 kDa. The predicted molecular mass for human HMGB1 is 25 kDa, however it is usually detected at 25-36 kDa on western blots. The difference between the predicted band size and the observed band size could be due to the post-translational modifications, which increases the size of the protein. HMGB1 undergoes a number of post-translational modifications, which determine its interactions with other proteins and modulate its biological activity (Sioud et al., 2007, Yang et al., 2013).

IR increased translocation of CRT to the surface of treated cells. The translocation of CRT to the surface of the cell membrane acts as an ‘eat me’ signal (Obeid et al., 2007a). One suggested mechanism for cell surface exposure of CRT involves the association of cytoplasmic CRT with phosphatidylserine (PS) on the inner leaflet of the plasma membrane, thereby allowing CRT to become exposed during apoptosis and necrosis (Raghavan et al., 2012). Sites of CRT exposure have been shown to significantly correspond with localized areas of PS exposure, suggesting there is a combined role for each in optimum apoptotic cell recognition and induction of uptake (Gardai et al., 2005). These data suggest that the increased exposure of CRT on irradiated DU145 cells could enhance cross-presentation of the 5T4 antigen by enabling increased uptake of irradiated tumour cells.

Hsp70 expressed on the cell surface may serve as a danger signal and induce DC activation. Hsp70 was significantly exposed on the surface of irradiated compared to non-irradiated DU145 cells. It also acts as a vehicle to deliver its associated antigen to DC, and then facilitates antigen cross-presentation to $CD8^+$ T cells. A suggested mechanism for Hsp70 externalisation is by binding to PS upon tumour cell death, similar to CRT (Schilling et al., 2009). Numerous studies have demonstrated the immunosuppressive effects of PS (Hoffmann et al., 2005). Therefore it is possible that the PS-binding function of CRT and Hsp70 influences immunogenicity by binding to SREC-I or CD91, thus blocking other PS-dependent interactions, such as those involving TIM-4 and TAM family of receptors, which are known to be tolerance inducing (Freeman et al., 2010, Lemke and Rothlin, 2008).

In the immature state, DC are specialized to recognize and capture specific antigens, including tumour antigens. Indeed, DC phagocytosed significantly more irradiated DU145 cells compared to the non-irradiated cells possibly due to the upregulated expression of the ‘eat me’ signal on the former compared to the latter group. However, although immature DC are highly phagocytic, they express relatively low levels of MHC and co-stimulatory molecules and are therefore unable to efficiently activate T cells resulting in T cell anergy (Tan and O'Neill, 2005).

Distinct DC development and activation plays a role in the induction of tolerance versus immunity. Activated DC are matured to become immunostimulatory. DC maturation is associated with the upregulation of the DC maturation marker (CD83), co-stimulatory (CD86) and MHC molecules as well as chemokine receptors such as CCR7. Primarily, in an *in vivo* tumour antigen cross-presentation setting, CCR7 enables DC to migrate from the tumour tissue to the tumour draining lymph node, where DC present peptides derived from antigen acquired from the tumour in the context of MHC class I molecules to naïve CD8 T cells (Breckpot and Escors, 2009). These phenotypic changes were displayed by DC following the uptake of irradiated DU145 cells compared to that of non-irradiated cells.

Activated and immunogenic DC produce pro-inflammatory cytokines such as IL-6 and IL-12 (Morelli et al., 2001, Lutz and Schuler, 2002). IL-6 which is also has an important role in T cell migration (Weissenbach et al., 2004), was highly produced by DC in response to irradiated compared to non-irradiated DU145 cells. IL-12 was only secreted by DC co-cultured with irradiated tumour cells. A study showed that IL-12 enhances cross-presentation of tumour antigens and reverses the immunosuppressive function of tumour-resident myeloid cells (Kerkar et al., 2011). The aim for carrying out the cytokine/chemokine array was to identify any other inflammatory mediators being secreted by DC, which may influence the cross-presenting ability of DC. DC downregulated CCL2 but upregulated CXCL10 following uptake of irradiated compared to non-irradiated DU145 cells. CCL2 within the tumour microenvironment has been associated with tumour progression and metastasis by inducing M2-type macrophage polarization (Roca et al., 2009) and prevents normal DC development (Spary et al., 2014). Conversely, CXCL10 is important for trafficking of T cells to the tumour microenvironment (Franciszkiewicz et al., 2012). $CD8^+$ T cell infiltration in the irradiated tumour tissue serves as a prognostic factor (Golden et al., 2013, Postow et al., 2012, Tabachnyk et al., 2012, Schmidtner et al., 2009, Suwa et al., 2006) indicating that radiation can switch the immunosuppressive tumour milieu to a pro-immune environment.

Co-culturing DC with tumour cells alone, supernatant alone, or tumour cells and supernatant provided an idea regarding which signals might be contributing to the immunogenicity of irradiated tumour cells. Signals translocated to the surface of

tumour cells upon IR treatment include CRT and Hsp70. Both have been shown to activate DC through binding to CD91 (Pawaria and Binder, 2011). Hsp70 also activates and matures DC through binding to TLR2 and TLR4 (Chen et al., 2009, Asea et al., 2002). Signals often released into the extracellular space by dying tumour cells include HMGB1, Hsp70 and ATP. Although the soluble factors were not able to activate DC, they did contribute to the enhanced cross-presentation of antigen from irradiated DU145 cells. HMGB1 can aid cross-presentation by preventing antigen degradation within the DC (Apetoh et al., 2007b), Hsp70 facilitates the transportation of antigen through the MHC class I pathway in DC (Kato et al., 2012), and ATP induces IL-1β production, which is required for efficient T cell priming (Ghiringhelli et al., 2009). Given that cross-presentation was only affected by supernatant from irradiated but not non-irradiated cells, it is conclusive that the signals induced by IR are responsible for improved cross-presentation of antigen from irradiated DU145 cells.

In conclusion, IR drives the immunogenicity of irradiated DU145 cells by inducing the type of cell death that provides immunogenic signals such as CRT, Hsp70 and HMGB1. IR also increases the expression of 5T4 on irradiated DU145 cells. Therefore, CRT might be triggering DC to take up more antigens from irradiated than non-irradiated cells. DC then become activated and subsequently process the antigen with the aid of the immunogenic signals thus allowing better cross-presentation of the 5T4 antigen to 5T4 specific $CD8^+$ T cells.

V. The Mechanism of Tumour Antigen Cross-Presentation from Irradiated Tumour Cells and the Role of TLR4 and TLR4 Polymorphism

INTRODUCTION

HMGB1 is a ligand for TLR4 and it is widely published that HMGB1 is released when cells undergo necrosis. HMGB1 was detected in the supernatant of irradiated DU145 cells. Hsp70, another ligand for TLR4, was highly expressed in irradiated DU145 cells. The presence of the TLR4 ligands in the experimental setting suggests that TLR4 contributes to the antitumor antigen T cell responses observed in the cross-presentation model.

DC require signalling through TLR4 and its adaptor MyD88 for efficient processing and cross-presentation of antigen from dying tumour cells (Apetoh et al., 2007b). The activation of tumour antigen-specific T cell immunity involved the ligation of HMGB1 with TLR4 expressed on mouse DC (Apetoh et al., 2007b). In the same study, DC from individuals bearing the Asp299Gly SNP showed impaired ability to cross-present MART1 from oxaliplatin-treated melanoma cells to MART1 specific $CD8^+$ T cells compared to normal DC in HMGB1-dependent manner (Apetoh et al., 2007b). However, a chemotherapeutic agent and not IR was used to induce cell death. Therefore, this chapter will investigate role of TLR4 and the TLR4 polymorphism in the cross-presentation of antigen from irradiated tumour cells.

Questions:

Does TLR4 play a role in the cross-presentation of antigen from irradiated tumour cells and does Asp299Gly SNP of TLR4 interfere with antigen cross-presentation?

Specific aims:

a) Block TLR4 and its potential ligands HMGB1 and Hsp70 in the tumour antigen cross-presentation model.
b) Identify donors with TLR4 Asp299Gly SNP.
c) Determine the characteristics of DC with TLR4 Asp299Gly SNP.
d) Determine if TLR4 Asp299Gly SNP impairs the ability of DC to cross present the 5T4 antigen from irradiated tumour cells better than the non-irradiated tumour cells.
e) If the role for TLR4 Asp299Gly SNP in the above experiments has been demonstrated, carry out SNP analysis from PCa patients with known clinical history post radiation therapy.

RESULTS

Analysis of TLR4 expression

TLR4 surface expression was evaluated on monocytes and DC. Monocytes expressed approximately 6 times more surface TLR4 molecules compared to DC (Figure 5.1). Total TLR4 expression levels were also compared to the surface levels after monocytes were incubated with GM-CSF alone (monocytes) or IL4+GM-CSF (DC) for different time periods (Figure 5.2). In DC, surface TLR4 was downregulated after 5 days while total levels remained unchanged. However, the level of TLR4 expressed on the surface of monocytes increased after 5 days. This suggests that the presence of IL4 in the culture results in the downregulation of TLR4 on the surface of monocyte-derived DC but their intracellular content did not change significantly.

LPS responsiveness and TLR4 blocking

In order to block TLR4, several approaches were used. Vaccinia virus encodes the A46 protein, which binds to multiple TIR-domain containing proteins, ultimately preventing TLRs from signalling. An 11 amino acid long peptide (KYSFKLILAEY) from A46 was termed *Viral inhibitory peptide of TLR4* (VIPER). When fused to a cell-penetrating delivery sequence (9R), VIPER specifically inhibited TLR4 mediated responses such as TNF-α production by RAW264.7 cells, THP-1 cells and PBMC. CXCL2, RANTES and IL-6 responses by murine immortalized bone marrow derived macrophages (iBMDM) (Lysakova-Devine et al., 2010) were also inhibited. VIPER binds to the TIR domains of the adaptor proteins, thereby inhibiting TLR4 signalling by interfering with TLR4-Mal and TLR4-TRAM interactions (Lysakova-Devine et al., 2010). As an attempt to block TLR4 function, I carried out an experiment to stimulate DC with LPS in the presence of VIPER. VIPER was added either at increasing concentrations to 10 ng/ml LPS stimulation (Figure 5.3). No inhibition of TNF-α production by any concentration of VIPER was observed. Instead of inhibition, VIPER alone was able to stimulate DC as determined by the significant expression of TNF-α even in the absence of LPS compared to the control without VIPER and LPS. Thus, the VIPER peptide was not used in the cross-presentation assay to investigate the role of TLR4.

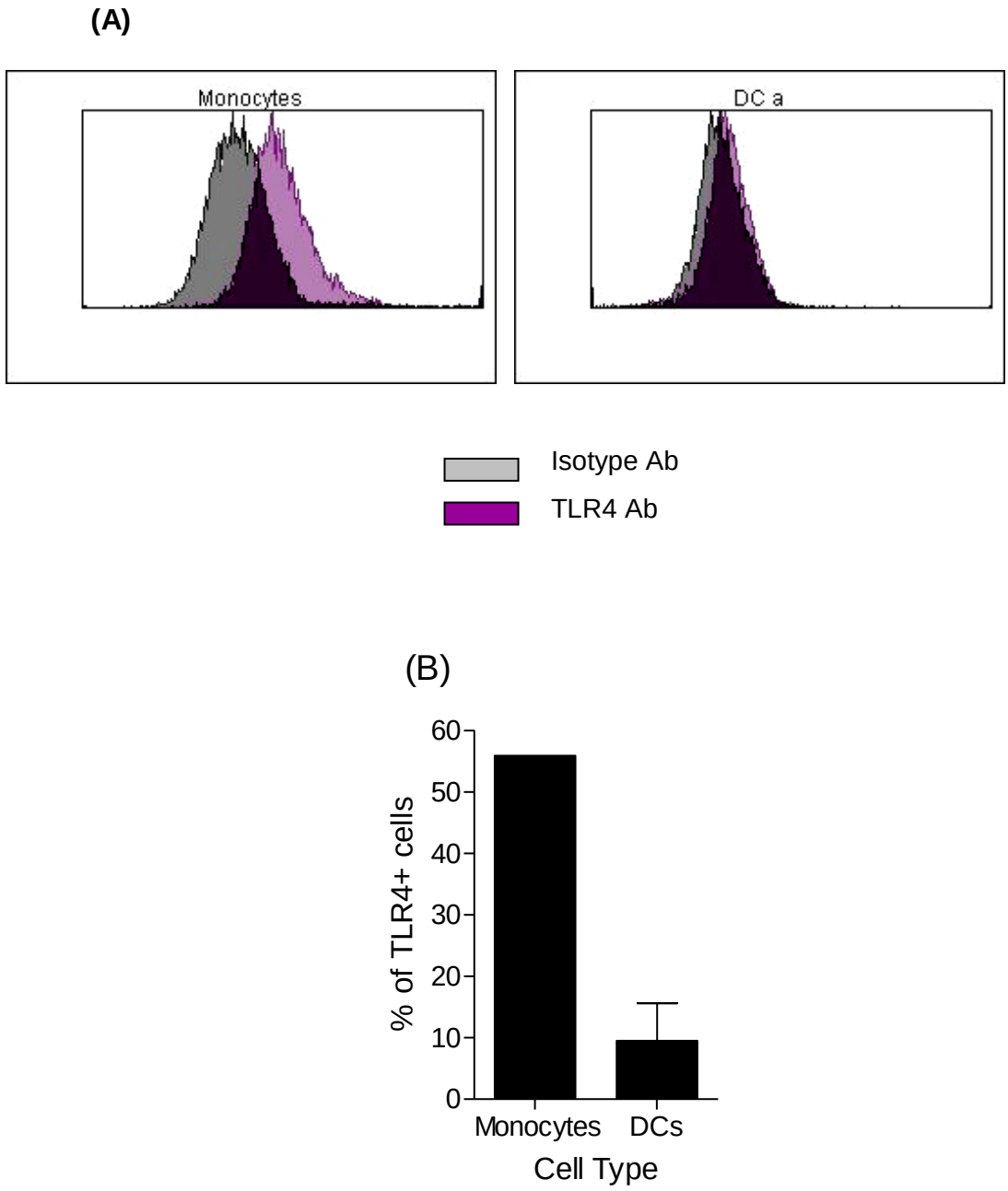


Figure 5.1: TLR4 surface expression on monocytes and DC. A) Histograms of TLR4 (Purple) and isotype control (Grey) B) Mean and SD of the percentage of TLR4 positive cells (%) from triplicate samples are shown. Antibody binding to monocytes (left) or DC (right)

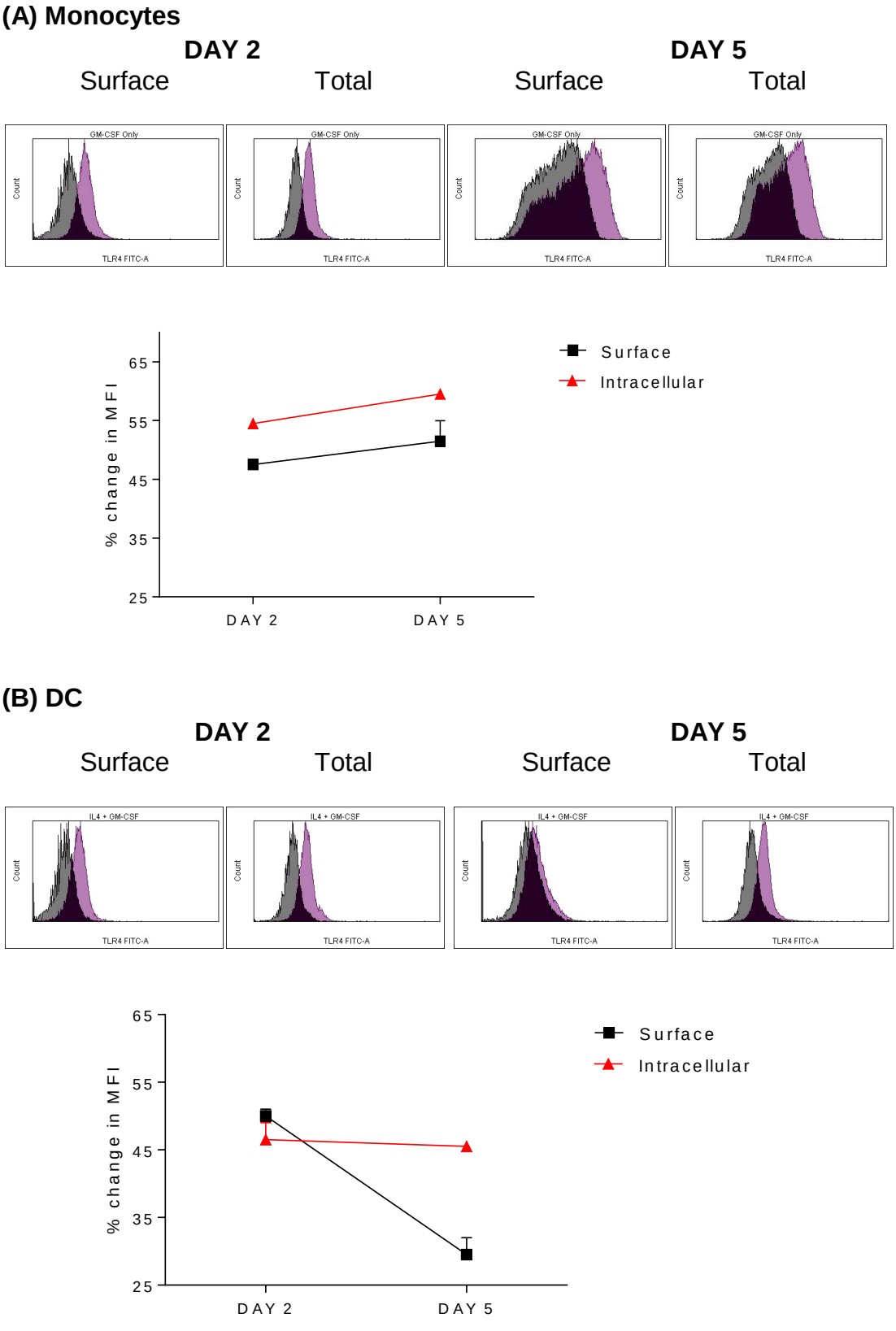


Figure 5.2: TLR4 expression on monocytes and DC *in vitro*. (A) Monocytes (GM-CSF) or (B) DC (IL4+GM-CSF). TLR4 expression was measured after 2 and 5 days of incubation in both groups. Representative histograms of cells (as indicated above the figures) labelled with TLR4 (Purple) or isotype control (Grey) antibodies. Means and SD of percentage change in MFI of TLR4 compared to isotype from duplicate samples are shown.

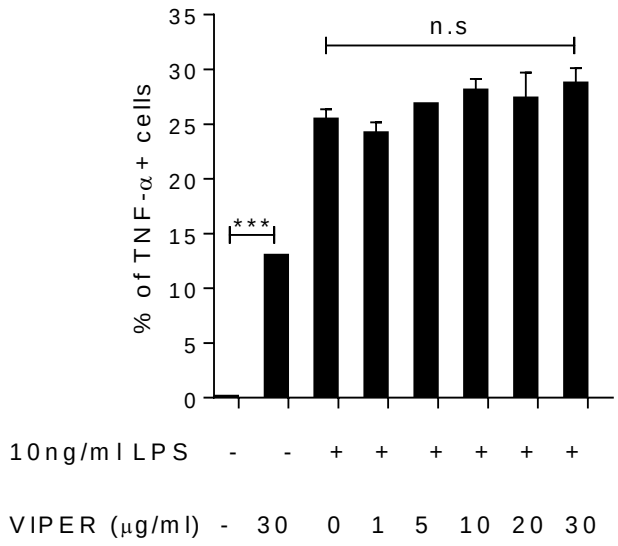


Figure 5.3: The effect of VIPER on DC stimulation. VIPER concentrations are shown in the x-axis. TNF-α production by DC in response to 10 ng/ml LPS is shown. Mean and SD of the percentage of TNF-α positive cells from triplicate samples are shown.

MyD88 and TRIF inhibition

Activation of cell surface or endosomal TLR4 drives the recruitment of the adaptor pairs Mal/MyD88 and TRAM/TRIF respectively, through the interaction of the TIR domain on TLR4 with the TIR domain on the adaptors via a loop referred to as the BB-loop (Figure 1.2). In order to investigate the TLR4 adaptor function, MyD88 and TRIF were blocked using peptides containing amino acids that correspond to the sequence of the BB loop of MyD88 (RDVLPGT) and TRIF (FCEEFQVPGRGELH). The peptides function as decoys by binding to their TIR domain and interfering with TLR-adaptor interactions (Loiarro et al., 2005, Toshchakov et al., 2005).

In order to determine the ability of the inhibitory peptides to block TLR4 signalling, DC were pretreated with different concentration of the peptides for 6 h before 100 ng LPS was added. The MyD88 inhibitory peptide set (both test and control) proved to be cytotoxic to DC when used at 50 µM and more (data not shown), therefore, lower concentrations, ranging from 5-20 µM, were used in the LPS stimulation assay. A dose dependent reduction in the percentage of cells producing TNF-α was observed. However, significant differences between the control and test peptide were only evident at 15 µM and 20 µM (Figure 5.4A). Peptide concentrations against TRIF were used from 5-50 µM. When compared to the control peptide, the test peptide inhibited TLR4 signalling via TRIF at all the concentrations used (Figure 5.4B).

In the cross-presentation model, DC were pretreated with 20 µM MyD88 test/control peptide (Figure 5.5A) or 10 µM TRIF (Figure 5.5B) test/control peptide for 6 h before adding the DC to the irradiated or non-irradiated DU145 cells. Blocking individual signalling pathways did not affect cross-presentation from irradiated cells, as there were no significant differences observed between the MyD88 or TRIF test and control peptides (Figure 5.5A and B). As there is a synergy between the adaptors (Meissner et al., 2013), DC were also pretreated with both the MyD88 and TRIF test or control peptides to ensure inhibition of both adaptor molecules (Figure 5.5C). Inhibition of both MyD88 and TRIF signalling pathways with the test peptide partially reduced T cell stimulation by cross-presented antigen from irradiated DU145 cells and this was significant compared to the control peptide. The treatment did not significantly affect T cell stimulation by antigen from non-irradiated cells

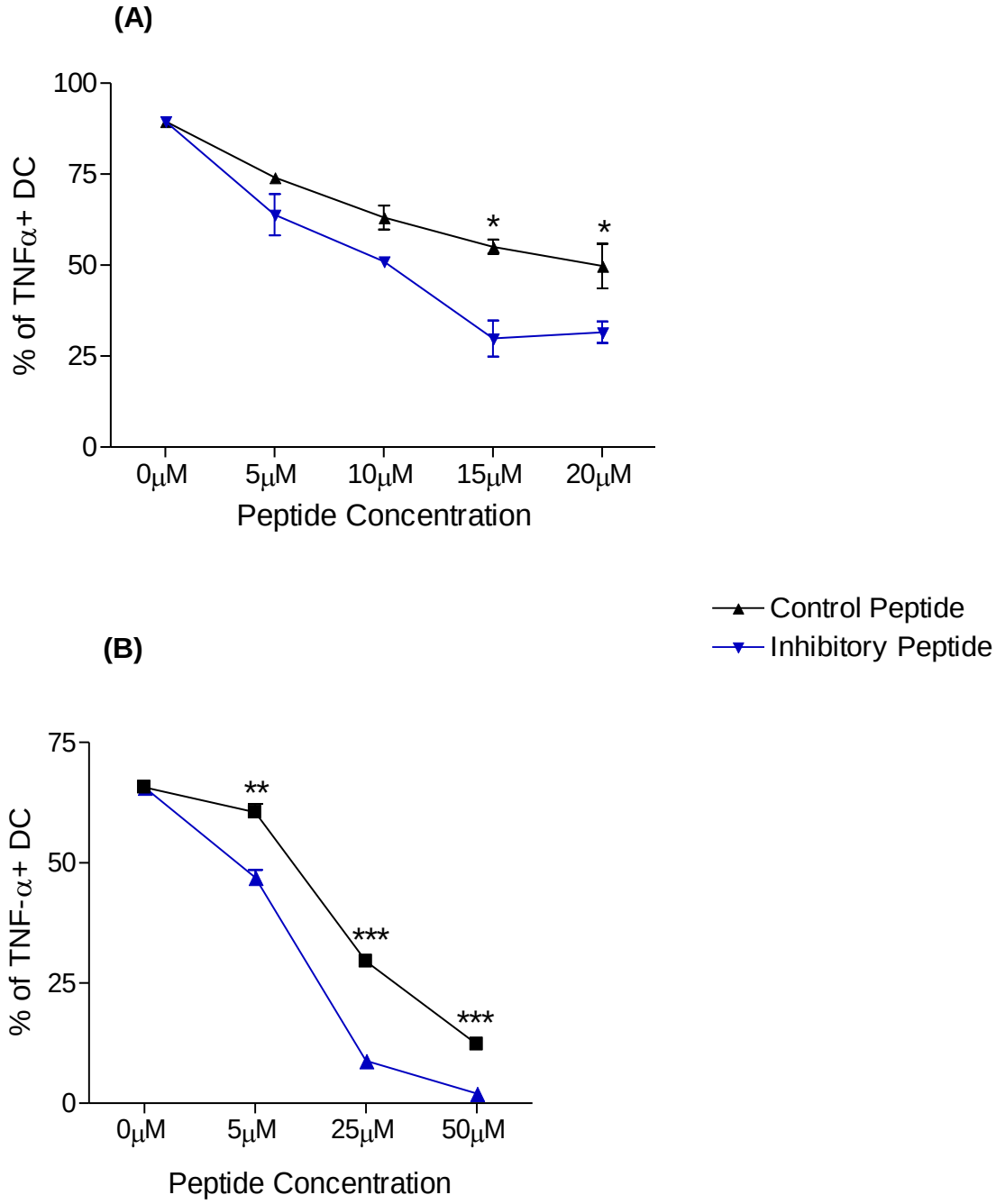


Figure 5.4: Dose dependent inhibition of TNF-α production in DC by (A) MyD88 or (B) TRIF inhibitors. MyD88 and TRIF inhibitory peptide or control peptide concentrations are shown on the x-axis. TNF-α production by DC in response to 100 ng/ml LPS is shown. Mean and SD of the percentage of TNF-α positive cells from triplicate samples are shown.

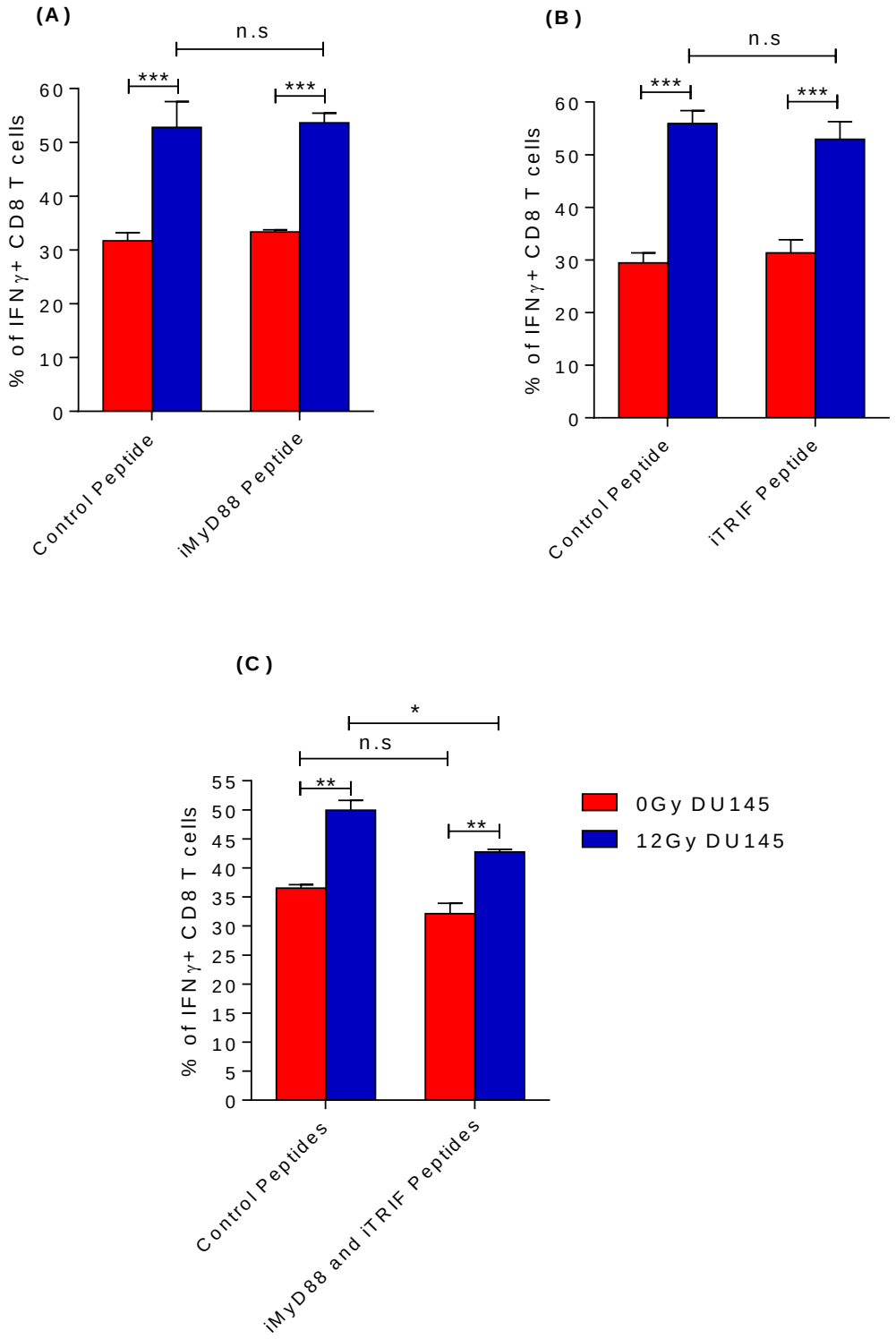


Figure 5.5: Inhibition of cross-presentation by MyD88 or TRIF inhibitors. 5T4 specific T cell stimulation after cross-presentation of antigen from irradiated and non-irradiated DU145 cells by DC in the presence of either control peptides or (A) MyD88 – 20 µM or (B) TRIF – 10 µM inhibitory peptides. (C) 25 µM of control peptide or MyD88 and TRIF inhibitory peptides together. Mean and SD of percentage of IFNγ positive $CD8^+$ T cells from triplicates samples are shown.

(Figure 5.5C). This illustrated that a ligand released by irradiated cells was partially contributing to TLR4 signalling in DC.

HMGB1 and Hsp70 inhibition

It has been demonstrated that ligation of TLR4 with HMGB1 (Messmer et al., 2004, Dumitriu et al., 2006, Apetoh et al., 2007b) and Hsp70 (Basu et al., 2000, Joly et al., 2010, Bendz et al., 2007) induces DC activation and improves antigen processing. Therefore, in order to determine if HMGB1 and Hsp70 might be contributing to the antitumor T cells responses being observed in our model, blocking experiments of the ligands were carried out. In this study, HMGB1 function was inhibited using glycyrrhizin. Glycyrrhizin has been shown to inhibit chemoattractant and mitogenic activities caused by HMGB1 binding to RAGE (Mollica et al., 2007) and to reduce cytokine production induced by HMGB1 binding to TLR4 (Wang et al., 2013). It binds directly to HMGB1 by interacting with two shallow concave surfaces formed by the two arms of both HMG boxes.

Glycyrrhizin (50 µM) was added to irradiated and non-irradiated DU145 cells once every 24 h over a 72 h incubation period before co-culturing them with DC. HMGB1 inhibition had no effect on DC activation, as CD86 remained constant in both the presence and absence of glycyrrhizin (Figure 5.6A). However, it did significantly reduce cross-presentation of the 5T4 antigen from irradiated tumour cells almost to the level observed with non-irradiated DU145 cells (Figure 5.6B).

In order to assess the function of Hsp70, VER 155008 (5 µM) an inhibitor for Hsp70 was added to irradiated and non-irradiated DU145 cells once every 24 h over a 72 h incubation period (Figure 5.7). The molecular chaperone activity of Hsp70 is conferred by two functional domains: a dedicated binding domain that seizes client polypeptides and an ATPase domain. HSPs are allosteric molecules, one domain reciprocally affecting the other, and when polypeptide moieties bind to the peptide binding domain, ATP is hydrolysed to ADP but when ATP binds, associated peptides are released (Massey, 2010). As such, VER 155008 functions as an ATP mimetic and binds to the ATPase pocket of Hsp70 (Massey et al., 2010), thereby inhibiting the activity of Hsp70.

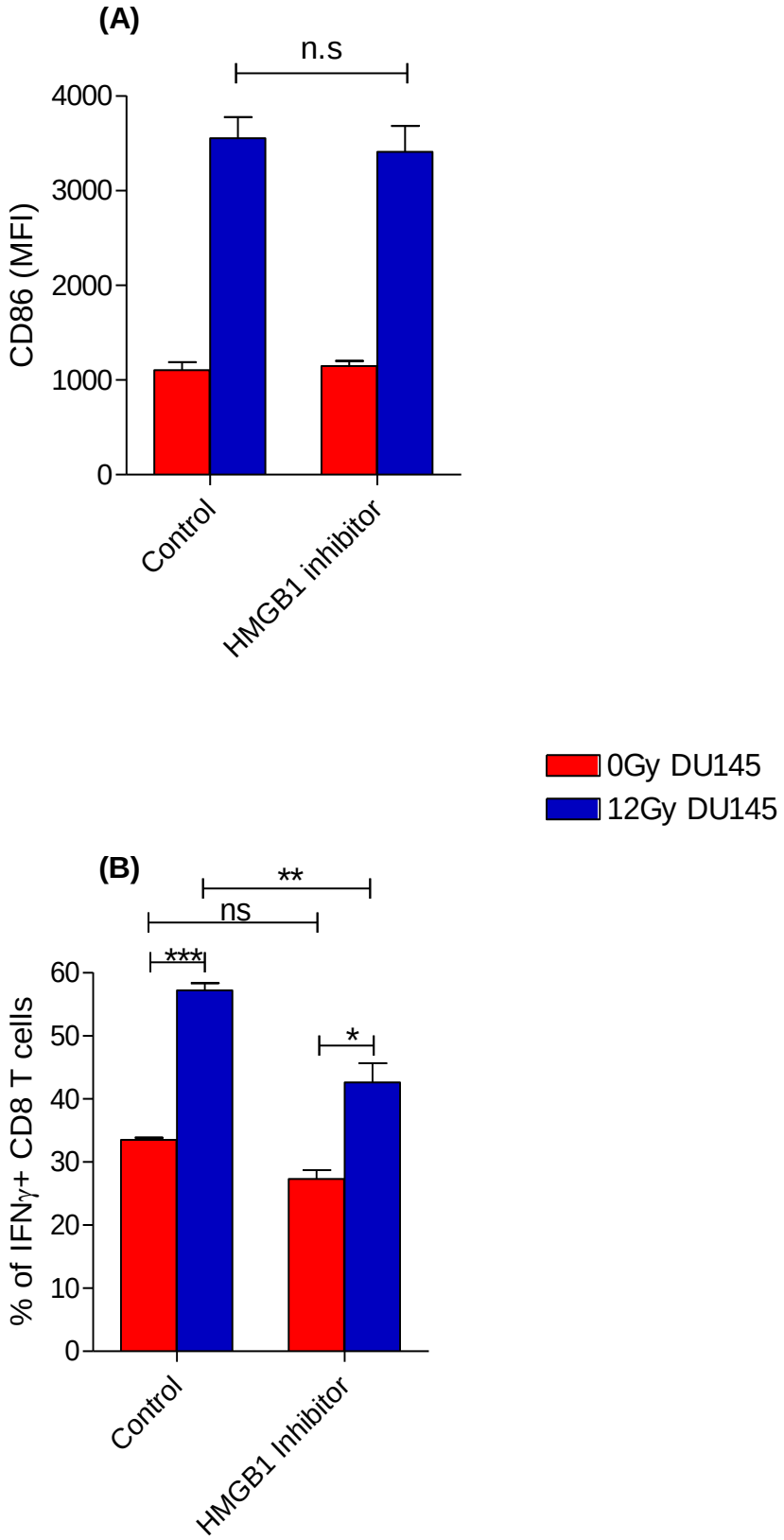


Figure 5.6: Effects of HMGB1 inhibition on (A) DC activation and (B) RLAR T cell activation in a cross-presentation experiment. (A) Mean and SD of MFI of CD86 on DC exposed to DU145 cells from triplicate samples are shown. (B) Mean and SD of the percentage of IFNγ positive $CD8^+$ T cells from triplicates samples. *(*p < 0.5; **p < 0.01; ***p < 0.001; Student's t test).* Representative data of several experiments.

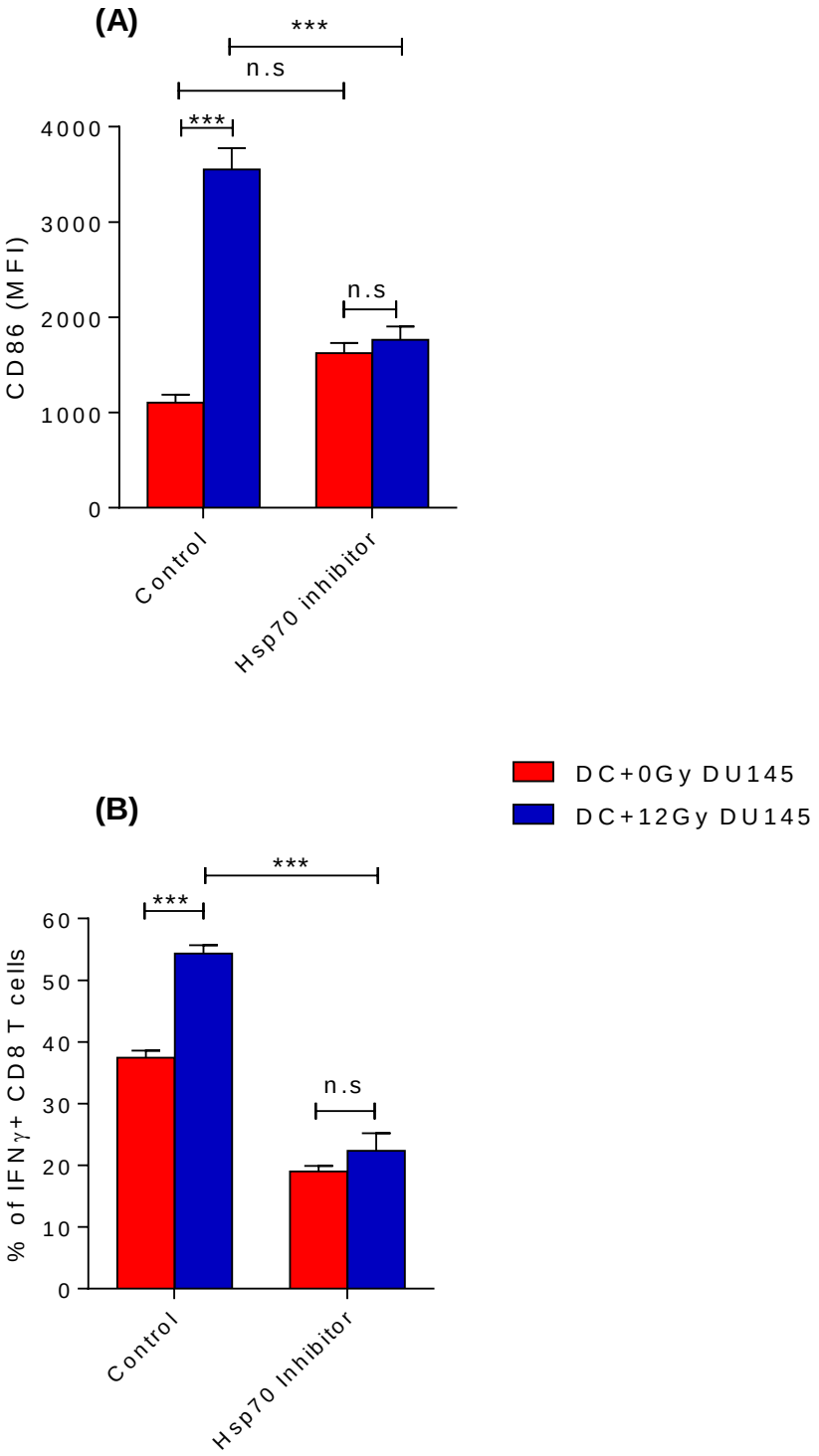


Figure 5.7: Effects of Hsp70 inhibition on (A) DC activation and (B) RLAR T cell activation in a cross-presentation experiment. (A) Mean and SD of MFI of CD86 on DC exposed to DU145 cells from triplicate samples are shown. (B) Mean and SD of the percentage of IFNγ positive $CD8^+$ T cells from triplicates samples. *(*p < 0.5; **p < 0.01; ***p < 0.001; Student's t test).* Representative data of several experiments.

Inhibition of Hsp70 significantly impaired the ability of irradiated DU145 cells to activate DC; CD86 remained low on DC co-cultured with irradiated tumour cells and the levels were similar to those expressed on DC co-cultured with non-irradiated cells (Figure 5.7A). In the cross-presentation experiment, T cell responses generated by antigen cross-presented from irradiated DU145 cells were not significantly different to those induced by non-irradiated cells (although both were reduced), demonstrating that inhibition of Hsp70 completely inhibited the advantage irradiated tumour cells had over the non-irradiated cells (Figure 5.7B).

Patients and healthy donors identified for TLR4 polymorphism

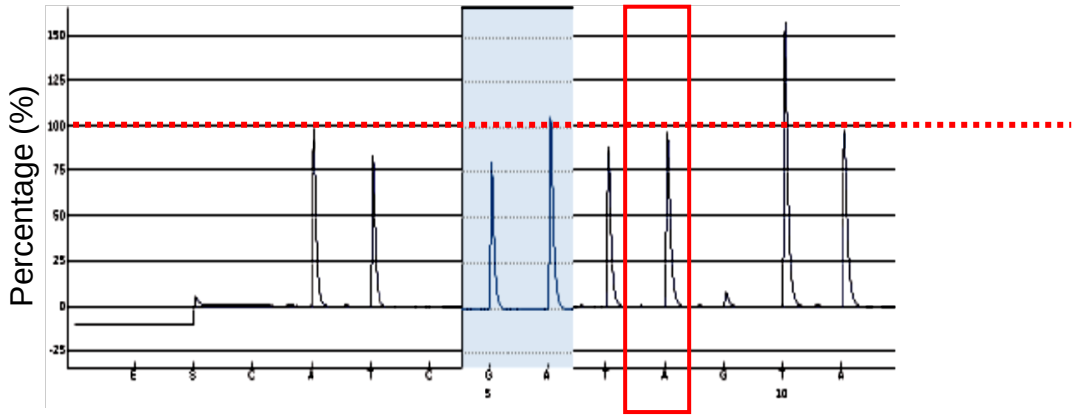
In the experiments shown in this chapter, we have demonstrated that TLR4 (and its potential ligands) play a role in antigen cross-presentation. Next, we wanted to analyse if TLR4 SNP affects this function. In order to identify individuals with the TLR4 Asp299Gly SNP, DNA amplification from blood or established BLCL cells was carried out by PCR followed by pyrosequencing in Dr Rachel Butler's laboratory (Cardiff and Vale NHS Trust, University Hospital of Wales). Samples from 50 health donors (HD) and 18 PCa patients were tested. The percentage of people with the TLR4 SNP was slightly higher in the population with PCa (11.1%) compared to the healthy individuals (6%) (Table 5.1).

In order to investigate if DC with TLR4 Asp299Gly SNP differs significantly from DC with normal TLR4 allele, I planned to carry out phenotypic and functional assays on DC from donors belonging to each group. A Taqman Predesigned SNP Genotyping Assay for SNP ID rs4986790 was done on 10 donors (5 SNP and 5 Normal) to confirm the results attained from the pyrosequencing. When the data from the two genotyping experiments were compared (Figure 5.8.1 and 5.8.2), it was evident that all the donors with the TLR4 Asp299Gly SNP were heterozygous. Analysis of the pyrosequencing data revealed that the A allele had 100% detection in the wild type donors, while for the SNP donors the G allele had a higher percentage of detection but did not reach 100%. Additionally, the TaqMan Predesigned Assay showed that wild type donors only had the A allele while the SNP donors had both the A and G alleles. In both assays, although the A allele was detected in the SNP donors, the levels were lower compared to the wild type. However, the TaqMan Predesigned Assay highlighted that one of the two the PCa patients initially shown to have the SNP based on the pyrosequencing data actually had a normal allele. This assay was repeated three times and the same result was attained each time. Therefore, the assays were carried out on 6 DC with the normal TLR4 allele and 4 DC with the TLR4 Asp299Gly SNP allele (unless otherwise stated).

	HD	PCa	Total
Normal	47	17	**63**
SNP	3	2	**5**
Percentage of SNP	6%	11.1%	**7.4%**

Table 5.1: TLR4 SNP results based on pyrosequencing. Data represents the TLR4 allele (as indicated on the left of the table) of healthy donors (HD) and patients with PCa

(A) Donor with normal/wild type TLR4 Asp299 allele



(B) Donor with the variant TLR4 Asp299Gly allele

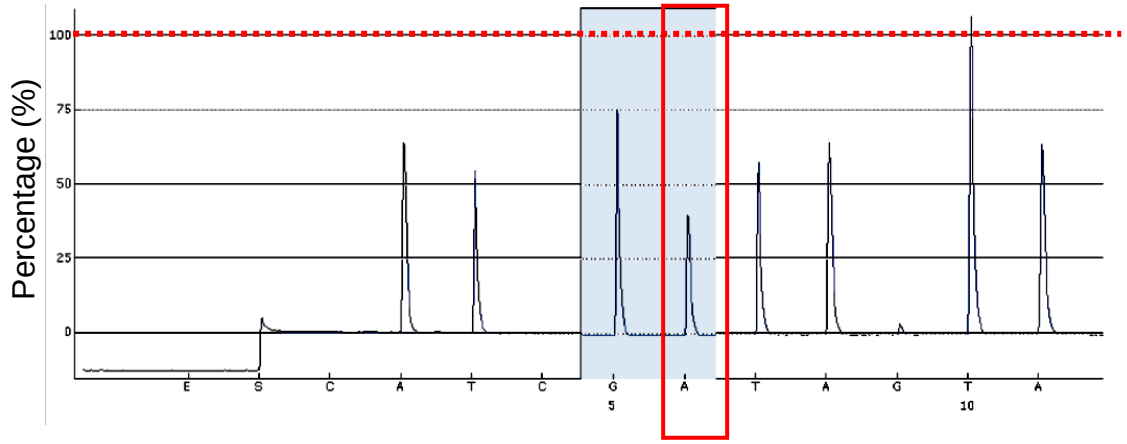


Figure 5.8.1: Pyrosequencing peaks. Representative peaks from 70 donors. (A) Donor with normal/wild type TLR4 Asp299 allele. (B) Donor with the variant TLR4 Asp299Gly allele. Red dotted line = 100% mark. Red Box = position 299

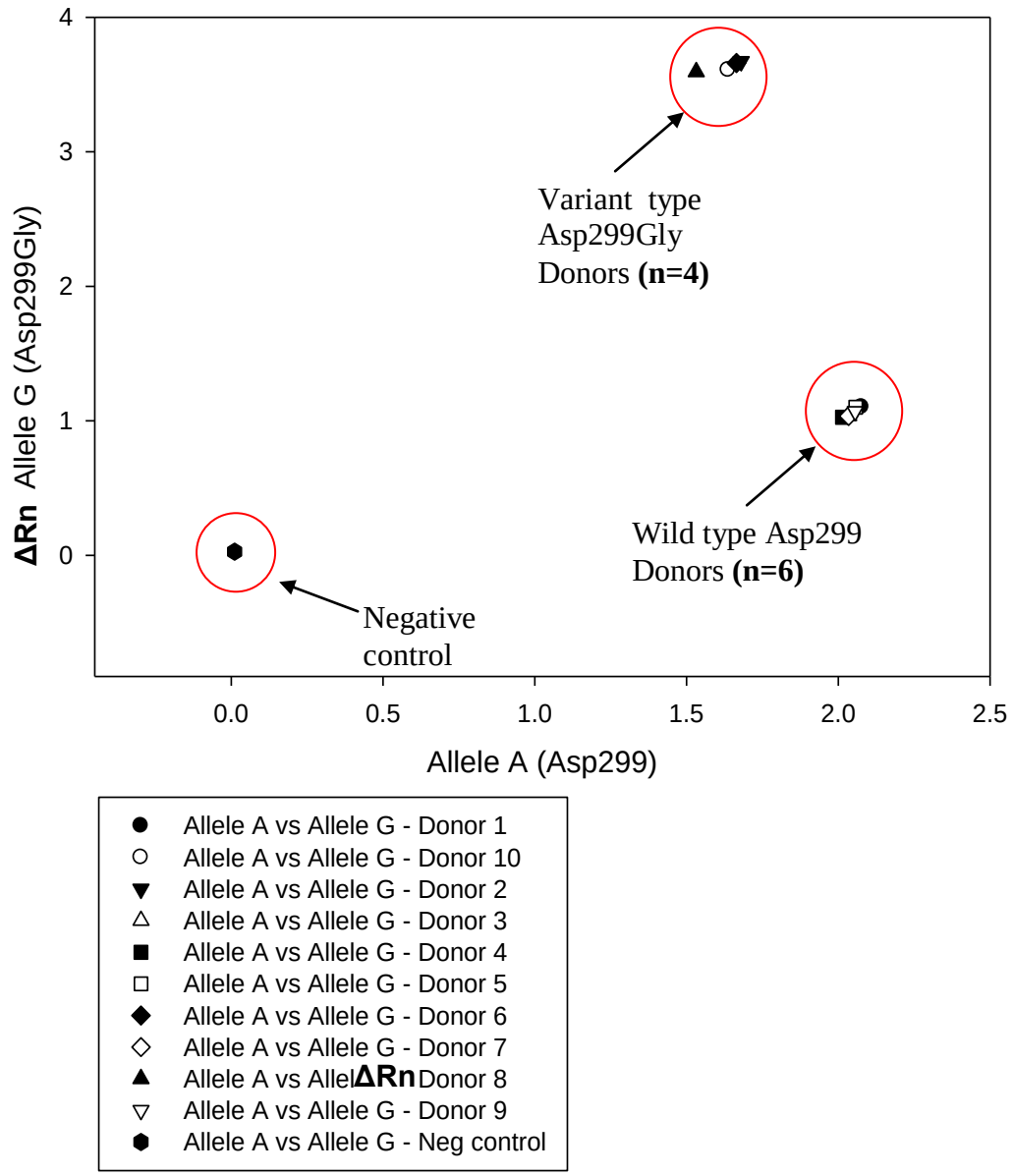


Figure 5.8.2: SNP genotyping: Allelic discrimination plots showing signal intensities (ΔRn) for Normal (Asp299) versus SNP (Asp299Gly) TLR4 alleles from 10 donors. Each shape represents the genotype of an individual sample. Variant type/SNP donors are heterozygous and contain both A and G allele and Wild type/normal donors are homozygous for the A allele. Data are an average of 3 assays.

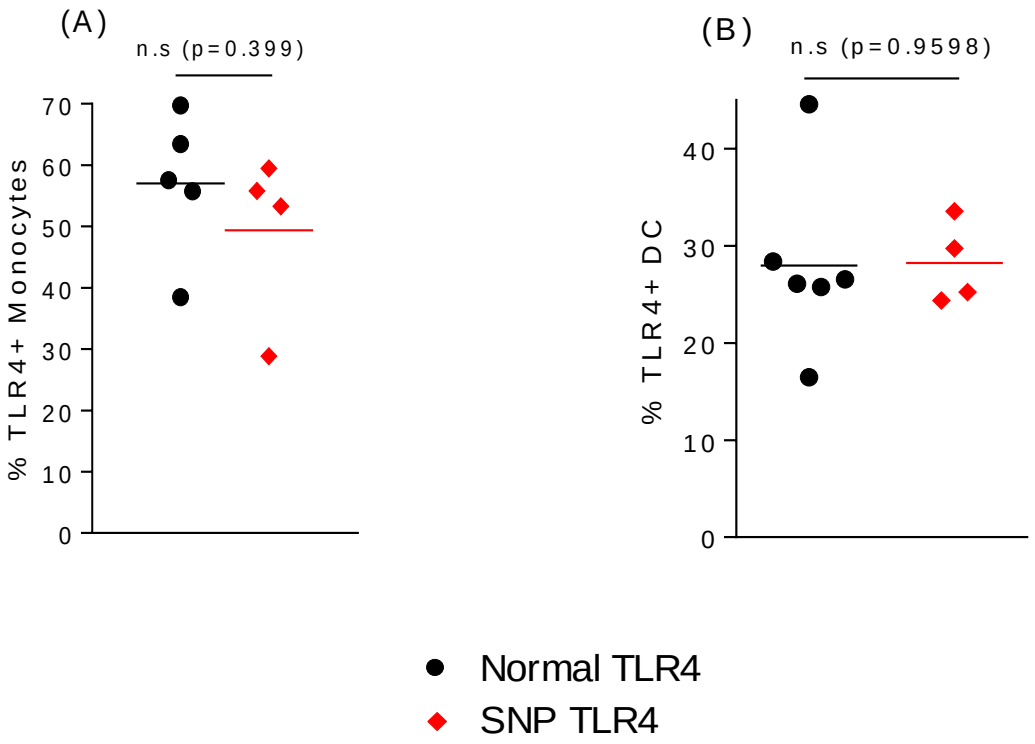


Figure 5.9: Total TLR4 expression in (A) monocytes and (B) DC carrying the normal (Black n=5 and n=6) **or SNP** (Red n=4) **TLR4 allele.** Percentage of TLR4 positive monocytes and DC are shown. Each symbol represents a different donor and is a mean of triplicate samples. The lines represent the mean of TLR4 expression in each group.

TLR4 polymorphism and LPS stimulation

Total TLR4 expression on monocytes and DC was assessed to evaluate if it differs between individuals with the normal allele vs. those with the polymorphic allele (Figure 5.9). Whilst varying levels of TLR4 expression were detected among all individuals, there were no significant differences observed between the two groups either on monocytes or DC.

To determine if the TLR4 Asp299Gly SNP affects DC responses to LPS stimulation, DC from individuals bearing the SNP and those with normal TLR4 allele were stimulated with LPS and TNF-α production or phenotypic changes were analysed by flow cytometry (Figures 5.10 and 5.11). DC responses were assessed by either calculating the percentage of DC producing TNF-α (Figure 5.10) or evaluating DC maturation based on the upregulation of HLA-DR, CD86 and CD83 (Figure 5.11). Both sets of DC produced similar levels of TNF-α in response to LPS. Furthermore, no significant differences were observed in the maturation of DC after stimulation with 100 ng/ml LPS for 24 h. This demonstrates that the TLR4 SNP does not affect the function and phenotype of DC in response to LPS.

TLR4 polymorphic DC maturation by irradiated tumour cells

As demonstrated in the previous chapter, irradiated DU145 cells provided maturation signals for DC. To determine if the TLR4 Asp299Gly SNP affects DC maturation by irradiated tumour cells, DC from individuals bearing the TLR4 Asp299Gly SNP and those with normal TLR4 allele were co-cultured with irradiated or non-irradiated DU145 cells for 48 h before phenotyping the cells for flow cytometry analysis (Figure 5.12). The SNP did not affect the maturation of DC by irradiated cells, as there were no significant differences in the MFI for CD86, CD83 and HLA-DR between the two DC groups. The differences between irradiated and non-irradiated cells were also evident in both SNP and normal DC.

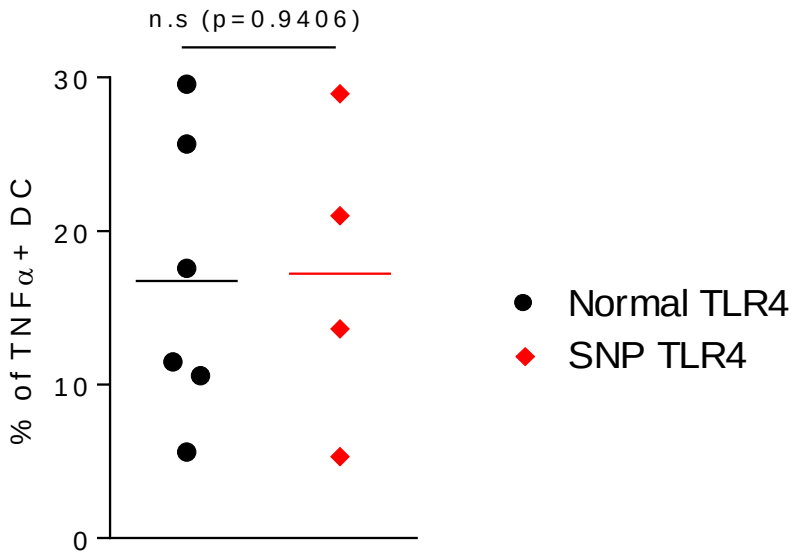


Figure 5.10: LPS stimulation of DC carrying the normal or SNP TLR4 allele. Percentage of TNF-α positive cells are shown. Each symbol represents a different donor and is a mean of triplicate samples. The lines represent the mean of TNF-α production in each group.

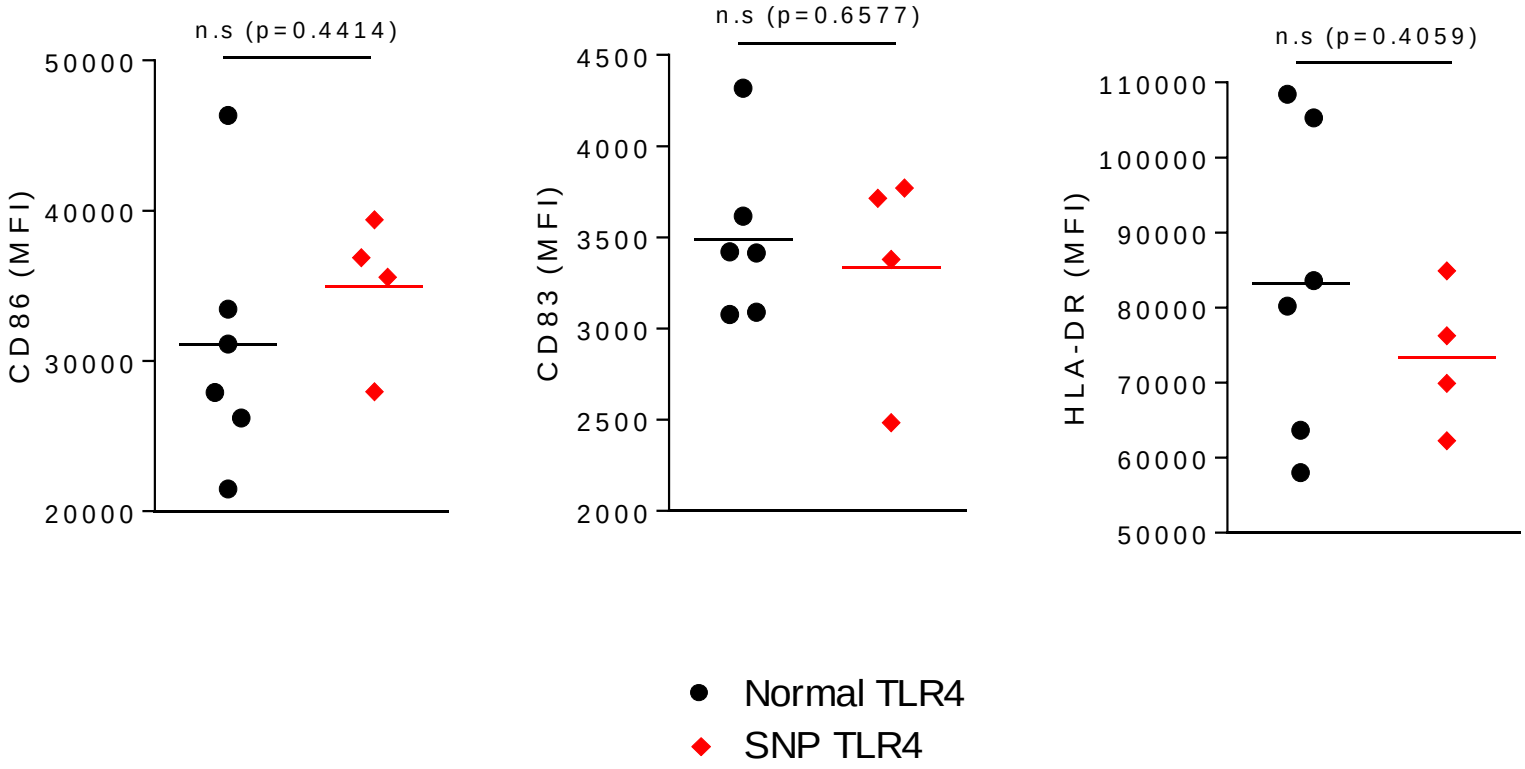


Figure 5.11: LPS stimulation of DC carrying the normal or SNP TLR4 allele. MFIs for CD86, CD83 and HLA-DR on DC are shown. Each symbol represents a different donor and is a mean of triplicate samples. The lines represent the mean of CD86, CD83 and HLA-DR expression in each group.

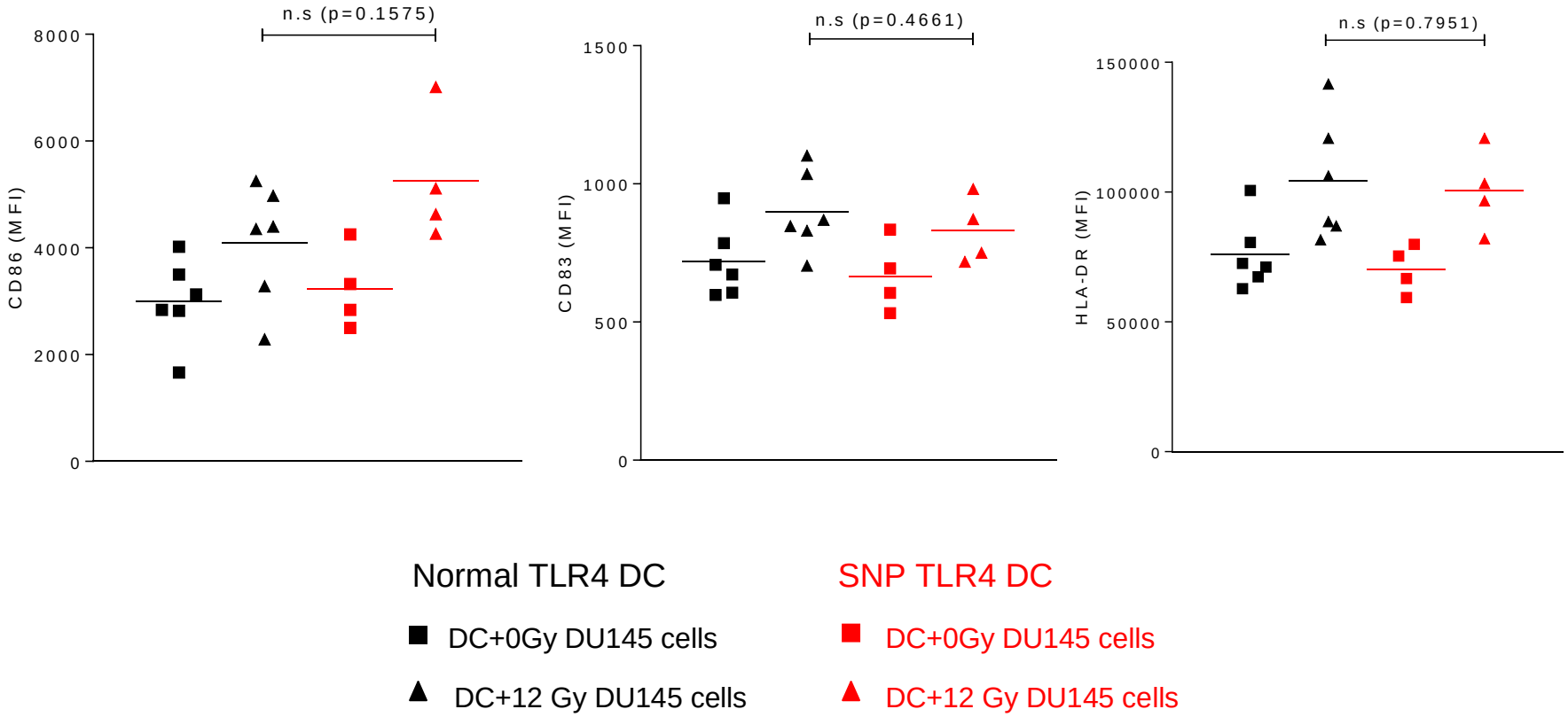


Figure 5.12: Maturation of DC carrying the normal or SNP TLR4 allele following co-culture with irradiated or non-irradiated DU145 cells. MFIs for CD86, CD83 and HLA-DR on DC are shown. Each symbol represents a different donor and is a mean of triplicate samples. The lines represent the mean of CD86, CD83 and HLA-DR expression in each group.

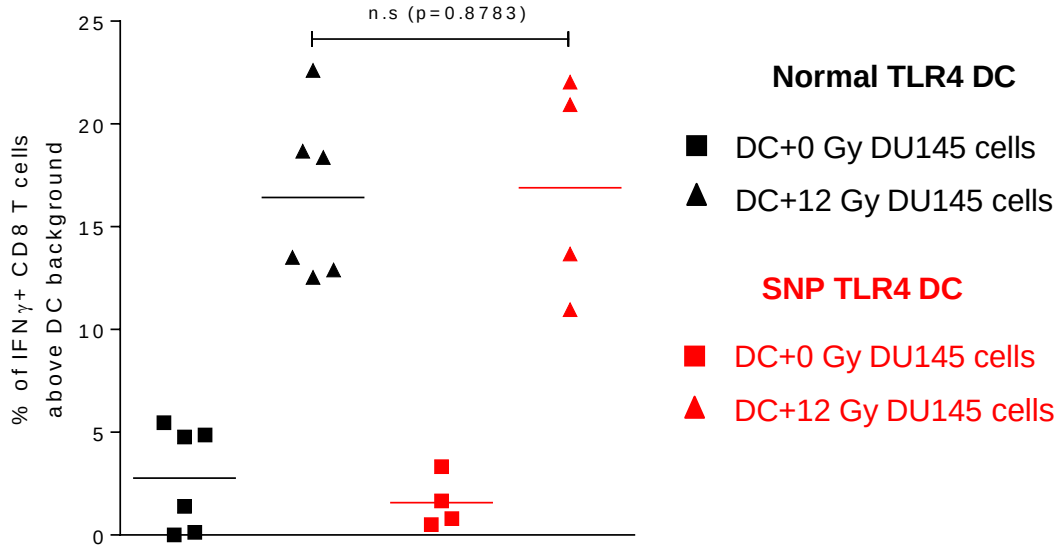


Figure 5.13: Cross-presentation of 5T4 antigen from irradiated and non-irradiated DU145 cells by DC carrying either normal or SNP TLR4 alleles. Percentage of IFNγ positive cells are shown. Each symbol represents a different donor and is a mean of triplicate samples. The lines represent the mean of IFNγ production in each group.

Cross-presentation of 5T4 antigen by TLR4 polymorphic DC to 5T4 specific T cells

To determine if the TLR4 Asp299Gly SNP impairs the ability of DC to cross-present antigen to tumour-specific T cells, DC from individuals bearing the TLR4 Asp299Gly SNP and those with normal TLR4 allele were co-cultured with irradiated or non-irradiated DU145 cells before addition of RLAR-T cells. DC with the TLR4 SNP stimulated approximately four times more T cells following uptake of irradiated tumour cells compared to non-irradiated cells. These responses were similar to those induced by DC with the normal TLR4 allele. Based on these results, we concluded that TLR4 SNP does not influence cross-presentation of tumour antigen from irradiated tumour cells (Figure 5.13).

Albeit the *in vitro* comparative analysis of DC function from Asp299 and Asp299Gly carrying alleles was carried out from a relatively small number of donors, the results suggest that if there were differences to be detected (especially in cross-presentation experiments), a large number of donors would be needed. As we only had access to approximately 200 PCa patients' DNA with post-RT clinical data for 10 years or more, it was concluded that it is unlikely that a correlation analysis between TLR4 SNP and post-RT clinical outcome from these patients would be conclusive. Thus, unlike in the original plan, TLR4 SNP screening was not carried out from PCa patients.

DISCUSSION

The questions in this chapter were whether TLR4 plays a role in the cross-presentation of antigen from irradiated tumour cells and if the effect of TLR4 SNP in this process. In order to answer this, inhibition of TLR4 via its adaptor molecules MyD88 and TRIF as well as via two TLR4 ligands (HMGB1 and Hsp70) was carried out. SNP analysis was carried out on cells from healthy and patient donors in order to identify individuals with the Asp299Gly TLR4 SNP.

TLR4 phenotyping revealed that its surface expression is high on monocytes while there is little to no surface expression on DC. However, intracellular expression is high in both monocytes and DC. This confirms the results by Uronen-Hansson et al., (2004) who demonstrated that TLR4 is highly expressed intracellularly but not on the surface of DC while monocytes express TLR4 both on the surface and intracellularly. IL-4 seems to be the key cytokine that downregulates TLR4 (Uronen-Hansson et al., 2004, Mita et al., 2002).

The attempt to inhibit TLR4 with the A46 VIPER peptide failed as LPS stimulation of DC in the presence of VIPER did not inhibit TNF-α production but rather stimulated it in our experimental setting. The study that identified VIPER demonstrated that it could inhibit TLR4 mediated responses such as pro-inflammatory cytokine and chemokine production, specifically upon stimulation of PBMC, THP-1 cells, RAW264.7 and iBMDM with LPS (Lysakova-Devine et al., 2010). To our knowledge, VIPER has not been tested in human DC. Therefore, it is possible that VIPER is not able to inhibit TLR4 function in DC. Furthermore, Oda et al., (2011) provided evidence demonstrating that VIPER does not interact with Mal *in vitro* contradicting the findings by Lysakova-Devine et al., 2010 (Oda et al., 2011, Lysakova-Devine et al., 2010).

Therefore, we turned our attention to block TLR4 signalling via the adaptor proteins MyD88 and TRIF. In the presence of both signalling adaptors, the immune response to ligands is determined by adaptor interplay, which can be synergistic or redundant. For example, synergistic adaptor interplay during LPS stimulation causes cytokine production to be reduced as long as one adaptor is absent. However, if there is

redundant adaptor interplay, cytokine production in the WT and single KOs is comparable and differences can only be observed in the double KOs. This may allow efficient mechanisms to achieve full responses regardless of the route that the signal travels (Meissner et al., 2013). During the cross-presentation experiments, inhibition of either pathway on its own did not affect T cell responses to antigen from irradiated cells even though the concentrations used had significantly reduced the outcome of LPS stimulation. T cell responses and cross-presentation of antigen was only reduced when both MyD88 and TRIF were inhibited at the same time. This indicates that dual signalling is required for efficient cross-presentation when using irradiated tumour cells and the output is likely to be determined by redundant adaptor interplay between MyD88 and TRIF.

Inhibition of HMGB1 revealed that while HMGB1 expressed or released by irradiated cells is unlikely to activate DC, it does however contribute to the enhanced cross-presentation of irradiated DU145 cells. Although other studies have demonstrated the ability of HMGB1 to activate DC, their studies used HMGB1 released by immune cell such as monocytes and macrophages and not from dying cells (Messmer et al., 2004, Dumitriu et al., 2006). Inhibition of the HMGB1 signalling pathway only inhibited cross-presentation from irradiated and not non-irradiated DU145 cells. This demonstrates that a TLR ligand released or highly expressed by irradiated cells is contributing to the enhanced cross-presentation of 5T4. However, because cross-presentation from irradiated cells was only partially reduced by HMGB1 inhibition and T cell responses were still significantly higher compared to that with non-irradiated cells, it suggests that other receptors or signalling pathways are also contributing to the enhanced cross-presentation. The function of HMGB1 in antigen cross-presentation but not DC activation is in agreement with the work by Apetoh et al (2007b), who found that HMGB1 was not required for the maturation of DC. Rather, their results suggested that ligation of TLR4 and HMGB1 prevented the accelerated degradation of the phagocytic cargo within the DC, thereby allowing for optimum cross presentation. Therefore, one of the conclusions from the experiments presented in this chapter is that TLR4 activity and HMGB1 from irradiated tumour cells contribute to enhanced antigen cross-presentation.

Hsp70 has been demonstrated to have dual roles as it is 1) a danger signal with the ability to induce DC maturation and 2) a peptide chaperone that protects peptides from degradation along the MHC class I pathway (Joly et al., 2010, Binder et al., 2012). Inhibition of Hsp70 with VER 155008 impaired DC activation by irradiated tumour cells and completely abrogated antigen cross-presentation. In responses to stress, Hsp70 can be translocated or mobilized to the plasma membrane for cell surface expression or even be released into the extracellular environment. In the previous chapter, it was demonstrated that supernatant alone from irradiated tumour cells was inadequate to activate DC maturation and irradiated tumours cells were required for the upregulation of CD86 on DC. Therefore, we can speculate that the cell surface membrane bound Hsp70 may play a role in DC maturation observed in our experimental setting and this function is inhibited by VER 155008. It has been shown that TLR4 either on its own or in combination with TLR2 is required for DC activation (Vabulas et al., 2002, Asea et al., 2002, Palliser et al., 2004). Since, according to our data, TLR4 contributes to the enhanced cross-presentation of irradiated tumour cells, it is possible that Hsp70 binds to TLR4 for the upregulation of CD86. However, Hsp70 also utilizes other receptors on DC such as CD91 to activate NF-κB and p38 MAPK for the release of a number of cytokines (Pawaria and Binder, 2011). The chemokine and cytokine profile observed in the previous chapter after DC stimulation with irradiated DU145 cells is similar to those activated via CD91 (published by Pawaria and Binder, (2011)), i.e. possess high CXCL10 and IL-6 expression. Hence, the possible contribution of other receptors beside TLR4 in association with Hsp70 cannot be ignored.

Due to the inherent chaperone activity of HSPs, Hsp70 can potently bind intracellular peptides, including peptides from tumour cells and "piggyback" them outside the cells. The initial interaction of APC with Hsp70 is mediated through binding to cell surface receptors like CD91, TLR4/TLR2, as well as LOX-1 (Delneste et al., 2002). Following binding, the HSP with the chaperoned peptide is internalized into endosomal vesicles. While HSP90 facilitates the translocation of antigen from the endosomal vesicles into the cytosol, Hsp70 facilitates the transportation of antigen to the proteasome for antigen degradation. Antigen-derived peptides generated by the proteasome enter the same endosome from which it dislocates to the cytosol through TAP molecules and associate with MHC I molecules for presentation to $CD8^+$ T cells

(Kato et al., 2012). Treating DU145 cells with VER 155008 could have inhibited the peptide-chaperoning ability of Hsp70, resulting in the failure to transport the antigen for presentation to $CD8^+$ T cells.

Since we attained data showing the influence of TLR4 activity in our cross-presentation experiments, the effect of the TLR4 Asp299Gly SNP was investigated. Our data demonstrated that the TLR4 Asp299Gly SNP does not influence TLR4 expression and DC function including cross-presentation of tumour antigen from irradiated tumour cells. This contradicts the findings by Apetoh et al., 2007 where cross-presentation of antigen from oxaliplatin treated cells was impaired in DC with the TLR4 Asp299Gly SNP and this was HMGB1 dependent. Firstly, the important thing to note is that cell death in our study was induced by IR and not oxaliplatin treatment. It is possible that these treatments induce a different type of cell death, which may activate different danger signals. Secondly, TLR4 signalling only partially affected cross-presentation of the irradiated DU145 cells and as mentioned above, TLR4 may work in combination with other receptors. Therefore, other receptors, such as RAGE for HMGB1, CD91 or LOX-1 for Hsp70 as well as TLR2 for both HMGB1 and Hsp70 may compensate for the loss of function in TLR4 signalling caused by the Asp299Gly SNP. Lastly, numerous other studies have found no association between the Asp299Gly SNP and cellular immune responses (Allen et al., 2003, Read et al., 2001, Feterowski et al., 2003, van der Graaf et al., 2005b).

In conclusion, dual signalling via MyD88 and TRIF partially contributes to enhanced cross-presentation of antigens from irradiated DU145 cells through potential binding of TLR4 with HMGB1 and/or Hsp70. However, the TLR4 Asp299Gly SNP does not affect T cell responses in our cross-presentation model.

General Discussion

Antigen cross-presentation has been indicated as an important mechanism for generating $CD8^+$ T cell responses against solid tumours, which do not migrate into lymph nodes or viruses, which do not infect professional antigen presenting cells. Our study addresses the question of antigen cross-presentation from irradiated human tumour cells, as 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 tumour cells (Golden et al., 2013, Postow et al., 2012). Our experiments employ a tumour-specific T cell line as a detector of cross-presentation thus, we demonstrate the key mechanism of antigen cross-presentation but not that of cross-priming. There is a paucity of information about the mechanism of radiation-mediated antigen cross-presentation, and thus there has been a need for mechanistic studies in order to better understand how cancer radiation therapy could be made more successful.

The radiation dose (12 Gy) used in these experiments reflects the continuously evolving field of radiation therapy in prostate cancer and other malignancies. High dose brachytherapy and intensity modulated radiotherapy offer fewer fractions with higher doses delivered more precisely to the cancer (Zaorsky et al., 2013). The effect of high dose (>2 Gy) radiation is complex as it results not only in different types of cell death but also in senescence and growth arrest. We observed cell cycle arrest at the G2/M phase, as reported by others (Janicke et al., 2001), and a gradual increase of cell death with time following radiation. However, in our model, the latter was predominantly of late apoptotic/necrotic type. The p53 gene is mutated in DU145 cells, which may impact on the radiation-mediated repair response and apoptosis (Lehmann et al., 2007). As p53 mutations are frequent in PCa (Ritter et al., 2002), our observations are likely to be representative of the physiological behaviour of the majority of tumour cells. However, hypoxia, which may occur in larger tumours, can increase tumour cell resistance to radiation (Marignol et al., 2008), with as yet unmapped immunological consequences. IR increases the expression of tumour antigens (Sharma et al., 2011). Therefore, it seems likely that the increased expression of 5T4 on DU145 cells in response to radiation provides DC with more

tumour antigen to process and present to T cells compared to that from non-irradiated cells.

IR induced the translocation of CRT, HMGB1 and Hsp70, which are potentially important contributors to immunogenic cell death. HMGB1 was also detected in the supernatant of irradiated DU145 cells. HMGB1 is a nuclear protein that signals tissue damage when released into the extracellular medium and thus works as a DAMP. Although extracellular HMGB1 can act as a chemoattractant for leukocytes and as a pro-inflammatory mediator, inhibition of HMGB1 function using Glycyrrhizin in our experimental setting did not inhibit DC activation. Recent studies have shown that the pro-inflammatory activity of HMGB1 depends on its redox state (Yang et al., 2012, Venereau et al., 2012) .

HMGB1 contributed to the enhanced antitumor T cell responses upon cross-presentation of antigen from irradiated tumour cells. This is in agreement with the work by Apetoh et al (2007b). It has been suggested that HMGB1 is preventing the accelerated degradation of the phagocytic cargo within the DC, thereby allowing for optimum cross presentation (Apetoh et al., 2007b), although its exact contribution has not been elucidated.

Cell surface CRT is an "eat me" signal that mediates phagocytic uptake and immunogenicity of dying cells (Gardai et al., 2005, Raghavan et al., 2012). Obeid et al., (2007) found that surface exposure of CRT allowed irradiated dying tumour cells to be efficiently engulfed by DC thereby setting the stage for efficient presentation of cancer specific antigen to $CD8^+$ T cells (Obeid et al., 2007a). A significant increase in surface CRT was observed after DU145 cells were treated with 12 Gy ionising radiation and uptake of the irradiated DU145 cells by DC was significantly enhanced compared to that of non-irradiated cells. Therefore, there is a possibility that the increased exposure of CRT on irradiated DU145 cells enhances cross-presentation of the 5T4 antigen by stimulating increased uptake of irradiated tumour cells.

The pathway by which surface CRT is exposed depends on the stage of cell death during which the exposure takes place. Depending on the cell death stage, one molecular pathway might exclusively execute the trafficking of surface CRT, or

several signalling pathway might co-exist, and depending on the cell death inducer, one pathway might dominate. CRT exposure in some cases precedes PS exposure and the morphological signs of apoptosis. The study by Obeid et al., (2007) showed that while irradiation had no or little effect on PS at 1 or 4 h post treatment, respectively, CRT exposure was observed as early as 1 h after treatment, as detected by immunofluorescence and microscopy. This demonstrates that irradiation-mediated CRT exposure occurs at the pre-apoptotic stage. The translocation of this pre-apoptotic surface CRT depends on the ER to Golgi transport, PERK-governed proximal and a PI3K-mediated distal secretory pathway for its trafficking (Garg et al., 2012b).

However, for later stages of cell death, another suggested mechanism for cell surface exposure of CRT involves the association of cytoplasmic CRT with PS on the inner leaflet of the plasma membrane, thereby allowing CRT to become exposed during apoptosis (Raghavan et al., 2012). Given that irradiation of DU145 with 12 Gy in our experimental setting, which included a 72 h incubation, predominantly resulted in late apoptotic/necrotic cell death, it likely that the association of cytoplasmic CRT with PS might be the main mechanism of CRT exposure in the treated cells.

Significant translocation of Hsp70 from the nucleus to the cytoplasm was observed in the DU145 cells treated with 12 Gy. Hsp70 is a stress-inducible protein and therefore the translocation observed is a stress response to irradiation. This nuclear-to-cytoplasmic translocation of Hsp70 has also been observed in response to heat shock treatment (Martin et al., 1993). Exogenous stress may also change the environment within the cytosol (e.g. induction of oxidative stress), which may cause Hsp70 to adopt a more structured conformation favourable to association with peptides. The gain of the Hsp70 secondary structure allows better accessibility of peptides to the peptide-binding pocket and therefore makes Hsp70 a more effective chaperone. The secondary structure was not observed in a resting cytosol (Callahan et al., 2002). As the damaged cells succumb to cell death, it is assumed that cytosolic Hsp70 could be transported to the cell surface in concert with other proteins possessing transmembrane domains that fulfil shuttle functions. Hsp70 has been shown to be externalised upon binding to PS upon tumour cell death (Schilling et al., 2009). Significantly more Hsp70 was detected on the surface irradiated DU145 cells

compared to the non-irradiated cells. Hsp70 expressed on the cell surface serves as a danger signal and interacts in different ways with the innate immune system. Firstly, they can act as a cytokine and induce DC activation. Secondly, due to their chaperone function, Hsp70 proteins can act as carriers that will deliver peptides to DC (Murshid et al., 2011).

Upon uptake of irradiated DU145 cells, DC were activated to express significantly higher levels of CD86 and HLA-DR as well as release more pro-inflammatory cytokines (IL-12 and IL-6) and chemokines (CXCL10) compared to the non-irradiated cells. The co-stimulatory molecules expressed on activated but not resting DC are needed to bind to the cell surface receptor CD28 on T cells for effective T cell activation. The secretion of mediators such as IL-12 aid in creating a pro-inflammatory environment required for the elicitation of antitumor T cell responses. Inhibition of Hsp70 function using VER 155008 inhibited the upregulation of CD86 on DC upon uptake of irradiated DU145 cells. This suggests that Hsp70 from irradiated tumour cells contributes to the activation of DC. Membrane-bound Hsp70 has been shown to activate macrophages in another study (Vega et al., 2008).

An investigation into the ability of irradiated tumour cells to activate DC confirmed that cell associated factors were responsible for DC activation because the supernatant alone from irradiated DU145 failed to upregulate CD86 on DC. Surface-bound immunogenic signals translocated as a result of IR in our system were CRT and Hsp70. While some work using CRT isolated from murine cells (Pawaria and Binder, 2011, Hong et al., 2010) or transfected HEK293 human cells have shown that CRT can activate APC, no studies have demonstrated the ability of surface-bound CRT to stimulate DC.

It is also suggested that in a cross-presentation setting, Hsp70 bound to peptides facilitates the transportation of antigen to the proteasome for antigen degradation. VER 155008 diminished the ability of DC to cross-present the 5T4 antigen highlighting the importance of Hsp70 in antigen cross-presentation. Given that inhibition of the MyD88 and TRIF pathway partially reduced the cross-presentation of antigens from irradiated DU145 cells, it can be concluded that TLRs are involved in our cross-presentation setting. TLR4 might be binding to HMGB1 and/or Hsp70

being released by the irradiated DU145 cells. However, because the effects of inhibition with the MyD88 and TRIF inhibitors were only partial, other receptors must also be involved. Since the HSPs in our system seem to have the most impact, we have examined the expression of potential HSP receptors on monocyte-derived DC used in our cross-presentation experiments. CD91, SREC-I and TLR2 are present on monocyte-derived DC and therefore might be working in an additive or synergistic manner with TLR4 to initiate DC activation and enhance antigen cross-presentation. Due to the lack of time, this part of the work has not been completed before submission of the thesis.

Furthermore, I studied the consequences of the Asp299Gly SNP of TLR4, which is associated with structural changes of the TLR4 extracellular domain, with a potential impact on LPS binding (Ohto et al., 2012). LPS-induced cytokine production has not been affected by this TLR4 SNP even when present in a homozygous form (van der Graaf et al., 2005a). However, Asp299Gly SNP was demonstrated to have a detrimental effect on antigen cross-presentation, similar to that observed in TLR4 -/- knockout mice (Apetoh et al., 2007b). While our experiments confirmed the lack of LPS-induced cytokine production effect by Asp299Gly SNP in DC, we observed no effect on antigen cross-presentation. Our donors were heterozygous for the SNP allele thus functionally not comparable to the TLR4-/- mice. However, the discrepancy of the human DC results with that observed by others (Apetoh et al., 2007b) calls for caution in generalizing antigen cross-presentation data regardless of the model they were obtained in.

Taken together, we have observed that ionising radiation induces immunologically relevant changes in DU145 cells. Upregulation of the tumour-associated antigen in question and radiation induced CRT and PS exposure are likely to work collectively to stimulate DC to take up dying/stressed DU145 cells and process them via the cytosolic cross-presentation pathway. Cytokine and chemokine production by DC indicates how radiation can switch the immunosuppressive tumor milieu to a pro-immune environment. Surface Hsp70 is also required for DC activation as well as for aiding antigen processing and/or presentation.

My work has some unfinished elements, due to time and funds coming to an end but it also raises some important questions, which may provide projects for future students. As Hsp70 and other HSP chaperones have been used successfully to immunise mice to a range of tumour types and Hsp70 and Grp94 are undergoing clinical trials (Murshid et al., 2011), it would be useful to have more information about their role in combination with RT. As an example, the uptake and signalling mechanism of Hsp70 could be further studied by blocking or silencing the receptors reported to be used by Hsp70. Other future work could investigate the effect of stromal cells in the cross-presentation model. Given that solid tumours are complex tissues with a local microenvironment made up of stromal and myeloid-derived cells that support growth and progression of transformed cells (Spary et al., 2014), multi-component conventional or tumour spheroid (MCTS) cultures should be used to assess if they are also able to generate immune responses. The model established as described in this thesis would be appropriate to study these and further questions about tumour antigen cross-presentation.

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