AUTOPHAGY AND ANTIGEN PROCESSING: STRATEGIES TO ENHANCE T CELL RECOGNITION OF CANCER CELLS

by

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Abstract

Recent studies have suggested an increasingly important and therapeutically relevant role for $CD4^+$ T cells as direct effectors in immunotherapy. It has also become clear that antigens can access the MHC-II pathway via non-classical endogenous routes such as autophagy. The aims of this project were to investigate endogenous MHC-II processing of tumour antigens and explore methods that, by enhancing such processing, could increase T cell recognition of tumour cells. The frequency and diversity of T cell responses to the tumour antigen WT1 in healthy individuals and cancer patients was investigated. T cell responses were rare and only one epitope-specificity was identified in these donors. The Epstein Barr Virus Nuclear Antigen 1 (EBNA1), a protein that becomes more efficiently processed by autophagy when relocalised from the nucleus into the cytoplasm, was also investigated. When EBNA1-positive cells were treated with the CDK inhibitor Roscovitine, previously reported to relocalise EBNA1 into the cytoplasm, presentation of an autophagy-dependent indicator epitope was decreased. Roscovitine did not affect autophagy activity but decreased cellular levels of several autophagy adaptor proteins. siRNA Knock down of each autophagy adaptor showed CALCOCO2, NBR1 and OPTN were involved in the generation of an autophagy-dependent epitope from EBNA1; a control epitope was unaffected. Finally, efforts were made to define fundamental rules determining why only a subset of potential MHC-II epitopes are generated from an antigen by autophagy. Resistance to degradation by lysosomal peptidases was shown to be an important factor determining whether an MHC-II epitope can be generated by autophagy.

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AEP	Asparagine-specific endopeptidase
APC	Antigen presenting cell
ATG	Autophagy-related gene
CMA	Chaperone mediated autophagy
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
FasL	Fas ligand
GAr	Glycine/alanine repeat
HSC	Haematopoietic Stem cell
IFN	Interferon
IGF	Insulin-like growth factor
Ii	MHC class II invariant chain
IL	Interleukin

LC3	Light chain 3
LCL	Lymoblastoid cell line
LIR	LC3 interacting region
LTa	Lymphotoxin-α
MBP	Myelin basic protein
MHC	Major histocompatibility antigen
NBR1	Neighbour of BRAC1
NDP52	Nuclear dot protein 52
NeoR	Neomycin phosphotransferase
NES	Nuclear export sequence
NK	Natural killer
NLS	Nuclear localisation sequence
OPTN	Optineurin
PDGF	Platelet derived growth factor
PMN	Piecemeal microautophagy of the nucleus
PTLD	Post transplant lymphoproliferative disorder
SQSTM1	Sequestersome 1

TA	Tumour antigen
TCR	T cell receptor
TEC	Thymic epithelial cell
TNF	Tumour necrosis factor
TRAIL	TNF related apoptosis induced ligand
UBA	Ubiquitin-associated
V(D)J	Variable, diverse and joining gene segments
WT1	Wilms' tumour gene

1. INTRODUCTION

The human immune system is of vital importance in protecting against disease. It functions by discriminating self from non-self and killing or disabling foreign agents. These agents may be invading pathogens but cancerous cells, which have undergone extensive genetic mutation due to failure of DNA repair mechanisms, can also be considered to be foreign to the host.

The immune system can be divided into two categories, innate and adaptive immunity

1.1 Innate immunity

The innate immune system acts as an initial defence against pathogens. It functions as a rapid but relatively non-specific method of responding to infection and is classically considered to contain no element of memory. Innate immunity is primarily driven by myeloid cells but the system comprises several different elements. First, pathogen access is prevented by physical barriers such as epithelia, which are sealed by tight junctions and skin that is keratinised (Citi and Cordenonsi, 1998; Friedman, 2006). Second, internal epithelia maintain a constant flow over their surfaces by peristalsis, cilia or fluids such as saliva to remove invading pathogens (Sheehan *et al.*, 2006). Third, serum proteins such as the complement system have multiple functions including causing direct killing of pathogens (Sarma and Ward, 2011). Fourth, phagocytosis of pathogens is performed by cells such as dendritic cells and neutrophils and is aided by binding of complement to pathogens. More recently, γδ-T cells, usually considered to be part of the adaptive immune system, have also been shown to be capable of phagocytosis (Wu *et al.*, 2009). Fifth, natural killer (NK) cells mediate spontaneous

cytotoxicity against infected cells but also play an important role in shaping adaptive immune responses (Laouar *et al.*, 2005). Other cells of the innate immune system include eosinophils, mast cells and basophils. As well as having a direct role in protection against pathogens a key role of the innate immune system is to alert and arm the adaptive immune system.

1.2 Adaptive immunity

Adaptive immunity is a system of much greater complexity having evolved later in the tree of life (Beutler, 2004; Pancer *et al.*, 2004). On first encountering a pathogen the adaptive immune response occurs more slowly than the innate. The adaptive immune system is truly differentiated from the innate system by its intrinsic ability to generate pools of cells specific to previous infections, so called memory cells. The maintenance of memory cells allows a much more rapid and robust immune response to be generated upon reencountering a pathogen. Adaptive immunity is driven mainly by lymphocytes and can be further subdivided into two categories: cellular immunity, driven mainly by T cells and humoral immunity driven by antibodies produced by B cells.

1.2.1 The role of T cells in immunity

T and B lymphocytes were discovered in the 1960s and fundamental differences between the two cell types were quickly identified. The activation of B cells *in-vitro* was shown to require antigen alone, while the activation of T cells required an accessory cell (Alter and Bach, 1970). In the mid-1970s it was discovered that T cells recognise antigens presented by MHC molecules on antigen presenting cells (Blanden *et al.*, 1975). This seminal observation showed that T cell recognition was fundamentally different

from B cell recognition, however it was not until the 1980s that T cells were shown to possess a unique receptor for this role known as the T cell receptor (TCR) (Allison *et al.*, 1982; Haskins *et al.*, 1983; Meuer *et al.*, 1983). The structure of the TCR was later resolved and revealed it consisted of a heterodimer of two different polypeptide chains. For the majority of T cells these two chains are the α and β chains and cells that express these chains are therefore known as αβ-T cells (hereafter referred to simply as T cells). A minority of T cells possess γ and δ delta chains instead and these are therefore known as γδ-T cells (Brenner *et al.*, 1986; Lew *et al.*, 1986). TCRs associate with two accessory molecules, the CD3- and ζ-chains forming the T cell receptor complex. Upon binding to an MHC-peptide complex the TCR signals via the two accessory chains to activate the cell. This activation triggers multiple immunological processes that can result in the killing of pathogens or pathogen-infected cells, either directly via a cytotoxic response or indirectly by providing help to other immune cells.

1.2.2 T cell development

T cell development is a highly complex process with multiple stages occurring in different areas of the body, each requiring signals and assistance from many different cell types. The process is unique among haematopoietic development in its requirement for a specific organ, the thymus (Miller, 1961) . Another unique property of T cell development is that the haematopoietic stem cells (HSCs) that T cells are derived from do not differentiate where they reside. Like all other HSCs they are produced throughout life and reside in the bone marrow. T cell development begins with progenitor T cells migrating from the bone marrow to the thymus under the direction of chemokines such as CCL21, which is produced by thymic epithelial cells (TECs) (Calderon and Boehm,

2011). Once in the thymus IL-7 and DL4 that are also produced by TECs induce proliferation and differentiation of the progenitor cells (Koch *et al.*, 2008). The progenitor cells then migrate through the thymus and development proceeds through a number of pre-T cell stages. During the first of these pre-T cell stages the progenitor cells, known as thymocytes, are negative for TCR, CD4 and CD8. T cell development switches on the expression of these molecules but also triggers changes within the genomes of thymocytes to generate a diverse repertoire of T cells each expression a distinct TCR.

1.2.3 Generation of the T cell repertoire

Enormous variety exists in the pathogens that the immune system can encounter. Pathogens are also constantly evolving in response to pressure exerted by immune responses. As pathogen encounters cannot be predicted, the immune system must prepare for every possible pathogen in advance of encountering it. To meet this challenge the immune system must generate an almost limitless repertoire of TCR specificities. However all TCRs are generated from a single region of the genome that contains a limited number of genes. To overcome this problem thymocytes employ a complex series of random genetic rearrangements to generate genetically different TCRs in each thymocyte. The TCR is encoded as non-contiguous segments in the genome of progenitor cells. Three gene segments within this region, the variable (V), diversity (D) and joining (J) gene segments are rearranged in a process known as V(D)J recombination. The random joining of any V, D, and J segments followed by insertion of non-germline encoded nucleotides at the joining regions is directed by recognition sequences that flank VDJ sections and act as substrates for V(D)J recombinase (Gellert,

2002). This process creates genetically distinct TCR chains. Finally the random combination of an α and β chain creates a fully functional TCR molecule that is unique to the individual thymocyte. Early, low level expression of the TCR provides stimulus to induce the expression of CD4 and CD8 co-receptors creating $TCR^{low}CD4^{+}CD8^{+}$ cells (Yamasaki and Saito, 2007).

V(D)J recombination creates an effectively limitless pool of TCR specificities. However, without control over this process not all T cells will express an immunologically relevant TCR. Some T cells will express TCRs that cannot bind MHC complexes and others will express TCRs specific to self-antigens, creating the potential to trigger autoimmunity. Therefore before naive T cells leave the thymus they are subject to positive and negative selection to remove redundant and autoreactive T cells respectively

The thymic cortex induces TCR mediated positive selection of thymocytes that engage MHC complexes at intermediate levels of avidity. MHC class I binding downregulates CD4 producing a $CD8^{+}$ T cell whereas MHC class II binding downregulates CD8 producing a $CD4^{+}$ T cell. Binding to MHC is vital as it triggers intracellular signals in developing thymocytes that overcome a default pathway of programmed cell death. This pathway "death by neglect" occurs in cells that do not bind to peptide-MHC complexes (Rosenheimer-Goudsmid *et al.*, 2000).

This process generates a pool of single positive thymocytes that is then subjected to negative selection to remove T cells that would react against self-antigens. Medullary TECs and dendritic cells present a diverse set of self antigens to single positive thymocytes. In the thymic medulla, high affinity binding to self peptide MHC complexes

triggers pro-apoptotic molecules that led to the death of the cell thereby removing self-reactive T cells (Suen and Baldwin, 2012).

1.2.4 T cell responses

As with all adaptive immune responses, the T cell response is initiated in peripheral lymphoid tissues. As infection can arise anywhere in the body, it is vital that dendritic cells (DCs) deliver pathogenic antigens to these lymphoid tissues in order to stimulate T cells (Reis e Sousa, 2004). Naïve T cells circulating in the blood stream enter lymph nodes, spleen and other lymphoid tissues where they come into contact with thousands of DCs and sample the peptide MHC complexes expressed on the surface of them. The original interaction between T cell and DC is transient and non-antigen specific. If the T cell does not encounter a peptide-MHC complex able to stimulate its TCR, it is released and continues to sample other DCs and eventually returns to the blood stream. When a T cell does encounter a peptide-MHC complex able to activate its TCR it stops migrating. Following this encounter the T cell receives a number of different signals. The first signal is a direct MHC-peptide to TCR signal; however this signal is not sufficient to stimulate a response. Costimulatory signals are also produced by DCs to stimulate T cell. DCs express the ligand B7 which acts on CD28 on T cells and causes proliferation via IL-2 production. Other signals also trigger differentiation of the T cell into the different effector phenotypes. It is this differentiation into effector phenotype that will allow the T cell to carry out its immune function upon subsequently encountering an infected target cell (Lanzavecchia and Sallusto, 2001). Once a T cell takes on an effector phenotype it can be activated by MHC I-peptide complexes and is no longer dependent on

costimulatory molecules expressed by antigen presenting cells (Kaech and Ahmed, 2001; van Stipdonk *et al.*, 2001).

1.2.5 Effector phenotypes

Naïve T cells are divided into two pools, $CD8^+$ and $CD4^+$. Naïve $CD8^+$ T cells are destined to become cytotoxic T lymphocytes (CTLs) that function to directly kill target cells. Naïve $CD4^+$ T cells can differentiate into a number of different effectors cells subsets. The main subsets are TH1, 2 and 17. Once differentiated into an effector phenotype, T cells will migrate to sites of infection to mount immune responses. Here they transiently bind target cells in a non peptide-specific manner. Then, if the cell expresses the correct peptide MHC complex the T cell will bind more tightly and initiate its effector function. Another important subset of $CD4^+$ T cells are the regulatory T cells (Tregs). These cells act to suppress the immune system and are important in establishing peripheral tolerance to self-antigens.

1.2.6 The $CD8^+$ T cell response

Activation of naive $CD8^+$ T cells results in their differentiation into CTLs. CTLs are marked by their ability to produce cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) and their ability to exhibit cytolytic activity (Bachmann *et al.*, 1999; Opferman *et al.*, 1999). These highly specialised cells recognise peptides presentenced on MHC class I molecules which are expressed on the surface of all nucleated cells. Therefore all proteins within cells are potentially available to display on the cell surface and interrogation by CTLs. CTLs that encounter a non-self peptide MHC complex are able to rapidly kill the presenting cell by two mechanisms: granule- or Fas-

mediated killing. Both these pathways trigger a caspase cascade resulting in apoptosis and death of the target cell. Granule mediated killing involves the exocytosis of effector proteins from cytotoxic granules in a calcium-dependent manner. Cytotoxic granules are modified lysosomes that contain at least three classes of effector proteins. The first, perforin, permits the delivery of the other two proteins into the cytoplasm of the infected cell (Lowin *et al.*, 1994). The second class, the granzymes, are a family of serine protease that act in the cytoplasm to induce the target cell to undergo apoptosis (Darmon *et al.*, 1995). The third class consists of granulysin that has a direct antimicrobial function but is also thought to be pro-apoptotic (Stenger *et al.*, 1998). In contrast to granule exocytosis, Fas mediated killing is triggered by the upregulation of a protein, Fas ligand (FasL), on the surface of the CTL. FasL binds to Fas on the surface of target cells and triggers caspase mediated apoptosis (Ju *et al.*, 1995).

In their role as CTLs, $CD8^+$ T cells are vital in the control of intracellular parasites, especially viruses. They are vital for the clearance of all acute viral infections as well as controlling reactivation of viruses in the case of persistent infection.

1.2.7 The $CD4^+$ T cell response

Upon antigen encounter, naive $CD4^+$ T cells also differentiate into effector cells. In the late 1980s Mossman and Coffman identified differences in these effector cells and described two phenotypes, Th1 and Th2 cells (Mosmann and Coffman, 1989). These two subsets are classified according to the cytokines they secrete. Th1 cells are marked by the secretion of IFN-γ, interleukin-2 (IL-2) and lymphotoxin-α (LTa), whereas Th2 cells are defined by the secretion of IL4, 5, 10 and 13 (Mosmann *et al.*, 2005). Th1 cells provide help to the cellular arm of the immune system. They stimulate other cells such

as macrophages and in instances where pathogen infection does not trigger a strong inflammatory response, Th1 cells are vital in the stimulation of the $CD8^+$ T cell response by providing signals such as IL-2 (Simon *et al.*, 1986). Th2 cells provide help to the humoral arm of the immune system. They stimulate B cells to upregulate antibody production and undergo class switching (Lebman and Coffman, 1988). More recently a third subset of T helper cells namely Th17 cells has been defined. These cells are marked by the secretion of IL17, 21 and 22. They are vital for pathogen clearance and inducing tissue inflammation (Veldhoen *et al.*, 2006).

$CD4^+$ T cells recognise peptides in the contexts of MHC class II complexes. Under normal circumstances expression of MHC class II molecules is limited to specialist antigen presenting cells (APC), meaning that $CD4^+$ T cells cannot bind directly to non-immune cells. This limitation has led to the establishment of the dogma that $CD4^+$ T cells function only to provide help to other cells of the immune system. However this is not the case and increasingly reports of $CD4^+$ T cells with direct effector function are appearing in the literature. Many different cell types have now been shown to upregulate the expression of MHC class II molecules under certain conditions, one such example being virus infection (Debbabi *et al.*, 2005). The expression of MHC class II molecules on virus infected cells suggests a direct role for $CD4^+$ T cells in anti-viral immunity. Indeed $CD4^+$ T cells have now been shown to control infection of many viruses including gamma herpes viruses, murine influenza and west Nile virus amongst others (Brien *et al.*, 2008; Sparks-Thissen *et al.*, 2004; Stuller *et al.*, 2010). A number of reports have shown isolation of virus-specific $CD4^+$ T cells, including cytotoxic $CD4^+$ T cells specific to viral antigens such

as those of Epstein-Barr virus (EBV) (Khanna *et al.*, 1997; Long *et al.*, 2005; Munz *et al.*, 2000).

Cytotoxic $CD4^+$ T cells are capable of killing target cells by the same two mechanisms employed by $CD8^+$ CTLs, namely granule-mediated cytolysis or FasL-Fas signalling or indeed both (Brown *et al.*, 2006; Brown *et al.*, 2012; Ishikawa *et al.*, 2009). Interestingly the cytotoxic activity of $CD4^+$ T cells does not depend on Th1 polarisation. As with Th1 cells, IL-2 is required as a differentiation signal but cytotoxic $CD4^+$ T cells do not use the transcription factor T-bet. Instead the T-box transcription factor Eomesodermin has been shown to be critical for inducing the cytotoxic marker granzyme B (Brown *et al.*, 2009; Qui *et al.*, 2011). These key differences have led to the suggestion that cytotoxic $CD4^+$ T cells should be considered a separate functional T cell subset (Swain *et al.*, 2012).

1.3 Antigen processing

As previously discussed, the ability of a T cell to function relies on its intrinsic capacity to recognise target cells that are infected by a pathogen or have internalised pathogen-derived proteins. To facilitate this recognition, cells express MHC molecules that bind peptide fragments derived from proteins contained within the cell. These peptide-MHC complexes are then displayed on the cell surface. In order for this to occur antigen processing is required to degrade proteins within cells and generate these peptide fragments.

In the classical model of antigen processing, MHC class I molecules bind peptides derived from endogenous nuclear and cytoplasmic proteins whereas MHC class II molecules bind peptides derived from extracellular proteins that have been

phagocytosed by the cell. However this model has proved to be an oversimplification of reality and additional non-classical processing pathways have been described. Both classical and non-classical processing will be discussed in the following section.

1.3.1 MHC class I processing

Classical MHC class I processing involves the degradation of proteins in the cytosol via the proteasome and subsequent transport into the endoplasmic reticulum (ER) for loading onto MHC class I molecules. MHC class I processing is therefore vital for the immune response against many intracellular parasites including viruses that hijack the cellular machinery to express viral proteins within the cytosol.

1.3.2 Generation of MHC class I epitopes

Intracellular proteins are constantly degraded as part of the normal process of turnover and recycling of proteins and organelles within cell. Degradation is a tightly regulated process and different proteins have different half-lives that can range from minutes to weeks. Within the cytosol degradation is mostly carried out by the 26S proteasome which targets proteins labelled by conjugation to ubiquitin moieties (Hershko *et al.*, 1980).

The 26S proteasome is a multi-catalytic protease complex. It contains a barrel-shaped proteolytic core complex, the 20S proteasome and is capped at either one or both ends by 19S regulatory complexes, which recognize ubiquitinated proteins (Voges *et al.*, 1999). The proteasome degrades proteins within its lumen and generates peptides suitable for binding to MHC class I molecules (Niedermann *et al.*, 1999).

In the context of infection, interferons in the cell alter the proteasome to increase levels of antigen processing. IFN-γ causes a conformational change in the proteasome and triggers the exchange of proteases in the complex, forming a new structure, the immunoproteasome (Aki *et al.*, 1994). The proteases in the immunoproteasome cleave peptides in a specific manner so that they are more readily transported into the antigen presentation pathway and bind MHC complexes with greater affinity. The conformational change in the barrel structure also increases the rate of peptide production meaning a greater number of peptides can be presented more rapidly (Cerundolo *et al.*, 1995).

The source of proteins for MHC class I processing is a widely contested issue. The proteasome is known to degrade stable, mature proteins and these proteins are represented in the repertoire of MHC class I peptides presented on the cell (Rock *et al.*, 2002). However a second source of peptides also exists. Defective ribosomal products (DRiPs) are the product of erroneous mRNA translation. Yewdell has proposed that the majority of peptides are not products of stable protein degradation, but instead come from the degradation of DRiPs (Yewdell *et al.*, 1996). This hypothesis is supported by the fact that MHC class I peptide complexes can appear rapidly after induction of antigen and peak before steady state levels of mature protein had been reached (Mackay *et al.*, 2009).

Once generated by the proteasome, peptides are released into the cytosol. These peptides must then bind MHC I molecules to be displayed on the surface of cells.

1.3.3 The MHC class I molecule

The MHC class I molecule is a heterodimer composed of an MHC heavy chain in association with a second, smaller protein, beta-2-microglobin (β2m). The extracellular region of the heavy chain folds into three domains, with β2m forming a fourth. Two of the extracellular domains form the peptide binding grove, which binds peptide epitopes about 8-11 amino acids in length (Garrett *et al.*, 1989). To generate diversity in the repertoire of presented peptides, MHC class I molecules are highly polymorphic. Heterozygous individuals can express up to 6 different alleles, that will each bind and display a different pool of peptides (Lund *et al.*, 2004).

1.3.4 Formation of MHC class I-peptide complexes

Upon synthesis, polypeptides of the MHC complex are translocated into the lumen of the endoplasmic reticulum (ER). Here they bind the chaperones calnexin and calreticulin that facilitate folding of the MHC molecule. Once the heterodimer is formed it is rapidly recruited into the peptide loading complex, a multi-protein complex that retains MHC molecules within the ER in a peptide receptive state.

To gain access to MHC class I complexes, peptides in the cytosol bind chaperones and are transported to the ER membrane. Here they bind a protein of the peptide loading complex, the transporter associated with antigen processing (TAP) and are transported into the ER lumen (Abele and Tampe, 1999). Though TAP-dependent transport is required for the majority of MHC class I peptides it should be noted that MHC class I processing can occur in the absence of TAP (Lautscham *et al.*, 2001).

Once within the ER, binding of high affinity peptides to peptide-receptive MHC class I molecules is catalysed by another protein contained within the peptide loading complex, tapasin. Peptide loading causes a conformational change in MHC class I molecules causing them to be released from the peptide loading complex. Once released they can be transported to the cell surface via the golgi network (Wearsch and Cresswell, 2008).

1.3.5 Alternative MHC class I processing: exogenous proteins

As previously discussed, $CD8^+$ T cells recognise pathogen infected cells through MHC class I molecules. However, CTLs cannot gain cytotoxic function without first being primed by DCs. This priming occurs through MHC class I but this creates a problem for the immune system: not all pathogens infect DCs. The solution to this fundamental problem is provided by cross priming, a non-classical processing pathway

Cross priming and was first described in the 1970s. It occurs in DCs and is of vital importance in priming $CD8^+$ T cells responses. Classically, cross priming is thought to take place via two routes. The first involves processing of antigens and peptide loading in endocytic vesicles using MHC class I molecules recycled from the plasma membrane. The second pathway involves retrograde translocation of proteins from endosomes into the cytosol where they can be processed by the classical MHC class I processing machinery. However more recently a more complicated mechanism involving interplay of endosomal and cytoslic pathways as well as hybrid compartments has been suggested (Segura and Villadangos, 2011).

1.3.6 MHC class II processing

Peptides from the cytosol of infected cells are presented on MHC class I molecules. However many pathogen-derived proteins do not enter the cytosol. Some parasites live in intracellular vesicles whereas others are completely extra cellular. Proteins that have been internalised into endocytic vesicles upon uptake by professional antigen presenting cells do not enter the cytosol and therefore never encounter free MHC class I molecules. A different system is therefore required to present them to the immune system, MHC class II presentation.

MHC class II molecules are expressed on the surface of a small subset of specialised cells, APCs. The main function of APCs is to sample the extra cellular environment as well as the contents of other cells and present peptides from all pathogens including those that subvert the MHC class I pathway. The process by which pathogenic proteins are presented as peptides on class II molecules is very different from the class I system and occurs mainly in the endocytic vesicles of APCs.

1.3.7 Antigen uptake

Exogenous antigens are taken into cells by many different endocytic pathways. Soluble antigens are most often internalised by clathrin mediated endocytosis. In this process cargo receptors bind antigens and cluster in clathrin coated pits. The pit then invaginates, pinches off from the membrane and forms a clathrin coated vesicle containing receptor and cargo. A small quantity of extra cellular fluid is also internalised in this process.

A second mechanism of soluble antigen uptake is macropinocytosis. This is a non-selective form of endocytosis and involves actin-dependent formation of large, endocytic vacuoles called macropinosomes. Macropinoctosis is highly active in macrophages and DCs where it is the major pathway for the capture of antigens (Norbury, 2006).

In contrast to soluble antigens, particulate antigens are internalised via a number of different phagocytic pathways. Fc-receptor mediated phagocytosis involves the extension of large membrane protrusions around antibody coated particles. A second mechanism complement mediated phagocytosis involves the invagination of the cell membrane to take in particles coated with complement proteins.

Once internalised, vesicles are directed into different stages of the endocytic pathway according to their contents. Vesicles can be targeted to early endosomes where they may be recycled back to the cell surface or they can be targeted for degradation in mature endosomes and other compartments with much lower pH (McMahon and Boucrot, 2011).

1.3.8 Generation of MHC class II epitopes

Proteins contained within the endocytic pathway are degraded by a range of acidic proteases. Cathepsins are a key group of acidic proteases that reside within endosomal/lysosomal compartments. They fall into two main categories, aspartyl and cysteine proteases. Many cathepsins have been implicated in MHC class II processing, either in peptide generation or MHC class II peptide loading (Table 1.1).

Table 1.1 Cathepsins involved in antigen processing

a. Cysteine Proteases

Protease	Proteolytic activity	Distribution	Location
AEP	Asparaginase	APCs	Lysosome
Cathepsin S	Endoprotease	APCs	Endosome (E)
Cathepsin L	Endoprotease	Widespread	Late E, lysosome
Cathepsin B	Carboxypeptidase	Widespread	Early E
Cathepsin H	Aminopeptidase	Widespread	Lysosome

b. Aspartyl Proteases

Protease	Proteolytic activity	Distribution	Distribution
Cathepsin D	Endoprotease	Widespread	Lysosome
Cathepsin E	Endoprotease	Restricted	Endosomes

Cathepsins B, D, E, F,K, L and S and the asparagine-specific endopeptidase (AEP) have all been shown to participate in antigen processing. The specific roles of many cathepsins are still poorly understood and it appears that redundancy exists within this repertoire, as individual cathepsins appear to be dispensable. Only Cathepsins L and S have been shown to have distinct and non-redundant roles in endosomal presentation *in-vivo*. Cathepsin L has been shown to be critical in the generation of some epitopes required for T cell maturation. Cathepsin S has been shown to be vital for the generation of both exogenous and self peptides in B cells and DCs.

Lessons from AEP have shown there is a fine balance between epitope peptide generation and epitope peptide degradation. As well as being required for the generation of some peptides, AEP is able to destroy epitopes of myelin basic protein (MBP)(Manoury *et al.*, 2002). Expression of cathepsins is tightly controlled and varies between cell lines. Differential expression of cathepsins between cell types may contribute to diversity of peptides presented by the MHC class II processing pathway.

1.3.9 The MHC class II molecule

MHC class II molecules are heterodimers like MHC class I. However, MHC class II molecules are composed of two homogenous chains, the MHC encoded alpha and beta chains. The two chains form an extracellular peptide binding groove and a membrane proximal region comprised of two conserved domains, homologous to Ig constant domains. Unlike the closed groove of the MHC class I molecule, the ends of the MHC-II groove are open meaning bound peptides can overhang at either end. This open conformation allows the binding of peptides much longer than the short peptides that are bound to MHC class I molecules. As with MHC class I, the MHC class II genes are

highly polymorphic. This variation allows for considerable variation in the peptides presented by the MHC-II molecules.

1.3.10 Formation of MHC class II-peptide complexes

Upon translation, the nascent MHC class II molecule forms a complex with a second protein the MHC class II invariant chain (Ii). After assembly, MHC class II-Ii complexes are trafficked to the endocytic pathway by the Ii di-leucine motif (Landsverk *et al.*, 2009). Once within an acidic endosomal compartment peptidases such as cathepsins cleave the Ii at two sites leaving a 20 amino acid peptide in the peptide binding groove. Removal of this peptide, known as CLIP (class II invariant chain peptide) is catalysed by another MHC-encoded heterodimeric glycoprotein, HLA-DM (Denzin and Cresswell, 1995; Morris *et al.*, 1994). HLA-DM is homologous to other MHC-class II molecules but it does not have an accessible peptide binding groove. Instead, DM binds to MHC-II molecules triggering a conformational change in the MHC-II molecule releasing CLIP. This opens the peptide binding groove allowing antigenic peptide binding. As well as removing CLIP, DM can remove any low affinity peptides. Therefore repeat DM-MHC-II interactions refine the repertoire of peptides bound to MHC-II molecules resulting in a pool of high affinity peptide-MHC complexes. This process is in part controlled by a third MHC encoded, MHC-II like molecule, HLA-DO. DO binds tightly to DM inhibiting DM-MHC-II binding. DO is expressed in B cells and some dendritic cells and it appears to alter the repertoire of antigenic peptides presented by MHC class II molecules. However, a clear biological role for HLA-DO had not yet been defined (Denzin *et al.*, 2005).

1.3.11 Alternative MHC class II processing: endogenous proteins

Although the MHC class II pathway classically presents peptides from extracellular proteins, it is now well established that endogenous proteins can also access this pathway. Analysis of peptides presented by MHC-II molecules reveals a significant portion of these peptides are derived from endogenous antigens (Rammensee *et al.*, 1999). As previously discussed, many $CD4^+$ T cells have been shown to be capable of direct cytotoxic activitiy, therefore the presentation of endogenous antigens to $CD4^+$ T cells via the MHC class II processing pathway could provide the immune system with another way to recognise and eliminate pathogen-infected cells.

To date, three pathways for MHC class II restricted presentation of endogenous proteins have been described and all have been implicated in the generation of epitopes from intracellular pathogens such as murine influenza and Epstein-Barr virus. Each pathway has been shown to generate a different repertoire of epitopes leading some to speculate that the pathways exist to maximize the diversity of MHC-II peptides available for $CD4^+$ T cell recognition (Eisenlohr *et al.*, 2011). A diverse repertoire of epitopes may be vital in controlling large viruses such as the herpesviruses and poxviruses. Although the differences in epitope generation between different pathways has been assessed, the extent to which they contribute to natural $CD4^+$ T cell responses has not been well studied.

One such pathway for MHC class II-restricted presentation of endogenous proteins was identified by studying the processing of an influenza epitope from the NA protein, NA79. The molecular mechanisms of this pathway are poorly understood but inhibitor experiments have shown this NA79 eptiope to be processed in a manner more similar to

the MHC class I pathway. NA79 is highly susceptible to endosomal proteases and is destroyed in acidic compartments and therefore would not have access to class II molecules within the endocytic pathway. Processing of NA79 requires delivery into the cytosol and activity of the proteasome, with loading most likely occurring in the ER or directly onto MHC-II molecule in endosomes via TAP (Eisenlohr *et al.*, 2011).

Two other mechanisms for MHC class II restricted presentation of endogenous proteins have recently been elucidated. These two mechanisms are much better defined and involve chaperone-mediated or macro-autophagy.

1.4 Autophagy

Autophagy is an evolutionarily conserved mechanism in eukaryotes, where cytoplasmic cargo is delivered to lysosomes for degradation. This process of self-eating functions to remove misfolded and long-lived proteins as well as redundant and damaged organelles. In addition to this catabolic function, autophagy provides nutrients and energy to the cell in response to various stresses.

Christian de Duve first described the process of self-eating in the 1960s and coined the term autophagy (De Duve and Wattiaux, 1966). Early studies on autophagy focussed on morphological analysis and demonstrated that autophagy occurred in normal cells but was enhanced under starvation conditions (Novikoff *et al.*, 1964). Further studies by Seglen and Gordon on the induction of autophagy identified the initial sequestering vesicle that develops into the autophagosome, the phagophore (Gordon and Seglen, 1988). As well as this, they identified 3-MA as the first specific inhibitor of autophagy (Seglen and Gordon, 1982).

The initial molecular analyses of autophagic processes were carried out in the 1990s. These studies focused on manipulation of genes in yeast and led to the identification of a number of key autophagy (Atg) genes, such as *ATG1* (Matsuura *et al.*, 1997). Later, studies in higher eukaryotes identified the first mammalian Atg genes such as Atg5 and Atg12. These studies also revealed many mammalian homologues of yeast genes, such as the Atg 8 homologue LC3 (Kabeya *et al.*, 2000; Mizushima *et al.*, 1998)

These early studies, particularly those carried out in yeast cells, focussed on macro-autophagy but in mammalian cells three autophagy pathways have been identified, namely macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). These three pathways are differentiated by the mechanisms by which they deliver cargo into the lumen of lysosomes. The contribution of each of these pathways to antigen processing and presentation will be discussed individually.

1.4.1 Microautophagy

By far the least studied and least well understood of the three autophagy pathways is microautophagy, a process in which the lysosomal membrane invaginates, capturing portions of cytoplasm. Microautophagy involves five sequential stages, demarcated by morphological changes. Firstly, segregation of lipids and exclusion of large transmembrane proteins from an area of the lysosomal membrane causes it to bulge inwards. This process is initiated by GTPases and requires the calcium binding protein Calmodulin (Uttenweiler *et al.*, 2005). This invagination forms a characteristic shape known as an autophagic tube. Secondly, due to a high density of lipids and low density of proteins, a vesicle forms at the top of the tube. Thirdly the vesicle expands due to further separation of lipids and proteins drawing cytoplasmic contents up the tube into the

vesicle. Fourthly, under the control of Atg1, enzymes bind to the inside of the vesicle and trigger vesicle scission (Bellu *et al.*, 2001). Finally V-ATPases acidify the vesicle by pumping H+ into the vesicle lumen. Lysosomal hydrolases then break down the membrane releasing its contents into the lumen of the lysosome (Muller O. *et al.*, 2000; Sattler and Mayer, 2000).

A recent discovery shows microautophagy does not appear to be exclusive to lysosomes. Sahu *et al.* showed cytosolic contents can be delivered into late endosomes during mulitvesicular body formation, in a process termed endosomal microautophagy (Sahu *et al.*, 2011)

Despite delivering many different proteins to multiple endocytic compartments, to date, no role for microautophagy in antigen processing and presentation has been revealed.

1.4.2 Chaperone-Mediated Autophagy

Chaperone mediated autophagy (CMA) is a selective process. It is very different from micro- and macroautophagy in that delivery of proteins to the lysosome does not involve the formation of autophagic vesicles. Instead, cargo is directly targeted to the lysosome membrane where it is transported directly into the lumen of the lysosome. Proteins are targeted according to the presence of a pentapeptide motif, KFERQ, within the protein. Around 30% of cytosolic proteins contain a KRERQ-like domain and therefore are potential targets for CMA (Dice J.F, 1990).

CMA substrates containing a KRERQ-like motif bind to a complex of proteins containing Hsc70, Hsp90 and several other co-chaperones associated with the lysosomal receptor

LAMP-2A (Chiang *et al.*, 1989). Cargo is transported directly through the membrane into the lumen of the lysosome for degradation. (Dice J. F., 2007).

CMA has been shown to generate MHC class II epitopes from intracellular proteins. Manipulating expression of LAMP-2A or heat shock cognate protein Hsc70 was shown to alter the presentation MHC class II epitopes derived from cytoplasmic proteins such as Glutamate decarboxylase 65(GAD65) (Zhou *et al.*, 2005). These results revealed a novel role for the lysosomal proteins LAMP-2A and Hsc70 in promoting antigen presentation. To date, no pathogen-derived antigens have been shown to be presented by CMA. This may be due to its requirement for a KFERQ-like motif that could be easily mutated in pathogen-derived proteins to allow them to avoid CMA-dependent antigen presentation.

1.4.3 Macroautophagy

Macroautophagy (hereafter referred to simply as autophagy) is the best described of the three autophagic pathways and can be divided into six sequential steps: initiation, nucleation, elongation, closure, maturation and degradation. Each step is a highly regulated process involving the coordinated action of numerous autophagy related proteins. Initiation occurs upon activation of the ULK1 and UKL2 complexes after release of their inhibition by the mTOR complex. Nucleation depends on Beclin-1, which modifies membranes which then act in the recruitment of multiple other autophagy-related proteins defining the site for the autophagic membrane to begin to form within the cytoplasm (Liang *et al.*, 1999). Elongation of the autophagic membrane around a portion of cytoplasm involves the recruitment of more proteins and lipidation of the microtubule associated protein, Light Chain 3 (LC3) by the E2-like enzyme ATG3 (Itakura and Mizushima, 2010). Closure of the membrane completes the formation of the

double-membrane vacuole, called an autophagosome, which contains a portion of cytoplasm within its lumen. The autophagosome then undergoes maturation by fusing with lysosomes to form an autophagolysosome (Gordon *et al.*, 1985). This process is again mediated by Beclin-1 (Kimura *et al.*, 2007). Following maturation, the captured cytoplasmic contents are degraded by lysosomal proteases for reuse by the cell.

Autophagy was originally considered to be a non-selective pathway for bulk degradation of cytosolic contents. However, mounting evidence now suggests that autophagy is a selective process. Recent reports have shown a number of different and distinct macrostructures within cells can be directly targeted for autophagic destruction. These specific degradative processes have been named according to the structures they target. Aggrephagy is the degradation of protein inclusions caused by aggregate-prone or misfolded proteins, mitophagy the degradation of mitochondria, pexophagy (perioxisome), reticulophagy (ER), ribophagy (ribosomes) and finally xenophagy describes the degradation of bacteria and viruses (Lamark and Johansen, 2012; Lemasters, 2005; Scott and Klionsky, 1998; Talloczy *et al.*, 2006; Tasdemir *et al.*, 2007; Yang and Chiang, 1997).

From these reports an important role for a new class of proteins, “autophagy adaptors” has emerged. Autophagy adaptors act as cargo receptors for the degradation of autophagy substrates and are themselves degraded by autophagy. The first discovered and most well studied autophagy adaptor is P62/SQSTM1 (sequestersome 1) (Shin, 1998). P62 is a 440 amino acid protein containing many functional domains. The key domains for its function as an autophagy adaptor are a PB1 domain, a ubiquitin associated (UBA) domain and an LC3 interacting region (LIR). The PBI domain facilitates

protein-protein interactions. It allows p62 to bind to protein kinases and other autophagy adaptors such as NBR1, as well as allowing homopolymerisation of p62 (Nakamura *et al.*, 2010; Wilson *et al.*, 2003). The UBA domain binds multiple different forms of ubiquitin (Ub) and is key in allowing p62 to target ubiquitinated proteins for degradation (Vadlamudi *et al.*, 1996). The LIR domain confers p62 binding to LC3. As LC3 is present on the internal membrane of the phagophore and retained within autophagosomes, binding to LC3 targets p62 for autophagic degradation along with any other proteins in complex with p62 (Ichimura *et al.*, 2008; Pankiv *et al.*, 2007). Interestingly p62 contains both a nuclear localisation sequence (NLS) and a nuclear export signal (NES), this allows p62 to shuttle between the nucleus and the cytoplasm (Pankiv *et al.*, 2010). Note that due to its rapid nuclear export, p62 appears to be wholly cytoplasmic in the absence of nuclear export inhibitors.

Increasing evidence shows that selective autophagic degradation of protein aggregates requires Ub as a signal (Kirkin *et al.*, 2009a). A simple model for selective degradation has recently been described and involves five sequential steps. First, substrates are identified by chaperones or other cellular sorting systems such as Hsc70 or Bag-3 (Gamerdinger *et al.*, 2009). Second, the substrate is ubiquitinated by E3 ligases (Lelouard *et al.*, 2004). Third, p62 is recruited, binds to Ub and aggregates substrates forming a large complex known as a p62 body (Komatsu *et al.*, 2007; Szeto *et al.*, 2006). Fourth, p62 recruits the phagophore to the site of aggregation through its interaction with LC3 (Ichimura *et al.*, 2008). Finally the autophagosome forms around the p62 body enclosing the substrates within.

To date, most models of selective autophagy been shown to require ubiquitination of substrates. However some examples of Ub-independent, selective autophagy have been observed. P62 has been shown to bind to some substrates directly, independently of Ub to degrade them by autophagy (Gal *et al.*, 2009). As well as Ub-independent, selective autophagy, some mechanisms of selective autophagy are p62-independent. Two pathways of mitophagy have been identified in mammals, one of which involves the autophagy adaptor Nix and does not require p62 (Novak *et al.*, 2010; Sandoval *et al.*, 2008) .

As previously mentioned p62 is the best-studied autophagy adaptor but many others have been discovered, including: neighbour of Brca1 (NBR1), nuclear dot protein 52 NDP52, also referred to as CALCOCO2 and optineurin (OPTN) (Figure 1.1).

NBR1 shows similar domain organisation to p62. It is recruited to Ub-positive protein aggregates and is degraded by autophagy according to its interaction with LC3 via its LIR domains (Waters *et al.*, 2009). NBR1 can interact and form aggregates with p62 but it is also able to function as an individual autophagy adaptor in the absence of p62 (Kirkin *et al.*, 2009b)

CALCOCO2 has been shown to bind to a number of different substrates and is able to bind to *Salmonella enteric* to target it for autophagic degradation (xenophagy). CALCOCO2 can bind Ub substrates or can act independently of Ub by binding a second protein Galectin 8 (Li *et al.*, 2013; Thurston *et al.*, 2009)

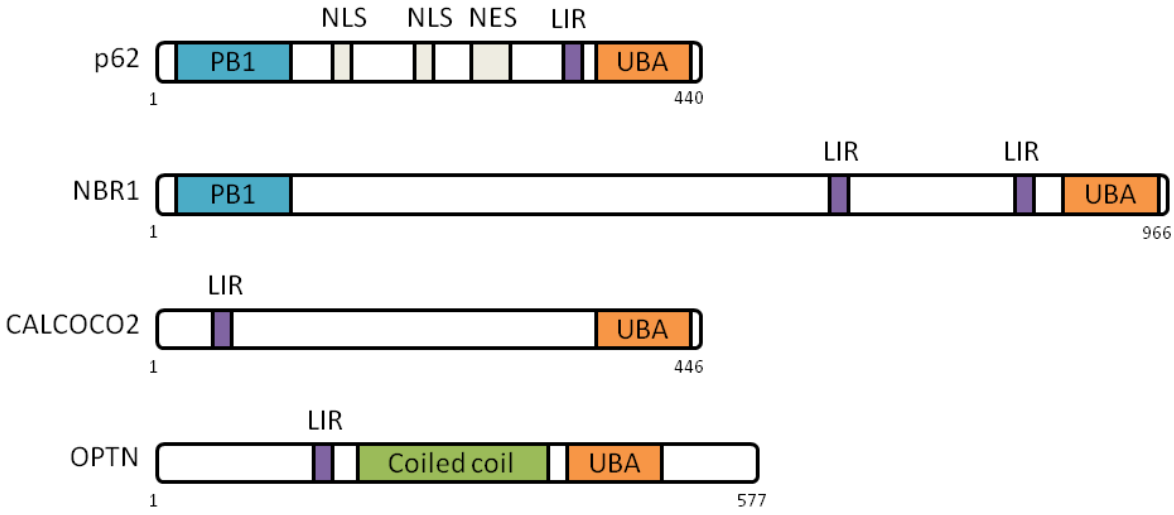


Figure 1.1 Domain architecture of mammalian autophagy adaptors

Architecture of major mammalian autophagy adaptor proteins with annotated domains: PB1 (Pox and Bern 1), NLS (nuclear localisation sequence), NES (Nuclear export sequence), LIR (LC3-interacting region), UBA (ubiquitin associated).

OPTN is another autophagy adaptor with both Ub-dependent and independent functions. Optineurin is present in many protein inclusion bodies and is required for degradation of such structures. More recently, Optineurin has shown to be able to bind directly to protein aggregates via a C-terminal coiled coil domain in a Ub-independent manner. This binding is required to target such aggregates for degradation (Korac *et al.*, 2013; Tumbarello *et al.*, 2012)

1.4.4 Macroautophagy and antigen processing

As previously described, a significant proportion of antigens presented on MHC class II molecules are derived not from extracellular proteins but from cytosolic or nuclear antigens. In 1997 Brazil and colleagues made the landmark observation that autophagy can generate MHC class II epitopes from a self-antigen (Brazil *et al.*, 1997). Since this time, mounting evidence now suggests that autophagy makes an important contribution to the presentation of a wide range of endogenous epitopes. Dengjel and colleagues showed that starvation-induced autophagy can upregulate MHC class II presentation of nuclear and cytosolic proteins in favour of membrane proteins. Among these proteins were the autophagy proteins LC3 and GABARAP (Dengjel *et al.*, 2005; Suri *et al.*, 2008). As LC3 and GABARAP are present on the inner membrane of autophagosome this suggests that autophagosomes fuse with MHC class II loading compartments (MIICs). In a later study Schmid *et al.* confirmed this result, showing that 70-80% of autophagosomes fuse with MIICs. Further to this, tagging antigens with LC3, targets proteins to autophagosomes and dramatically enhances their presentation on MHC class II molecules (Comber *et al.*, 2011; Schmid *et al.*, 2007).

In addition to self-antigens being processed by autophagy, pathogen derived antigens are also processed by autophagy. The classic example of autophagic presentation of a pathogen derived antigen is Epstein Barr Virus Nuclear antigen 1 (EBNA1). EBNA1 is the genome maintenance protein of Epstein Barr Virus (EBV). It is expressed in all known states of EBV expression and is a key viral antigen in that it contains multiple $CD4^+$ and $CD8^+$ T cell epitopes (Hislop *et al.*, 2007; Long *et al.*, 2005). EBNA1 has been shown to be processed by an intracellular route for MHC class II processing (Munz *et al.*, 2000). Paludan *et al.* later showed the presentation of EBNA1 requires autophagy (Paludan *et al.*, 2005).

Other pathogen-derived proteins have also been shown to be presented by autophagy. Neomycin phosphtransferase (NeoR) has been shown to be sequestered in autophagsomes resulting in the presentation of a NeoR epitope via MHC class II (Nimmerjahn *et al.*, 2003) A third example comes from the mycobacterium protein Ag85B. Autophagy stimulation causes this protein to be better presented by MHC class II molecules on DCs (Jagannath *et al.*, 2009).

As shown by these examples, autophagy makes a significant contribution to the presentation of MHC class II epitopes. Both macroautophagy and CMA generate MHC class II epitopes from self-proteins. But only macroautophagy has so far been shown to generate MHC class II epitopes from pathogen derived proteins (Figure 1.2).

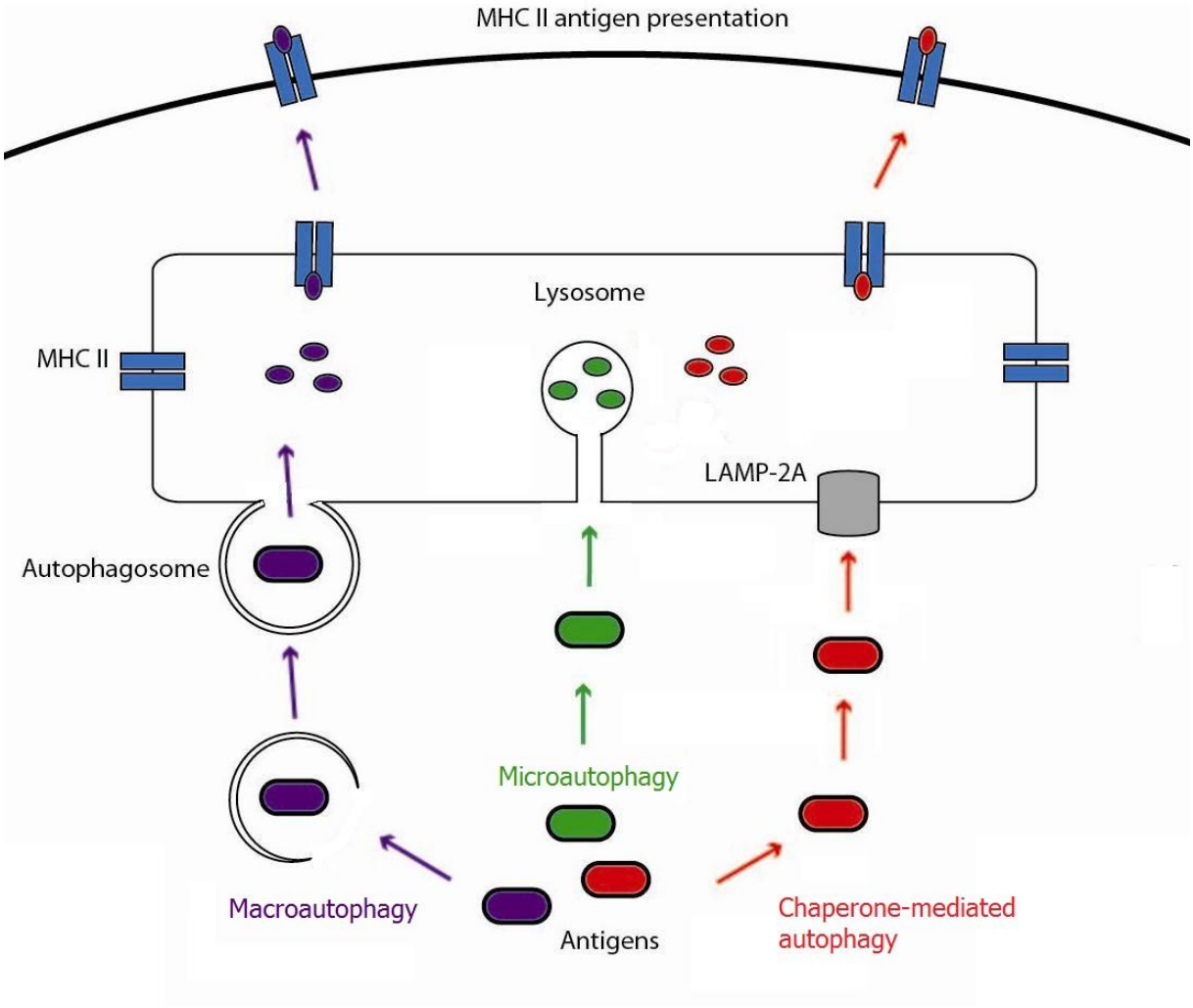


Figure 1.2 Generation of MHC class II epitopes via the different autophagy pathways

Three autophagy pathways exist to import proteins into the endocytic pathway. Macroautophagy (left) has been shown to generate MHC-II epitopes from both self- and pathogen-derived proteins. Chaperone mediated autophagy (right) has been shown to generate MHC-II epitopes from self-derived proteins. A role for microautophagy (centre) in the production of MHC class II processing has not yet been revealed.

As well as these examples of MHC class II presentation, autophagy has now been shown to play an important role in the presentation of some MHC class I epitopes. In 2009 English *et al*. showed that presentation of an MHC class I restricted epitope from the viral glycoprotein B of herpes simplex virus 1 (HSV-1) was sensitive to bafilomycin and 3-MA. This suggests a role for the endosomal processing pathway and autophagy in the generation of this epitope. This work also identified a novel form of autophagy marked by four layered membrane structures, containing LC3 and originating from the nucleus (English *et al.*, 2009). In support of this role of autophagy in MHC class I processing, Tey and Khanna recently showed that an epitope from Human Cytomegalovirus (HCMV) latency-associated protein, pUL138 required autophagy for its presentation via a TAP-independent class I processing pathway (Tey and Khanna, 2012).

Although autophagy is able to generate a wide range of epitopes, the factors that determine which peptides are generated from an antigen have not been examined. Studies on EBNA1 showed that some but not all EBNA1 MHC class II epitopes were generated by autophagy. Leung and colleagues showed that $CD4^+$ T cell clones specific for two epitopes could recognise LCLs expressing natural levels of the EBNA1 protein, while clones specific for a third epitope could not (Leung *et al.*, 2010). The avidity of the clones was not responsible for these differences and experiments showed that the two epitopes presented by LCLs were indeed endogenously accessing the MHC-II pathway, confirming previous findings (Paludan *et al.*, 2005). Interestingly, only one of these two epitopes was autophagy-dependent. Over-expression of EBNA1 was shown to increase the presentation of both epitopes, but this did not affect processing routes. However, relocalising EBNA1 to a cytoplasmic protein, through mutation of its nuclear localization

sequence (NLS), caused all three epitopes to be processed by autophagy. This striking result was shown to be a direct result of EBNA1's cytoplasmic localisation since introducing a heterologous NLS reversed the effect (Leung *et al.*, 2010). This work suggests that nuclear localisation confers some protection to EBNA1 from autophagic MHC class II presentation. However whether other nuclear-localised proteins are similarly protected from autophagy is still debated.

The huntingtin and ataxin-1 proteins each contain a large poly-glutamine repeat that can cause these proteins to aggregate in cells. When these proteins aggregate in the cytoplasm they are degraded by autophagy, but aggregates in the nucleus are not degraded by autophagy (Iwata *et al.*, 2005). Further support for this nuclear protection comes from the work of Wang *et al*. They showed that a mutant form of the cellular protein, CDC27, was better recognised by $CD4^+$ T cells. This increased recognition was due to a mutation in the protein's nuclear localisation sequence which caused the protein to relocalise to the cytoplasm (Wang R. F. *et al.*, 1999). Furthermore, antigens fused to YopE, a Yersinia protein that is injected into the cytosol, are have been shown to be processed for MHC class II presentation by a mechanism involving lysosomes and autophagy (Russmann *et al.*, 2010).

In contrast to these examples, presentation of an autophagy-dependent epitope from the NeoR protein was not reduced by the addition of a heterologous NLS (Riedel *et al.*, 2008). The reason for this different result is unclear, but one possibility is that it might reflect different rates of nuclear import between EBNA1 which is rapidly imported into the nucleus and NeoR where import rates were not studied but may be much slower

since only a minimal NLS was used; flanking sequences can increase dramatically the rate of nuclear import.

The results of EBNA1 relocalisation suggest that manipulation of antigen processing could increase $CD4^+$ T cell responses to pathogens. This hypothesis is supported by the work of Jagannath and co-workers who showed that stimulating autophagy caused DCs to better present epitopes and upon adoptive transfer of these DCs, $CD4^+$ T cell responses were more efficiently primed (Jagannath *et al.*, 2009).

Manipulation of antigen processing to increase T cell presentation could be used to improve the clearance of pathogens but could also be exploited in cancer therapy where $CD4^+$ T cell responses have been shown to be very important.

1.5 Cancer Immunology

The first evidence for an immunological role in the control of cancer was revealed in the late 19^{th} century when William Coley, an American physician, began treating cancer patients with a "mixed bacterial vaccine". This vaccine, termed Coley's toxins, made of live *Streptococcus pyogenes* and *Serratia marcescens*, gave a generic boost to the immune system and triggered regression in 18 cancer patients (Coley, 1893). In 1957, Burnet and Thomas developed the immune surveillance theory of cancer. They postulated that an immunological mechanism existed to eliminate or inactivate malignant cells (Burnet M., 1957). Later they suggested that lymphocytes carried out this function (Burnet F. M., 1970).

Later experiments in mice showed animals could be immunised against transplanted, syngeneic tumour cells, revealing tumours were immunologically distinguishable (Old

and Boyse, 1964). These experiments and others established the existence of tumour-specific antigens (Klein, 1966). If it was possible to immunise against tumours there must be tumour-specific antigens that elicit a memory response. Early work on tumour transplants in mice revealed that tumours are immunogenic and could be eradicated by the immune system.

This theory of immunesurveillance was thoroughly tested and many results supported a role for the immune system in protection against cancer. Further experiments in mice showed that IFN-γ was key in controlling tumours. Neutralising IFN-γ with specific antibodies was shown to cause tumours to grow more rapidly and IFN-γ (-/-) mice developed more tumours more regularly (Dighe *et al.*, 1994; Kaplan *et al.*, 1998). Later experiments revealed perforin, a key molecule in T and NK cell killing was also vital, in that perfornin (-/-) mice showed higher rates of spontaneous tumours (Street *et al.*, 2001).

Support for the immune surveillance theory in humans began in the 1970s. Multiple studies showed that patients who were immunodeficient or immunosuppressed after transplant showed higher relative risk of developing cancer (Gatti and Good, 1971; Penn *et al.*, 1971). It was later noted that many of these tumours were of viral aetiology (Penn, 1999). And later, after the AIDs epidemic many of these viral associated malignancies became common in AIDs patients (Boshoff and Weiss, 2002).

Mechanistic evidence supporting immune surveillance came later. It was shown that a positive correlation between lymphocyte infiltration in tumours and increased survival existed (Clemente *et al.*, 1996). Later work then showed that this was linked specifically to infiltrating $CD8^+$ T cells (Naito *et al.*, 1998).

Many studies in human cancer immunology originate from studies on melanoma. Rosenberg *et al*. showed that adoptive transfer of tumour infiltrating lymphocytes and IL-2, a key cytokine for T cell function, resulted in the regression of tumours in 30% of melanoma patients, providing strong evidence that T cells play a critical role in tumour regression (Rosenberg *et al.*, 1988).

Since these early experiments in melanoma, similar results have been observed in the context of many other cancers. To further understand the molecular basis of immune-dependent regression of tumours, much effort has been devoted to the identification of tumour antigens recognized by T cells with an antitumor activity *in-vivo*.

1.5.1 Tumour antigens

A key hallmark of cancer is genomic instability (Hanahan and Weinberg, 2011). This results in aberrant gene expression of normal as well as mutated proteins. These proteins are often not represented in the array of proteins presented in the thymus during the establishment of central tolerance and are therefore often immunogenic. The first tumour antigen specifically recognised by T cells was Mage a1 which encodes the antigen MZ2E (van der Bruggen *et al.*, 1991). Melanoma was the first tumour model to show $CD4^+$ and $CD8^+$ T cell specificity to tumour antigens gp100 and tyrosinase (Topalian *et al.*, 1994; Touloukian *et al.*, 2000).

Since these early studies tumours antigens have been identified in many self-proteins and can roughly be separated into four groups. The first group are the tissue-specific tumour antigens. These antigens are derived from proteins that are expressed at low levels in a small subset of tissues but not in other normal healthy tissues. They are

expressed at high levels in a small subset of cancers often linked to the organ of restriction. Examples of such antigens include Melan-A and gp100 which are both overexpressed in melanoma (Kawakami *et al.*, 1994a; Kawakami *et al.*, 1994b).

The second group of antigens, similar to the tissue-specific antigens, are the tumour-specific shared antigens otherwise known as Cancer-testis (CT) antigens. These antigens are expressed in a wide range of tumours as well as the immune-privileged site of normal testis. One such CT antigen is NY-ESO-1. This gene is expressed in melanoma, breast, prostate, bladder and lung carcinoma as well as others (Chen *et al.*, 1997; Lee L. *et al.*, 1999; Wang R. F. *et al.*, 1998).

The third group of tumour antigens are the mutated antigens. Genomic instability in tumours results in the expression of many mutated proteins which have the potential to be highly immunogenic as they are specific to the tumour itself. Surprisingly due to their highly immunogenic potential, mutated proteins represent a very small subset of tumour antigens. Many CTLs raised against mutated proteins such as Ras and p53 fail to recognise tumours (Cheever *et al.*, 1995). However some epitopes have been identified. One example being cyclin-dependent kinase 4, where a point mutation results in a new epitope recognized by CTLs (Wolfel *et al.*, 1995)

Proteins in the fourth group of tumour antigens contain T cell epitopes derived from alternative open reading frames. TRP-1/gp75 represents the first demonstration that T cells may respond to gene products derived from alternative open reading frames (Wang R. F. *et al.*, 1996).

As well as tumour antigens that originate from self-proteins some tumours express antigens from pathogens. This is best represented in the case of virus-associated cancers. Many of these cancers express viral proteins and the immune system can recognise these proteins in the context of cancer in the same way it would recognise them in the context of viral infection. These viral tumour antigens therefore have the potential to serve as excellent therapeutic targets as high avidity T cells should be present in donors, though if virus-associated tumours have arisen it is clear the immune response to these antigens has failed in some way.

Most work to date has focussed on harnessing $CD8^+$ T cells as the effectors of anti-tumour immunity. $CD4^+$ T cells, which provide immune help to support the generation of sustained effective immune responses, are now also starting to be seen as effectors against tumours that bear MHC-II molecules (Mumberg *et al.*, 1999; Wang L. X. and Plautz, 2010). Since inflammatory cytokines can upregulate MHC-II expression a wide range of malignancies are potentially targetable via $CD4^+$ T cells (Garrido *et al.*, 1993; Wang L. X. and Plautz, 2010).

The evidence presented so far shows that tumours can be highly immunogenic and T cells are able to eradicate tumour cells in certain settings; despite these facts cancers still arise. Many immunotherapies are able to improve immune recognition of cancer but often these therapies ultimately fail and patients succumb to their disease. Recent attempts to improve immunotherapies have focussed on increasing the efficacy of T cells in the tumour microenvironment. However an alternative does exist, if tumours could be manipulated to increase antigen processing of tumour antigens, these tumours may be more susceptible to immunotherapies.

Therefore in this study I will examine the antigen processing and presentation of two tumour antigens with the ultimate aim of improving the presentation of these antigens to increase T cell recognition of tumour cells. The two tumour antigens I will focus on are WT1 and EBNA1.

1.5.2 WT1 - a cellular tumour antigen

The Wilms' tumour gene 1 (*WT1*) is the gene believed to be responsible for the childhood renal neoplasm, Wilms' tumour. Wilms' tumours arise from an inactivation of both alleles of the WT1 gene; this fact led to WT1 first being classified as a tumour suppressor gene. Unlike more classical tumour suppressor genes such as p53, which are ubiquitously expressed, WT1 is restricted to a limited set of tissues, including gonads, uterus, kidney and mesothelium.

More recently an alternative role for WT1 as an oncogene in leukaemia and various solid tumours has been suggested. This is supported by a number of recent findings. WT1 expression is high in leukemic blast cells as well as many solid tumours including: breast, lung, gastric and bladder. Increased levels of WT1 expression in leukaemia correlates with poor disease prognosis and WT1 expression is further increased in relapsed patients. Further to this, inhibition of WT1 using anti-sense oligomers and WT1-specific siRNAs leads to reduction in leukaemogenesis and growth of solid cancer cells. Forced expression of the WT1 gene also promotes cell growth, suppresses apoptosis and induces leukaemia in transgeneic mice.

High levels of WT1 in leukaemia and solid tumours led to WT1 being investigated as a potential tumour antigen. Gaiger *et al.* showed that immunisation with WT1 peptides

induced WT1-specific responses in mice and that these T cells were capable of killing WT1-expressing tumour cells (Gaiger *et al.*, 2000). Others have shown that immunised mice can reject challenge with WT1-expressing leukemic blasts and importantly that WT1 immunisation shows no damage to WT1 expressing organs or haematopoietic stem cells.

Since these original experiments peptide stimulation of WT1-specific T cells in humans has been widely studied. Oka *et al.* and others first identified class I epitopes in the WT1 protein by peptide stimulation of PBMCs. WT1 peptides were shown to induce WT1-specific $CD8^+$ T cells in a number of donors (Gao *et al.*, 2000; Ohminami *et al.*, 2000; Oka *et al.*, 2000). These T cells were shown to recognise naturally expressed WT1, to kill WT1 expressing leukemic cells, caused no damage to haematopoietic stem cells and did not inhibit colony formation of normal bone marrow.

Research into the identification of class II epitopes in WT1 has been slower than that of class I epitopes. Knights *et al.* first identified an MHC class II epitope. Peptide stimulation showed this epitope stimulated $CD4^+$ T cells capable of producing IFN-γ in response to WT1 (Knights *et al.*, 2002). Since then multiple MHC class II epitopes have been identified (Guo *et al.*, 2005; Kobayashi *et al.*, 2006; May *et al.*, 2007; Muller L. *et al.*, 2003).

1.5.3 EBNA1 - a viral tumour antigen

As described previously EBNA1 is the genome maintenance protein of EBV. EBV is a ubiquitous γ-herpes virus; following primary infection in childhood or early adulthood it establishes a lifelong asymptomatic infection in B-cells. In the vast majority of EBV-

infected individuals, T cell responses successfully control infection and limit replication and growth-transforming ability of the virus. However in a small subset of individuals a number of malignancies can arise in association with EBV. These malignancies include post-transplant lymphoproliferative disease (PTLD), Burkitt's lymphoma, Hodgkin's lymphoma, natural killer/T cell lymphoma, nasopharyngeal carcinoma and gastric carcinoma. In some cases such as PTLD, development of these cancers is directly caused by the growth transforming ability of EBV and occurs as a failure of the T cell response to control the virus (Young and Rickinson, 2004).

In its role as the genome maintenance protein, EBNA1 is the one EBV antigen expressed in all EBV-infected cells, including all EBV-associated malignancies. As EBV is able to persist in humans, EBNA1 was long thought to go undetected by the immune system. However this was shown not to be the case when EBNA1-specific $CD4^+$ T cells were identified (Khanna *et al.*, 1997). Since this first study $CD4^+$ T cell responses to EBNA1 have been shown to be very common in healthy individuals(Leen *et al.*, 2001; Munz *et al.*, 2000). EBNA1-specific $CD4^+$ T cells are often of Th1 phenotype and are able to directly recognise and kill EBV-infected cells (Bickham *et al.*, 2001; Munz *et al.*, 2000). As well as $CD4^+$ T cells, EBNA1-specific $CD8^+$ T cells have been identified (Lee S. P. *et al.*, 2004; Tellam *et al.*, 2004; Voo *et al.*, 2004). EBV's requirement for EBNA1 may represent an Achilles heel for the virus in that efficient targeting of EBNA1 could be an effective immunotherapy against EBV-associated malignancies.

1.6 Aims

The main aim of this body of work was to study MHC class II processing of tumour antigens and subsequent presentation to $CD4^+$ T cells. A particular focus will be placed

on the non-classical MHC-II processing pathways involved in processing of endogenous antigens. I will aim to apply the knowledge of MHC class II processing to develop novel strategies to improve both the range and levels of MHC class II epitopes presented to tumour-specific $CD4^+$ T cells.

2. MATERIALS AND METHODS

2.1 Eukaryotic cell culture

2.1.1 Cell culture media and reagents

RPMI-1640 supplemented with 0.2M L-glutamine (Gibco) was used for the culture of all lymphocyte lines and stored at 4°C.

DMEM (Gibco) was used for the culture of all epithelial lines and stored at 4°C.

EBSS (Gibco) was used for culturing cells under starvation conditions and stored at room temperature.

Foetal calf serum (FCS) (PAA) was stored at -20°C.

Penicillin-streptomycin solution (pen/strep) (Gibco) containing 5000IU/ml penicillin and 5000µg/ml streptomycin was stored at -20°C.

Optimen (Gibco) was stored at 4°C.

Phosphate buffered saline solution (PBS) (8.2mM Na_2HPO_4, 1.5mM KH_2PO_4, 139mM NaCl, 3mM KCl) was prepared using Dulbecco PBS tablets (Oxoid)

Cyclosporin A (Sandimmun) was reconstituted in RPMI 1640 to 10µg/ml and stored at 4°C.

MLA-144 supernatant (MLA) was derived from Gibbon derived MLA-144 cell line. Cells were cultured in RPMI 1640 + 10% FCS and 1% pen/strep. After two weeks the cell supernatant was harvested by centrifuging at 1600rpm for 10min, followed by filtration

through Millipore Steritop 0.22µm vacuum driven disposable bottle top filters and stored at -20°C.

Recombinant interleukin-2 (IL-2) (Peprotech) was reconstituted in water to 10^5IU/ml and stored at -20°C.

Doxycycline (dox) was prepared from doxycycline hyclate powder (Sigma) dissolved in water to 100µg/ml and stored in the dark at 4°C.

Synthetic peptides (Alta bioscience, Birmingham, UK) were dissoloved in dimethyl sulphoxide (DMSO) (Fisher Scientific) and stored at -20°C.

LCLs media: RPMI 1640 + 10% FCS + 1% pen/strep

Epithelial cell culture media: DMEM + 10% FCS + 1% pen/strep

T cells media: RPMI 1640 + 30% MLA + 10% FCS + 1% human serum + IL-2 (50IU/ml) + 1% pen/strep

2.1.2 Tissue culture techniques

LCLs and epithelial cells were grown in culture flasks (Iwaki or Corning) and T cells were grown in 24-well culture plates (Iwaki). Cells were incubated in a cell culture incubator with 5% CO_2 at 37°C. Cells were fed with fresh media twice weekly and split depending on confluency. Cell manipulation was performed in a class II biological safety cabinet.

2.1.3 Mycoplasma testing

MycoAlert Mycoplasma detection assay kit (Cambrex) was used to routinely test for mycoplasma infection in cell cultures according to the manufacturer's instructions.

2.1.4 Cryopreservation of cells

Cells to be preserved were pelleted, resuspended in freezing media (RPMI-1640 + 10% DMSO + 20% FCS) and transferred into sterile 1ml cryovials (Nunc). These were then stored at -80°C for 16h. Cryovials were then transferred into liquid nitrogen freezers

To revive cells, 1ml cryrovials were placed into a 37°C waterbath to thaw. Following this cells were washed with standard media (RPMI-1640 + 10% FCS), resuspended in appropriate media and transferred into an appropriate plate or flask, for culturing in a 37°C, 5% CO_2 incubator

2.1.5 Blood donors

Blood was taken from healthy donors and multiple myeloma patient samples were obtained from Birmingham Queen Elisabeth Hospital with ethical consent according to the human tissue act. Peripheral blood was collected from donors into a syringe with heparin and then diluted with 1 part RPMI-1640.

2.1.6 Preparation of peripheral blood mononuclear cells (PBMCs)

PBMCs were separated by layering 35ml blood/RPMI onto 15ml lymphoprep and centrifugation at 1800rpm with no break. Cells were then washed and either used for

downstream experiments or cryopreserved. All experiments were approved by an ethics committee and donors provided consent.

2.2 Cellular Assays

2.2.1 Peptides

Peptides were synthesised by either peptide 2.0 or Alta biosciences, dissolved in DMSO to obtain a concentration of 5mg/ml and stored at -20°C.

Pepmixes spanning Actin, WT1 and influenza A nuclear protein were acquired from JPT, Berlin.

2.2.2 T cell assay

T cell recognition of target antigen presenting cells was measured by IFN-γ production in an ELISA assay. T cells (0.5 or $1x10^4$/well) were incubated with target cells ($5 x10^4$) at 37°C, 5% CO_2 in RPMI + 8%FCS. After 16 hours culture supernatant was collected and IFN-γ was measured using IFN-γ ELISA kit according to manufactures protocol.

2.2.3 IFN-γ ELISA

MaxiSorp plates were washed with wash buffer (PBS-0.05% tween-20) and then blocked with 200µl of blocking buffer (PBS-0.05% tween-20 + 10% BSA) for two hours at room temperature. Following this, plates were washed four times with wash buffer and then 100µl of cell supernatant from the overnight assay was harvested and added to the MaxiSorp plates. 100µl of an IFN-γ standard was also added, in triplicate, this was done using doubling dilutions (2000pg/ml – 31.25pg/ml) of recombinant IFN-γ

(peprotech). Plates were then incubated at room temperature for 3-4 hours after which, plates were washed four times with wash buffer. 50µl of biotinylated anti-human IFN (Sigma), diluted in blocking buffer, was added to each well and plates were incubated for 1 hour at room temperature. Following this, plates were washed 4 times in wash buffer and then 50µl of streptavidin-peroxidase (ExtraAvidin-Peroxidase, Sigma), diluted 1/1000 in blocking buffer, was added to each well and plates were incubated at room temperature for 30min. Plates were then washed 8-times in wash buffer and 100µl of peroxidase substrate (3, 3', 5, 5'-tetramethylbenzidine (TMB) solution, Tebu-bio Laboratories) was added and the plates incubated for 30 minutes to allow for colour development. To stop the reaction, 100µl of 1M hydrochloric acid was added, resulting in a soluble yellow product. Plates were then read using dual wavelengths of 450nm and 695nm. Using the results from the IFN-γ standard curve, the level of IFN-γ release could be calculated as IFN-γ release in pg/ml.

2.2.4 ELIspot assay

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor and multiple myeloma patient blood samples. $5x10^5$PBMCs/well were stimulated with pepmixes or phytohaemagglutinin for 24 hours at 37°C, 5% CO_2 in RPMI + 8% foetal calf serum (FCS). The frequency of IFN-γ producing cells was then detected with an IFN-γ ELIspot kit (Mabtech, MAriemont) according to manufactures protocol

2.2.5 Tetramer assay

A pretitrated volume of MHC-II tetramer (0.5–1.0 µl) was added to relevant cells, or no tetramer to control tubes, which were incubated at 37°C for 2 h during which the cells

were resuspended periodically by agitating the tubes. After an additional two washes, the cells were stained for surface markers by incubation for 30 min on ice with predetermined saturating amounts of FITC-conjugated CD4 mAb (BD), After two subsequent washes, stained cells were either analyzed immediately or fixed in 2% paraformaldehyde for later analysis, on an LSRII flow cytometer (Beckman Coulter). $CD3^+CD4^+$ cells falling within the lymphocyte gate were analyzed for tetramer and surface antigen staining. All data were processed using FlowJo software (Tree Star).

2.2.6 Overexpression of EBNA1 constructs

To transfect LCLs, cells were washed in 10ml PBS and resuspended in 0.3ml OPTI-MEM (Gibco) containing 15μg plasmid DNA. The cells were transferred to 4mm electroporation curvettes and electroporated at 230V and 0.975mF using a Gene Pulser II (Bio-Rad).

Protein expression from dox-regulated plasmids in stably transfected cell lines was achieved by the addition of dox to the cell cultures.

MJS cells were transfected with specified quantities of plasmid DNA using lipofectamine (Life technologies) according to the manufacturer's protocol.

2.2.7 siRNA Knock down

siRNA oligos (Sigma) were electroporated (5μM) into LCLs according to the previous protocol. siRNAs were delivered to MJS cells using oligofectamine (Life technologies) according to the manufacturer's protocol.

2.3 Biochemical methods

2.3.1 Preparation of cell lysates

$1x10^6$ cells were pelleted in 1.5ml eppendorf tubes and washed twice with PBS. Pellets were immediately frozen at -80°C. Pellets were later resuspended in 5M Urea buffer and sonicated for 30s at 50% power in a misonix microson ultrasonic cell disruptor. Insoluble material was removed by centrifugation and protein concentrations in the lysates determined by BCA protein determination assay kit (Pierce).

2.3.2 Nuclear cytoplasmic fractionation

Lysis of cells was performed using the NEPER nuclear and cytosolic extraction reagents (Pierce Biotechnology). CERII was added to samples, vortexed and incubated for on ice for an additional minute. Lysates were then centrifuged for 5 minutes at 13000rpm and 4°C, the pellet contained the nuclei and the supernatant, which was removed and transferred to a clean eppendorf, the cytosolic fraction. The nuclear pellet was resuspended in 50µl of NERI on ice and vortexed for 15 seconds every 10 minutes for 40 minutes. Samples were then centrifuged as before and the supernatant containing the nuclear fraction removed and transferred to clean pre-chilled 1.5ml eppendorf tube.

2.3.3 Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis

Cell lysates were separated in either 4-12% or 12% Tris SDS-PAGE gels under reducing conditions using the Life technologies precast gel system.

2.3.4 Western Blotting

Polypeptides separated by SDS-PAGE were transferred to a nitrocellulose membrane. Membranes were blocked in 5% milk, PBS tween and probed with specific antibody. After washing, the membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit IgG and subjected to chemiluminescent detection using the millipore ECL detection kit (Millipore, Billerica).

2.3.5 RNA extraction

Up to $5x10^6$ cells were washed in PBS by centrifuging at 1600rpm for 5 minutes, cells were then resuspended in 1ml PBS, transferred into a 1.5ml eppendorf and pelleted. Cellular RNA was then extracted and purified using RNAeasy kit (Nugen), according to the manufacturer's protocol. RNA was quantified using a Nanodrop machine (thermo scientific) and stored at -80°C until needed.

2.3.6 Quantitative reverse transcriptase-polymerase chain reaction

The presence of mRNA transcripts in cell lines was measured by qRTPCR. cDNA was produced by incubating RNA with reverse transcriptase and random hexamer primers. transcripts were detected by qRTPCR using WT1 or p62-specific primers and probes (BD biosciences, New Jersey). All results were expressed relative to levels of GAPDH

2.3.7 RT-PCR

Five microliters cDNA preparation was used for polymerase chain reaction (PCR) amplification in a 50-μL volume of final reaction mixture containing 2.5 U Taq DNA polymerase (Qiagen), 1mmol/L dNTP and 20 OD/ml primer. Amplification of the human

WT1 coding region was achieved using sense primers located in exon 7 (21 mer 58-ggc atc tga gac cag tga gaa-38) and antisense primers in exon 10 (22 mer 58-gag agt cag act tga aag cag t-38). RNA integrity was verified by amplifying the human c-ABL gene in every sample using intron-spanning primers: 22 mer sense 58-ccc aac ctt ttc gttgca ctg t-38; 22 mer antisense 58-cgg ctc tcg gag gag acg atg a-38. Hot-start PCR was performed for 35 cycles with a thermal cycler under the following conditions: denaturing at 95°C for 1 minute, primer annealing at 56°C for 1 minute and chain elongation at 72°C for 2 minutes. The cycling was initiated by a 5-minute denaturation step at 95°C to heat inactivate the reverse transcriptase and it was terminated by a 10-minute final extension at 72°C.PCR products were electrophoresed through 1.5% agarose gels.

2.3.8 Isolation of lysosomes

$1x10^8$ – $7x10^9$ cells were isolated and washed three times in fractionation buffer: 10mM tris(hydroxymethyl)aminomethane/acetic acid pH7.0, 250mM sucrose. Pellets were resuspended in 1.5ml fractionation buffer and homogenised using 30 strokes in a dounce homogeniser. Cell homogenate was centrifuged at 1000 X g (2500 RPM GH3.8 rotor) for 10 min to pellet debris and undestroyed cells. Supernatant were transferred to a ultracentrifuge tube, pellets were washed with twice the pellet weight of fractionation buffer and centrifugation repeated. Supernatants were combined in the ultracentrifuge tube and centrifuged at 4000 X g (9500 RPM TLA55 rotor) for 10 min to pellet a crude fraction containing plasma membrane and nuclei. Supernatant were transferred to a fresh tube and pellets washed with twice the pellet weight of fractionation buffer and centrifugation repeated. Supernatants were combined and centrifuged at 50,000 X g (33500 RPM TLA55) for 10 min to pellet mitochondria,

endosomes and lysosomes The pellet was resuspended in three to five times the pellet volume of distilled water depending on cells and incubated for 10min. Tubes were centrifuged at 50,000 X g for 10min to pellet mitochondria and endosomes and supernatants containing lysosomal material were transferred to a fresh tube.

2.4 Molecular biological methods

2.4.1 Bacterial culture media and reagents

LB (Luria Broth) media was prepared by dissolving 20g/L of LB powder (Invitrogen) in SDW. This was then sterilised by autoclaving at 121°C for 20 minutes at 15psi.

LB agar was prepared by dissolving 20g/L of LB agar powder (Invitrogen) in SDW and sterilising by autoclaving at 121°C for 20 minutes at 15psi.

Ampicillin was made up as a 1000x stock at 100mg/ml in distilled water and stored at -20°C.

2.4.2 Preparation of plasmid DNA

For generation of plasmids, competent bacteria (XL-1 blue) were transformed using purified plasmid DNA (50-100ng). This was added to 200µl of competent bacteria in a 1.5ml eppendorf and incubated on ice for 30 minutes. Following this, bacteria was heat shocked for 90 seconds at 42°C and then placed briefly on ice. 800µl of LB broth was then added and samples were incubated in a shaker at 37°C for one hour. For each transformation the bacteria were plated by spreading 200µl and 20µl onto two different agar plates (containing the appropriate antibiotic) and a final plate of concentrated bacteria. Plates were then incubated overnight at 37°C.

2.5 Microscopy

LCLs were harvested, washed and resuspended in PBS, spotted onto glass slides and air dried or cytopspun onto slides. Cells were then fixed in 4% paraformaldehyde (PFA) and permeabilised in 0.5% Trition X-100. Residual PFA was absorbed by incubating slides in 20μM glycine. Cells were then incubated with antibodies for 16 hours at 4°C. After rinsing with PBS, slides were incubated with alexa-fluor 594-conjugated goat anti-rabbit antibody for 1 hour at 37°C. Vectorshield + dapi mounting solution (vector labs Peterborough), was applied and cells were analysed by fluorescent microscopy.

2GL9 cells were cultured on microscopy slides fixed in 4% paraformaldehyde (PFA). Residual PFA was absorbed by incubating slides in 20μM glycine. Vectorshield + dapi mounting solution (vector labs Peterborough), was applied and cells were analysed by fluorescent microscopy.

3. ANALYSIS OF WILM'S TUMOUR 1 AS A TARGET ANTIGEN FOR T CELL RECOGNITION

As previously discussed, WT1 is a classic example of a cellular tumour antigen. It is expressed at low levels in a small subset of normal cells but is over expressed in a wide range of tumours and is therefore an attractive target for immunotherapy. Indeed, WT1 has recently been listed as the number one antigen in a priority-ranked list of cancer vaccine target antigens (Cheever *et al.*, 2009). WT1 contains both $CD8^+$ and $CD4^+$ T cell epitopes and has been used as a tumour antigen in many clinical trials.

Although it is considered to be an excellent therapeutic target, relatively few $CD8^+$ and $CD4^+$ T cell epitopes have been defined in WT1. Furthermore, little is known about how WT1 is processed and presented within tumour cells. As well as being expressed in primary tumour tissue a number of papers have reported that WT1 is expressed in transformed cell lines. One such paper by Kobayashi *et al.* reported that WT1 is expressed in EBV transformed lymphoblastoid cells lines (LCLs) (Kobayashi *et al.*, 2006). These cells are transformed B cells that express both HLA class I and class II molecules and have intact and fully functioning antigen processing and presentation pathways. LCLs therefore represent an ideal model in which to study the processing and presentation of natively expressed WT1.

A second aim of this work was to further study the expression of WT1 in other types of cancer. It is known that EBV transformation of B cells is concurrent with the initiation of WT1 expression *in-vitro* but WT1 expression in EBV-related malignancies has not been

well studied. Therefore I aimed to analyse the level of expression of WT1 in a range of EBV-associated malignancies.

A third aim of this study was to further investigate the range and frequency of WT1 T cell responses in healthy individuals as well as cancer patients. This work may identify new $CD8^+$ and $CD4^+$ T cell epitopes in the WT1 protein since few have been described in the literature.

The ultimate aim of this work was to develop strategies to enhance the immunogenicity of cellular tumour antigens using WT1 as a therapeutically relevant model. In this regard, WT1 is located in the nucleus of normal cells and some tumours but in other tumours WT1 has been observed in the cytoplasm (Drakos *et al.*, 2005). As previously discussed, protein subcellular localisation may be an important factor in the recognition of antigens by $CD4^+$ T cells. A previous report indicates that the pharmacological compound forskolin can relocalise WT1 from the nucleus to the cytoplasm (Ye *et al.*, 1996). I aim to further study this finding in WT1 positive antigen presenting cells and investigate how this relocalisation affects the processing and presentation of WT1. I hypothesise forskolin-mediated relocalisation of WT1 will enhance presentation of the antigen to WT1-specific $CD4^+$ T cells.

3.1 Expression of WT1 in LCLs

Kobayashi *et al.* detected WT1 protein in three LCLs, each derived from a different donor. It is not however known if WT1 is expressed in all LCLs. Therefore in order to further define the experimental system, the variability of WT1 expression between different LCLs was tested. The expression of WT1 in seven LCLs, each derived from a

different donor was assessed by western blotting using the WT1-specific antibody 6F-H2 (Figure 3.1a). The CML line K562 was included as a positive control as it had previously been reported to express WT1 (Kobayashi *et al.*, 2006). A band with approximate molecular weight 52kDA was detected in four of the seven LCLs (LCLs 2-5) and was also detected in K562. This was considered to be the 497 amino acid, canonical WT1 isoform. Levels of expression of this canonical WT1 isoform varied between the four positive LCLs, with LCL3 showing the lowest level of expression. The remaining three LCLs showed no expression of canonical WT1; however bands of different molecular weight were detected in all seven LCLs. These extra bands could reflect the presence of alternative transcripts as well as post transcriptional modification of WT1 or they may represent non-specific recognition by the 6F-H2 antibody.

To confirm this result, the same seven LCLs were tested for WT1 expression using a second WT1-specific antibody, C-19. This time the melanoma cell line, 624-mel was used as a negative control and again, K562 was used as a positive control (Figure 3.1b). As previously seen, four LCLs (LCLs 2-5) showed expression of canonical WT1. These were the same four LCLs that showed expression of canonical WT1 using 6F-H2. Bands of different molecular weight were again detected in all LCLs with the exception of LCL 7 where no bands were observed. One of these alternative bands, at a low molecular weight, was also detected in 624-mel. This suggests this band was due to non-specific recognition by the antibody. It should be noted that the band detected in K562 using C-19 appeared to be of slightly lower molecular weight than the canonical WT1 band detected in LCLs. This could suggest that C-19 was detecting a different WT1 isoform in the different cell lines.

These data show that WT1 expression varies between LCLs however it is not known if WT1 expression is stable or if the variation between LCLs observed is due to WT1 expression changing over time. To investigate the stability of WT1 expression, samples of cells were taken over 14 days from two LCLs, one of which had tested positive for WT1, the other had not. The expression of WT1 in each sample was assessed by western blotting using the WT1-specific antibody 6F-H2. Figure 3.2a shows WT1 expression in LCL 4, previously shown to be WT1 positive. Canonical WT1 was detected at all time points across 14 days and level of expression appeared to be similar. Figure 3.2b shows expression in LCL 1, previously shown to be WT1-negative. No canonical WT1 expression was detected at any time point, though it should be noted bands of low molecular weight were observed at all time points.

These two experiments show that levels of canonical WT1 remained constant over all time points showing that these differences in expression are fixed for each LCL.

3.2 Expression of WT1 in EBV-associated malignancies

The previous experiments confirm that WT1 is expressed in some LCLs; such LCLs therefore could be used to study the processing and presentation of WT1.

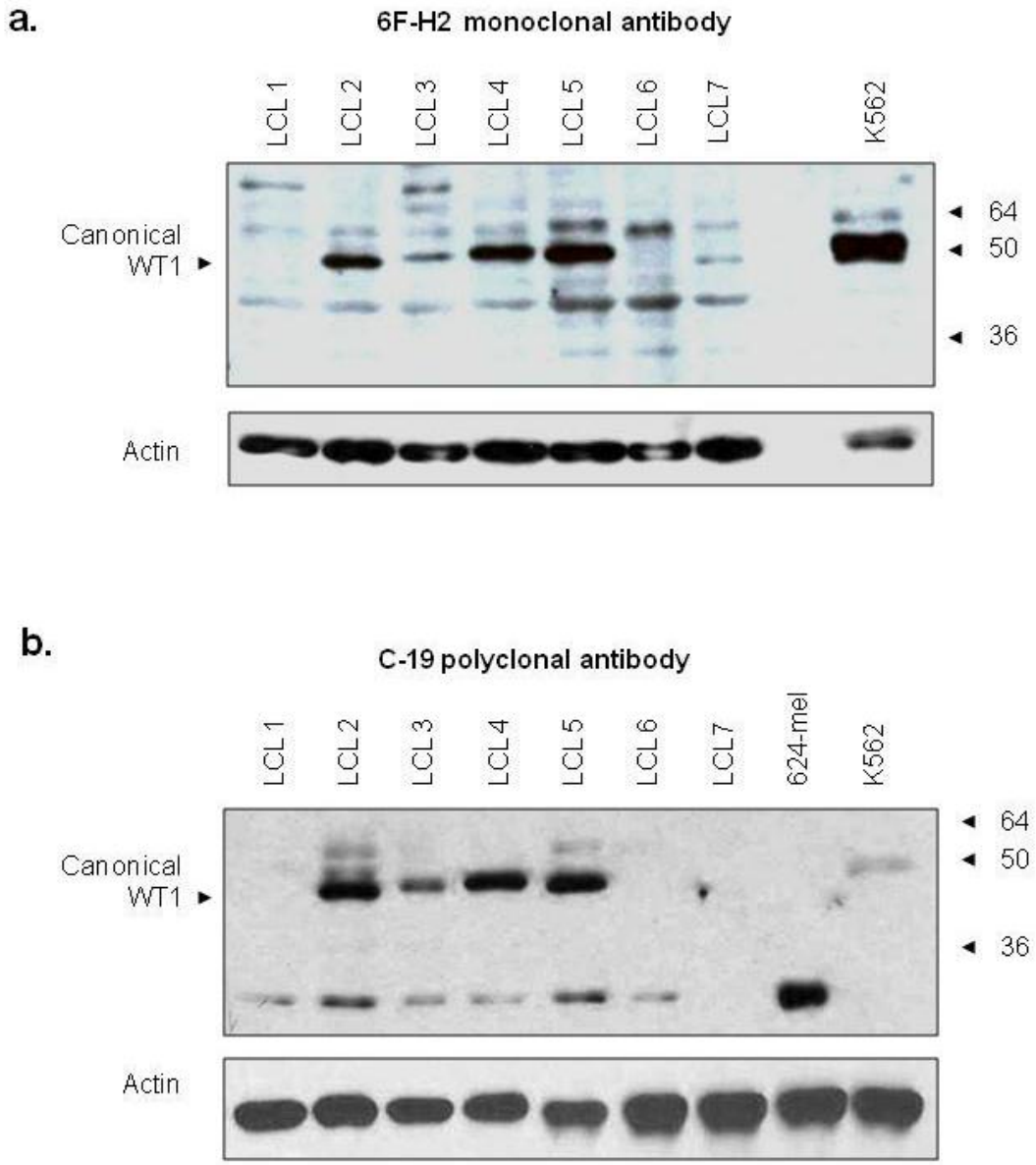


Figure 3.1 Expression of WT1 protein in LCLs

Western blot analysis of WT1 protein expression in seven different LCLs. **a**. WT1 protein was detected using the WT1-specific monoclonal antibody 6F-H2. Canonical WT1 was detected in four of seven LCLs. Alternative bands were detected in all seven LCLs. **b**. WT1 protein was detected using the WT1-specific polyclonal antibody C-19. Canonical WT1 was detected in the same four LCLs. Alternative transcripts were detected in all LCLs except LCL7. 624-mel was used as a negative control and K562 as a positive control. Expression of actin protein was used as a loading control.

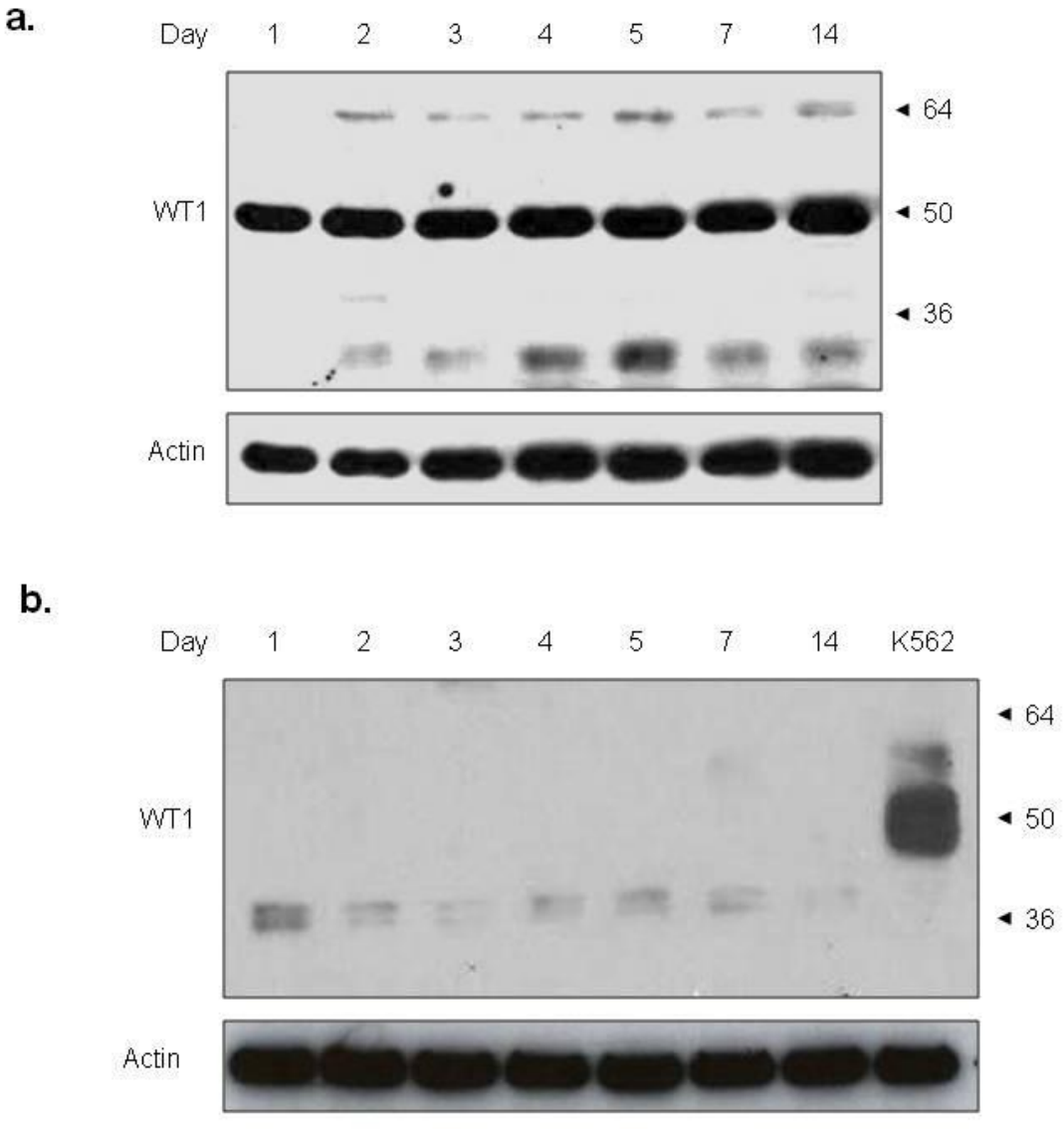


Figure 3.2 Expression of WT1 protein in LCLs over time

Western blot analysis of WT1 protein expression in two different LCLs, using the WT1-specific monoclonal antibody 6F-H2. **a**. Expression of WT1 protein in the previously WT1-positive LCL, LCL4. **b**. Expression of WT1 protein in the previously WT1-negative LCL, LCL1. Expression of WT1 protein remained constant in both LCLs across all time points. K562 was used as a positive control. Expression of actin protein was used as a loading control.

I next examined whether other EBV-associated malignancies express WT1.

To determine if WT1 is expressed in other EBV-associated malignancies, a range of EBV-transformed NPC and NK/T cell lymphoma cell lines were assayed by western blot analysis for expression of WT1 protein. As WT1 expression varied between LCLs multiple cell lines for each malignancy were tested. WT1 expression in three NPC lines and five NK/T cell lymphoma cell lines was tested using the WT1-specific antibody 6F-H2 (Figure 3.3a). The three NPC cell lines HK1, HONE-1 and HOU-1 showed no expression of WT1 protein. The five NK/T cell lymphoma lines MEC04, NK1, SNK6, SNK10 and SNT16 were also negative for WT1. The negative control 624-mel showed no expression and canonical WT1 was detected in the positive control K562 cells. This result showed that WT1 is not expressed in any of the EBV-transformed cell lines established from NPC or NK/T cell lymphoma.

3.3 Expression of WT1 in other haematological malignancies

As cell lines from EBV-associated malignancies proved to be negative for WT1 protein, cell lines from a wider range of non-EBV-associated haematological malignancies were tested for WT1 expression. Previous reports have shown WT1 is expressed in both acute myeloid leukaemia (AML) and myeloma (Hatta *et al.*, 2005; Schmid *et al.*, 1997). Cell lines from these malignancies were therefore analysed for WT1 expression with the aim of further studying WT1 expression in these malignancies as well as identifying tumour cell lines that could act as targets in T cell assays.

Four AML lines and three myeloma lines were analysed for WT1 protein expression by western blotting, using the 6F-H2 antibody (Figure 3.3b). Canonical WT1 was detected

in three of four AML lines, HL60, KG1a and NB4. Levels of expression were high and comparable to levels in the positive control K562. The fourth AML line NOMO1 showed no expression of WT1. No WT1 expression was observed in any of the three myeloma lines tested, H929, JJN3 and U266.

3.4 WT1 expression by qRT-PCR

As shown in Figure 3.1, when LCLs are subjected to western blot analysis a different pattern of bands was observed when two different WT1-specific antibodies were used. WT1 is known to have many different splice variants (Figure 3.4). The 6F-H2 antibody is monoclonal and specific for a peptide in exon 1, whereas the C-19 antibody is polyclonal and raised against a peptide at the C-terminus of WT1. It is possible that the different antibodies detect different isoforms of WT1 and it is also possible that some isoforms are not detected due to alternative exon usage and amino acid changes in the C-terminus. Due to the ambiguous nature of the western blot analysis, qRT-PCR was carried out on matched samples to confirm the data obtained.

mRNA was extracted from the same samples of LCLs used in previous western blotting experiments. Levels of WT1 mRNA were then assayed by a commercial qRT-PCR assay spanning exon 3 and 4, designed to detect all known transcripts of WT1 (Figure 3.5a). Interestingly no WT1 transcripts were detected in any of the seven LCLs tested despite previous results showing WT1 protein was expressed in four of these lines. WT1 transcripts were detected in the positive control cell line K562 showing the assay was functioning correctly and capable of detecting WT1 mRNA.

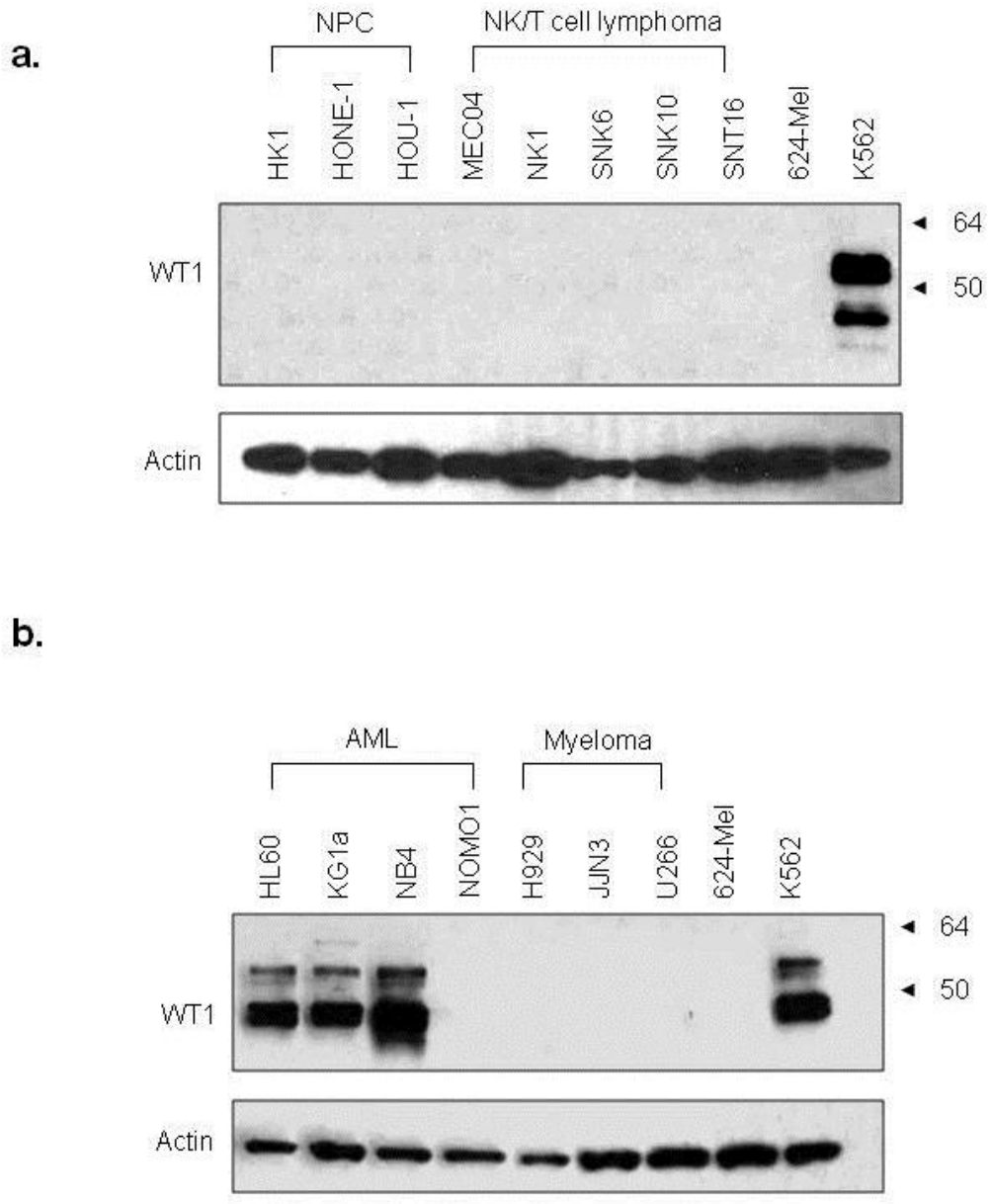


Figure 3.3 Expression of WT1 protein in tumour cell lines

Western blot analysis of WT1 protein expression in tumour cell lines, using the WT1-specific monoclonal antibody 6F-H2. **a**. Expression of WT1 protein in cell lines from EBV-associated malignancies. WT1 protein was not detected in any of the lines tested. **b**. Expression of WT1 protein in cell lines from other haematological malignancies. WT1 was detected in three of four AML lines tested. No WT1 was detected in any Myeloma lines. 624-mel was used as a negative control and K562 as a positive control. Expression of actin protein was used as a loading control.

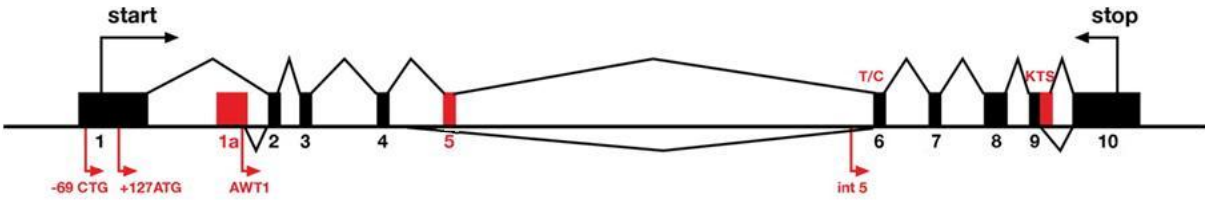


Figure 3.4 Human WT1 gene locus

Schematic of the human WT1 gene locus on chromosome 11 at 32409325 – 32457087. To date 26 isoforms of WT1 have been descirbed. The locus contains four alternative start codons -69 CTG, +127ATG, AWT1 and int 5. Exon variants result from the use of an alternative exon 1a or skipping exon 5. KTS isoforms exclude three amino acids (KTS) in exon 9. A final alternative isoform is generated through RNA editing in exon 6 (T/C). (Adapted from Hohenstein and Hastie 2006)

To further investigate this difference between results obtained by western blotting and those obtained by qRT-PCR all other cell lines previously tested for WT1 protein expression were assayed for WT1 mRNA transcripts (Figure 3.5b and c).

As previously shown, all NPC and NK/T cell lymphoma lines were negative for any WT1 transcripts (Figure 3.5b). qRT-PCR data for AML and myeloma lines matched that obtained by western blotting in that three AML lines were WT1 positive and the remaining lines including all myeloma lines were negative (Figure 3.5c). However subtle differences were observed; the AML line NB4 showed the highest level of protein expression but the lowest level of WT1 mRNA transcript.

Taken together these data could suggest that an alternative mRNA transcript is being expressed in LCLs and perhaps in AML lines also.

3.5 WT1 expression by an alternative RT-PCR assay

As the commercial qRT-PCR assay was unable to detect WT1 mRNA in lines where WT1 protein had previously been detected an alternative RT-PCR assay was used to confirm the presence of WT1 mRNA in these lines. Kobayashi *et al.* previously detected WT1 mRNA in multiple cell lines, including LCLs, using an RT-PCR assay. The assay uses a forward primer in exon 7 and a reverse primer in exon 10.

To confirm WT1 expression in LCLs the same mRNA samples used in qRT-PCR experiments were used in the "Kobayashi" RT-PCR. Figure 3.6a shows control experiments conducted to confirm the assay's specificity. Lane one shows a negative control where water rather than RNA was added to the cDNA synthesis reaction.

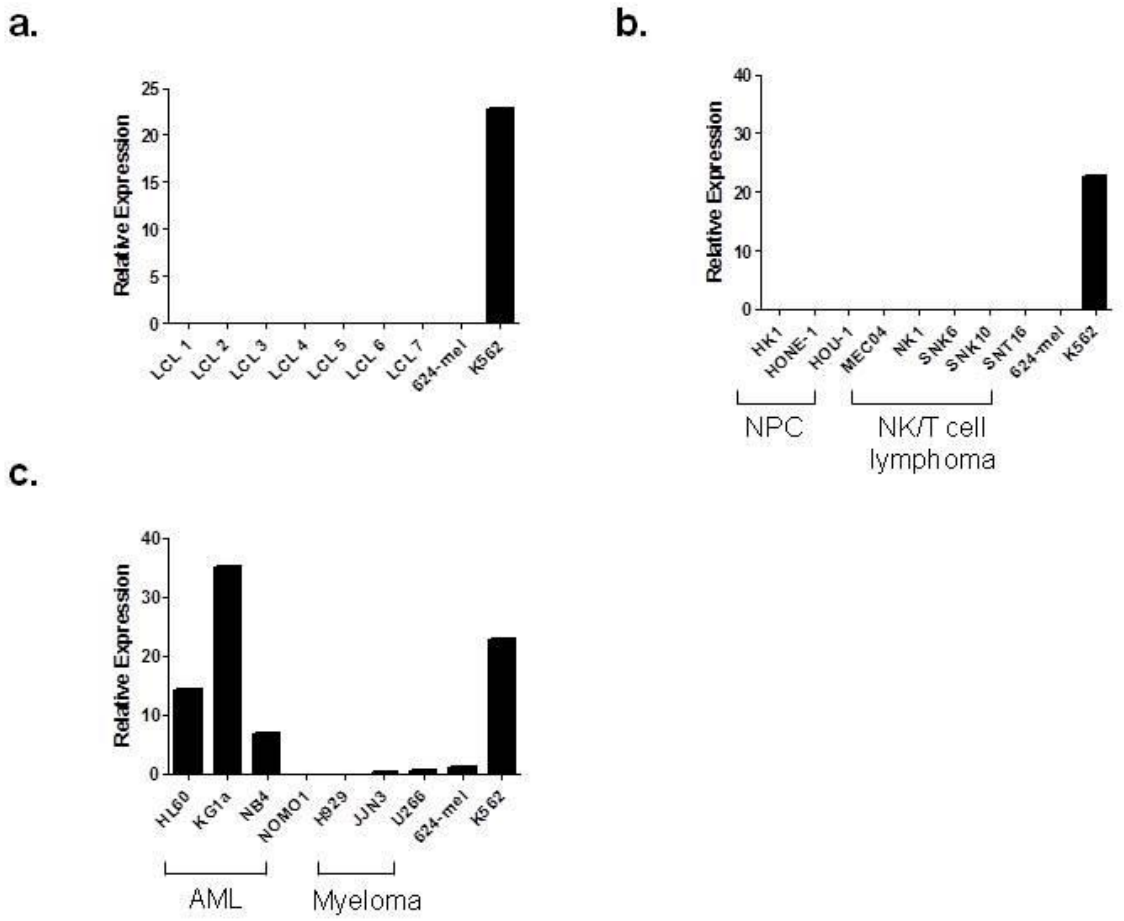


Figure 3.5 Expression of WT1 mRNA in LCLs and tumour cell lines by qRT-PCR

Quantitative RT-PCR analysis of WT1 mRNA in cell lines. **a.** WT1 mRNA levels in seven different LCLs. WT1 mRNA was not detected in any LCLs. **b**. WT1 mRNA levels in cell lines from EBV-associated malignancies. No WT1 mRNA was detected in any of the lines tested. **c**. WT1 mRNA levels in cell lines from other haematological malignancies. WT1 mRNA was detected in three of four AML lines. Levels in myeloma lines were below that of the negative control. All results are expressed relative to the level of the housekeeping gene GAPDH. 624-mel was used as a negative control.

No RT controls, where the cDNA synthesis reaction was carried out without reverse transcriptase are shown in lanes two and three. As the K562 no RT control was negative this shows the assay does not detect genomic DNA and only detects mRNA. No WT1 mRNA was detected in the negative control 624-mel. WT1 mRNA was detected in K562; a PCR amplicon of approximately 500bp was observed as would be expected. The sensitivity of the assay was also tested by diluting the quantity of cDNA in the PCR reaction. Standard cDNA input was 5µg of cDNA, when K562 cDNA input was reduced to 40ng and then 8ng a PCR amplicon could still be detected.

Figure 3.6b shows the results of Kobayashi RT-PCR assay to detect WT1 mRNA in the same seven LCL mRNA samples used in previous experiments. Four out of seven LCLs gave a PCR amplicon of the expected size. These were the same four LCLs that showed expression of WT1 protein when analysed by western blot.

The cell lines from the EBV-associated malignancies were again tested for WT1 expression and were again all negative (data not shown).

Figure 3.6c shows the results of Kobayashi RT-PCR assay to detect WT1 mRNA in the same four AML cell lines previously tested. Three of the four lines gave a PCR amplicon of the expected size. These were again the same three lines that showed expression of WT1 protein when analysed by western blot. The fourth line, NOMO1, was again negative for WT1 mRNA confirming all previous findings.

Interestingly, when the myeloma lines were assayed for WT1 mRNA by the Kobayashi RT-PCR, two lines, H929 and U266 gave a PCR amplicon of the expected size. This is despite them both testing negative for WT1 in all previous experiments.

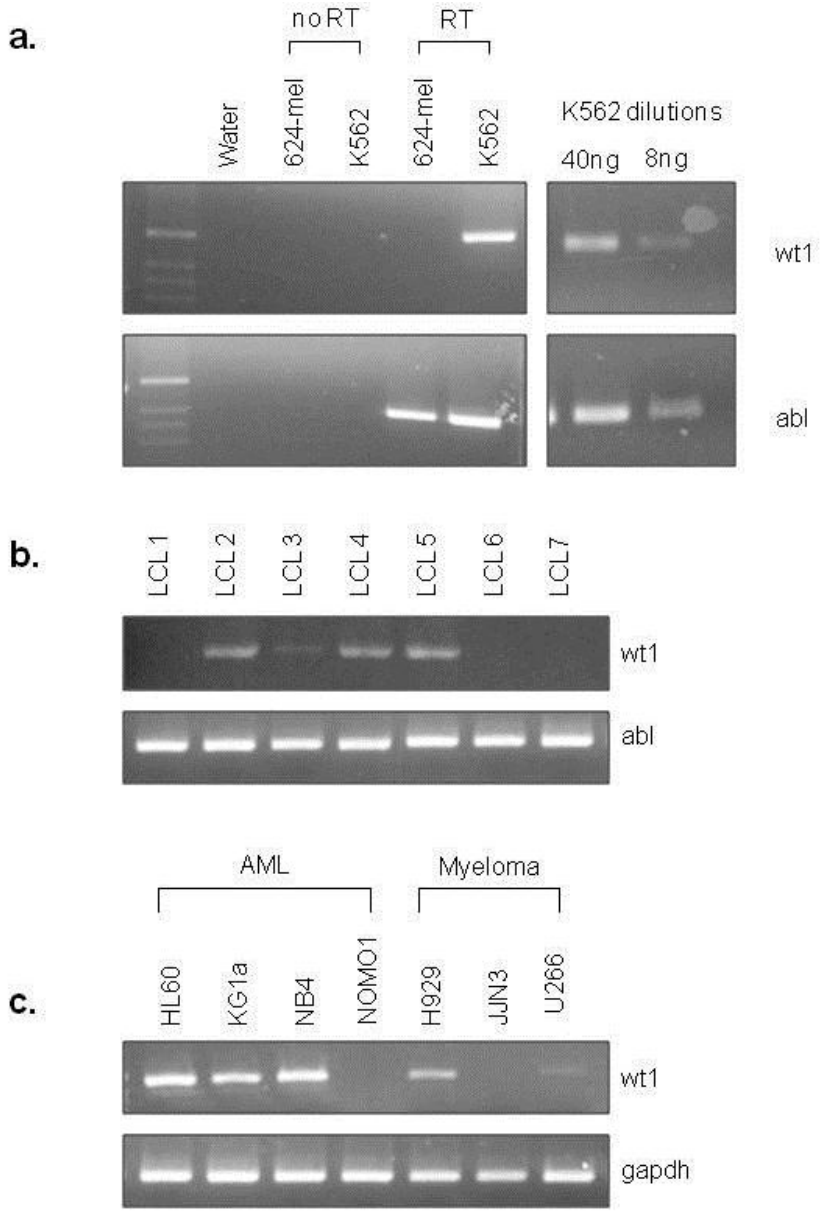


Figure 3.6 Expression of WT1 mRNA in LCLs and tumour cell lines by an alternative RT-PCR assay

Analysis of WT1 mRNA in cell lines using an alternative "Kobayashi" RT-PCR assay. Standard cDNA input was 5µg. **a.** A PCR amplicon of size 482 bp was detected in K562 but was absent in water, no reverse-transcriptase (RT) and 624-mel negative controls. The same amplicon was observed when K562 cDNA input was reduced to 40 and then 8ng. **b.** Four of seven LCLs tested were WT1 positive. **c.** Three of four AML lines and two of three myeloma lines were also WT1 positive. Abl or gapdh were used as housekeeping gene controls for RT-PCR.

These data suggest that the Kobayashi RT-PCR assay can: i) detect an alternative WT1 transcript in LCLs, one that is not detected by a commercial qRT-PCR assay and ii) can detect an mRNA transcript in myeloma cell lines that is not detected by two different antibodies in western blot analysis or by the commercial qRT-PCR assay.

3.6 The effect of forskolin on WT1 expression and localisation

As described in the introduction to this section, one of the aims of this work was to study how the subcellular localisation of WT1 affects the processing and presentation of WT1-specific epitopes. A previous paper reported that forskolin can relocalise WT1 (Ye *et al.*, 1996). Ye *et al.* showed that 24h forskolin treatment caused cytoplasmic localisation of WT1 in 98% of C2 myoblast cells. It was not known if forskolin will relocalise WT1 in other cell types or what effect such relocalisation would have on the processing of WT1. To begin to address these questions the effect of forskolin upon WT1 expression in LCLs was investigated.

An LCL previously shown to be WT1 positive by western blotting was treated with forskolin and then analysed for WT1 expression by immunofluorescence microscopy, using the WT1-specific antibody 6F-H2. To allow greater differentiation of nuclear and cytoplasmic regions of the LCLs (which have relatively small amounts of cytoplasm) the cells were cytospun onto microscopy slides. Cytospinning causes cells to flatten and spread out permitting easier visualisation of their cytoplasm. Figure 3.7 shows the results of this experiment. Bright WT1 staining was observed in the majority of cells. All WT1 staining was concurrent with Dapi staining showing WT1 was localised in the nucleus. Following treatment with 50μM forskolin levels of WT1 expression were

greatly reduced in LCLs. Some dim staining was observed in cells but no staining was observed in the cytoplasm.

One explanation for this result could have been that WT1 was relocalised into the cytoplasm whereupon it was degraded. To test this theory forskolin-treated LCLs were incubated with 50mM chloroquine, a chemical that raises the pH of endosomes and lysosomes thereby inhibiting degradation of proteins by cathepsins. Addition of chloroquine increased levels of WT1 protein, reversing the effect caused by forskolin. No clear WT1 staining was observed in the cytoplasm but this may be due to the difficulty of imaging cytoplasm in LCLs cells.

3.7 Measuring WT1-specific T cell responses in healthy donors and Multiple Myeloma patients by IFN-γ ELIspot assay

In order to examine the antigen processing and presentation of WT1 it was necessary to generate T cell clones specific for epitopes within the protein. Several reports in the literature suggest WT1-specific $CD4^+$ T cell clones can be isolated, not only from patients with malignancies such as multiple myeloma, but also from healthy donors (Azuma *et al.*, 2002; Doubrovina *et al.*, 2004; Kobayashi *et al.*, 2006). Although WT1-specific T cell clones have been generated from multiple donors the number of epitope-specificities of WT1-specific T cells is relatively low. Also the range and frequency of WT1 T cell responses in healthy donors and patients has not been studied. Therefore I first aimed to measure the frequency of WT1 T cell responses in a number of donors.

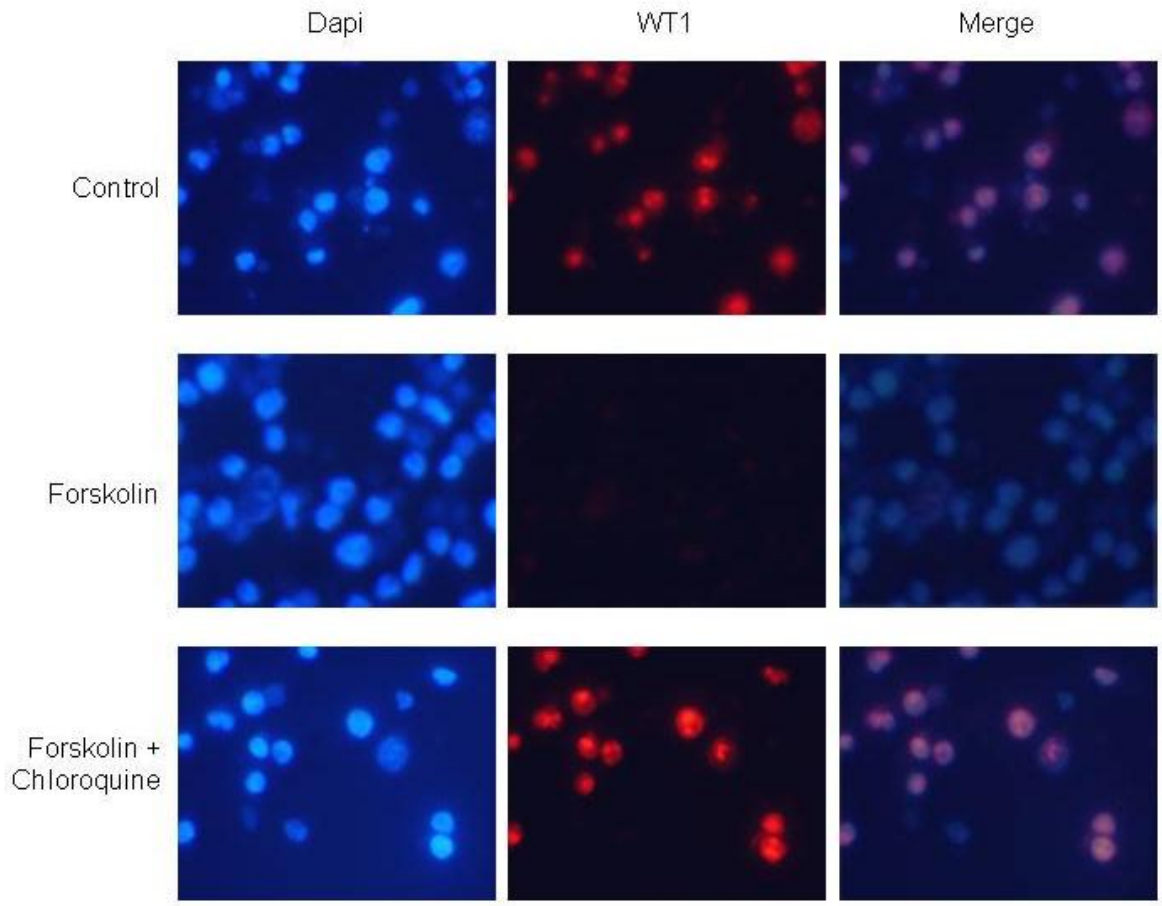


Figure 3.7 Expression and localisation of WT1 in LCLs.

WT1 positive LCLs were fixed and stained with the WT1-specific antibody 6F-H2 (red). The nucleus of cells was stained with Dapi (blue). The cells were analysed by immunofluorescence microscopy. Bright, WT1 staining was observed in the nucleus of control cells. 24h, 50μM Forskolin reduced WT1 staining. Treatment of forskolin incubated LCLs with 50mM chloroquine reversed this effect and bright WT1 staining was observed in the nucleus of these cells.

To examine whether WT1 T cell responses could be detected *ex-vivo,* I used a highly sensitive IFN-γ ELIspot assay. In these experiments pepmixes were used to provide the antigen stimulus to the T cells. Pepmixes consist of 15mer peptides that span the entire primary sequence of an antigen; each peptide overlaps the previous one by 10 amino acids. The advantage of using pepmixes is that they allow all responses to an antigen to be stimulated in a single ELIspot well, increasing the sensitivity of the assay.

In cases where several weak T cell responses exist, combining them together in a single well may allow them to be detected, whereas individually each may be below the limit of detection for the assay. This pepmix ELIspot assay has been shown to be highly sensitive in detecting weak responses in previous reports (Hui *et al.*, 2013).

T cell responses were measured in 16 healthy donors and 18 multiple myeloma patients. A pepmix of the self-protein actin was used as a negative control. PHA, a non-specific stimulator of T cells was used as a positive control. A pepmix of Influenza A nuclear protein was used as a second positive control as most donors should have a detectable T cell response to this protein. A pepmix of WT1 was used to test for WT1 T cell responses.

Figure 3.8a shows representative ELIspot results from three healthy donors and three myeloma patients. Figure 3.8b shows a summary off all 16 healthy donors (left) and all 18 myeloma patients (right). Responses to the negative control actin were low in all donors, with a mean of 6 spots/10^6 PBMCs in healthy donors and 3 spots/10^6 PBMCs in patients. Responses to PHA were high and in many cases separate spots were impossible to count as they merged together. In such cases responses to PHA were reported as 1000 spots/10^6 PBMCs. Responses to Influenza A nuclear protein varied between all donors. In healthy donors responses were relatively high with a mean of 214 and a range 24 to

870 spots/10^6 PBMCs. Responses to Influenza were much lower in multiple myeloma patients with a mean of 22 and a range 5 to 51 to 5 spots/10^6 PBMCs.

Responses to WT1 protein were not detected in any of the 16 healthy donors or 18 multiple myeloma patients tested in the *ex-vivo* assay. Responses were below the level of the actin negative control in all cases.

3.8 Measuring WT1 T cell responses by cultured ELIspots assay

The lack of a detectable WT1 T cell response in the *ex-vivo* ELIspot assay may be due to responses being too weak to measure directly. Another possibility is that the response is confined to central memory T cells which do not respond immediately following antigenic stimulation. *Ex-vivo* ELIspots are limited to the detection of effector memory T cells, which are generated following antigen-induced clonal expansion *in-vivo.*

Therefore although *ex-vivo* ELIspot assays revealed no responses to WT1 in either healthy donors or patients it is possible that these individuals did possess WT1-specific T cells but they were not detected due to limitations in the methods applied. I therefore performed cultured ELIspots on PBMCs from the same previous donors. This involved stimulating PBMCs with specific antigens prior to testing in IFN-γ ELIspot assays. This procedure expands all antigen-specific T cell responses and aids in the detection of low frequency as well as central memory or naive T cells.

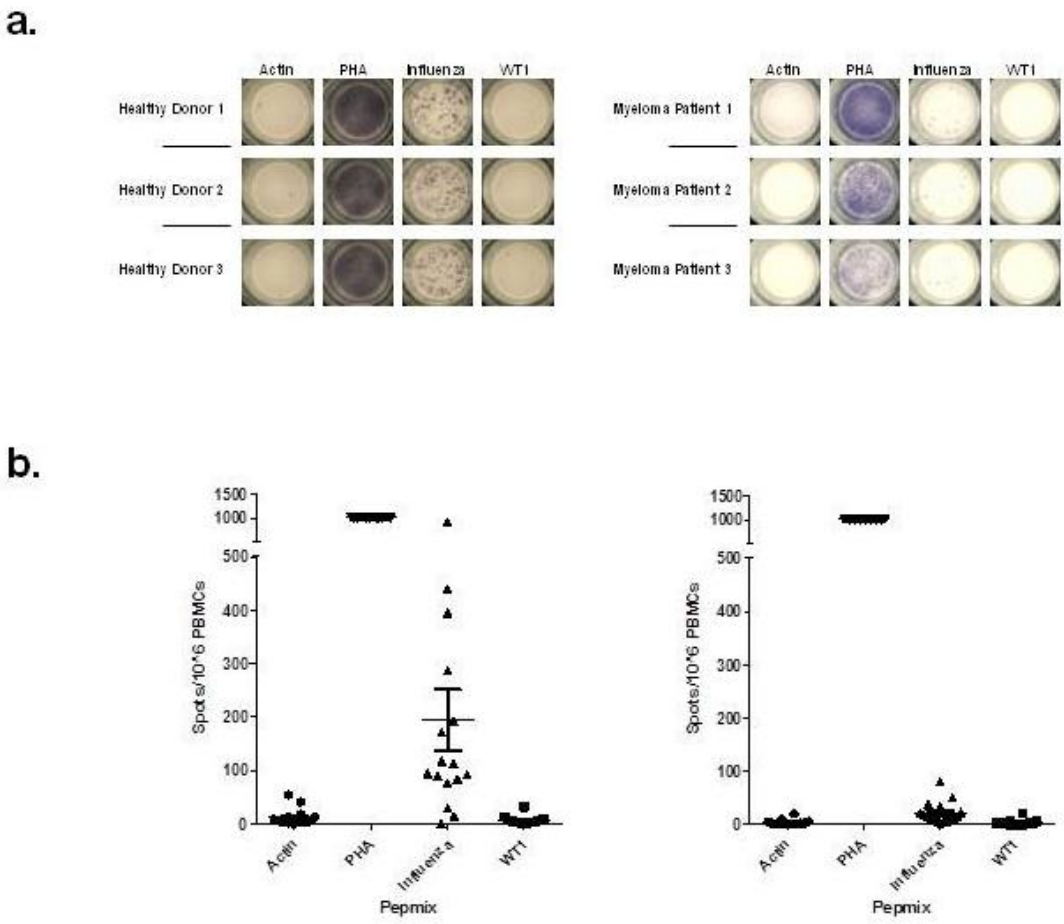


Figure 3.8 T cell responses in healthy donors and multiple myeloma patients by *ex-vivo* ELIspot

PBMCs from healthy donors and multiple myeloma patients (500,000/well) were tested in ELIspot assays using indicated pepmixes or PHA as a control. **a.** Representative ELIspot assay from three healthy donors and three myeloma patients are shown. T cell secretion of IFN-γ is visualised as dark spots. **b**. Results from ELIspot assay in healthy 16 donors (left) and 18 multiple myeloma patients (right). T cell responses were detected against influenza A nuclear protein in the majority of healthy donors and some patients. WT1 responses were below the level of actin controls in all donors. Bars represent mean +/- standard error.

PBMCs from a selection of the same healthy donors and myeloma patients were stimulated with pepmixes. As a positive control PBMCs from each donor were stimulated with a pepmix of the EBV protein EBNA1. A second peptide-stimulated line was established from each donor using a WT1 pepmix. Cells were then cultured with IL-2 and IL-7 for 12 days. Cells were then tested for antigen specificity in a standard IFN-γ ELIspot assay.

Pepmixes were dissolved in dimethyl sulfoxide (DMSO), so lines were tested against DMSO as a negative control. PHA was used as a positive control. EBNA1 and WT1 pepmixes were used to test for antigen-specific T cell responses.

Figure 3.9a shows ELIspot results from EBNA1 pepmix stimulated lines derived from five healthy donors (left) and five myeloma patients (right). Responses to DMSO negative control were low in all lines, with a mean of 45 spots/10^4 cells in healthy donors and 22 spots/10^4 PBMCs in patients. Responses to PHA were high and again in many cases separate spots were impossible to count. In such cases responses to PHA were reported as 1000 spots/10^4 PBMCs. Responses to EBNA1 pepmix protein were high in all 10 lines, with a mean of 262 spots/10^4 cells in healthy donors and 117 spots/10^4 PBMCs in patients.

Figure 3.9b shows ELIspot results from WT1 pepmix stimulated lines derived from five healthy donors (left) and five myeloma patients (right). Responses to DMSO negative control were low in all lines, with a mean of 11 spots/10^4 cells in healthy donors and 17 spots/10^4 PBMCs in patients. Responses to PHA were again reported as 1000 spots/10^4 PBMCs. Responses to WT1 pepmix protein were below or comparable to negative

controls in all 10 lines, with a mean of 10 spots/10^4 cells in healthy donors and 20 spots/10^4 PBMCs in patients.

3.9 Isolation of WT1-specific T cells

Results from the ELIspots assays suggested that WT1 T cell responses were less common in our donors than might have been expected based on reports in the literature. Despite this fact, because these reports suggested WT1-specific T cell clones could be isolated from healthy donors and cancer patients I attempted to prepare such clones from the healthy donors and multiple myeloma patients I had screened. A number of different techniques were used to try to stimulate and isolate WT1-specific T cells, a summary of which is shown in Table 3.1.

A number of different sources of WT1 antigen were used. Peptides representing previously defined WT1 T cell epitopes as well as a peptide library spanning the whole WT1 protein were first used to stimulate PBMCs. The pepmixes used for ELIspot assays were also used as a source of antigen. These were either added to PBMCs to stimulate WT1-specific T cells directly or loaded onto APCs such as B cells and DCs. Peptide loaded APCs were then cultured with PBMCs to stimulate responses. Two different markers of T cell activation were used to define peptide-specific T cells; these were IFN-γ and CD137. IFN-γ production was monitored by ELISA and CD137 positive cells were isolated by magnetic enrichment of cells labelled with a CD137-specific antibody. As controls, peptides and pepmixes of the two EBV antigens EBNA1 and LMP1 were used to stimulate separate PBMCs from the same donors. In all cases EBNA1 and LMP1-specific T cells were isolated from all healthy donors. While all these methods were capable of generating EBV-specific T cell clones, no WT1-specific T cell clones were isolated.

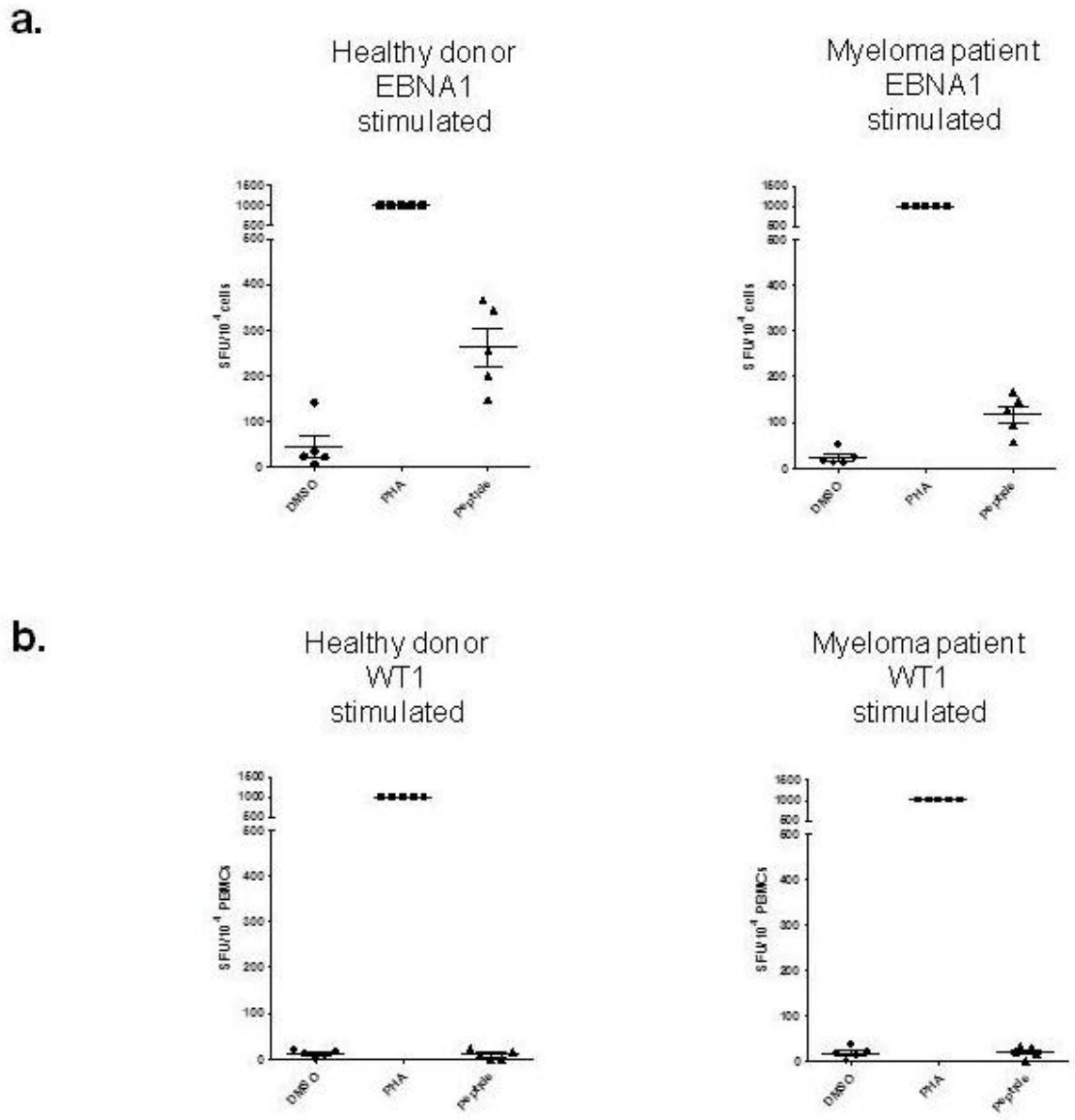


Figure 3.9 T cell responses in healthy donors and multiple myeloma patients by cultured ELIspot assay.

PBMCs from healthy donors and multiple myeloma patients were stimulated with EBNA1 or WT1 pepmixes. After 12 days stimulated lines were tested in ELIspot assay using 5000 cells/well **a.** EBNA1 stimulated lines from 5 healthy donors and 5 multiple myeloma patients showed responses to EBNA1 peptides. **b.** WT1 stimulated lines from 5 healthy donors and 5 multiple myeloma patients showed no responses to WT1 peptides. Bars represent mean +/- standard error.

As attempts to generate WT1-specific clones using peptides and pepmixes had been unsuccessful an alternative antigen stimulation mechanism was sought. DNA and RNA transfection of APCs causes expression of target antigens within APCs. The antigens can then be processed and presented to T cells in a physiologically relevant manner.

PBMCs and APCs such as B cells and DCs were transfected with WT1 DNA or mRNA. This led to expression of WT1 in APCs which was confirmed by western blotting (data not shown). These WT1 positive APCs were then used to stimulate PBMCs from lab donors and myeloma patients. As a control, APCs were transfected with EBNA1 DNA or mRNA and used to stimulate PBMCs. The activation of peptide-specific T cells was monitored by IFN-γ ELISA. Control APCs were able to stimulate EBNA1-specific T cells but no WT1-specific T cells were generated by stimulation with WT1 positive APCs.

It was possible that over-expressed WT1 in APCs was poorly processed and presented by the endogenous class II pathway, which would limit the generation of WT1-specific $CD4^+$ T cells. To improve the processing of over-expressed WT1, an invariant chain (Ii) sequence was added to the WT1 DNA producing a WT1-Ii fusion protein. The Ii sequence should cause the WT1 protein to be rapidly imported into the endocytic pathway of APCs and should therefore significantly increase the processing of WT1, resulting in higher levels of WT1 peptide-MHC-II complexes on the surface of the APCs. APCs transfected with the Ii-WT1 fusion were used to stimulate PBMCs from healthy donors, but were still unable to generate WT1-specific T cells.

3.10 Tetramer detection of WT1-specific CD4$^+$ T cells

All previous attempts to study WT1-specific T cells have focussed on identifying them on the grounds of functional output. As these experiments all failed to identify WT1-specific T cells a different experimental method was sought.

MHC Tetramer detection involves the direct detection of epitope-specific T cells by distinguishing TCR specificity solely by its binding to peptide:MHC complexes. MHC tetramer technology has been used to great effect in studying the size, kinetics and phenotype of epitope-specific T cell responses. MHC-II tetramers have been used to investigate CD4$^+$ T cell responses to HIV, influenza, Hepatitis C and more recently EBV (Long *et al.*, 2013). MHC-II tetramers should therefore be able to identify WT1-specific T cells regardless of the T cell phenotype or functional ability.

Table 3.1 Methods for stimulation and isolation of WT1-specific T cells

Antigen source	Antigen presenting cell	Isolation marker	WT1 T cell clones generated	Control T cell clones generated
Peptide	non (direct stimulation)	IFN-γ	0	EBNA1 / LMP1
	PBMCs	IFN-γ	0	EBNA1 / LMP1
	LCLs	IFN-γ	0	EBNA1 / LMP1
	B cell blasts	IFN-γ	0	EBNA1 / LMP1
	DCs	IFN-γ	0	EBNA1 / LMP1
Pepmix	non (direct stimulation)	IFN-γ / CD137	0	EBNA1 / LMP1
	LCLs	IFN-γ / CD137	0	EBNA1 / LMP1
	DCs	IFN-γ / CD137	0	EBNA1 / LMP1
DNA	PBMCs	IFN-γ	0	EBNA1
	B cell blasts	IFN-γ	0	EBNA1
	DCs	IFN-γ	0	EBNA1
RNA	PBMCs	IFN-γ	0	EBNA1
	B cell blasts	IFN-γ	0	EBNA1
	DCs	IFN-γ / CD137	0	EBNA1

Three different tetramers were designed using previously published MHC class II epitopes from WT1. These tetramers were then used to measure the frequency of tetramer-binding $CD4^+$ T cells in $CD8^+$ T cell–depleted PBMCs, from healthy donors. Figure 3.10 shows an example of one such experiment. An MHC-II tetramer specific for the PRS epitope of the EBV antigen EBNA2 was used as a positive control and a clearly defined population of tetramer positive, $CD4^+$ T cells was detected (Figure 3.10a). However, when PBMCs were stained with three different WT1 MHC-II tetramers, no tetramer positive populations were detected (Figure 3.10b).

These experiments were repeated using PBMCs from 5 healthy donors and 5 multiple myeloma patients and in all cases WT1 tetramer-specific populations were below the level of no tetramer controls (data not shown).

3.11 Tetramer-associated magnetic enrichment of WT1-specific $CD4^+$ T cells

Though MHC-II tetramer detection of epitope-specific T cells is a highly sensitive method there is a lower limit to the method. It is possible that WT1 tetramer experiments failed to detect WT1-specific T cells because the T cells exist in very small numbers in the donors tested. One possible method for increasing the sensitivity of tetramer detection is Tetramer-Associated Magnetic Enrichment (TAME).

TAME enables detection of extremely low frequency epitope-specific T cells in PBMCs by enriching the tetramer-specific populations through magnetic sorting (Figure 3.11). PBMCs are first stained with MHC tetramers and then tetramer positive cells are stained with magnetically labelled antibodies specific for the tetramer fluorophore.

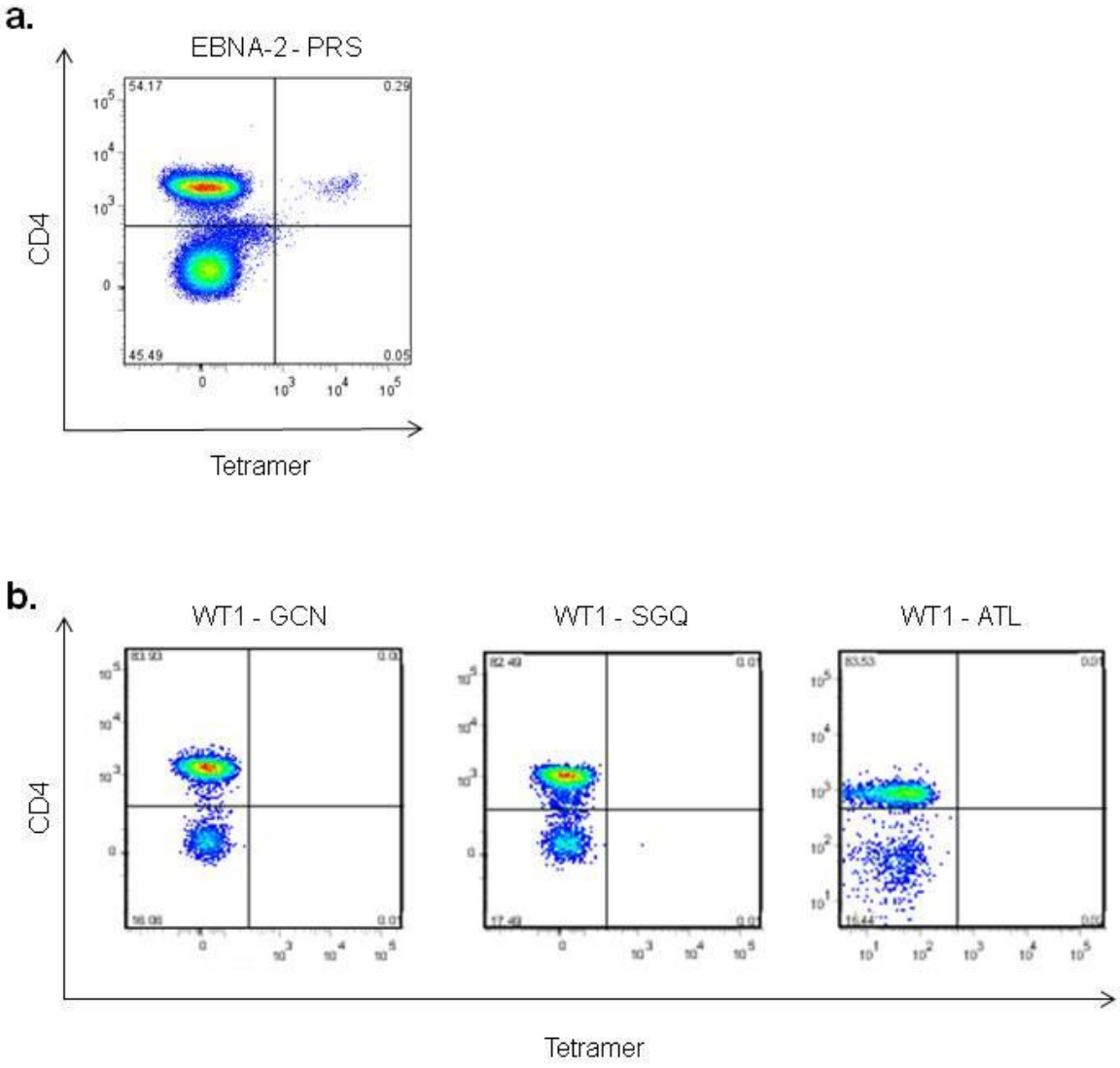


Figure 3.10 Tetramer detection of antigen-specific CD4⁺ T cells

The frequency of tetramer-binding CD4⁺ T cells in CD8⁺ T cell–depleted PBMCs, from healthy donors was assessed. **A.** A clearly defined population of cells was detected by the control EBNA-2 PRS tetramer. **B.** No tetramer binding cells were detecting in cells stained with WT1 tetramers

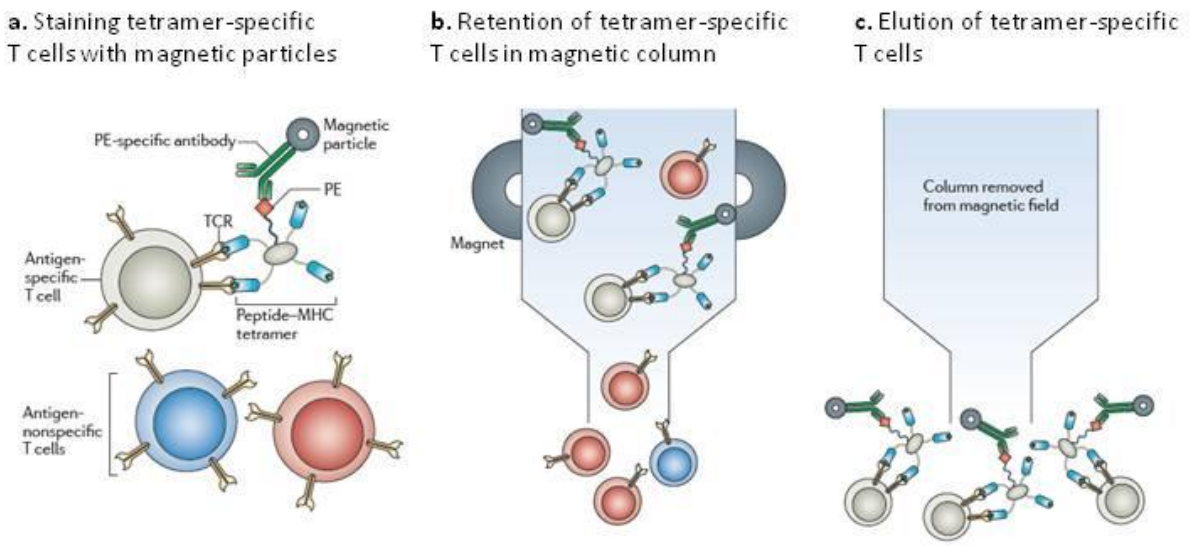


Figure 3.11 Tetramer associated magnetic enrichment

TAME enables detection of extremely low frequency epitope-specific T cells in PBMCs by enriching the tetramer-specific populations through magnetic sorting.(Figure adaptor from Davis *et al.*, 2011)

Stained PBMCs can then be magnetically sorted, enriching tetramer-specific T cells prior to flow cytometry analysis of cell populations.

PBMCs from healthy donors were stained with a control EBNA2 MHC-II tetramer or WT1 MHC-II tetramers and then magnetically sorted into two populations, negative and positive. A third population of unsorted cells was also included and all populations were analysed by flow cytometry (Figure 3.12). The EBNA2 MHC-II tetramer revealed a clearly defined population of $CD4^+$, tetramer-specific T cells in unsorted cells. After magnetic sorting this population had been enriched over 20-fold, 5% of cells in the positive sort were tetramer positive.

As seen in previous experiments, staining with the WT1–ATL MHC-II tetramer revealed no population of tetramer-positive cells. However after magnetic sorting a small population of tetramer positive $CD4^+$ T cells was observed in the positive sort fraction. The positive fraction contained 1% tetramer positive cells.

This result confirms the existence of a very rare population of $CD4^+$ T cells specific for the WT1 epitope ATL, in a single donor. This experiment was repeated on multiple donors using all three WT1 tetramers but tetramer positive cells were not detected in any other experiments.

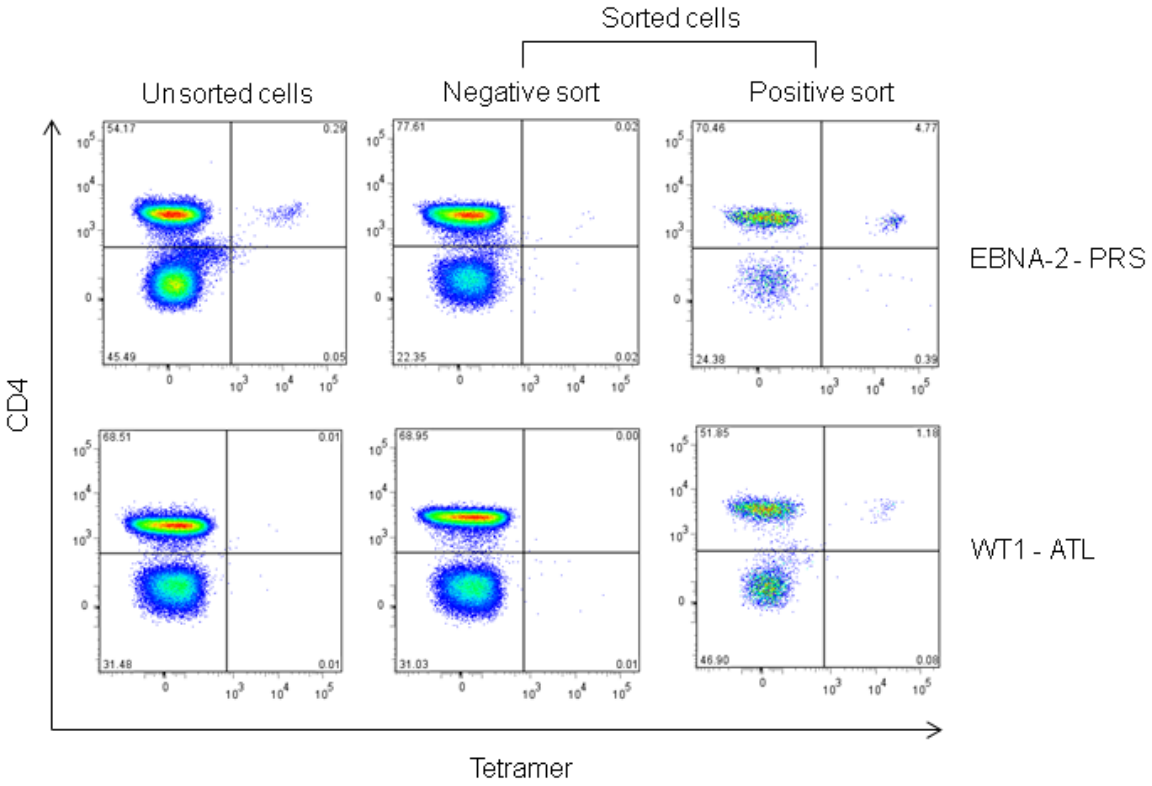


Figure 3.12 Identification of antigen-specific CD4+ T cells by TAME assay

PBMCs from healthy donors were stained with a control EBNA2 MHC-II tetramer or WT1 MHC-II tetramers and then magnetically sorted into two populations, negative and positive by TAME. Unsorted cells , negative and positive cells were then analysed for tetramer positive cells. TAME using the PRS tetramer enriched a population to 5% tetramer positive. TAME using the ATL tetramer enriched a previously undetectable population to 1% tetramer positive.

3.12 Discussion

WT1 is a highly promising tumour antigen and WT1-specific T cells show great potential to produce therapeutic benefit in cancer patients. However, many questions about the immune response to WT1 remain to be answered. Very little is known about the processing mechanisms that generate WT1 peptide MHC complexes. Expression of WT1 has been identified in particular tumour types but a definitive description is lacking and the ability of WT1-specific T cells to recognise WT1-positive cells has been poorly investigated. Finally, although WT1-specific T cells have been identified by multiple groups, the frequency and breadth of the WT1 T cell response in the population has not been explored.

The work in this chapter sought to answer some of these questions with the overall aim of applying knowledge about the processing of WT1 to develop strategies to improve the processing of WT1 to enhance T cell recognition of WT1 positive cell lines.

3.12.1 WT1 expression

LCLs were chosen as a model system in which to study the processing of WT1. These transformed B cells have functional MHC I and MHC-II processing pathways and are an established model for studying antigen processing. Kobayshi *et al.* had shown multiple LCLs express WT1, but it was not known if this was the case for all LCLs.

Monitoring the expression of WT1 presented a challenge as many different WT1 isoforms have been identified. Protein expression was examined with two different antibodies specific for different regions of the WT1 protein. These two antibodies confirmed WT1 was expressed in some but not all LCLs. Temporal studies confirmed the

expression pattern in different LCLs was stably maintained over time. These differences would seem to suggest that WT1 expression is not triggered by EBV-infection *per se* but may occur as a result of another event that occurs for some LCLs during their establishment *in-vitro*. Although WT1 expression was not examined on a per cell basis, the fact that WT1 expression is essentially binary, with some LCLs strongly expressing the protein and others not, suggests that in the case of WT1 positive LCLs, all cells in the culture are expressing the protein. During the establishment of an LCL *in-vitro*, the initially polyclonal collection of B cells evolves rapidly into an essentially monoclonal LCL (Ryan *et al.*, 2006). WT1 expression has been shown to activate genes that confer a growth advantage to haematopoietic stem cells (Alberta *et al.*, 2003). Thus, activation of WT1 in a subset of B cells in an establishing LCL would lead to the final monoclonal LCL being WT1 positive. The reasons why some LCLs are WT1 positive and others not are currently unknown but they could start to be addressed by examining WT1 expression during formation of an LCL. Whether WT1 is expressed in particular cases of post-transplant lymphoproliferative disease, the tumours of which are essentially LCLs that have arisen in immunosuppressed patients, is an important question for future work, particularly given the association of WT1 with poor disease-free and overall survival in AML and ALL (Bergmann *et al.*, 1997; Chiusa *et al.*, 2006; Garg *et al.*, 2003; Inoue *et al.*, 1994).

The fact that WT1 was expressed in LCLs raised the question of whether EBV-positive malignancies, which express a smaller subset of EBV genes, express WT1. Western blotting revealed that the cell lines tested did not express WT1 protein. This again suggests that EBV infection *per se* is unable to trigger WT1 expression. However, as cell

lines rather than primary tumour tissue were tested, it remains possible that EBV-positive tumours express WT1 *in-vivo.* This means that EBV tumours may still be targeted by WT1-specific T cells. The expression of WT1 in some LCLs suggest that WT1 may be expressed in EBV-related, post-transplant lymphoproliferative disorder where B cells show an LCL-like phenotype.

Attempts were next made to identify other tumour cells lines that could act as targets for WT1-specific T cells. Three out of four AML lines tested showed expression of WT1 protein. These cell lines could act as targets in T cells assays to study the phenotype and functional abilities of WT1-specific T cells. The results also suggest that AML may be an appropriate setting in which to study WT1 T cell responses *in-vivo.*

Due to the polymorphic nature of WT1, expression was also monitored by two RT-PCR assays. A commercial qRT-PCR assay designed to identify all known transcripts of WT1 by amplify a region spanning exons 3 and 4 failed to identify WT1 mRNA in LCLs previously shown to be WT1 positive. A second RT-PCR assay designed across exons 7-10 did detect WT1 mRNA in the same samples.

Exons 3 and 4 are WT1 are thought to be conserved in all transcripts of WT1. However as the commercial qRT-PCR failed to detect WT1 in LCLs this suggests that some LCLs express a WT1 transcript with different exon usage or with mutations in one or both of exons 3 and 4.

WT1 mRNA was detected in AML lines by the commercial qRT-PCR assay. However levels of mRNA did not correlate with protein expression observed by western blotting. Two possibilities exist here. First, it is possible that intrinsic differences within the AML

lines such as variations in rates of protein translation or degradation of WT1 account for these discrepancies. Second, western blotting may detected WT1 protein transcribed from an alternative WT1 mRNA transcripts that are not detected by the commercial qRT-PCR.

Another final interesting finding from the analysis of WT1 expression in cells lines came from using the same RT-PCR assay employed by Kobayashi and colleagues. This assay detected WT1 mRNA in two myeloma lines that had tested negative for WT1 in all previous experiments. This suggests these lines express a highly different WT1 transcript that is not detected by either antibody or the commercial qRT-PCR assay.

Taken together these data suggest that no single current assay can identify all forms of WT1. This means that the expression of WT1 may have been underestimated in some studies. This only adds weight to the fact that WT1 is a promising tumour antigen and immune responses to WT1 could have therapeutic benefit in a wide range of tumour settings. However as WT1 appears to be even more polymorphic than previously understood, a wide range of WT1-specificities may be required to effectively target WT1 positive tumours.

3.12.2 WT1 and forskolin

As described above, the processing and presentation of WT1 has not been well studied. Work on other nuclear-resident tumour antigens has shown that relocalisation of a protein to the cytoplasm can increase generation of MHC class II epitopes from that protein. WT1 is a nuclear-resident tumour antigen and previous reports have suggested

it can be relocalised to the cytoplasm using forskolin. The effect of forskolin on WT1 localisation in EBV-positive cells was therefore determined.

Studying cellular localisation in LCLs can be difficult as they contain very little cytoplasm. Nevertheless, the results clearly showed that in untreated cells WT1 appeared to be entirely nuclear. Furthermore, levels of nuclear WT1 decreased following forskolin treatment. Although there could be several explanations for such a decrease, reduced transcription or translation for example, the fact that chloroquin inhibited this decrease was intriguing. Although no cytoplasmic localised WT1 protein was observed in cells treated with chloroquine and forskolin, this may be due to technical difficulties in defining the small cytoplasmic area in LCLs. It should also be noted that given the exquisite sensitivity of T cells, only a small amount of WT1 protein would need to be relocalised into the lysosomal pathway to render the WT1 positive cell sensitive to T cell attack. Taken together, these observations were consistent with the hypothesis that WT1 is relocalised to the cytoplasm by forskolin whereupon it is degraded by a chloroquine-sensitive degradative pathway.

3.12.3 T cell responses to WT1

Using the *ex-vivo* ELI-spot assay this study showed that the numbers of WT1-specific T cells was below the level of detection of this sensitive assay for both healthy donors and multiple myeloma patients. T cells specific for influenza A nuclear protein were readily detected in these assays confirming the sensitivity of the ELISPOT screen. WT1-specific T cells are therefore present at very low numbers in healthy donors and patients, which would make the generation of WT1-specific T cell clones more challenging than EBV-specific clones that can be readily detected by the ELIspot assay.

Interestingly, T cell responses to the influenza antigen were lower in multiple myeloma patients compared to healthy donors. Impaired immunity has been observed previously in multiple myeloma patients although the mechanisms responsible are poorly understood (personal communication - Frank Mussai, the University of Birmingham, UK).

Although others have reported the detection of WT1-specific T cells in *ex-vivo* assays, it is perhaps not surprising that WT1-specific T cells were not detected in healthy individuals in these experiments. *Ex-vivo* ELIspots are designed to detect effector memory T cells. These cells only develop after antigen induced clonal expansion of T cells. As a tumour antigen, WT1 should not be expressed in healthy individuals and therefore antigen exposure is unlikely to occur.

The reasons for the lack of WT1 T cell responses in myeloma patients are unknown. Reports have shown that T cell responses to tumour antigens can vary drastically over time and this can be linked to disease burden (Goodyear *et al.*, 2005). The status of multiple myeloma patients used in this study was unknown. It is possible that a low disease burden in all patients accounted for the lack of effector memory T cell responses to WT1.

In an attempt to expand and detect low frequency and central memory T cells, a cultured ELIspot protocol was used. However, this method also failed to identify any WT1-specific T cells in either healthy donors or multiple myeloma patients. Despite this finding, attempts were still made to isolate WT1-specific T cell clones, based on successful reports of their isolation by other groups. Methods used by these groups to generate WT1-specific clones were used but all were unsuccessful. Additional

methodologies were also explored, such as expressing WT1 in DCs via RNA electroporation. Again, all failed to generate WT1-specific T cell clones. It should be noted that these methods were able to reliably and reproducibly generate T cell clones specific to multiple control EBV antigens, demonstrating their effectiveness.

In a final attempt to identify WT1-specific T cell clones, MHC-II tetramers specific for known WT1 epitopes were used. These studies confirmed previous findings and WT1-specific T cells were not detected. Tetramer-associated magnetic enrichment was employed to increase the sensitivity of tetramer detection. Using TAME, a single population of T cells specific to the WT1 MHC-II tetramer ATL was detected. This population was only observed in a single donor.

All attempts to culture these T cells were unsuccessful. The TAME protocol involves sorting cells at low temperatures in low nutrient conditions. It is possible that this protocol damaged the tetramer-specific cells resulting in the failure to culture these cells. However TAME has previously been used to culture other T cells so this is unlikely. WT1-specific T cell clones appear to be extremely difficult to produce, a finding confirmed by another worker in the field who found they do not proliferate for more than a few days and are often not of an effector phenotype (personal communication - Graham Pawlec, University of Tübingen, Germany). It is therefore more likely that an intrinsic property of these WT1-specific T cells was responsible for the failure to culture these cells. The few reports of WT1 clones in the literature therefore likely reflect the tip of an iceberg, with many more unsuccessful attempts remaining unpublished due to publication bias.

3.12.4 Future work

As I was unable to generate WT1-specific T cells I was unable to study the antigen processing of WT1. Though inhibitor experiments suggested forskolin may cause WT1, to relocalise into the cytoplasm, this finding was not pursued because without WT1-specific T cell clones it would be difficult to demonstrate increased epitope presentation. While WT1 levels increased in cells treated with chloroquine, this increase occurred only in the nucleus with no staining evident in the cytoplasm. One possible model to explain this result would be that WT1 is predominantly nuclear-localised but with a proportion of protein constantly shuttling between the nucleus and cytoplasm. If the cytoplasmic fraction was being continually degraded by autophagy, then this shuttling would result in overall lower levels of WT1 in the cell as a whole. Following an inhibition of autophagy mediated by chloroquine, levels of WT1 in the cytoplasm would increase and this protein could re-enter the nucleus. Further experiments using inhibitors of nuclear import or export, such as ivermectin or leptomycin B respectively in addition to inhibitors of autophagy would be needed to test this model.

4. THE EFFECT OF ROSCOVITINE ON EBNA1 ANTIGEN PROCESSING

Failure to generate WT1-specific T cell clones meant I was unable to study the antigen processing and presentation of WT1 epitopes. I was therefore unable to test the hypothesis that relocalisation of WT1 may increase its presentation to $CD4^+$ T cells. This hypothesis however, should be applicable to many nuclear resident antigenic proteins. I therefore turned to a different nuclear-localised tumour antigen to test the hypothesis that pharmacological alteration of subcellular localisation could affect the generation of MHC class II epitopes by autophagy.

As previously discussed in chapter 1, the original observation that subcellular localisation is important for presentation of MHC class II epitopes came from the work of Leung *et al*. (Leung *et al.*, 2010). EBNA1 contains many MHC class II epitopes, however EBV transformed B cells (LCLs) are poorly recognised by EBNA1-specific $CD4^+$ T cells. Leung and colleagues showed that this poor recognition was at least in part due to EBNA1's nuclear localisation. Expression of a mutant EBNA1 with a deleted nuclear localisation sequence created LCLs expressing EBNA1 in the cytoplasm, enhancing their recognition by EBNA1-specific $CD4^+$ T cells.

This work was of great importance in understanding the antigen processing of EBNA1 and highlighted another mechanism by which EBV might evade the immune system. However Leung used mutation of the EBNA1 NLS in order to achieve the required relocalisation. As such, it is difficult to imagine how the findings could be directly applied in a clinical setting to improve immunological recognition of EBV infected cells.

Identifying a pharmacological compound to relocalise EBNA1 would allow this strategy to be exploited therapeutically to boost the recognition of tumour cells by $CD4^+$ T cells.

Coincidentally, whilst considering EBNA1 as an alternative experimental system, Kang *et al.* reported that Roscovitine, a clinically approved CDK inhibitor, was able to relocalise EBNA1 to the cytoplasm (Kang *et al.*, 2011). I hypothesise that Roscovitine mediated relocalisation of EBNA1 should increase the recognition of EBV infected cells. If correct, Roscovitine could be used in a clinical setting to increase the recognition of EBV-associated malignancies by the immune system.

I therefore decided to investigate the effect of Roscovitine on EBNA1 and any subsequent effect on $CD4^+$ T cell recognition of EBV infected cells.

4.1 Localisation of EBNA1 in Roscovitine treated cells

Previous experiments conducted by Kang *et al.*, focussing on sub-cellular localisation of EBNA1 were carried out in HeLa cells. In these experiments EBNA1 was artificially overexpressed, treatment of these cells with Roscovitine was shown to cause cytoplasmic localisation of EBNA1 in the majority of cells. It is not known however if Roscovitine will have this same effect on naturally expressed EBNA1 in an EBV infected cell. I therefore investigated Roscovitine's effect on the subcellular localisation of EBNA1 in a LCL which naturally expresses nuclear EBNA1.

LCLs were treated with Roscovitine for 72h, harvested and nuclear and cytoplasmic extracts were obtained from cells using a nuclear/cytoplasmic fractionation kit. The expression of EBNA1 in these fractions was then assessed by western blotting using the EBNA1-specific antibody IH-4. As a control for separation of fractions the expression of a

nuclear resident transcription factor Sp-1 and a cytoplasmic resident protein, α-tubulin were also measured. Figure 4.1 shows protein expression in nuclear and cytoplasmic fractions of control and Roscovitine treated LCLs. A band representing EBNA1 with a molecular weight of 75kD was detected in all samples. Untreated control cells contained high levels of EBNA1 in the nucleus as would be expected. A small quantity of EBNA1 was detected in the cytoplasmic fraction of the untreated cells. This may be due to contamination of the fraction with a small number of nuclei or this may suggest that some EBNA1 is localised in the cytoplasm of LCLs. It should be noted that Sp-1, a control nuclear resident protein was only detected in the nuclear fraction suggesting separation of fractions was carried out successfully.

Surprisingly, and contrary to the results of Kang *et al*., no change in EBNA1 expression was detected in either the nuclear or cytoplasmic fraction after treatment with Roscovitine. Roscovitine treated cells contained high levels of EBNA1 in the nucleus, comparable to that of the untreated control. Most importantly, only very small amounts of EBNA1 were detected in the cytoplasm and these levels were the same detected in the cytoplasm of untreated control cells.

In summary, western blot analysis of nuclear and cytoplasmic fractions from LCLs suggested that Roscovitine did not affect the localisation of endogenous EBNA1. This result was unexpected given the report from by Kang *et al*. Since this report studied the effects of Roscovitine upon over-expressed EBNA1 I altered the experimental system to allow me to examine the effect of Roscovitine upon overexpressed EBNA1.

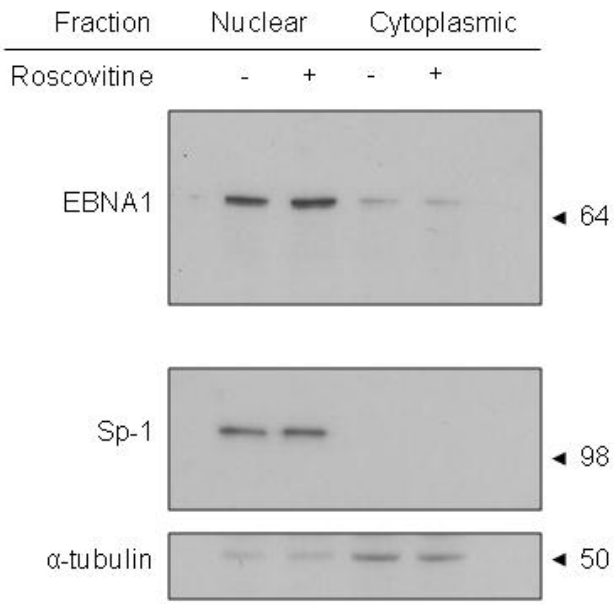


Figure 4.1 Expression of EBNA1 in nuclear and cytoplasmic fractions of Roscovitine treated LCLs

Western blot analysis of EBNA1 protein expression in nuclear and cytoplasmic fractions of Roscovitine treated LCLs. LCLs were treated with 50μM Roscovitine for 72h. Cells were then subjected to nuclear/cytoplasmic fractionation and protein expression assayed by western blot. EBNA1 protein was detected using the EBNA1-specific antibody IH-4. EBNA1 was detected in nuclear fractions of both control and Roscovitine treated cells. EBNA1 was detected in cytoplasmic fractions at very low level. Roscovitine had no effect on the level of EBNA1 in the cytoplasm of cells. Expression of Sp-1 protein served as control for nuclear fractionation and α-tubulin served as control for cytoplasmic fractionation.

HeLa cells were transfected with a vector expressing GFP tagged EBNA1. Cells were then treated with Roscovitine and the subcellular localisation of EBNA1 was studied by detection of GFP using fluorescent microscopy.

As expected, control cells showed EBNA1-GFP coincided with DAPI staining. This suggests EBNA1 was totally confined to the nucleus in these cells. As with previous experiments conducted in LCLs but again contrary to the results of Kang *et al*, Roscovitine appeared to have no effect on the sub-cellular localisation of EBNA1-GFP in HeLa cells. Cells treated with Roscovitine showed very similar staining and fluorescent pattern to untreated cells with GFP signal being detected only in the nucleus.

It may be possible that Roscovitine does relocalise EBNA1 but this may not be detected as EBNA1 may be rapidly degraded once in the cytoplasm. EBNA1 is known to be degraded by autophagy and cytoplasmic EBNA1 is more rapidly degraded than nuclear EBNA1 (personal communication – Graham Taylor, the University of Birmingham, UK). To investigate this possibility HeLa cells expressing EBNA1-GFP were again treated with Roscovitine, however the cells were also treated with chloroquine to raise lysosomal pH thereby inhibiting any cathepsin-mediated degradation of EBNA1. Fluorescence microscopy of chloroquine treated cells is shown in Figure 4.2b. Chloroquine treated control cells, showed EBNA1-GFP only in the nucleus. When treated with Roscovitine no change in the subcellular localisation was observed, EBNA1-GFP signal was only observed in the nucleus. Taken together the results in Figure 4.1 and Figure 4.2 suggest strongly that Roscovitine is unable to relocalise either stable endogenous EBNA1 or over-expressed GFP-EBNA1.

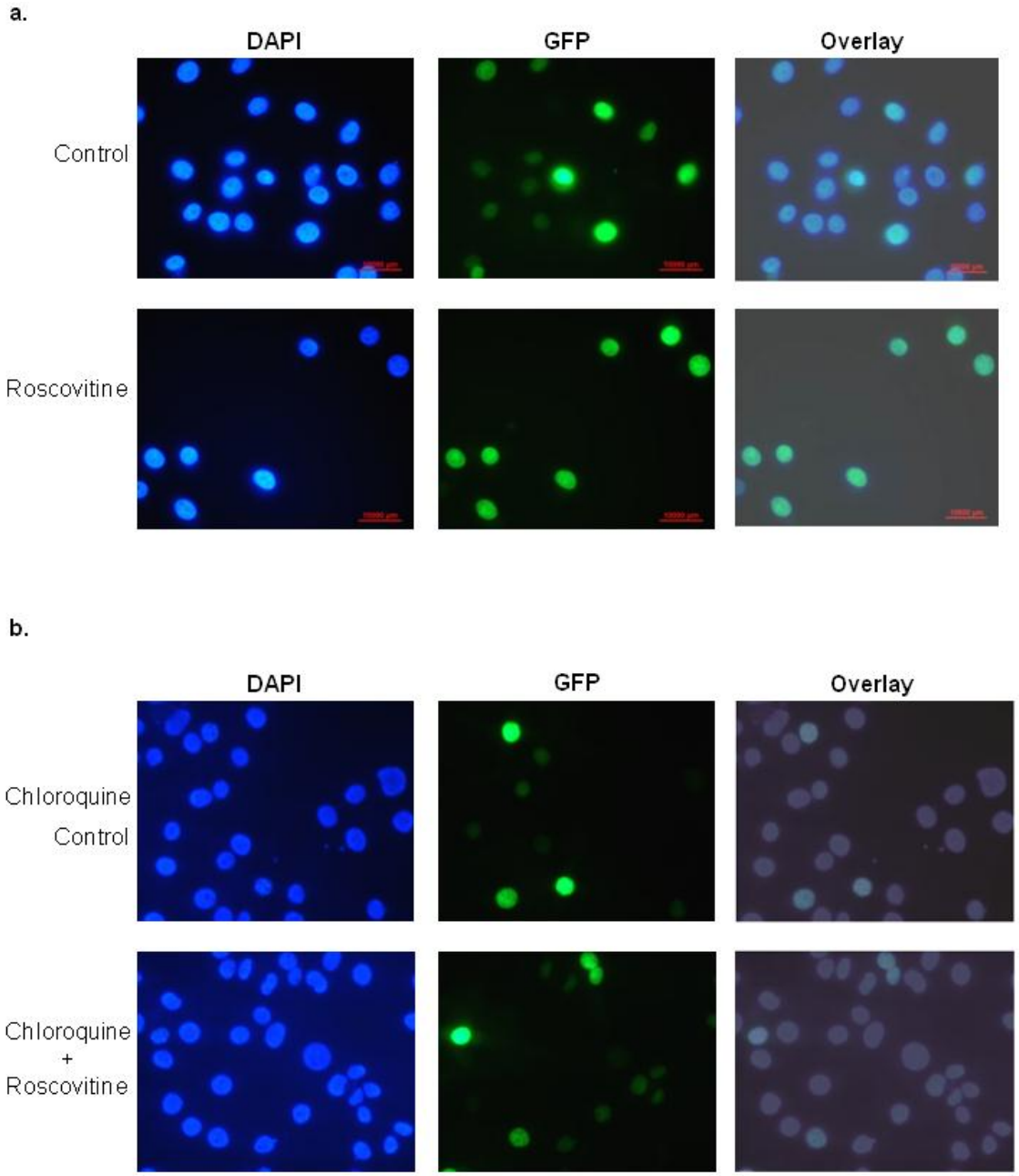


Figure 4.2 Localisation of GFP-EBNA1 in Roscovitine treated cells

Hela cells were transfected with EBNA1-GFP and localisation monitored by fluorescence microscopy. **a**. Control cells shows GFP fluorescence is concurrent with DAPI staining showing EBNA1 is present in the nucleus. 50μM Roscovitine had no effect on EBNA1 localisation. **b.** 50mM chloroquine treated cells also showed the same pattern. Chloroquine alone and chloroquine with Roscovitine had no effect on EBNA1 localisation. EBNA1 remained nuclear in all conditions.

4.2 Roscovitine reduces cellular proliferation

As well as showing Roscovitine relocalises EBNA1, Kang *et al.* showed that Roscovitine can reduce the growth of LCLs, an effect that they ascribed to Roscovitine-mediated relocalisation of EBNA1. However, these experiments carried out in an LCL do not prove the effect of reduced proliferation was dependent upon EBNA1. As Roscovitine is a potent inhibitor of multiple cellular CDKs it is possible that the drug inhibits proliferation by inhibiting CDKs rather than by relocalising EBNA1.

To investigate this possibility, the effect of Roscovitine upon proliferation of multiple cell lines both EBV-positive and EBV-negative was tested. Equal numbers of different cell lines were treated with increasing concentrations of Roscovitine for 96 h, after this time total cell numbers were counted, the results of which are displayed in Figure 4.3a. Two different LCLs were treated with Roscovitine, LCL 1 was transformed with B95.8 strain of EBV. LCL 2 is transformed with a strain of EBV isolated from a Chinese donor. Control populations showed both cell lines had proliferated over 96 h. LCL 1 reached nearly $2x10^6$ cells whereas LCL 2 proliferated faster and resulted in a total cell count of 4 $x10^6$. Importantly as the concentration of Roscovitine increased total cell numbers decreased. In both cell lines 30μM Roscovitine inhibited proliferation completely and was toxic to the cells resulting in no live cells remaining after 96h. These results are similar to those of Kang *et al.* and suggest that Roscovitine inhibits proliferation in EBNA1 expressing cells. It is important to note however, that this result does not show that changes in growth are caused by relocalisation of EBNA1 or even that it is dependent upon EBV being present in the cell.

To test if this effect was EBNA1- and EBV-specific, the effect of Roscovitine on cellular proliferation was tested on two different cell lines derived from the same parent line with differing EBNA1 expression status. Akata BL is a Japanese Burkitt's Lymphoma cell line infected with EBV and therefore expresses EBNA1. Akata a3 is a mutant strain of this cell line that has lost the EBV virion and therefore does not express EBNA1.

Again equal amounts of both cell lines were treated with increasing concentrations of Roscovitine and after 96h total cell numbers were counted. When untreated the two lines proliferated to similar numbers. At low levels of Roscovitine (1-10μM) the drug seemed to have little effect on cell number. In fact, 5μM Roscovitine seemed to increase proliferation of the EBV-positive Akata line. At 20μM Roscovitine both EBV-positive and EBV-loss Akata showed a decrease in total cell number as observed for LCLs. 30μM Roscovitine was toxic and resulted in no live cells being detected after 96h. The key result was that the effects of Roscovitine were similar regardless of EBV status of the cells.

To examine the effects of Roscovitine on cellular proliferation more closely, cells were again treated with Roscovitine and total cell numbers measured by WST assay. This assay measures cellular metabolic activity via oxidoreductase enzymes and can be used to determine the number of viable cells. This improved technique allowed a more accurate assay for cellular proliferation. Again the effect of Roscovitine on two LCLs and two Akata lines was measured. Figure 4.3b shows the results of this experiment. In LCLs concentrations of Roscovitine above 5μM reduced the proliferation of cells. In both EBV-positive and EBV-negative Akata lines proliferation of cells was reduced by 20μM Roscovitine.

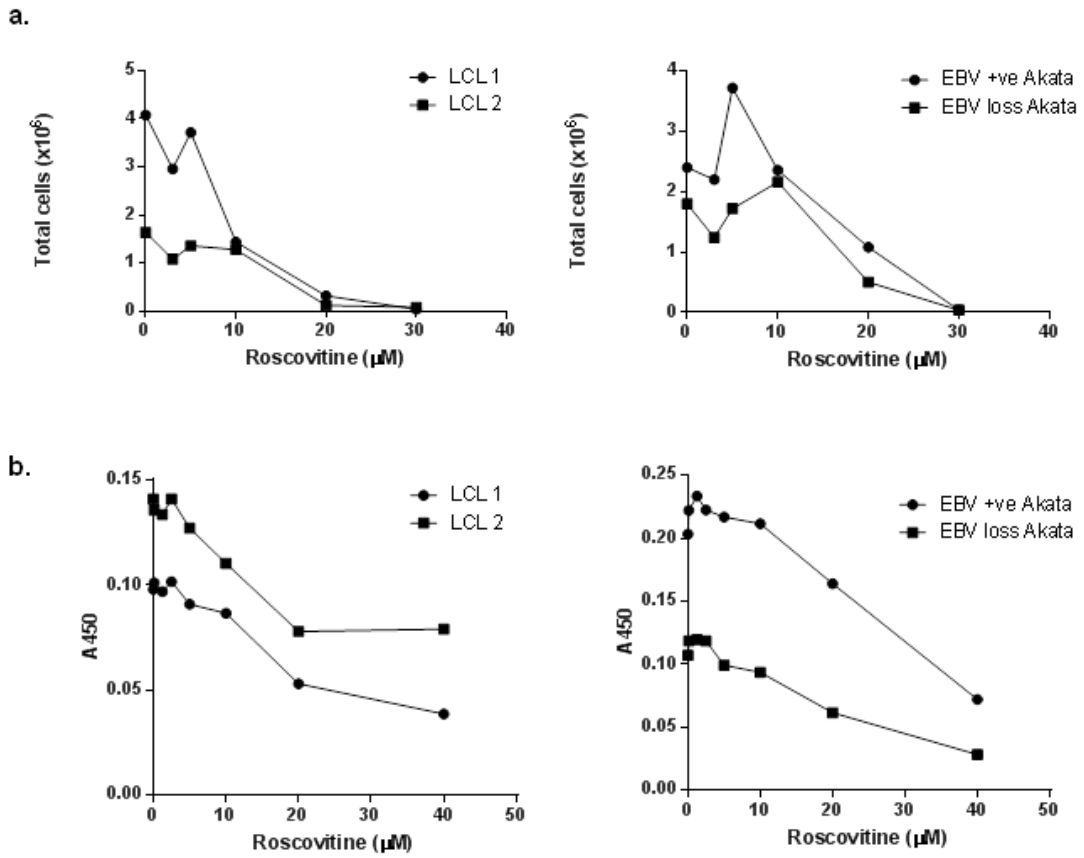


Figure 4.3 Roscovitine inhibits cellular proliferation in EBNA1 positive and EBNA1 negative cells

Four different cell lines were treated with increasing concentrations of Roscovitine and the effect on proliferation of these lines was assayed. **A.** Cells were treated with Roscovitine for 96h, proliferation was assessed by counting cell numbers after this time. High doses of Roscovitine inhibited proliferation in all cell lines regardless of EBV/EBNA1 status. **B.** Again cells were treated with Roscovitine for 96h and proliferation was measured by colormetric WST assay. Again Roscovitine appeared to inhibit proliferation in all cell lines regardless of EBV/EBNA1 status

These data show that although Roscovitine is able to inhibit cellular proliferation, the effects were the same in all cell lines regardless of EBV status and EBNA1 expression.

Taken together, these results suggested that decreases in cell proliferation caused by Roscovitine are not mediated through any effect the drug may have upon EBNA1.

4.3 The effect of Roscovitine on the recognition of EBNA1 by $CD4^+$ T cells

Despite results suggesting Roscovitine does not relocalise EBNA1, it is possible that EBNA1 is being relocalised to the cytoplasm in small quantities that cannot be detected by the methods used so far. If even small amounts of EBNA1 were being relocalised to the cytoplasm this should result in an increase in the amount of EBNA1 entering the autophagic pathway. This should in turn increase the amount of EBNA1 MHC class II epitopes being produced. T cells have previously been shown to be very sensitive to small changes in epitope levels and should therefore be able to accurately detect any EBNA1 relocalisation and subsequent change in epitope display.

Our laboratory has previously isolated multiple $CD4^+$ T cells specific for a number of different epitopes within EBNA1. A summary of these T cells is shown in Table 4.1 and locations of the epitopes within the EBNA1 protein is shown in Figure 4.4. How EBNA1 class II epitopes are generated differs between epitopes. The SNP epitope is known to be generated by an autophagy-dependent pathway whilst all other epitopes are generated in an autophagy-independent manner. I hypothesise that Roscovitine relocalisation of EBNA1 should alter the generation of EBNA1 MHC class II epitopes and this should be detected by an increase in LCL recognition by SNP-specific T cells.

Table 4.1 List of EBNA1-specific CD4+ T cells

EBNA1 CD4+ T cell clone	Clone Number	Epitope Coordinates	HLA Restriction	Functional Avidity [nM]	LCL recognition (% of max)
SNP	115	474-493	DR15 and 51	200	2.0
GLR	46	484-503	DQ6	180	1.4
VYG	7	509-528	DR11	0.5	2.0
KTS	11	514-533	DR1	400	0.6
PQC	82	529-548	DR11	2	0.0

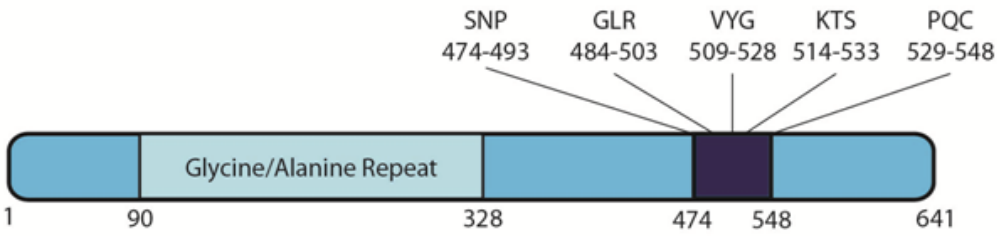


Figure 4.4 EBNA1 MHC-II epitopes

Representation of EBNA1 protein showing the position of MHC class II epitopes.

To test this hypothesis, LCLs were treated with Roscovitine and the levels of EBNA1 MHC class II epitopes were measured in T cell recognition assays. Following their treatment with Roscovitine, the LCLs were washed and co-cultured with EBNA1-specific $CD4^+$ T cells for 16h without the drug. After this time, supernatants were harvested from the LCL:T cell co-cultures and IFN-γ production by the T cells was measured by ELISA.

Figure 4.5a shows the results of three different T cell recognition assays, using three EBNA1-specific $CD4^+$ T cell clones against LCLs treated with increasing concentrations of Roscovitine. If Roscovitine were relocalising EBNA1 to the cytoplasm then there should be an increase in LCL recognition by the SNP specific T cells, resulting in increased levels of IFN-γ. This was not the case. Indeed, all three concentrations of Roscovitine tested decreased recognition of the LCLs by the SNP-specific T cells. In contrast, recognition of the same LCLs by T cells specific for VYG, an EBNA1 epitope that is not processed by autophagy showed the reverse effect; all doses of Roscovitine caused an increase in VYG T cell recognition of the LCL. DR1 positive LCLs were also treated with Roscovitine to study the effect on a third epitope TSL. LCL recognition by TSL T cells mirrored that of VYG; all doses of Roscovitine caused an increase in T cell recognition.

To further investigate this effect, aliquots of LCLs were treated with Roscovitine on subsequent days. After 4 days cells were used as targets in T cell assays. Again IFN-γ production was measured by ELISA (Figure 4.5b). As seen previously SNP T cell recognition was decreased upon Roscovitine treatment. The decreased recognition was seen to occur as early as 24 hours, was increased at 48 hours and shown to be sustained out to 96h. The increase in recognition seen in VYG T cells took slightly longer to occur. An increase was seen at 48 and 72 h and again was sustained out to 96 h.

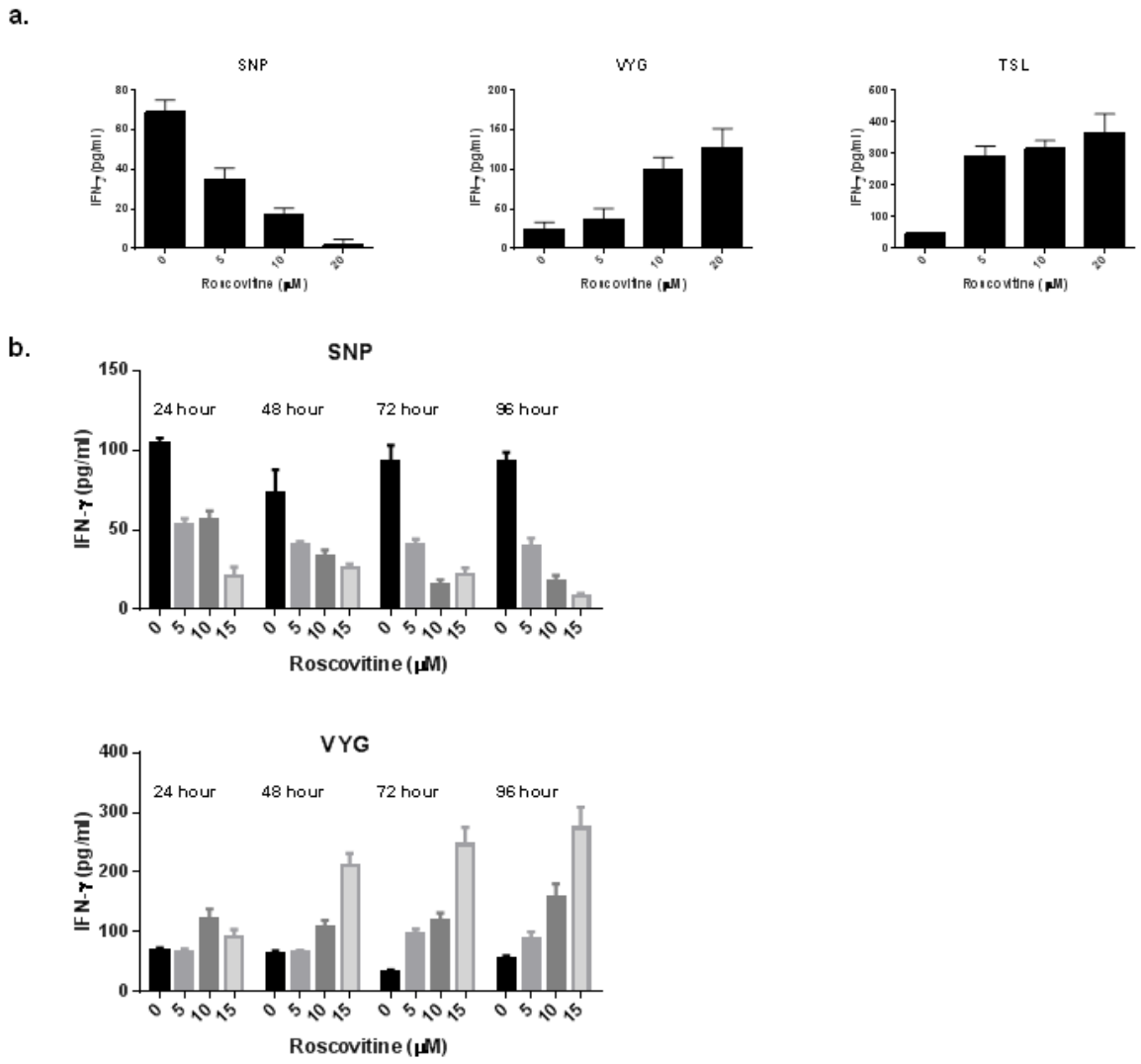


Figure 4.5 T cell recognition of Roscovitine treated LCLs

LCLs were treated with Roscovitine and the levels of EBNA1 MHC class II epitopes were measured in T cell recognition assays. **A.** T cell recognition assays, using three EBNA1-specific $CD4^+$ T cell clones show Roscovitine decreases presentation of SNP while increasing presentation of VYG and TSL. **B.** T cell recognition assays conducted on subsequent days.

As these results were unexpected a number of control experiments were carried out to further elucidate Roscovitine's effect on EBNA1 and to investigate any further effect Roscovitine has upon antigen processing and presentation. Two experiments were conducted to determine if Roscovitine has any effect on levels of MHC Class II molecules at the surface of LCLs. Firstly Roscovitine treated LCLs were incubated with EBNA1 Class II epitope peptides and subsequently used as targets in a T cell assay. Recognition of these peptide loaded LCLs is shown in Figure 4.6a. Recognition of LCL by two different EBNA1-specific T cells, SNP and VYG is shown and no effect of Roscovitine was observed at any dose. This suggests surface class II levels are unaffected by Roscovitine. Secondly LCLs were treated with increasing amounts of Roscovitine and stained for surface class II levels. Figure 4.6b shows flow cytometry data of LCLs surface stained with an antibody against the HLA-DR class II molecule . A very small decrease in DR staining was observed at 20μM but all other doses of Roscovitine (0-10 μM) appeared to have no effect on DR levels. These data suggest that Roscovitine is not having a general effect on class II processing but is specifically effecting the generation of EBNA1 class II epitopes.

4.4 The effect of Roscovitine on autophagy

The different results obtained for the autophagy-dependent epitope SNP and the autophagy-independent epitope VYG suggested that the effects of Roscovitine upon T cell recognition were nevertheless related to autophagy. Recognition of the autophagy-dependent epitope SNP seemed to decrease whereas all other class II epitopes appeared to increase.

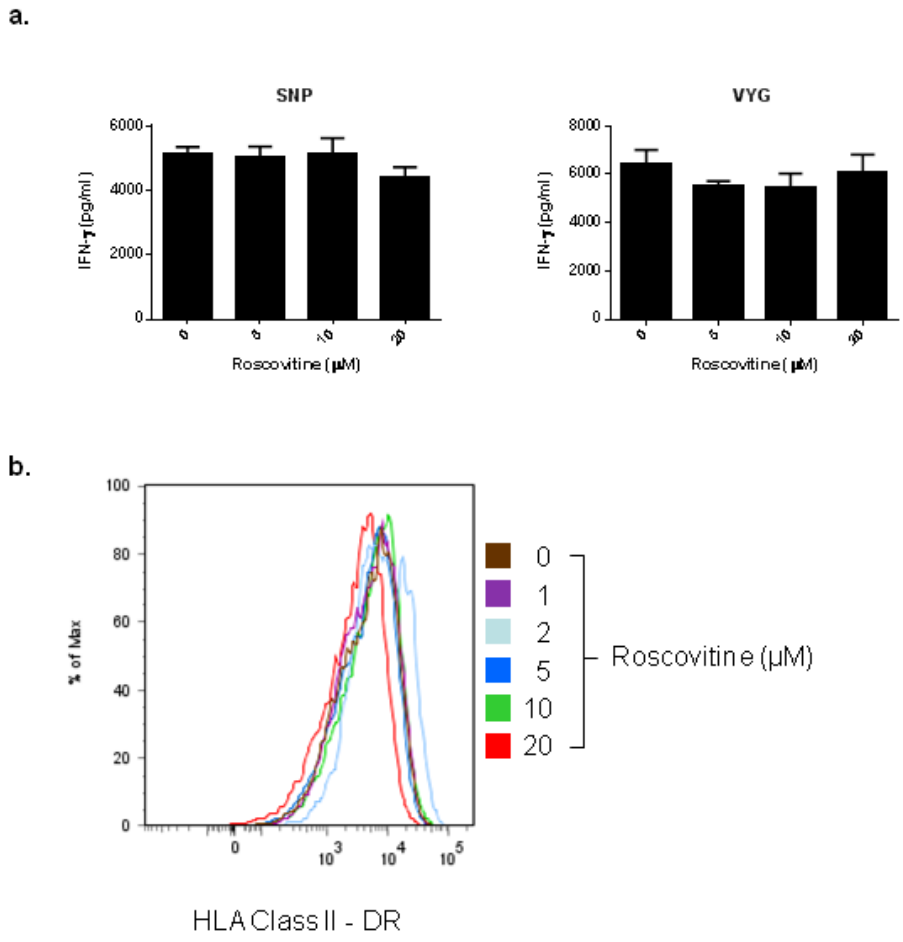


Figure 4.6 The effect of Roscovitine on surface MHC class II levels

Experiment to measure the effect of Roscovitine on surface MHC class II levels. **A.** Roscovitine treated LCLs were loaded with MHC class II epitope peptides and T cell recognition measured. No effect of Roscovitine was detected by this method. **B**. Roscovitine treated LCLs were stained with an antibody specific to HLA-DR and analysed by flow cytometry. Roscovitine had no significant effect on the levels of surface HLA-DR.

This mirrors the effect seen when LCLs are treated with inhibitors of autophagy such as 3-MA and wortmannin. It was therefore possible that Roscovitine was inhibiting autophagy causing decreased T cell recognition of the autophagy-dependent epitope.

Measuring autophagy in cells presents a number of technical difficulties. A number of methods quantify certain autophagic elements such as autophagosomes or autolysosomes at any stage of the autophagic process. These methods often give an inaccurate measure of autophagy as they do not measure the activity of the complete autophagy pathway, a term known as autophagic flux. Autophagic activity includes not just the increased synthesis of autophagy proteins such as LC3 or an increase in the formation of autophagosomes, but, most importantly, flux through the entire system. Therefore accurate measurements of autophagy require the monitoring of autophagic substrates dynamically over time to verify that they have reached the lysosome and, when appropriate, are degraded. One of the most accurate and commonly used methods of determining autophagic flux is to measure the lipidation and degradation of LC3 by western blotting (Klionsky *et al.*, 2012). The non-lipidated and lipidated forms of LC3 are termed LC3-I and LC3-II respectively. To measure autophagic flux, a comparison must be made between the amount of LC3-II in the presence and absence of inhibitors that prevent degradation of the protein. Preventing the degradation of LC3-II allows the quantification of the amount of LC3-II transiting through the autophagic pathway. If flux is occurring, the amount of LC3-II will be higher in the presence of the inhibitor. Smaller increases in LC3-II in the presence of the inhibitor represent lower levels of flux and larger increases represent greater levels of flux.

To investigate the effect of Roscovitine on autophagy, LCLs were treated with increasing concentrations of Roscovitine for 72h. For the final 6h cells were cultured in the absence and presence of Bafilomycin A1, to raise lysosomal pH and inhibit the degradation of LC3-II. After this time the amounts of LC3-II in control and Roscovitine treated cells cultured in the absence and presence of Bafilomycin A1 was measured by western blotting using an antibody specific to both forms of LC3. Expression of binding immunoglobulin protein (BiP), a housekeeping protein was monitored as a control. The results of this experiment are shown in Figure 4.7. A band at 16kDA was detected in the presence of bafilomycin A1, this represents LC3-II. Levels of LC3-II in control cells in the absence of Bafilomycin A1 were very low, the band is barely detectable. Control cells cultured in the presence of Bafilomycin A1 showed high levels of LC3-II. This increase represents the level of autophagic flux in the cells. Cells treated with Roscovitine showed similar levels of LC3-II. Levels were always very low in the absence of Bafilomycin A1 and increased in the presence of the inhibitor. The increase was comparable at all concentrations of Roscovitine. This suggests that levels of autophagic flux were the same in all conditions and therefore Roscovitine is not affecting autophagy.

As previously mentioned, autophagy can be technically difficult to study. Measuring multiple autophagy parameters is often required to confirm results. Therefore a second method of measuring autophagy was used to confirm that Roscovitine does not affect autophagy. 2GL9 cells are a cell line derived from 293 cells engineered to express a GFP tagged LC3 protein.

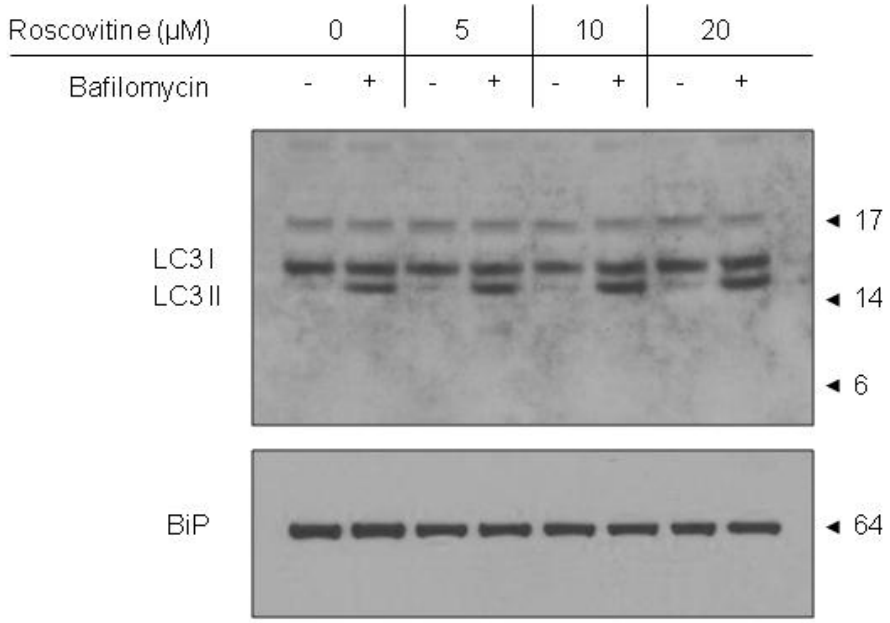


Figure 4.7 Western blot of LC3-II in Roscovitine treated LCLs to measure autophagic flux.

LCLs were treated with increasing concentrations of Roscovitine for 72h. For the final 6h, cells were cultured in the absence and presence of an inhibitor of lysosomal degradation, Bafilomycion A1 (100nM). Levels of LC3-II were then measured by western blot using an LC3-specific antibody. A band was detected at 16kDA representing LC3-II. Control cells showed an increase in the amount of LC3-II in the presence of Bafilomycin A1. The same level of increase was observed in cells treated with all concentrations of Roscovitine showing Roscovitine does not affect autophagic flux in LCLs. Expression of BiP protein was monitored as a control.

When autophagy is triggered in these cells autophagic vesicles form and LC3-GFP will cluster in the membranes of these vesicles. Autophagic vesicles can then be observed as bright GFP puncta within the cytoplasm of cells. A change in the number of puncta in a cell can be interpreted as a change in the level of autophagy.

The effect of Roscovitine on the number of autophagic vesicles within a cell was determined. Two separate experiments were conducted; the first to determine if Roscovitine could upregulate autophagy in resting cells and a second to determine whether Roscovitine could inhibit autophagy in cells where levels of autophagy were high. In the first experiment a control population of cells was grown in full growth media to determine the baseline level of autophagy in a cell. A second population, grown in full media was treated with 20μM Roscovitine to determine whether Roscovitine could trigger autophagy. As a control for activation of autophagy a third population was cultured in EBSS, a simple salt solution containing no amino acids or growth serum. This starvation should increase autophagy and cause an increase in the number of autophagic vesicle. Representative images are shown in Figure 4.8a. Control and Roscovitine treated cells show few puncta whereas puncta can clearly be seen in starved cells, one of which is marked by a white arrow. Total puncta per cell were counted in 20 fields from each condition and the results are shown in Figure 4.8b. Control populations showed a mean of less than 1 visible puncta per cell. When cells grown in full media were treated with Roscovitine no significant increase in puncta compared to untreated cells was observed. When starved, as suspected, numbers of visible puncta per cell increased significantly to over 10 puncta per cell. This suggests Roscovitine does not increase autophagy in a resting cell.

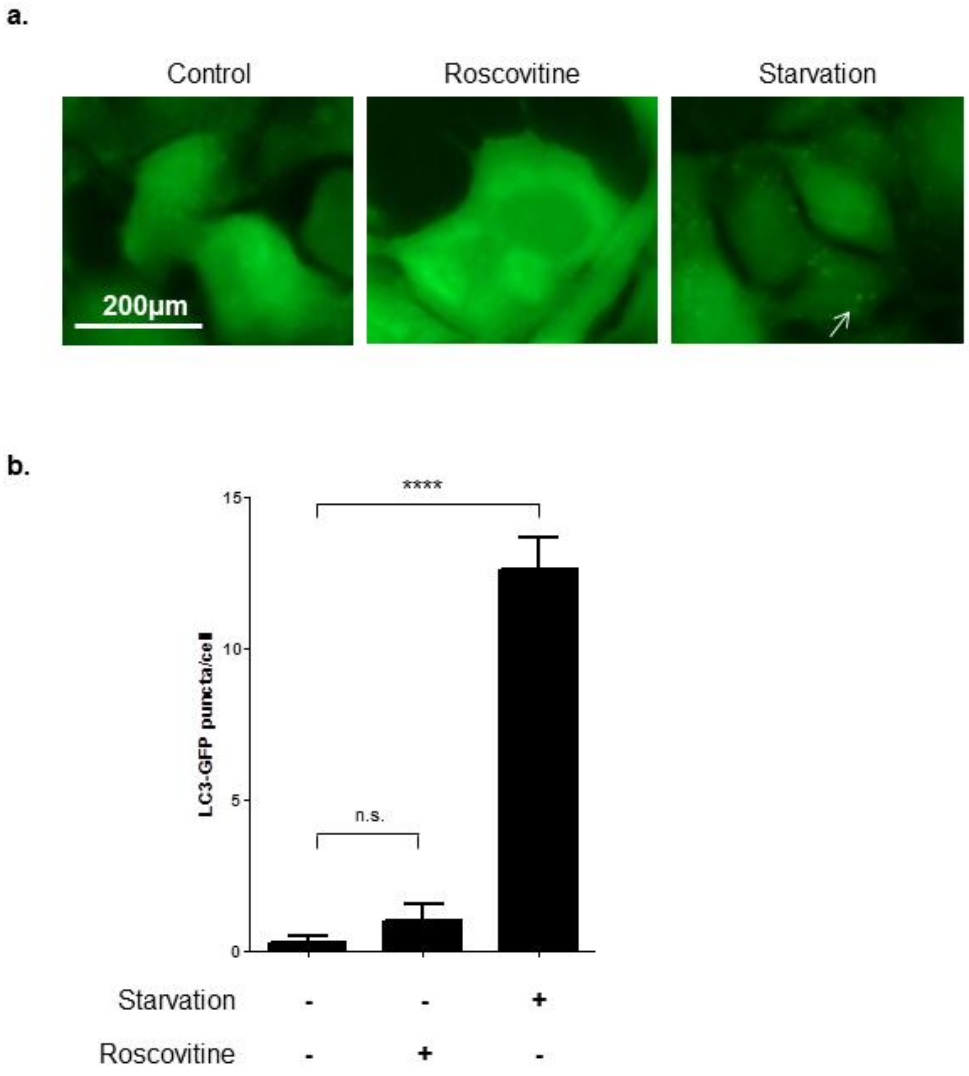


Figure 4.8 Quantification of LC3-GFP puncta to measure autophagy induction in Roscovitine treated cells

The effect of Roscovitine on numbers of LC3-GFP puncta were measured in 2GL9 cells. **A.** Representative images (a puncta is marked by the white arrow). **B.** Puncta were counted in cells. Starvation was able to increase the number of puncta. No effect of Roscovitine was observed. Statistical significance was determined by ANOVA and subsequent bonferroni multiple comparison analysis (****P< .0001).

To determine whether Roscovitine could inhibit autophagy in cells where levels of autophagy were high, a similar second experiment was conducted.

Again a control population of cells was grown in full growth media. Three populations of cells were cultured in EBSS for 4 h to induce autophagy. One starved population was untreated as a control. Another population of cells was treated with Roscovitine and as a control for autophagic inhibition, a third starved population of cells was treated with 5μM 3-MA. Figure 4.9a shows representative images and the results from counts in multiple fields are shown in Figure 4.9b. The results show that starvation significantly increased the number of puncta in the cells. As would be expected, 3-MA inhibited autophagy and caused a decrease in the number of puncta visible. Cells treated with Roscovitine had high levels of puncta in the cells comparable to those of starved untreated cells. This result shows that Roscovitine does not decrease autophagy. When taken together with the results of Figure 4.8, this suggests that Roscovitine does not affect autophagy in 2GL9 cells, a result that accords with the earlier data obtained by measuring LC3 flux.

While both autophagy assays clearly showed that Roscovitine was not decreasing autophagy in cells, these results directly contradicted the results of the T cell assays that showed a decrease in the generation of an autophagy-dependent epitope. Therefore a third method of determining levels of autophagy in cells was used. As discussed in chapter 1, p62 is an adaptor protein, selectively degraded by autophagy. Therefore p62 levels can be used to determine the amount of autophagy in a cell. Decreases in p62 can be interpreted as an increase in autophagy.

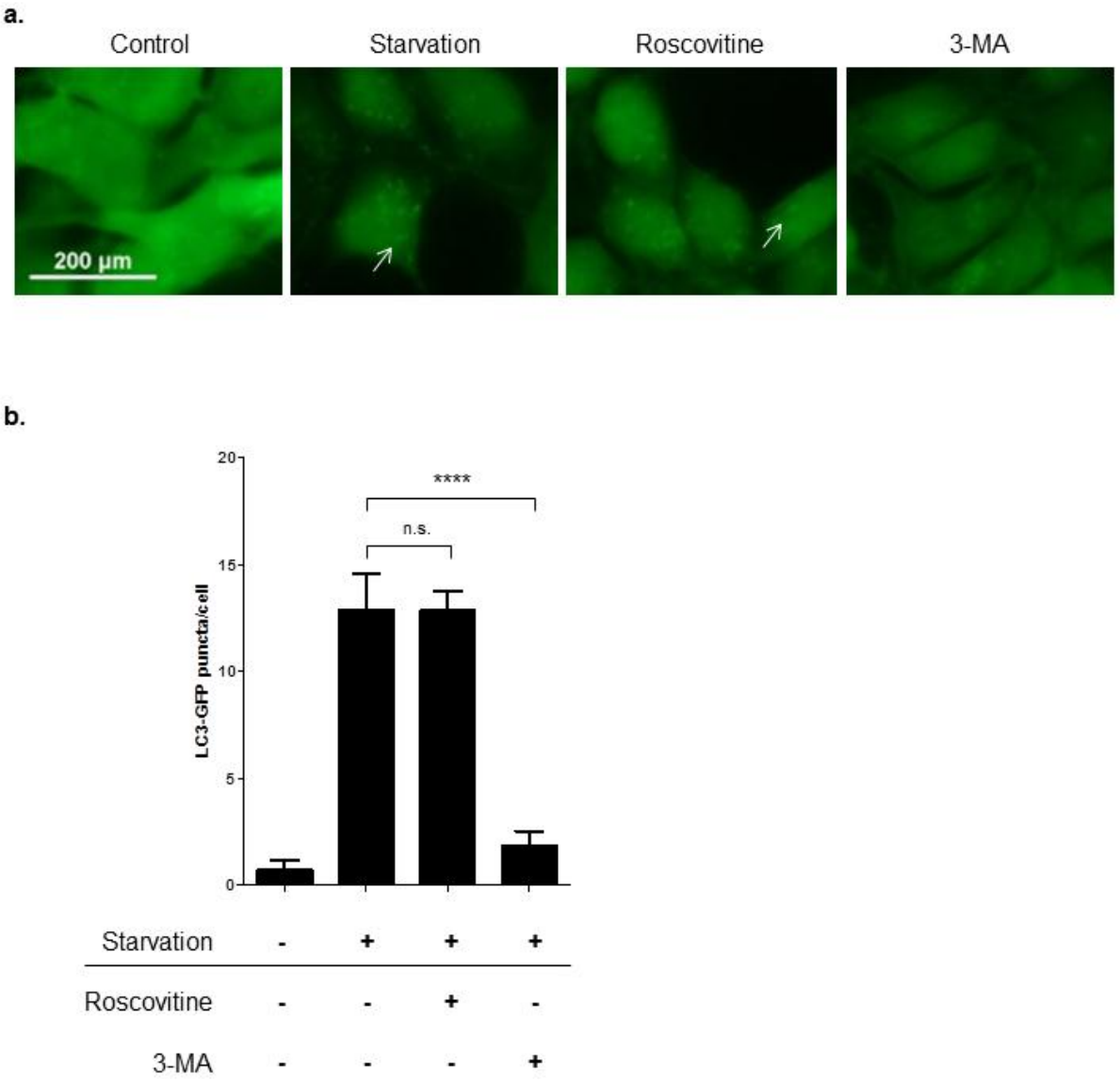


Figure 4.9 Quantification of LC3-GFP puncta to measure autophagy inhibition in Roscovitine treated cells

The effect of Roscovitine on numbers of LC3-GFP puncta were measured in 2GL9 cells. **A.** Representative images (puncta are marked by the white arrows). **B.** Puncta were counted in cells. Starvation was able to increase the number of puncta an effect which was ablated by 10mM 3-MA. No effect of Roscovitine was observed. Statistical significance was determined by ANOVA and subsequent bonferroni multiple comparison analysis (****P< .0001)

LCLs were treated with increasing amounts of Roscovitine and the amount of p62 in cells was then determined by western blot using a p62-specific antibody. Figure 4.10 shows the results of this western blot. Expression of p62 was detected in control cells. Remarkably, as the concentration of Roscovitine increased the level of p62 protein decreased.

The result of the p62 assay could be interpreted as Roscovitine decreasing the autophagy activity of cells. However, this conclusion conflicts with the results obtained by measuring LC3 flux and GFP-LC3 puncta that both showed Roscovitine did not affect autophagy.

4.5 Mechanism of Roscovitine-mediated reduction in p62 protein

Although data clearly showed that p62 levels were decreased upon Roscovitine treatment, it was not known how Roscovitine caused this reduction. It is possible that an increase in autophagy was responsible, although the earlier experiments showed Roscovitine did not affect autophagy. It is therefore possible that degradation by an alternative pathway, such as proteasomal-mediated degradation, was causing the reduction in p62. A third possibility was that decreased levels of p62 reflected decreased synthesis of p62. I conducted experiments to investigate all three possibilities.

To quantify p62 degradation by autophagy, in the presence or absence of Roscovitine, LCLs were treated with two different inhibitors of autophagic/lysosomal degradation, Bafilomycin A1 or chloroquine. Western blots were carried out and levels of p62 determined. The results of this are shown in Figure 4.11.

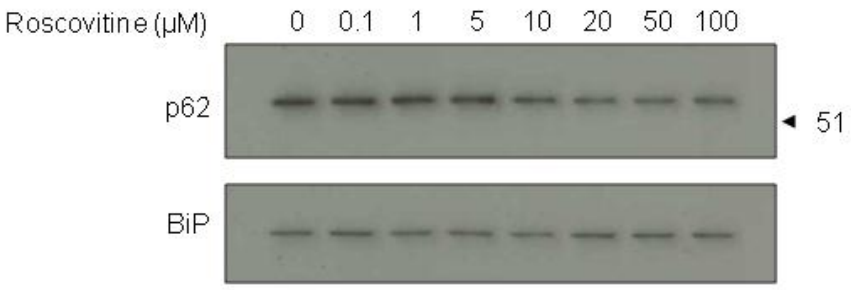


Figure 4.10 p62 expression in Roscovitine treated LCLs

LCLs were treated with increasing amounts of Roscovitine and the amount of p62 in cells was then determined by western blot using a p62-specific antibody. Expression of p62 was detected in control cells. As the concentration of Roscovitine increased the level of p62 protein decreased. The expression of BiP protein was monitored as a control.

In control populations of cells, both bafilomycin and chloroquine caused an increase in p62 levels compared to untreated controls in lane one. This suggests that p62 is indeed being degraded by autophagy under control conditions. Cells were also treated with Roscovitine prior to treatment with inhibitors to determine whether Roscovitine can inhibit autophagic degradation of p62. Again both bafilomycin and chloroquine treatment of these cells caused an increase in p62 levels suggesting that even with Roscovitine treatment, p62 is degraded by autophagy. It is however important to compare control and Roscovitine treated cells directly. In the absence of inhibitors Roscovitine causes a decrease in p62 protein as seen previously. But in the presence of bafilomycin and chloroquine this decrease is still observed. This suggests that even when autophagy is inhibited Roscovitine still causes a decrease in the levels of p62.

It is possible that the decrease in p62 is due to degradation by another pathway. To determine this, cells were treated with a proteosome inhibitor Bortezomib, results of which are shown in Figure 4.12a. Again western blots were carried out and p62 levels determined. In control population where proteosomal degradation was not inhibited p62 levels decreased upon Roscovitine treatment. When LCLs were treated with Bortezomib the amount of p62 protein was increased. This suggests that p62 is degraded by the proteosomal pathway. In the presence of Bortezomib, Roscovitine still caused a decrease in p62 levels. This shows that the decrease in p62 caused occurs when proteosomal degradation is inactivated and therefore suggests proteosomal degradation is not a significant pathway in Roscovitine-mediated reduction of p62 levels. These results rule out the possibility that the decrease in p62 being caused by Roscovitine is due to changes in levels of p62 degradation.

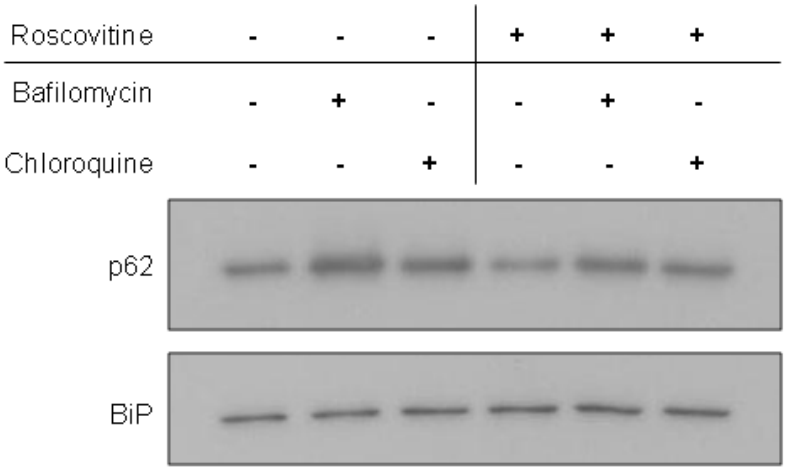


Figure 4.11 p62 expression in the absence and presence of inhibitors of autophagic degradation in Roscovitine treated LCLs

To quantify p62 degradation by autophagy, in the presence or absence of Roscovitine, LCLs were treated with two different inhibitors of autophagic/lysosomal degradation, Bafilomycin A1 (100nM) or chloroquine (50mM). P62 protein was detected by p62-specific antibody. Results show that levels of p62 protein are reduced by Roscovitine in the absence and presence of inhibitors.

Therefore I hypothesised that Roscovitine may be decreasing levels of p62 by inhibiting *de novo* synthesis of the protein.

To determine whether this was correct cells were treated with the protein synthesis inhibitor anisomycin. Again western blots were carried out and p62 levels determined. Figure 4.12b shows the results of this experiment. In control populations where protein synthesis was not inhibited, p62 levels decreased upon Roscovitine treatment. In the presence of anismoycin however Roscovitine had no effect on levels of p62 protein. This shows that when *de novo* protein synthesis is inhibited by anisomycin, Roscovitine is unable to reduce levels of p62 protein. This means that Roscovitine mediated reduction of p62 protein seen in control conditions is due to Roscovitine inhibiting synthesis of p62.

Roscovitine is a known RNA pol II inhibitor. I therefore hypothesize that Roscovitine reduces levels of p62 protein by inhibiting the synthesis of p62 mRNA. To determine if this is the case, levels of p62 mRNA transcript were measured by qRTPCR. Cells were treated with increasing amounts of Roscovitine. mRNA was then extracted from the cells and levels of p62 mRNA transcript were determined. As is clearly shown in Figure 4.13 and as would be expected from previous experiments, concentrations of Roscovitine above 1.0μM and up to 100μM all caused a decrease in p62 mRNA expression. This proves that Roscovitine mediated reduction of p62 protein is due to reduced synthesis of p62 and not related to autophagic degradation.

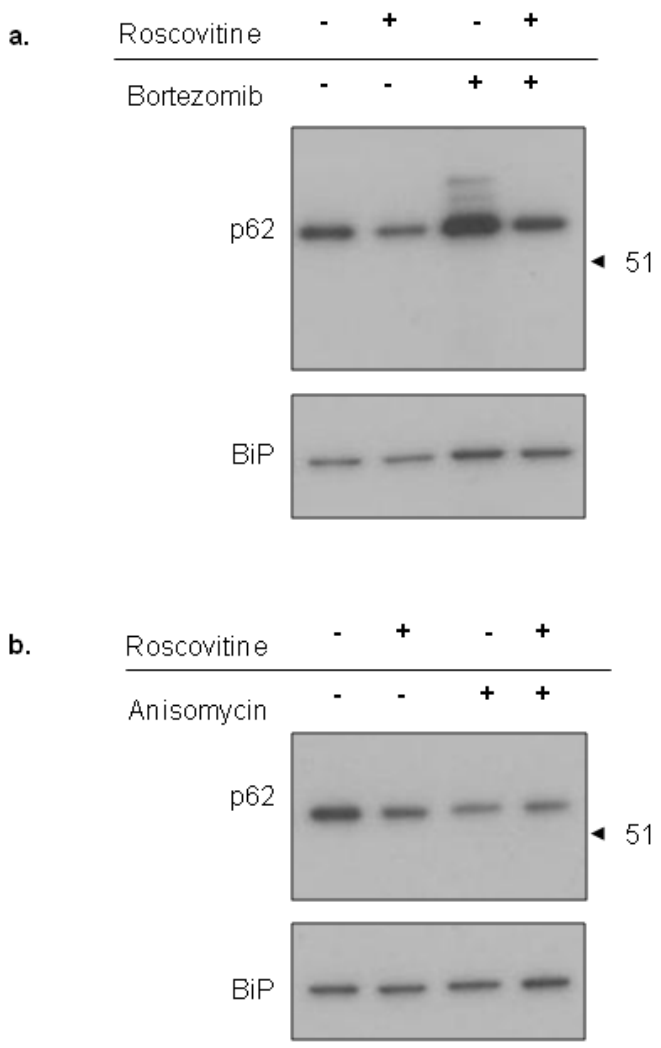


Figure 4.12 p62 expression in the absence and presence of proteosome and protein synthesis inhibitors in Roscovitine treated LCLs

The effect of Roscovitine on levels of p62 was monitored in LCLs in the absence and presence of inhibitors of proteasomal degradation and protein synthesis. **A.** Roscovitine caused a decrease in p62 protein in the absence and presence of Bortezomib **B.** Roscovitine mediated decrease in p62 protein was ablated by anisomycin. In the presence of anisomycin Roscovitine did not affect p62 protein levels. BiP protein expression was measured as a control.

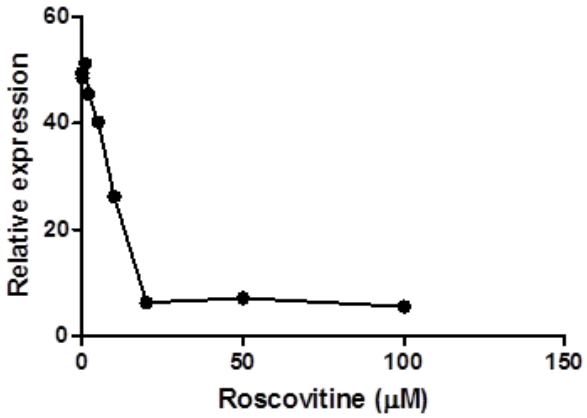


Figure 4.13 Expression of p62 mRNA in Roscovitine treated LCLs

Cells were treated with increasing amounts of Roscovitine and levels of p62 mRNA transcript were measure by qRT-PCR. Expression was compared to that of the the housekeeping gene GAPDH. Increasing concentrations of Roscovitine caused decrease in p62 mRNA expression.

4.6 The effect of Roscovitine on other short-lived, autophagy related proteins

RNA pol II is required for synthesis of all mRNA. Roscovitine inhibits RNA pol II causing a decrease in the short lived protein p62. I therefore hypothesise that Roscovitine will also cause a decrease in other short lived proteins, which could include other autophagy adaptor proteins.

To test this hypothesis the effect of Roscovitine on a number of short lived proteins was tested. These included the autophagy adaptors p62, CALCOCO2, NBR1 and OPTN. A fifth autophagy associated protein Bag 3 was also included. Bag-3 is a chaperone that functions with p62 to degrade ubiquitinated substrates (Carra *et al.*, 2008; Gamerdinger *et al.*, 2009).

LCLs were treated with increasing concentrations of Roscovitine. Western blots were carried out on all samples and blots were then probed with antibodies against a range of autophagy adaptors. Figure 4.14 shows the results of this experiment. The autophagy adaptors p62, CALCOCO2, NBR1, OPTN and BAG-3 were all detected in control cells. As previously seen, increasing concentrations of Roscovitine decreased the amount of p62 protein expressed. This result was replicated across all proteins assayed. Roscovitine causes a decrease in the amount of all five autophagy adaptor proteins.

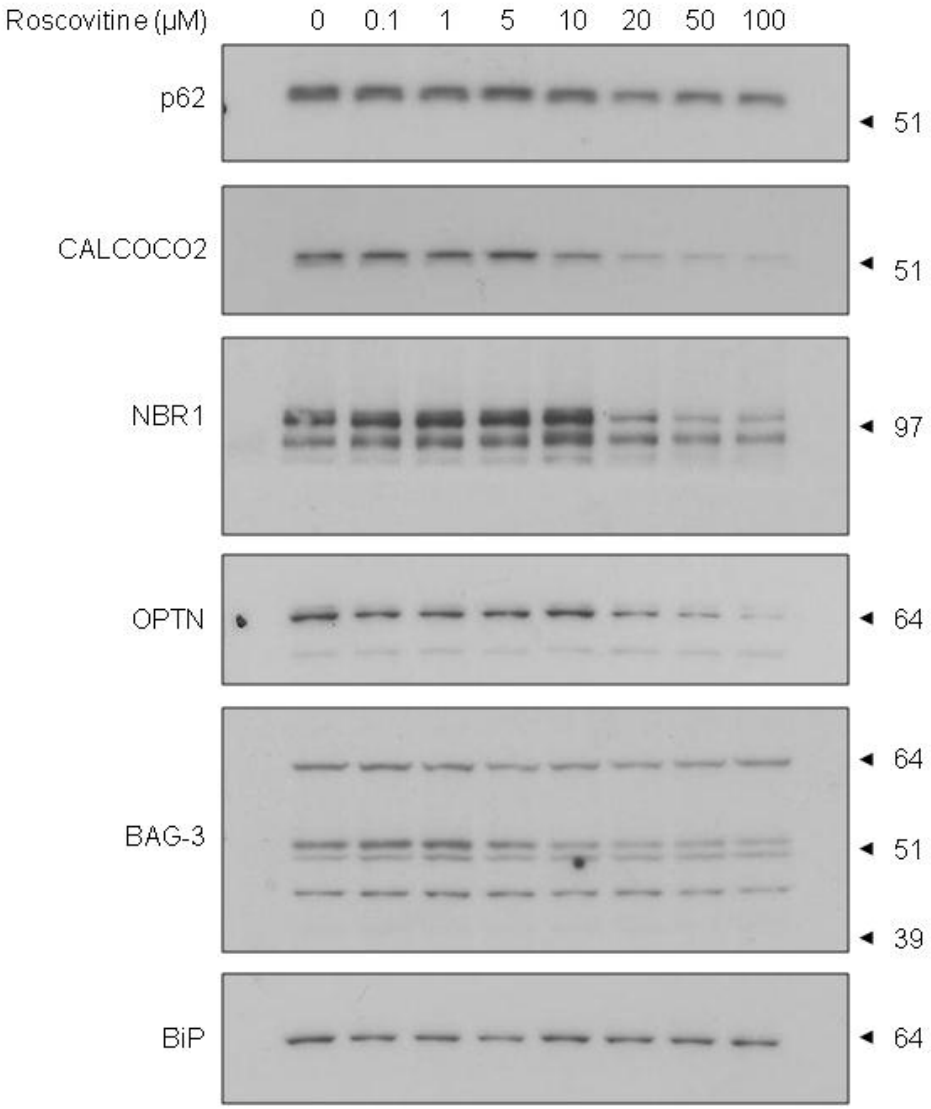


Figure 4.14 Protein expression of autophagy adaptors in Roscovitine treated LCLs

Expression of autophagy associated proteins was measured in Roscovitine treated LCLs with antibodies specific to p62, CALCOCO2, NBR1, OPTN and BAG-3. Expression of BiP was monitored as a control. Roscovitine caused a decrease in all proteins.

4.7 Discussion

Failure to generate WT1-specific T cell clones in the previous chapter led me to investigate the relocalisation hypothesis in an alternative system. Previous work by Leung and colleagues showed that a mutation of EBNA1 that relocalised this protein from the nucleus into the cytoplasm could increase the presentation of MHC class II epitopes from the protein. Although this mutation was deliberate, an example of such relocalisation occurring naturally has been reported. Enhanced $CD4^+$ T-cell recognition of a melanoma cell line was shown to result from a mutation in the target antigen's NLS (Wang R. F. *et al.*, 1999). Although cancer cells have high rates of mutation, such fortuitous events will occur only rarely. A pharmacological method to relocalise EBNA1 into the nucleus was therefore sought. This literature search coincided with the publication of a paper reporting Roscovitine relocalised EBNA1 into the cytoplasm. The effects of Roscovitine-mediated relocalisation of EBNA1 upon T cell recognition were examined in this study. In contrast to WT1, the majority of healthy EBV-positive donors have readily detectable EBNA1-specific T cell responses in their blood (Leen *et al.*, 2001; Munz *et al.*, 2000). Thus EBNA1 was likely to be a more amenable system in which to explore my hypothesis that pharmacological modification of subcellular localisation could enhance the presentation of MHC class II epitopes.

4.7.1 Relocalisation of EBNA1

The effect of Roscovitine on localisation of endogenous EBNA1 was tested by nuclear cytoplasmic fractionation. Results showed that Roscovitine was unable to relocalise EBNA1 in LCLs. At first this result appeared to contradict to the report of Kang *et al.* but

this fundamental difference may have been due to the different experimental systems used.

Kang *et al.* suggested that Roscovitine's mechanism of action was through reduced phosphorylation of the EBNA1 nuclear localisation sequence leading to reduced EBNA1 import into the nucleus. In contrast to my experiments which used the more physiologically relevant approach of studying endogenously-expressed EBNA1, Kang *et al.* conducted their experiments using over-expressed EBNA1 in HeLa cells. Accordingly, there will be high-level ongoing transcription, translation and then nuclear import of *de novo* synthesised protein. Here Roscovitine's effects on NLS phosphorylation and nuclear import would be substantial. In contrast, in cells that endogenously express EBNA1, most EBNA1 will already be in the nucleus, where it will be retained by binding to chromatin. A much smaller level of *de novo* EBNA1 synthesis will be occurring and any interference of nuclear import by Roscovitine will affect a much smaller amount of protein. Overall, my results suggest that Roscovitine may inhibit nuclear import of newly synthesised EBNA1 but may not actually relocalise a pre-existing pool of nuclear EBNA1 to the cytoplasm.

Levels of EBNA1 synthesis are very low in LCLs as the protein's Gly-Ala repeat inhibits its translation by different but complementary mechanisms. Consequently, only a small amount of EBNA1 will be present in the cytoplasm to be retained by Roscovitine-mediated effects on NLS phosphorylation. This would suggest that Roscovitine would not have a detectable effect on endogenous EBNA1; this was indeed shown to be true.

I repeated my experiments using over-expressed EBNA1 but again saw no effect of Roscovitine. These results directly contradict those of Kang *et al*. The reason why no effect of Roscovitine was observed in these experiments is not known.

4.7.2 Roscovitine and EBNA1 MHC class II processing

Despite the negative results of the previous experiments the effect of Roscovitine on the MHC class II processing of EBNA1 was explored. These experiments produced unexpected but none the less intriguing results. My original hypothesis was that by relocalising EBNA1 into the cytoplasm, Roscovitine would increase the presentation of EBNA1 MHC class II epitopes in an autophagy-dependent manner. My biochemical data suggested that Roscovitine did not relocalise EBNA1 and would therefore have no effect. However, my experimental data showed Roscovitine decreased the generation of an autophagy-dependent MHC class II epitope from EBNA1, a result seen previously using the autophagy inhibitor 3-MA.

4.7.3 Roscovitine, autophagy and p62

The decreased presentation of an autophagy-dependent epitope by Roscovitine suggested that, like 3-MA, Roscovitine may have been inhibiting autophagy. To examine this theory the effect of Roscovitine on autophagy was examined using two standard and widely-used approaches: measuring autophagic flux by LC3 blotting and quantifying autophagosome numbers in cells stably-expressing an LC3-GFP fusion protein (Klionsky *et al.*, 2012). Surprisingly, these experiments suggested Roscovitine had no effect on levels of autophagy. As these results directly conflicted with the results of the T cell assays a third method of studying autophagy was used. P62 is an adaptor protein that is

degraded by autophagy; measuring changes in cellular p62 levels is again a widely used technique to measure autophagy activity (Klionsky *et al.*, 2012). Upon treatment with Roscovitine the amount of p62 protein in LCLs decreased. Taken by itself, this result would be interpreted as an increase in the levels of autophagy. However, given the contradictory results from the three assays I decided to explore further the mechanism of how Roscovitine decreased p62 levels without apparently altering autophagy activity.

The Roscovitine-mediated reduction in p62 I observed could have been caused by i) increased autophagic degradation of p62 ii) increase proteosomal degradation of p62 or iii) reduced protein synthesis of p62. Inhibitor studies showed that the Roscovitine-mediated decrease of p62 levels was not affected by inhibition of autophagy- or proteosome-mediated degradation. However, following inhibition of protein synthesis Roscovitine no longer decreased p62 levels.

As protein synthesis inhibition ablated Roscovitine's effect on p62, I hypothesised that Roscovitine was inhibiting synthesis of p62 mRNA. In support of this hypothesis is the fact that Roscovitine is a known RNA pol II inhibitor. This theory was confirmed by assessing levels of p62 mRNA by qRT-PCR.

In total, the data discussed above show Roscovitine does not affect autophagy despite the reduction in p62. This clearly demonstrates that measuring a single component may not give an accurate measurement of autophagy activity and that autophagy flux should always be measured by assessing the change in these components over time in the absence and presence of inhibitors of their degradation.

4.7.4 Roscovitine and autophagy adaptors

In light of the knowledge that Roscovitine inhibited synthesis of p62 the effect of Roscovitine on the expression of other autophagy adaptors was measured. The expression of all tested proteins was shown to be decreased by Roscovitine.

The fact that Roscovitine decreased levels of p62 without decreasing autophagy *per se* presented a possible explanation for the results observed for Roscovitine treated LCLs in the T cell assays. If EBNA1 was dependent upon an autophagy adaptor such as p62 to be degraded by autophagy, then decreasing cellular levels of the adaptors would decrease the generation of epitopes without affecting autophagy. Several autophagy adaptor proteins were tested and levels of all were decreased following Roscovitine treatment. Although pathogens such as *Salmonella* have been shown to be targeted for autophagic degradation by various adaptor proteins no viral protein has before been shown to be processed in this way and there are no examples of MHC class II epitopes being generated via autophagy-adaptor mediated autophagy. If EBNA1 was indeed being degraded via autophagy adaptor proteins then it would represent the first example of a viral antigen being degraded by this pathway.

5. PROCESSING OF EBNA1 VIA AUTOPHAGY ADAPTOR PROTEINS

The data presented in Chapter 4 showed that Roscovitine specifically decreased the presentation of an MHC class II, autophagy-dependent epitope from EBNA1. This decrease occurred despite Roscovitine having no effect on levels of autophagy. However, Roscovitine does inhibit the expression of multiple autophagy adaptors. I hypothesised that autophagic degradation of EBNA1 is mediated through one or more of these autophagy adaptors and that Roscovitine-mediated decreases in levels of these proteins inhibited the generation of the autophagy-dependent MHC class II epitope, SNP.

To test this theory expression of individual autophagy adaptors and other autophagy related proteins were inhibited and the effect on the presentation of the SNP epitope as well other autophagy-independent epitopes was measured

5.1 siRNA Knock down of p62

I began this work by testing whether a previously published siRNA sequence designed to target p62 would be able to Knock down the protein in LCLs. LCLs are a notoriously difficult cell line to transfect and standard siRNA/lipofectamine protocols are usually ineffective. High doses of siRNA (5μM) were therefore electroporated into cells using a previously optimised protocol. Following transfection, samples were harvested every 24h for four days. Levels of p62 protein within cells were then determined by western blot using a p62-specific antibody. BiP protein was again measured as a control using a BiP-specific antibody. Figure 5.1 shows the amount of p62 protein in control and siRNA treated cells over 96h and a band representing p62 was detected in all samples. p62-specific siRNA caused a substantial reduction in the level of p62 protein at 24, 48 and

72h. The effect of the siRNA was reduced at 96h but a small decrease in p62 levels could still be observed. This shows that electroporation of this siRNA is able to Knock down p62 in LCLs.

5.2 Overexpression of EBNA1 using a doxycycline regulated system

As siRNA only causes a transient decrease in protein levels, the effect of MHC class II epitope generation can only be studied over a short period of time. This creates a problem as pre-existing MHC class II peptide complexes will be present on the surface of LCLs before siRNA transfection. MHC class II complexes are relatively stable and can be maintained at the cell surface for multiple days (Mackay *et al.*, 2009). These pre-existing complexes may make it difficult to study any changes in the production of new complexes, over a short period of time. It is possible to remove these complexes with acid treatment; however results are unreliable and vary between epitopes.

I therefore derived a system whereby EBNA1 could be over-expressed in LCLs causing a readily detectable increase in the generation of MHC class II epitopes over a short period of time. Knock down of autophagy adaptors could then be carried out to see if this would interfere with the increase in epitope generation.

To this end, I used a recently developed episomal vector, pRTS1 which is stably maintained in silent form in LCLs but from which antigen expression can be induced to high levels by doxocycline (dox) (Leung *et al.*, 2010).

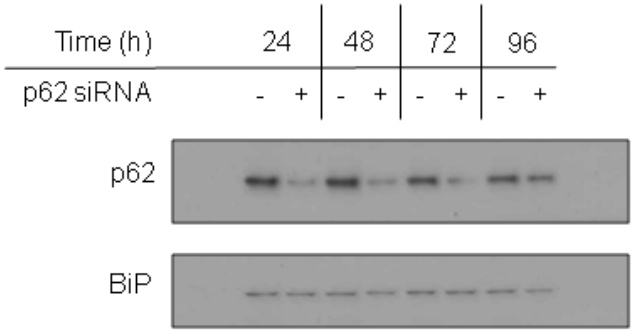


Figure 5.1 Expression of p62 protein in LCLs following siRNA treatment

Western blot analysis of p62 protein expression in siRNA treated LCLs. P62 protein was detected using a p62-specific monoclonal antibody. siRNA treatment reduced levels of p62 protein at all time points compared to untreated cells at the same time point. Expression of BiP protein was used as a loading control

Figure 5.2a shows expression of this construct in LCLs. Full length EBNA1 is detected in both the control empty vector and the construct encoding a modified EBNA1 protein with a deleted Glycine/Alanine region (E1ΔGA) but not in the EBV-negative line DG75. Dox induction of E1ΔGA induces high levels of the smaller-sized EBNA1 protein.

To test the effect on generation of MHC class II epitopes, control vector and E1ΔGA LCLs were again dox induced for 72h and used as targets in T cell assays using a $CD4^+$ T cell clone specific for the EBNA1 epitope SNP. IFN-γ release by T cells was assayed by ELISA. Recognition was compared to an uninduced control. Figure 5.2b shows results of this experiment. As expected T cells were able to recognise control LCLs expressing normal endogenous levels of EBNA1. This recognition was increased upon over-expression of E1ΔGA triggered by dox addition.

5.3 p62 siRNA Knock down and the effect on EBNA1 MHC class II epitopes

The dox regulated system provides an excellent model in which to study MHC-II epitope generation over short periods of time. Therefore this system was used to study the effect of p62 Knock down upon generation of such epitopes.

LCLs stably expressing the E1ΔGA vector were transfected with p62 siRNA or a control siRNA targeting GFP. Cells were then incubated for 24h to allow intracellular protein levels of p62 to decrease. Doxycycline was then added to the media to induce expression of E1ΔGA and cells incubated for a further 72h.

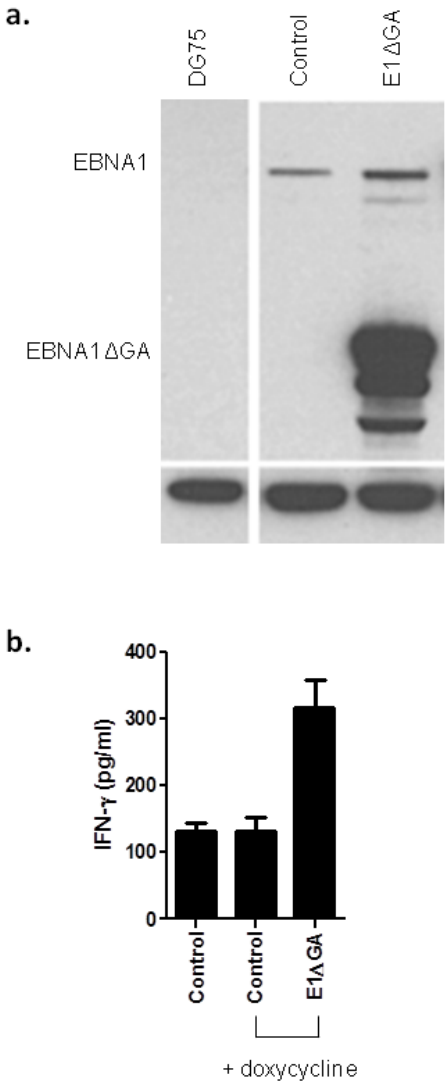


Figure 5.2 Overexpression of EBNA1 ΔGA and the effect of T cell recognition

Analysis of overexpression of EBNA1 ΔGA. **A.** EBNA1 protein was detected using the EBNA1-specific antibody IH-4. EBNA1 was detected in control and dox induced LCLs. E1 ΔGA was detected in dox induced LCLs. Expression of actin protein was used as a loading control (figure adapted from Leung *et al.*, 2010). **b.** Over expression of E1 ΔGA caused an increase in LCL recognition by SNP-specific $CD4^+$ T cells.

An aliquot of cells was harvested for western blot analysis to determine efficacy of Knock down. The remaining cells were used as targets in a T cell assays using the EBNA1-specific $CD4^+$ T cell clone SNP.

Figure 5.3a shows that p62 levels were reduced by siRNA transfection, note the blot only shows p62 levels at the 96h time point at which cells were used in T cell assays. Expression of p62 would have been lower at earlier time points as shown in Figure 5.1.

Figure 5.3b shows recognition of LCLs by SNP-specific T cells. As seen previously, T cells were able to recognise control LCLs and this recognition was increased upon over-expression of E1ΔGA, triggered by dox addition. SNP T cell recognition of LCLs transfected with p62 siRNA and treated with dox is comparable to that of LCLs transfected with control siRNA and treated with dox. This result suggests that reduction of p62 protein in antigen presenting cells using siRNA has no effect on the generation of the SNP epitope and subsequently recognition by SNP-specific T cells is unchanged.

5.4 siRNA Knock down of alternative autophagy related proteins and the effect on EBNA1 MHC class II epitopes

Though p62 was the first identified and most well studied autophagy adaptor, other proteins have also been shown to have similar functions to p62, as previously discussed. The previous result ruled out a role for p62 in the processing of EBNA1 but showed the combination of siRNA and EBNA1 over-expression provides a versatile system for measuring changes in the generation of class II epitopes. I therefore decided to repeat these experiments using siRNAs targeting the other autophagy adaptors CALCOCO2, NBR1 and OPTN as well as the autophagy chaperone BAG-3.

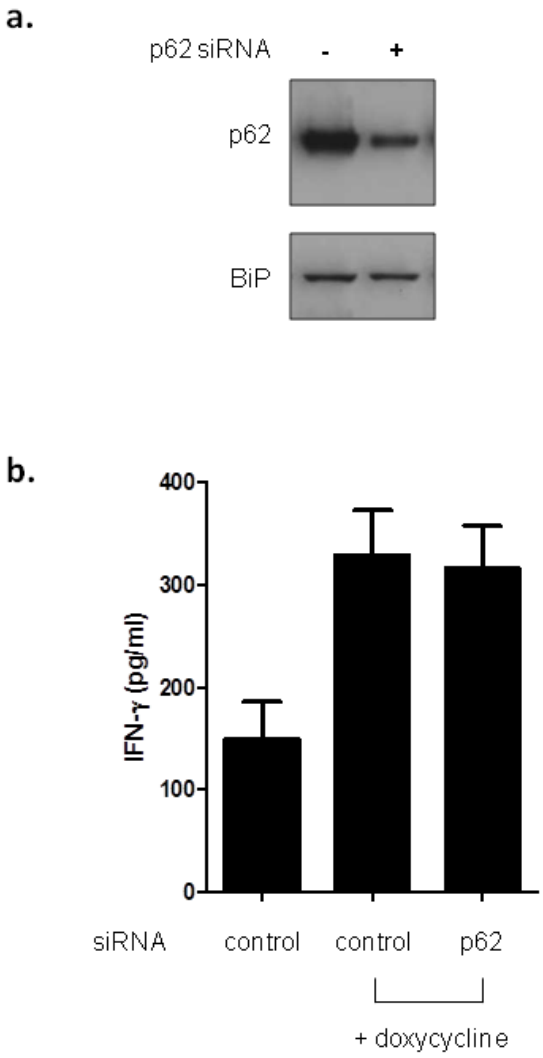


Figure 5.3 siRNA Knock down of p62 in LCLs and subsequent T cell recognition

Analysis of p62 siRNA Knock down. **A.** P62 protein was detected using a p62-specific antibody. P62 was detected in control cells , levels of protein were reduced by siRNA treatment. **b.** Over expression of E1 ΔGA caused a increase in LCL recognition by SNP-specific $CD4^+$ T cells. P62 siRNA had no effect on this recognition.

A fifth autophagy related protein was also included in these experiments. ALFY, a large phospoinositide-binding protein is recruited to p62 bodies as well as nuclear promyleocytic leukemia (PML) bodies and is required for the degradation of such aggregates by autophagy (Clausen *et al.*, 2010; Simonsen *et al.*, 2004). ALFY may therefore also be required for the generation of autophagy-dependent MHC class II epitopes.

siRNAs designed to target five different autophagy adaptors or chaperones were identified from the literature. Again these had been used in easily transfectable lines. To test the potency of these siRNAs in LCLs, cells were transfected with siRNA targeting a single autophagy adaptor or siRNA targeting GFP as a control. After electroporation, aliquots of cells were again harvested every 24h for four days. Knock down of the different proteins was assessed by western blotting using antibodies specific to each autophagy protein.

Figure 5.4 shows the expression of four different autophagy related proteins treated with control or target siRNA over 96h using antibodies specific for the proteins. Expression of BiP or actin was also measured as a control. Expression of the three autophagy adaptors CALCOCO2, NBR1 and OPTN was decreased by specific siRNA treatment at all time points. It should be noted that the levels of these proteins in GFP siRNA treated LCLs decreased over 96h. This may be due to an increase in autophagy, due to decreasing nutrient availability over this time. This did not seem to reduce any effect of target siRNAs.

Unlike other siRNAs, BAG-3 siRNA did not seem to reduce levels of BAG-3 protein. Further experiments were conducted using alternative siRNA sequences or higher doses

of siRNA but these were also unsuccessful in reducing BAG-3 levels (data not shown). All BAG-3 siRNAs used had previously been proven to Knock down BAG3 in other cell lines, the failure of these experiments highlights the difficulty of knocking down proteins in LCLs.

A fifth siRNA specific to the protein ALFY was also tested for its ability to Knock down protein in LCLs. ALFY is a high molecular weight protein and all attempts to resolve proteins of this molecular weight were unsuccessful and therefore Knock down of ALFY could not be confirmed.

Having demonstrated that siRNA could Knock down four autophagy adaptors in LCLs I next investigated the effect of this Knock down on T cell recognition. siRNA against BAG-3 and ALFY were included in these experiments despite Knock down not being confirmed in these cells: identification of an effect on T cell recognition would have led me to focus on these proteins again.

The design of the experiments was the same used previously for studying p62. LCLs were transfected with an siRNA targeting one of the six autophagy related proteins. Cells were incubated for 24h to allow protein levels of autophagy adaptors to decrease. After this time doxycycline was added to the media and cells incubated for a further 72h to allow processing and presentation of over-expressed E1ΔGA. An aliquot of cells was harvested for western blot analysis to confirm Knock down. The remaining cells were used as targets in T cell assays using SNP-specific $CD4^+$ T cells.

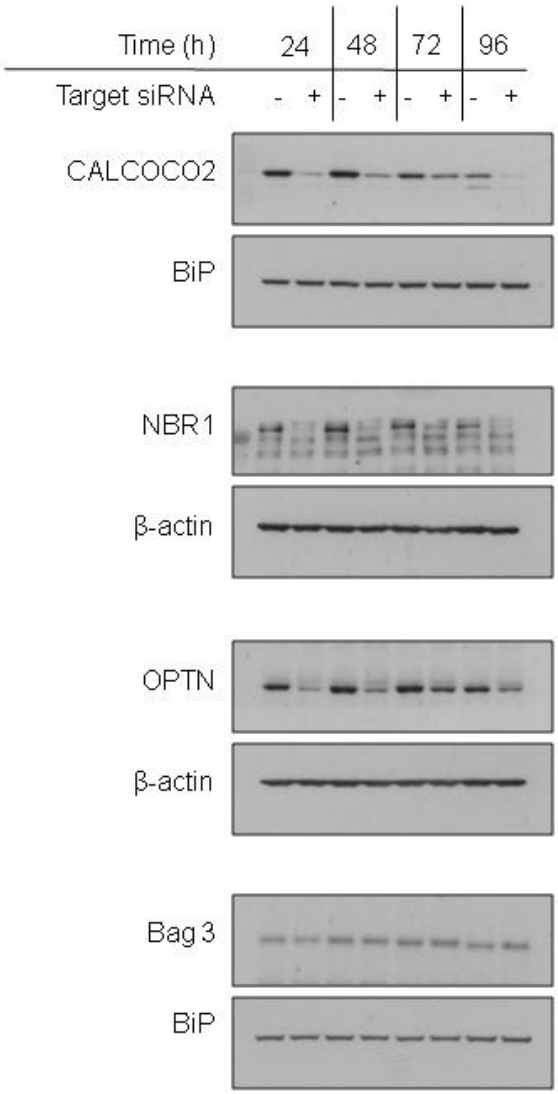


Figure 5.4 Expression of autophagy related proteins in LCLs following siRNA treatment

Western blot analysis of autophagy protein expression in siRNA treated LCLs. Protein was detected using specific monoclonal antibodies. siRNA treatment reduced levels of proteins at all time points compared to untreated cells at the same time point (with the exception of BAG3 siRNA which did not affect levels of BAG3 protein. Expression of BiP or actin protein was used as a loading control

Figure 5.5 shows the results of this experiment. Again as expected, T cell recognition of control siRNA transfected cells increased upon the addition of dox. As seen earlier, p62 siRNA had no effect on LCL recognition. No effect of ALFY or BAG-3 siRNA was observed either. However Knock down of three other autophagy adaptors did reduce T cell recognition. Recognition of CALCOCO2, NBR1 and OPTN siRNA treated LCLs were all below that of GFP siRNA treated cells. CALCOCO2 and OPTN showed similar levels of decrease whereas NBR1 showed the greatest decrease, completely ablating any increase in recognition caused by dox addition. NBR1 siRNA reduced recognition to below the level of uninduced cells.

To confirm this effect was specific to the generation of the autophagy-dependent epitope SNP, the same LCLs were used in parallel T cell assays using $CD4^+$ T cells specific to the autophagy-independent MHC class II epitope VYG. Figure 5.5b shows siRNA Knock down had no effect on presentation of the VYG epitope showing the previous effect was specific to the SNP epitope and not a general effect on class II processing.

5.5 Presentation of endogenous EBNA1 following siRNA Knock down of autophagy adaptors

The previous experiments suggest the three autophagy adaptors, CALCOCO2, NBR1 and OPTN are required for recognition of over-expressed EBNA1. Whether they are required for recognition of endogenously expressed EBNA1 is unknown. As previously mentioned the presence of pre-existing MHC-II peptide complexes on the surface of LCLs could mask a change in the generation of new complexes. This make changes in recognition of normal levels of endogenous protein more difficult to detect.

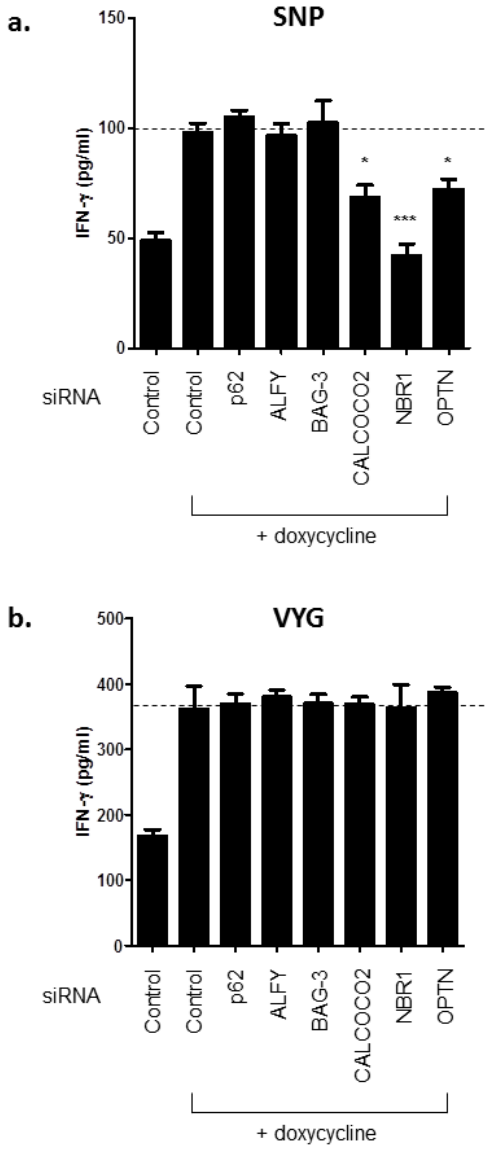


Figure 5.5 T cell recognition of LCLs following siRNA Knock down of autophagy-related proteins

Analysis of autophagy protein siRNA Knock down. **A.** Over expression of E1 ΔGA caused an increase in LCL recognition by SNP-specific $CD4^+$ T cells. siRNA specific to CALCOCO2, NBR1 and OPTN reduced this T cell recognition. Statistical significance was determined by ANOVA and subsequent bonferroni multiple comparison analysis (*P< .05; ***P<.001) compared with control siRNA + doxycycline. **B.** Over expression of E1 ΔGA caused an increase in LCL recognition by VYG-specific $CD4^+$ T cells. siRNA had no effect on this recognition.

Despite these possible difficulties, experiments were conducted on LCLs to determine the effect of protein Knock down by siRNA on T cell recognition of endogenous EBNA1. LCLs were transduced with siRNA and incubated for 96 h. After this time an aliquot of cells was removed for western blot analysis to determine level of Knock down. The remaining cells were used as targets in T cells assays using SNP-specific T cells. Figure 5.6a shows a typical example of one such experiment. Figure 5.6b shows results of multiple experiments, where T cell recognition has been standardised to the level of recognition of control LCLs. Both CALCOCO2 and NBR1 siRNA significantly decreased recognition of endogenous EBNA1 by SNP-specific $CD4^+$ T cells. These results confirm data obtained using E1ΔGA and support the theory that these proteins are involved in the generation of the SNP epitope. The over-expression system also implicated OPTN in the generation of SNP however in this experiment LCLs treated with OPTN siRNA showed only a very slight decrease in T cell recognition that was not significant.

5.6 Processing of EBNA1 MHC-II epitopes in epithelial cells

Previous experiments have been conducted in B cells as EBV is naturally maintained in these cells. However, EBV also infects epithelial cells. In fact EBV infection of epithelial cells causes a bigger disease burden in that nasopharyngeal carcinoma and the 10% of gastric carcinomas associated with EBV arise from epithelial cells. However $CD4^+$ T cell recognition of epithelial cells is very poorly studied and it is not known if findings from experiments conducted in B cells and other APCs apply in epithelial cells. I therefore investigated $CD4^+$ T cell recognition of E1ΔGA expressed in an epithelial cell line.

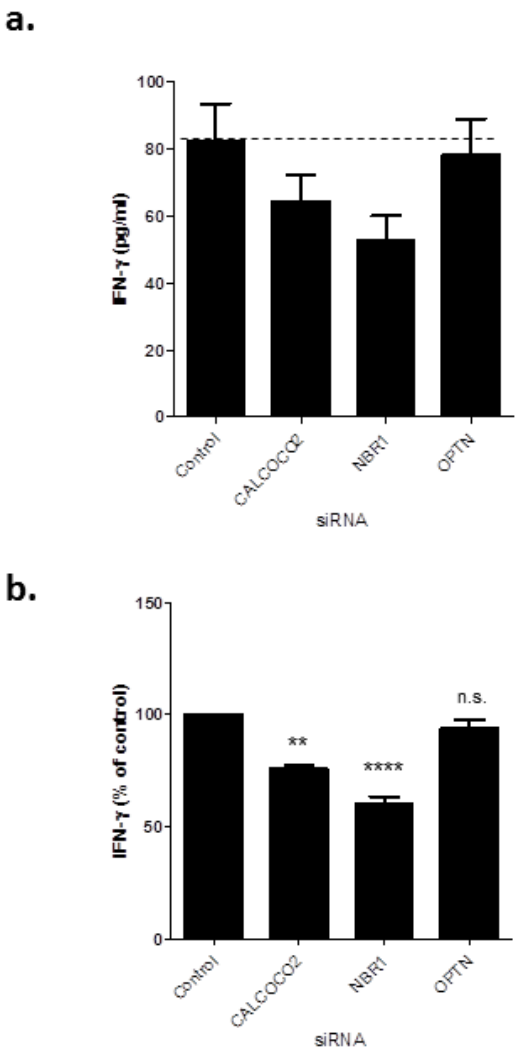


Figure 5.6 SNP T cell recognition of endogenous EBNA1 following siRNA Knock down of autophagy adaptors

Analysis of autophagy protein siRNA Knock down. **A.** siRNAs specific to CALCOCO2, NBR1 and OPTN reduced T cell recognition of endogenous EBNA1 from LCLs by SNP-specific T cells. **B.** Repeat experiments revealed the reduction observed by CALCOCO2 and NBR1 was significant by ANOVA and subsequent bonferroni multiple comparison analysis (**P< .01; ****P<.0001) compared with control siRNA.

Previously Zuo *et al.* have used the EBV-negative melanoma line MJS to study antigen processing of multiple EBV proteins including EBNA1 (Zuo *et al.*, 2011). This work showed that MJS cells stably transduced with the relevant HLA class II alleles could present EBNA1 class II epitopes when EBNA1 was over expressed following transient transfection. These experiments shows that MJS has a normal and functioning class II antigen processing and presentation pathway, this and the ease of which protein expression can be manipulated within these cells suggests that the MJS system would be perfect for studying a potential role of autophagy adaptors in the generation of class II epitopes from EBNA1. To ensure efficient processing of EBNA1, Zuo *et al.* used a modified EBNA1 protein localised to the cytoplasm by mutation of the proteins NLS. In B cells this mutation enhances epitope generation and affects the processing routes used. It is not known if MJS cell are able to present MHC class II epitopes from nuclear resident EBNA1. To investigate this, MJS cells were transfected with decreasing amounts of plasmid DNA encoding E1ΔGA and incubated for 72h. After this time the generation of the SNP peptide was measured using SNP-specific T cells. Figure 5.7a shows recognition of MJS cells transfected with E1ΔGA. The MJS cells were recognised by SNP T cells showing MJS cells can efficiently generate class II epitopes from nuclear EBNA1. To confirm the SNP epitope was still being processed through autophagy, MJS cells were transfected with E1ΔGA and incubated for 72h. For the final 24h cells were incubated with the autophagy inhibitor 3-MA. The generation of the SNP peptide was again measured using SNP-specific T cells. Figure 5.7b shows that 3-MA treatment significantly decreased SNP T cell recognition. This suggests that the SNP epitope is generated by autophagy in MJS system as it is in LCLs.

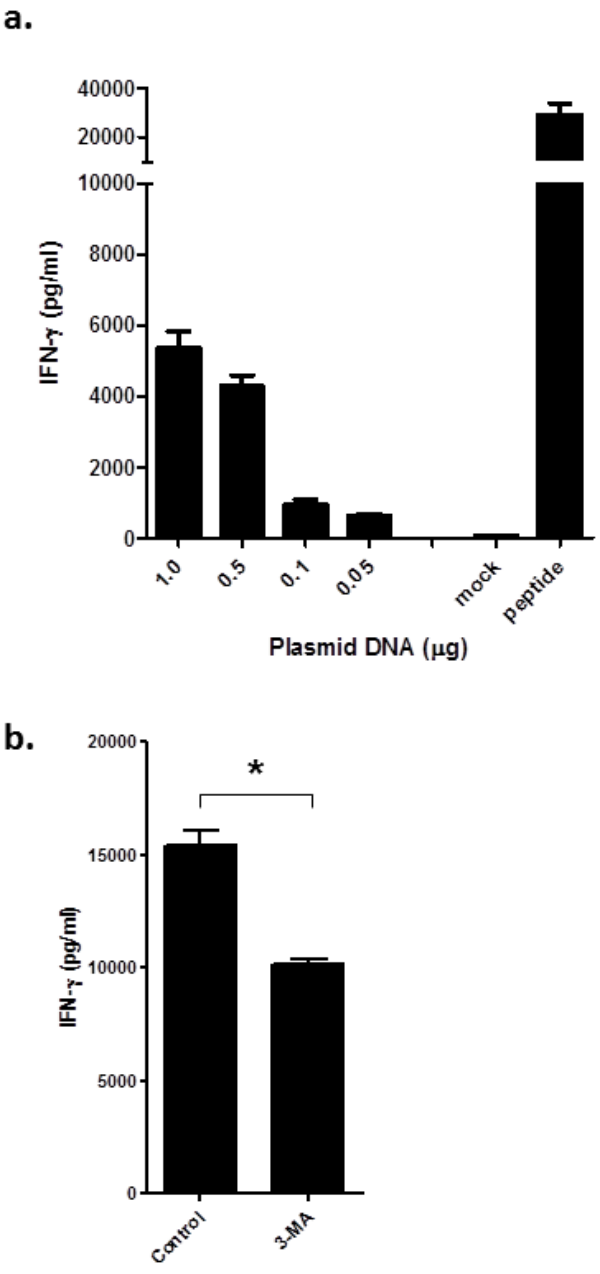


Figure 5.7 CD4⁺ T cell recognition of E1ΔGA in epithelial cells

SNP T cell recognition of MJS cells transfected with E1ΔGA. **A.** SNP T cells recognised MJS cells transfected with decreasing concentrations of E1ΔGA. **B.** 10mM 3-MA reduced SNP T cell recognition of MJS transfected with 0.1µg E1ΔGA DNA. Statistical significance was determined by a paired Student *t* test (*P< .05).

As autophagic presentation of SNP was shown to require autophagy adaptors in B cells, it is possible that this is also the case in epithelial cells. It is not however known, if autophagy adaptors are expressed in MJS cells. To investigate this important question, expression of each autophagy adaptor was measured in MJS cells by western blot. MJS cells were also treated with bafilomycin A1 to inhibit autophagic degradation of autophagy adaptors to confirm their degradation by autophagy. Results of this are shown in Figure 5.8. Results show that p62, CALCOCO2, NBR1 and OPTN are all expressed in MJS cells. Cells treated with Bafilomycin A1 for 4h showed increased expression of autophagy adaptors. This suggests that these proteins are being degraded by autophagy under normal conditions and therefore that MJS cells possess a fully functioning autophagic pathway.

5.7 MHC class II presentation of EBNA1 following siRNA knock down of autophagy adaptors in epithelial cells

Having confirmed that MJS cell present the SNP epitope in an autophagy-dependent manner from nuclear EBNA1, I next investigated whether autophagy adaptors were involved in this presentation as has been observed in previous experiments using B cells.

MJS cells were transfected with siRNA specific for a single autophagy adaptor and incubated for 24h to allow knock down to occur. Cells were then transfected with the E1ΔGA expressing vector and incubated for a further 72h. After this time, cells were assayed for antigen presentation to $CD4^+$ T cells specific to the SNP epitope.

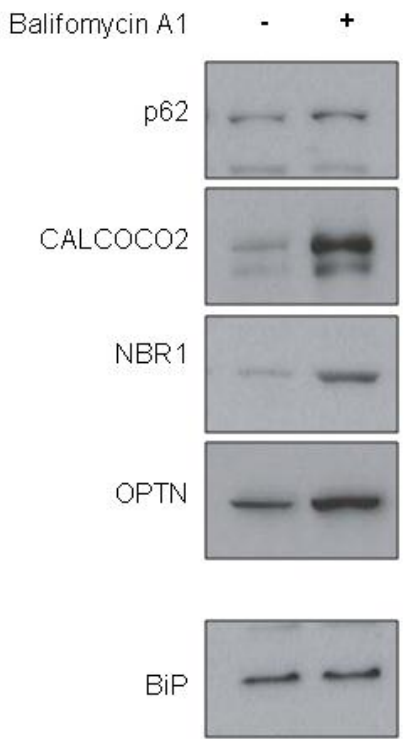


Figure 5.8 Expression of autophagy adaptor proteins in epithelial cells

Western blot analysis of autophagy adaptor protein expression in bafilomycin A1 (100nM) treated MJS cells. Protein was detected using specific monoclonal antibodies. All autophagy adaptors were detected in samples. Bafilomycin A1 treatment increased levels of protein of all autophagy adaptors. Expression of BiP protein was used as a loading control

As expected and because MJS cells do not naturally express EBNA1, mock transfected cells were not recognised by SNP-specific T cells (Figure 5.9). Good T cell recognition was observed of MJS cells co-transfected with EBNA1 and control (GFP) siRNA. As had been previously observed in LCLs knock down of p62 and ALFY had no effect on the generation of the SNP epitope. In MJS cells, Knock down of BAG-3 was successfully achieved using an siRNA that had previously failed to knock down the protein in LCLs. However knock down of BAG-3 appeared to have no effect on the recognition of the SNP epitope.

Importantly, and in agreement with previous experiments conducted in LCLs, recognition of E1ΔGA was significantly reduced when target cells were transfected with siRNA specific to CALCOCO2, NBR1 and OPTN. This result confirms the experiments conducted in LCLs and show that all three autophagy adaptors play an important role in the generation of the SNP epitope, in that knock down of these proteins causes a significant reduction in recognition of target cells by SNP-specific T cells.

Results of these siRNA experiments have been confirmed on both endogenous EBNA1 as well as over-expressed E1ΔGA in two different cells lines. It is however important to rule out any possible off-target effects that single siRNAs might be having. To this effect, two different siRNAs for each autophagy adaptor were designed. Off-target effects of siRNA tend to be different between different siRNAs. Targeting the same protein using different siRNA duplexes should have different off-target effects. If the results of reduced recognition are repeated when different siRNAs are used to Knock down the protein of interest then the effect is likely to be as a result of specific down-regulation of the target gene rather than off-target effects.

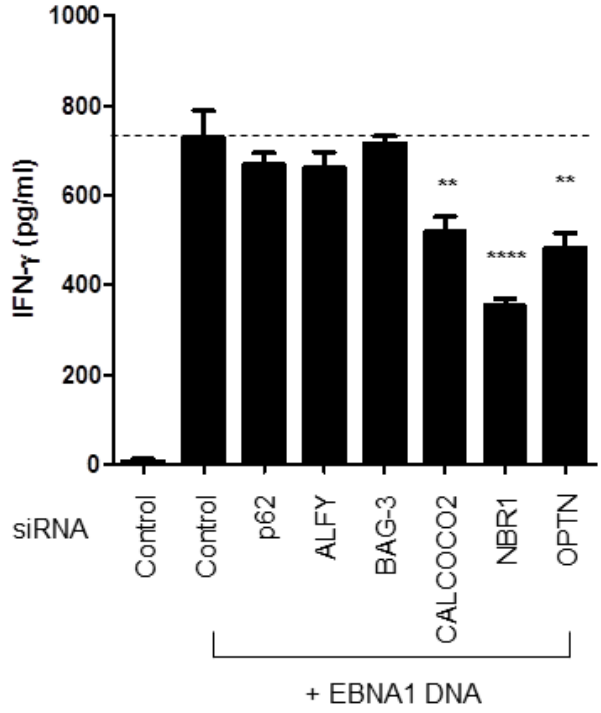


Figure 5.9 T cell recognition of E1ΔGA expressing MJS cells following siRNA knock down of autophagy related proteins

Analysis of autophagy protein siRNA Knock down on the effect of SNP-specific T cell recognition of MJS cells overexpressing E1ΔGA. siRNAs specific to CALCOCO2, NBR1 and OPTN reduced SNP-specific T cell recognition of E1ΔGA. Statistical significance was determined by ANOVA and subsequent bonferroni multiple comparison analysis (**P< .01; ****P<.0001) compared with control siRNA + EBNA1 DNA.

MJS cells were co-transfected with an siRNA and an EBNA1 contruct according to previous protocols. Cells were again assayed for epitope presentation to SNP T cells (Figure 5.10). Mock transfected cells were not recognised by T cells and E1ΔGA/control siRNA transfected cells were recognised well by SNP T cells. All other siRNAs, two specific to each autophagy adaptor, CALCOCO2, NBR1 and OPTN caused a significant reduction in T cell recognition. These results confirm that autophagy adaptor knock down by siRNA does cause a reduction in T cell recognition and rules out the possibility that any previous results are caused by off-target effects of the siRNA.

5.8 Autophagy adaptor – EBNA1 interactions

Classically autophagy adaptors sort cargo for degradation by binding to ubiquitin moieties on target proteins. However EBNA1 is not thought be ubiquitinated under normal conditions (personal correspondence – Lori Frappier, the University of Toronto, Canada). Some adaptors are known to be able to function in a ubiquitin-independent manner though these mechanisms are poorly understood. It is possible that the autophagy adaptors involved in the generation of the SNP epitope target EBNA1 to the autophagic pathway by binding directly to EBNA1. If this is the case EBNA1 should contain one or more consensus sequences where autophagy adaptors can bind. If autophagy adaptors are binding directly to EBNA1 via a consensus sequence then removal of this sequence through deletion should impair the generation of the SNP epitope.

To investigate this possibility a number of EBNA1 mutants containing specific deletions of the EBNA1 protein were obtained (Figure 5.11). MJS cells were transfected with the different constructs and generation of the SNP epitope was assayed.

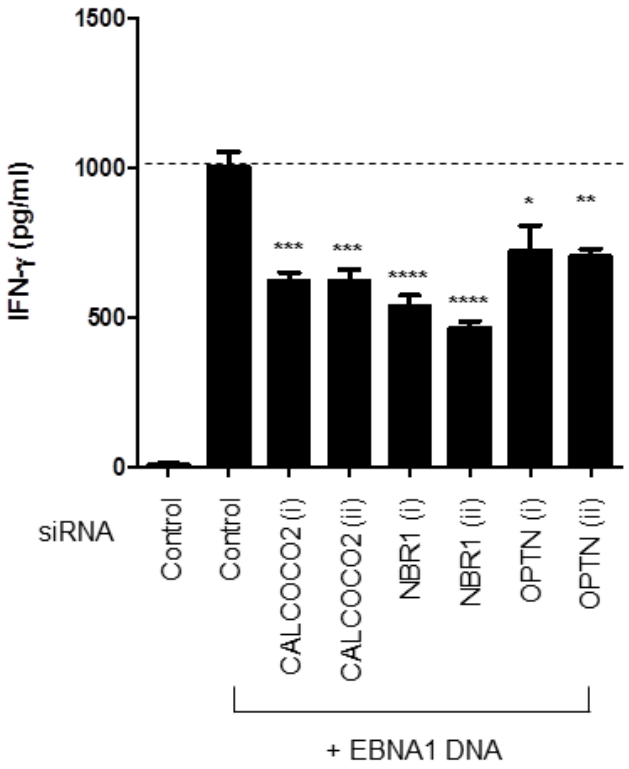


Figure 5.10 T cell recognition of E1ΔGA expressing MJS cells following knock down of autophagy adaptors by multiple siRNAs

Analysis of autophagy protein siRNA Knock down on the effect of SNP-specific T cell recognition of MJS cells overexpressing E1ΔGA. siRNAs specific to CALCOCO2, NBR1 and OPTN reduced SNP-specific T cell recognition of E1ΔGA. This result was confirmed with multiple siRNAs to each protein. Statistical significance was determined by ANOVA and subsequent bonferroni multiple comparison analysis (*P< .05; **P< .01; ***P<.001; ****P<.0001) compared with control siRNA + EBNA1 DNA.

Recognition of the different EBNA1 deletion mutants by SNP-specific T cells is shown in Figure 5.12a. Recognition of E1ΔGA was used as a control and good recognition of this construct was observed. Contrary to what might have been expected, recognition of EBNA1 Δ8-67 was higher than recognition of the E1ΔGA. Recognition of Δ41-376 and Δ395-450 was comparable to levels of control recognition. Interestingly Δ387-394 caused a marked decrease in recognition by SNP T cells. This suggests that the deleted section of EBNA1 contains a region important for EBNA1 processing and presentation.

If this region contained an autophagy adaptor binding domain then the reduction in recognition should be specific to the autophagy-dependent epitope SNP. Presentation of other EBNA1 class II epitopes should be unaffected. To test this, MJS cells transfected with the different EBNA1 constructs were assayed for their presentation to VYG-specific T cells (Figure 5.12b). VYG expression of all constructs was similar with the exception of Δ387-394. As observed with SNP T cells, VYG recognition of this construct was markedly lower than controls.

This excludes the possibility that Δ387-394 contains an autophagy adaptor binding region, but perhaps more interestingly, does suggest that the region is important for overall processing and presentation of multiple EBNA1 class II epitopes.

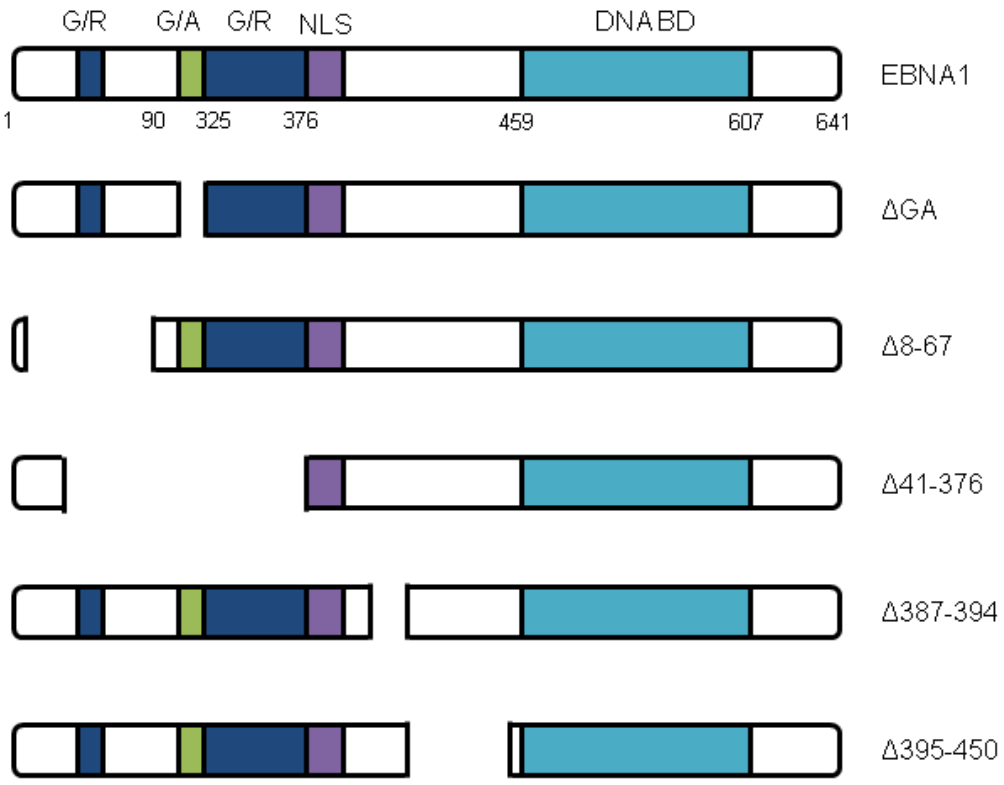


Figure 5.11 EBNA1 deletion mutants

Schematic of EBNA1 deletion mutants used in T cell recognition assays

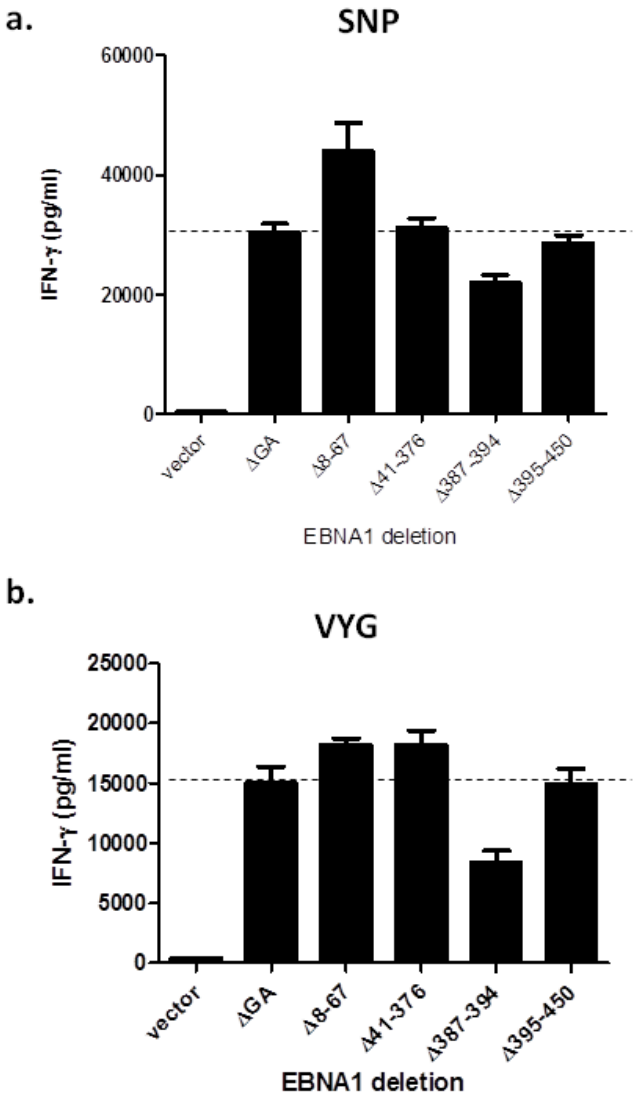


Figure 5.12 T cell recognition of EBNA1 deletion mutants

EBNA1-specific $CD4^+$ T cell recognition of MJS cells transfected with EBNA1 deletion mutants. **A.** SNP-specific T cell recognition. Recognition of the EBNA1 deletion mutant Δ8-67 was higher than control recognition of E1ΔGA. Recognition of the EBNA1 deletion mutant Δ387-394 was lower than control recognition of E1ΔGA. **b.** VYG-specific T cell recognition. Recognition of the EBNA1 deletion mutant Δ387-394 was lower than control recognition of E1ΔGA

5.9 Discussion

The aim of the work described in this chapter was to test the hypothesis that autophagy adaptor proteins were involved in the processing of EBNA1 by autophagy. This hypothesis was formed on the basis of Roscovitine-mediated decrease in adaptor proteins being coincident with decreased presentation of the autophagy-dependent MHC class II-restricted SNP epitope from EBNA1. Because Roscovitine could be having multiple effects on cells I decided to test the hypothesis by selectively decreasing cellular levels of each autophagy adaptor proteins and examining the effects on presentation of the epitope peptide.

5.9.1 Autophagy adaptors in the processing of E1ΔGA in LCLs

Cells that are endogenously expressing EBNA1 are pre-coated with MHC-peptide complexes. When supply of the epitope peptide is interrupted it can take several days for them to dissociate from MHC-II molecules (Mackay *et al.*, 2009). This slow dissociation makes it difficult to measure the effects of inhibitors, since it can take days before there is sufficient decrease in MHC class II-peptide complexes to be robustly and reliably detected in T cell assays. This problem is compounded for experiments reliant upon siRNA since its effects upon target protein levels i) are transient, lasting only 24-48 hours and ii) depend upon natural turnover of the target protein in order to become effective and thus do not act until sometime after their introduction into cells. Although long-term Knock down of cells can be achieved using shRNA, establishing Knock down in LCLs takes considerable time, meaning there is considerable scope for changes to occur in levels of autophagy activity and the antigen expression between the control and Knock down lines. For MHC-I peptides, stripping the pre-existing peptides from the cells

using acidic conditions can circumvent this problem. The denuded cells can then be treated with inhibitors and the effect upon reconstitution of the MHC-I peptide complexes is readily apparent. However, this technique cannot be used for studying MHC class II peptides because they are much less susceptible to elution under acidic conditions. Initial experiments were therefore conducted using an EBNA1 over-expression system, looking for changes in the increase in epitopes from the over-expressed protein. In these experiments, Knock down of three of the five autophagy adaptors was shown to affect the generation of the autophagy-dependent epitope SNP. The fact that some, but not all autophagy adaptors effected epitope presentation suggested that it was a specific effect on EBNA1 presentation rather than a general effect upon autophagy *per se*. To confirm this decrease in SNP epitope presentation was due to an alteration in EBNA1's processing via autophagy, the effect on the generation of the VYG epitope, which is not generated by autophagy from nuclear-localised EBNA1, was also examined. Generation of this autophagy-independent epitope was unaffected by knock down of any autophagy adaptors. These results provide the first example of autophagy adaptors being involved in the autophagy-dependent generation of a MHC class II epitope from an endogenous protein.

The processing of MHC class II epitopes from E1ΔGA is thought to follow the same pathways as naturally expressed EBNA1 (Leung *et al.*, 2010; Mackay *et al.*, 2009). However, I cannot exclude the possibility that these findings are an artefact of studying the processing of over-expressed antigen. Confirming these results in a more physiologically-relevant system therefore became a priority.

5.9.2 Autophagy adaptors in the processing of natural EBNA1 in LCLs

To confirm the results observed using over-expressed E1ΔGA were physiologically relevant, the siRNA Knock down experiments were repeated in LCLs expressing natural levels of endogenous EBNA1. As previously described, studying the processing of naturally expressed protein is made more difficult by the presence of pre-existing peptide-MHC complexes at the cell surface. Despite this fact, Knock down of NBR1 and CALCOCO2 caused similar effects for LCLs. Generation of the SNP T cell epitope was reduced by Knock down of these proteins, although as might be expected for the reasons described above, the magnitude of the effect was reduced compared to the over-expression system. These results were repeated and the decrease was shown to be statistically significant.

Knock down of the third autophagy adaptor, OPTN did not cause a significant decrease in the generation of the SNP epitope in the LCL system. This difference could be for two reasons. First, it is possible that the effect of OPTN knock down observed in the over-expression system was an artefact of the system and OPTN is not involved in the processing of natural EBNA1. Second, it is possible that there is some redundancy in the system and that EBNA1 can be targeted by CALCOCO2, NBR1 or OPTN. Therefore when OPTN was knocked down it is possible that the other two autophagy adaptors were in excess and they were able to compensate for any effect OPTN may have on EBNA1 processing.

Regardless of the differences observed for OPTN, it is clear that at least two autophagy adaptors, CALCOCO2 and NBR1, are important for the processing of EBNA1 via autophagy in B cells.

5.9.3 EBNA1 processing in epithelial cells

In this chapter, experiments were also conducted to study MHC class II processing of EBNA1 in epithelial cells. These experiments were conducted for two reasons. Firstly, siRNA Knock down of proteins is technically much easier to achieve in epithelial cells compared to LCLs. Secondly, and more importantly, a great disease burden arises from EBV infected epithelial cells and despite their importance very little is known about antigen processing in epithelial cells or about their recognition by EBV-specific T cells.

Previous experiments conducted by Zuo *et al.* have shown EBNA1-specific $CD4^+$ T cells can recognise MJS cells, an epithelial line originally derived from a melanoma patient, transfected with DNA encoding the cytoplasmic-localised EBNA1 protein. However, whether MJS cells can generate EBNA1 MHC-II peptides from nuclear EBNA1 was unknown.

By over-expressing EBNA1 in MJS cells I was able to show that these cells were also able to generate MHC class II epitopes from nuclear-localised EBNA1. I also investigated the mechanisms by which the SNP epitope was generated. Using inhibitors of autophagy I was able to confirm SNP is generated in an autophagy-dependent manner similar to that seen in B cells.

Because EBNA1 processing in these epithelial cells was similar to that of B cells I next attempted to confirm the previous result that showed production of the SNP epitope required autophagy adaptors. siRNA experiments were conducted again and results confirmed that the three autophagy adaptors, CALCOCO2, NBR1 and OPTN all contribute to the generation of the SNP epitope in epithelial cells.

As a final experiment to confirm these results, two different siRNAs for each autophagy adaptor were used to rule of any possible off-target effects of individual siRNAs. These data again confirmed that specific Knock down of autophagy adaptors was responsible for the effects observed and were not due to off-target effects from the siRNA originally-used.

Taken together these data represent strong evidence to support the hypothesis that EBNA1 is specially targeted for autophagic degradation by the action of CALCOCO2, NBR1 and OPTN and this targeting is required for the generation of the autophagy-dependent epitope SNP.

I next conducted experiments to identify a possible mechanism for this targeting. The most well documented method of cargo identification by autophagy adaptors is via ubiquitination of proteins. Ubiquitin-independent mechanisms have been identified but these are currently less well understood. As EBNA1 is not thought to be ubiquitinated under normal conditions (personal communication – Lori Frappier, the University of Toronto, Canada) I hypothesised that autophagy adaptors may be binding a specific region of EBNA1, either directly or via an intermediate protein.

To test this theory, generation of MHC class II epitopes from a range of EBNA1 constructs each carrying specific deletions was examined. If an individual deletion mutant showed decreased presentation via autophagy, it would suggest that region could be involved in the binding of EBNA1 to an autophagy adaptor. Such an effect was not observed and the mechanism by which autophagy adaptors and EBNA1 are able to interact is yet to be elucidated.

Though no deletion mutant decreased presentation in an autophagy-dependent manner, two interesting differences were observed. Firstly, deletion of amino acids 8-67 from EBNA1, a region that contains a Gly/Arg rich sequence caused an increase in the generation of the SNP epitope. As the generation of VYG was unaffected this effect was likely specific to autophagy-dependent epitope generation. The mechanism behind this increase was not investigated; however one possibility might be that this region is important for inhibiting the presentation of EBNA1 through autophagy.

The second interesting finding from these experiments was that deletion of amino acids 387-394 caused a decrease in epitope generation. This deletion was shown to confer reduced T cell recognition by both SNP-specific and VYG-specific $CD4^+$ T cells. EBNA1 has been shown to interact with the host protein, Casein Kinase 2 (CK2) via this region of EBNA1. Through this interaction EBNA1 has been shown to recruit CK2 to PML bodies, triggering the poly-ubiquitination and degradation of PML bodies (Sivachandran *et al.*, 2010).

I hypothesis that EBNA1 binding to CK2 is important for MHC class II processing of EBNA1. One possible explanation for the decrease in presentation of Δ387-394 is that this mutant protein is unable to disrupt PML bodies. This could possibly increase the amount of EBNA1 sequestered within PML bodies and therefore decrease the amount of EBNA1 available for MHC class II processing.

6. MACROAUTOPHAGY AND ENDOGENOUS MHC CLASS II ANTIGEN PROCESSING AND PRESENTATION

As discussed in Chapter 1, MHC class II epitopes are classically generated from exogenous proteins, however in certain cases class II epitopes can be generated from endogenous proteins. Multiple examples of endogenous MHC class II processing have now been identified but the factors that cause certain epitopes to be generated endogenously are poorly understood. One crucial factor defining endogenous class II processing is access to the endocytic compartment. Endogenous proteins have been shown to access this compartment though multiple pathways, including macro- and micro autophagy. However access to this compartment alone is not sufficient to allow processing and the generation of MHC class II epitopes. This is highlighted clearly in the example of MHC class II processing of EBNA1.

In B cells EBNA1 is known to be processed endogenously and can gain access to the MHC class II processing pathway via autophagy (Leung *et al.*, 2010; Paludan *et al.*, 2005). This processing pathway generates an autophagy-dependent, MHC class II epitope, SNP. Although EBNA1 contains multiple MHC class II epitopes, only SNP is generated in this manner. Another epitope, VYG is generated by a second, as of yet undiscovered endogenous processing pathway, whereas a third, PQC is not generated at all in B cells.

The rules defining how and why certain epitopes are processed endogenously and others not are poorly understood. These will be investigated in this chapter using EBNA1 MHC class II epitopes as a model.

6.1 The contribution of autophagy to the generation of EBNA1 class II epitopes

EBNA1 expression in LCLs presents an excellent model for studying how epitope peptides are generated, allowing the factors that affect whether an epitope can be generated through autophagy or not to be investigated. Work was carried out to study the differences between three key EBNA1 class II epitopes SNP, VYG and PQC.

As previously described, SNP is an autophagy-dependent epitope, VYG is processed and presented through an alternative pathway and PQC is not naturally processed and presented within B cells. Key experiments were conducted to confirm the differences in processing between the three epitopes. Results of these experiments are shown in Figure 6.1.

To confirm the dependence upon autophagy, LCLs were treated with 3-MA, a known autophagy inhibitor and presentation of the three different epitopes to T cells was measured. Figure 6.1a shows that untreated LCLs are recognised by SNP T cells. 3-MA caused a reduction in SNP-specific T cell recognition of LCLs, 10mM 3-MA reduced recognition by over 80%. The same 3-MA treated LCLs were used in parallel T cell assays using VYG-specific T cells. Untreated LCLs were again recognised by VYG T cells; however 3-MA caused an increase in VYG-specific T cell recognition of LCLs.

The effect of 3-MA was also tested on presentation of the third epitope PQC. PQC is not naturally presented in the B cell system, this result was confirmed in these experiments. Untreated LCLs were not recognised by PQC-specific T cells. Treatment with both 5 and 10mM 3-MA had no effect on this and all populations of LCLs were not recognised (Figure 6.1a).

These data confirmed that generation of the SNP epitope required autophagy, whereas VYG did not. The data also confirmed PQC is not presented in B cells regardless of autophagy status.

To further investigate the processing of these three peptides, T cell recognition of over-expressed EBNA1 was also studied. E1ΔGA was over-expressed in LCLs using the pRTS1, dox inducible system described in chapter 5

As has been previously shown, over-expression of E1ΔGA, triggered by dox addition causes a sharp increase in the generation of the SNP epitope (Figure 6.1b). When dox induced LCLs were treated with 10mM 3-MA this increase in T cell recognition was ablated. Recognition of the same LCLs by VYG-specific T cells was also studied. Over-expression of E1ΔGA caused a similar increase in the generation of the VYG epitope and as before 3-MA treatment increased VYG T cell recognition. PQC recognition was also measured in this system and again the PQC epitope was not generated under any conditions. This confirms that over-expression of E1ΔGA does not affect the processing route of individual MHC class II epitopes of EBNA1.

As previously discussed, work carried out by Leung *et al.* has showed that localisation of EBNA1 is key to defining the processing route for individual class II epitopes. Leung *et al*. showed that when EBNA1 was relocalised to the cytoplasm by mutation of EBNA1's NLS all epitopes were able to be presented thorough autophagy. Experiments were carried out to confirm this finding, the results of which are shown in Figure 6.1c.

The cytoplasmic EBNA1 variant E1ΔGA ΔNLS was over-expressed in LCLs again using the dox inducible system and generation of all three epitopes monitored in T cell assays

(Figure 6.1c). The pattern of SNP T cell recognition of E1ΔGA ΔNLS was similar to that of E1ΔGA. Dox induction caused a sharp increase in T cell recognition and 3-MA treatment completely ablated this recognition. Though the pattern was the same it should be noted that E1ΔGA ΔNLS was better recognised than E1ΔGA.

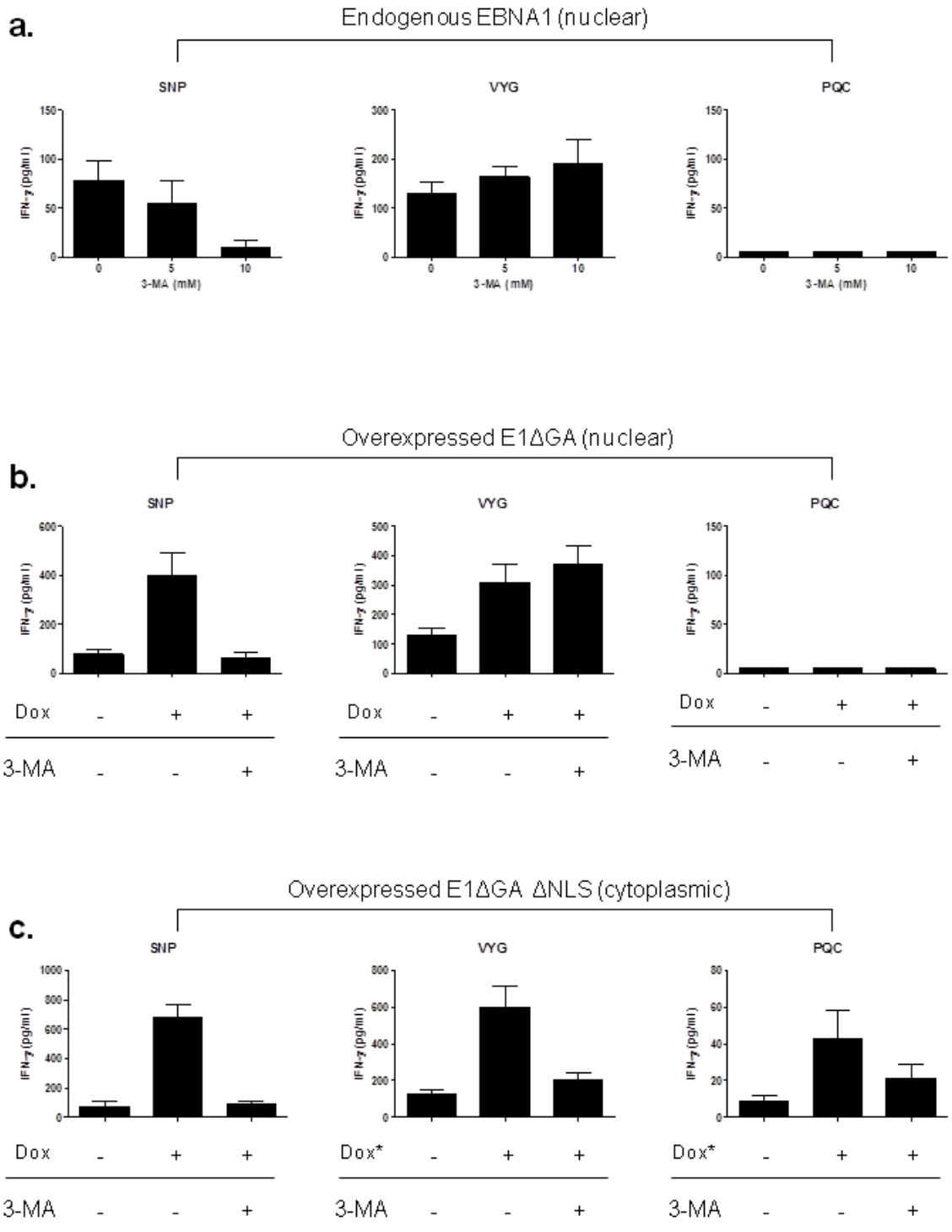
When VYG and PQC recognition of E1ΔGA ΔNLS was studied marked differences between nuclear and cytoplasmic EBNA1 were observed. As with previous results dox induction caused a sharp increase in recognition by VYG-specific T cells however this time 3-MA treatment ablated this increase. PQC recognition of E1ΔGA ΔNLS was also very different to recognition of E1ΔGA. Over expression of E1ΔGA ΔNLS caused LCLs to be recognised by PQC-specific T cells. This recognition was decreased by 3-MA treatment.

These results show that endogenous processing and presentation of cytoplasmic EBNA1 can generate all three EBNA1 MHC-II epitopes in an autophagy-dependent manner which suggests localisation is important to this process.

It should be noted that autophagy-dependent generation of the VYG and PQC epitopes from E1ΔGA ΔNLS required a longer period of time, compared to that required for the generation of the SNP epitope. It is possible therefore that the amount of EBNA1 in the cytoplasm is a limiting factor in the generation of these two epitopes by autophagy.

Figure 6.1 CD4$^+$ T cell recognition of EBNA1, E1ΔGA and E1ΔGA ΔNLS.

T cell recognition of endogenous EBNA1, E1ΔGA and E1ΔGA ΔNLS by the three EBNA1-specific CD4$^+$ T cells was investigated. **A.** LCLs were treated with 3-MA and used in T cell assays. Results show 3-MA inhibits the presentation of SNP. **B.** E1ΔGA was over-expressed in LCLs by the addition of 100nM dox and cells used in T cell assay. Over-expression increased presentation of SNP and VYG. SNP presentation was again inhibited by 3-MA. **C.** E1ΔGA ΔNLS was over-expressed in LCLs by the addition of 100nM dox and cells used in T cell assays using SNP T cells after 48 h, VYG T cells after 72h and PQC T cells after 120h. Over-expression increased the presentation of all three epitopes and the 3-MA inhibited this increase in all cases.


a.
Endogenous EBNA1 (nuclear)
SNP
VYG
PQC
IFN-γ (pg/ml)
3-MA (mM)
b.
Overexpressed E1ΔGA (nuclear)
SNP
VYG
PQC
IFN-γ (pg/ml)
Dox
3-MA
c.
Overexpressed E1ΔGA ΔNLS (cytoplasmic)
SNP
VYG
PQC
IFN-γ (pg/ml)
Dox
Dox*
3-MA

6.2 The effect of increased antigen supply on the generation of EBNA1 MHC class II epitopes

To further study these differences between epitopes, LCLs were induced with different concentrations of dox and the generation of class II epitopes was studied over time. Figure 6.2 shows the results of one such experiment. LCLs transduced with E1ΔGA ΔNLS were induced by addition of 50nM dox and the generation of the three epitopes SNP, VYG and PQC was observed over 5 days by measuring recognition of induced LCLs by EBNA1-specific $CD4^+$ T cells. To allow comparisons between epitopes, recognition was standardised to maximum recognition of each T cell, measured by recognition of LCLs pre-incubated with epitope peptides (% of peptide pulsed).

LCL recognition by SNP-specific T cells remained at baseline, around 2% of peptide max for the first 48h. After 72h of dox treatment, recognition rose sharply and after 120h had reached nearly 80% of peptide loaded LCLs. VYG-specific T cells showed a similar effect, however the increase was delayed and less dramatic. An increase in T cell recognition was not observed until 96h. After 120h recognition was only 10% of peptide loaded max. PQC-specific T cells again showed an increase after dox addition but this effect occurred later than both SNP and VYG and was smaller in magnitude. LCL recognition was not seen until 120h and was less than 2% of that of recognition of peptide loaded LCLs (Figure 6.2).

This experiment, along with the data from Figure 6.1 shows that all three epitopes can be generated by autophagy from E1ΔGA ΔNLS. However the generation of VYG and PQC epitopes is delayed.

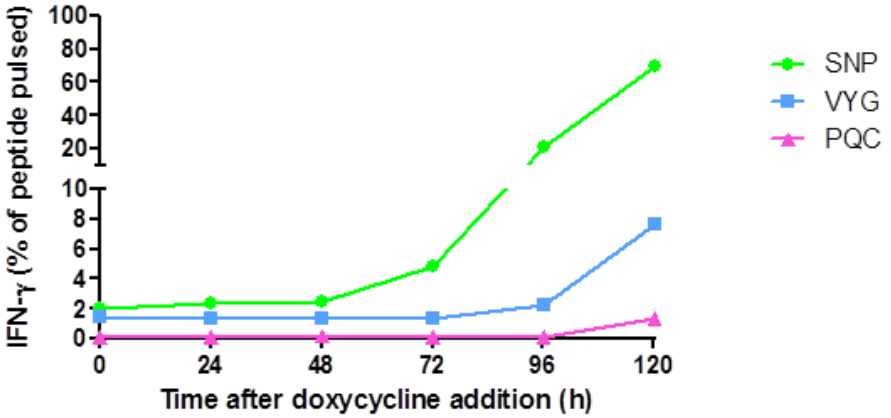


Figure 6.2 Generation of EBNA1 MHC class II epitopes from E1ΔGA ΔNLS over time

LCLs stably transduced with E1ΔGA ΔNLS were assayed for epitope presentation to EBNA1-specific $CD4^+$ T cells following 50nM dox induction. T cell recognition of LCLs was standardised to maximum recognition, measured by recognition of peptide loaded LCLs. T cell recognition of LCLs was increased after dox induction in all cases. The increase in recognition by VYG-specific T cells was delayed and smaller in magnitude than that of SNP-specific T cells and the increase in recognition by PQC-specific T cells was further delayed and smaller than seen with both SNP- and VYG-specific T cells.

It is known that long term dox addition causes a constant increase in the levels of EBNA1 protein expressed from the pRTS1 vector (unpublished data). It is therefore possible that the limiting factor delaying the increase in PQC and VYG-specific T cell recognition is the amount of EBNA1 protein present in cells. It is possible that to generate VYG epitopes through autophagy requires more protein than that required to generate the SNP epitope and that the generation of the PQC epitope requires more protein still. Increases in dox concentration causes a linear increase in the levels of protein expressed in LCLs, therefore dox concentration can be varied to study levels of protein required to induce epitope generation.

To determine if the differences observed in Figure 6.2 were due to amounts of EBNA1 expressed in LCLs, recognition of LCLs induced with increasing amounts of dox was measured at a single time point and again standardised to levels of maximum recognition. LCLs were treated with increasing concentrations of dox for 120h and presentation of epitopes to EBNA1-specific T cells was observed.

Figure 6.3 shows the results of this experiment. Uninduced LCLs were recognised by SNP-specific T cells, this recognition increased as dox concentration increased. In this experiment recognition peaked around 50% of peptide loaded LCLs at 20nM dox. Further increases in dox concentration caused no further increase in T cell recognition.

A similar effect was observed with VYG T cells but importantly the effect was smaller and occurred at higher concentrations of dox. LCL recognition increased sharply and began to plateau at 50nM. VYG recognition was much lower than SNP recognition reaching only just over 10% of peptide loaded LCL.

PQC recognition was again lower than that of SNP and VYG and no plateau was observed. 100nM dox caused only 4% T cell recognition.

These data show that all three EBNA1 MHC-II epitopes, SNP, VYG and PQC can be generated from E1ΔGA ΔNLS and that the generation of all three epitopes is dependent upon autophagy. The results also indicate that different levels of protein expression are required for the generation of each epitope. Generation of the PQC epitope requires the highest level of E1ΔGA ΔNLS expression and SNP, the epitope naturally processed by autophagy requires the lowest.

As a single molecule of E1ΔGA ΔNLS should generate one molecule each of the SNP, VYG and PQC epitope peptides, it might be assumed the levels of protein required for the generation of the different epitopes would be the same. However, as this is clearly not the case, it is possible that VYG and PQC epitope peptides are being destroyed before they can be displayed on the cell surface in complex with MHC class II molecules. Previous work by Leung *et al.* using crude cellular extracts containing lysosomal proteases suggests that PQC may be more susceptible to destructive processing by such proteases though this finding was never fully investigated (Leung *et al.*, 2010).

I therefore hypothesise that destructive processing of epitopes is limiting the presentation of VYG and PQC through the autophagic pathway. That is to say, that equal amounts of SNP, VYG and PQC epitopes will be generated from the degradation of EBNA1 within lysosomes but increased sensitivity to destructive processing by lysosomal proteases means greater numbers of VYG and PQC epitopes will be destroyed before they are loaded onto MHC class II molecules and exported to the cell surface.

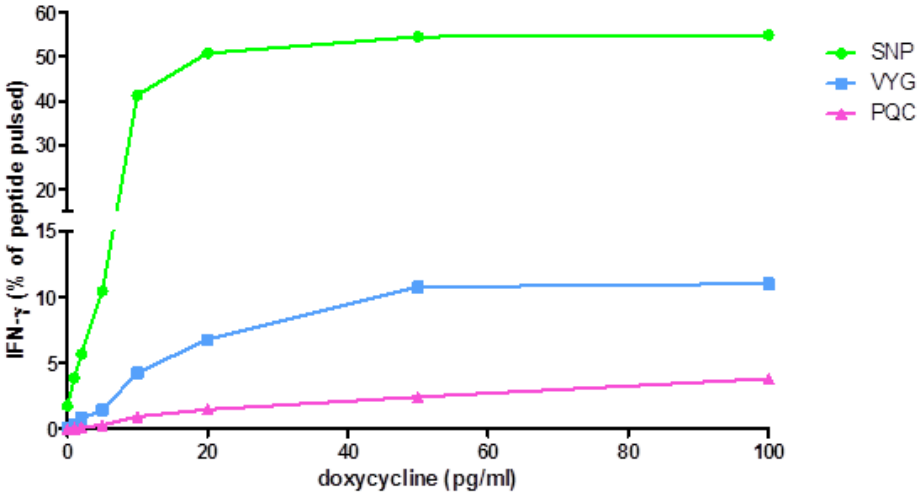


Figure 6.3 Generation of EBNA1 MHC class II epitopes from increasing amounts of E1ΔGA ΔNLS

LCLs stably transduced with E1ΔGA ΔNLS were assayed for epitope presentation to EBNA1-specific $CD4^+$ T cells following induction with increasing concentrations of dox. T cell recognition of LCLs was standardised to maximum recognition, measured by recognition of peptide loaded LCLs. T cell recognition of LCLs was increased after dox induction in all cases. The increase in recognition by VYG-specific T cells was smaller in magnitude than that of SNP-specific T cells and the increase in recognition by PQC-specific T cells was smaller than seen with both SNP- and VYG-specific T cells.

6.3 Destructive processing – a possible mechanism causing differential epitope display

Leung *et al*. noted that when EBNA1 was provided to LCLs as an exogenous antigen the PQC epitope was presented less efficiently than other epitopes and was first to be lost when antigen supply became limited. It was also shown that PQC was more susceptible to lysosomal degradation than SNP and VYG. These data suggests an important role for destructive processing in defining which epitopes are generated by autophagy.

I hypothesised that PQC is more susceptible to destructive processing than VYG and that in turn, VYG is more susceptible than SNP. To test this hypothesis a highly purified lysosomal fraction was isolated from multiple LCLs using density centrifugation and hypotonic shock to remove endosomes. Lysosomes were then lysed by freeze thawing to yield a pure lysosomal extract. These extracts were assayed for acid phosphatase and cathepsin activity.

Lysosome extracts were tested for acid phophatase activity using a commercial assay. Acid phosphatase is a lysosomal resident acid hydrolase and activity of this enzyme is a marker for the identification of lysosomes in sub-cellular fractionations. Figure 6.4a shows the results of this test. Extracts from three different LCLs were tested and lysosomal activity was detected in all three extracts. This shows that lysosomal contents were successfully isolated from all three cell lines. As cathepsins are crucial lysosomal proteases for antigen processing and presentation extracts were also assayed for activity of a number of different cathepsins. Figure 6.4b shows activity of different cathepsins was variable between LCLs but that all cathepsins showed some.

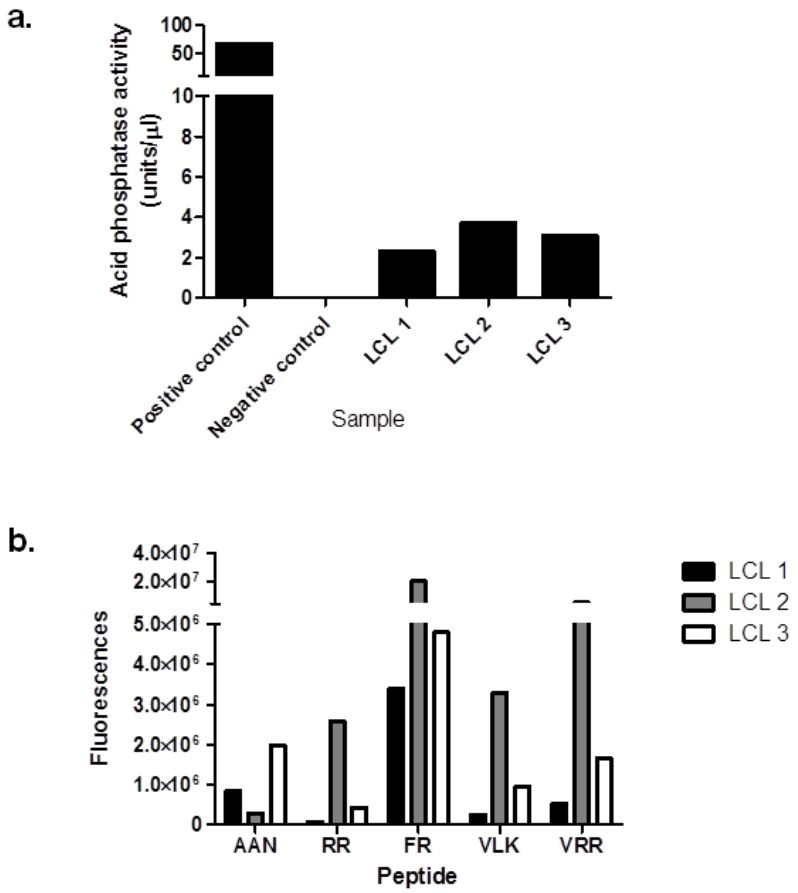


Figure 6.4 Acid phosphatase and cathepsin activity in lysosomal extracts from LCLs

Lysosomal extracts from three different LCLs were assayed for enzymatic activity. **A.** Acid phosphatase activity was measured by a commercial colourmetric assay. Results show all three extracts are positive for acid phophatase. **B.** Cathepsin activity was monitored by the cleavage of fluorescent tagged substrate peptides. AAN is a substrate for AEP. RR a substrate for cathepsin B. FR – cathepsin B and L. VLK – cathepsin K. VRR – Cathepsin S. Results show levels of cathepsins differed between all extracts but that cathepsin activity was detected in all lines.

6.4 Sensitivity of EBNA1 class II epitope peptides to destructive processing

To test the hypothesis that destructive processing limits the generation of epitopes through autophagy, the sensitivity of the three epitopes SNP, VYG and PQC to degradation by lysosomal proteases was measured.

A standard peptide degradation assay protocol was devised to measure degradation of EBNA1 peptides by lysosomal proteases. Limiting concentrations of the three peptides were exposed to lysosomal extracts for differing lengths of time. LCLs were then pulsed with these lysosome treated peptides and used as targets in T cell assays using $CD4^+$ T cells specific to the three different epitopes. Recognition of these pulsed LCLs was measured by IFN-γ ELISA. Lysosomal degradation of peptides will reduce the amount of epitopes loaded onto the surface of LCLs and will therefore decrease T cell recognition of LCLs. Higher T cell recognition can therefore be seen as greater resistance to degradation.

The results of peptide degradation assays using SNP, VYG and PQC peptides are displayed in Figure 6.5. To allow comparison of all three epitopes, IFN-γ release is displayed as a percentage of maximum achieved by pulsing LCLs with control untreated peptides (0h). As was expected, and matching previous results from Leung *et al.*, the SNP peptide appeared more resistant to degradation by lysosomal proteases than both VYG and PQC. Exposure to lysosomal extract did decrease presentation to SNP-specific $CD4^+$ T cells but after 8h of exposure recognition was still 50% that of maximum recognition seen at 0h. In contrast to this, both PQC and VYG appeared to be degraded by lysosomal extract much more rapidly. Recognition of LCLs by VYG and PQC-specific T cells was reduced to around 50% of maximum after 2h incubation with lysosomal extract. Though

VYG and PQC showed similar results it appeared that PQC is slightly more susceptible to degradation that VYG. After 8h incubation VYG recognition had fallen to 13% of maximum whereas PQC fell to 2%.

These data fit with the hypothesis that epitopes that are naturally generated through autophagy are more resistant to lysosomal proteases than those that are not processed in this manner. It also correlates with the differences seen in presentation of the three epitopes from E1ΔGA ΔNLS in that PQC generation required the highest level of expression and was the most susceptible to destructive processing.

6.5 Protecting EBNA1 MHC class II epitopes from destructive processing to increase T cell recognition

Efficient presentation of EBNA1 MHC class II epitopes requires the epitope peptides to be resistant to destructive processing. It would appear that both VYG and PQC are not efficiently presented through the autophagy pathway as the epitope peptides are degraded by lysosomal peptidases in this pathway.

I hypothesise that protecting VYG and PQC from destructive processing may cause these epitopes to be presented through autophagy. This would increase the levels and diversity of EBNA1 MHC class II epitopes displayed on the surface of cells and should increase T cell recognition of such cells.

This could be an effective mechanism to increase T cell recognition of EBV-positive cells and could potentially be translated to improve immune recognition of EBV-associated malignancies.

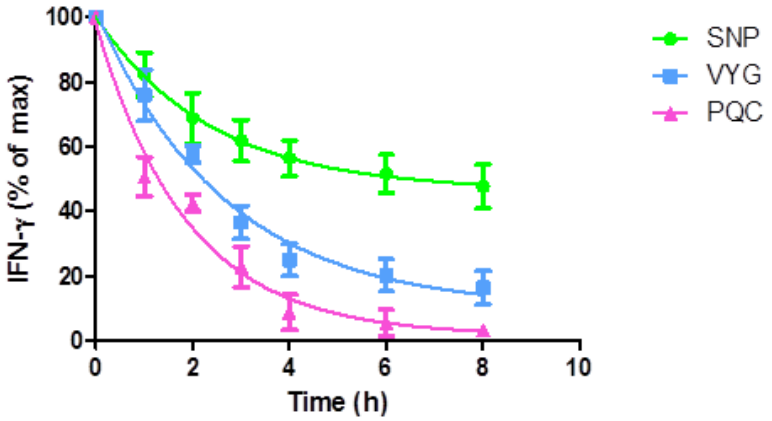


Figure 6.5 Degradation of EBNA1 MHC class II epitope peptides by lysosomal peptidases

Limiting doses of EBNA1 MHC class II epitope peptides were incubated with lysosomal extracts for increasing amounts of time. Peptides were then loaded onto LCLs and T cell recognition of LCLs was assayed. Peptide degradation is observed by decrease in T cell recognition. SNP T cell recognition was decreased as incubation time increased. After 8h recognition was 50% of that observed at 0h. A similar but greater effect was observed with VYG T cell recognition. After 8h recognition was 20% of that at 0h. Again a similar but greater effect was observed with PQC T cell recognition. After 8h recognition was 2% of that at 0h.

As a simple proof of principle experiment, attempts were made to protect the VYG and PQC epitopes from destructive processing by mutating the sequence of the epitope peptides. Cathepsins target specific sequences with proteins, as VYG and PQC are both degraded by lysosomal proteases I conclude that they must contain such cathepsin target sequences.

An alanine scan library of peptides was generated for each peptide. This comprises a library of peptides with individual point-mutation of each amino acid from the natural amino acid to an alanine. As these alanine scan peptides would be used in T cell assays following incubation with lysosomal extracts it was important to check that they were recognised by T cells. Some peptides would contain mutations crucial for MHC class II binding or TCR recognition and therefore would not be recognised by T cells and would not be suitable for use in degradation assays using T cells.

To ascertain which peptides T cells would recognise, LCLs were pulsed with individual peptides from VYG and PQC alanine scan peptide libraries. T cell recognition of peptide pulsed LCLs was then measured and compared to recognition of natural peptides (Figure 6.6). VYG peptides with alanine mutations at positions 9, 12, 14 and 15 were poorly recognised by VYG-specific $CD4^+$ T cells. Peptides with alanine mutations at positions 10, 11 and 13 were not recognised at all by VYG-specific $CD4^+$ T cells. PQC peptides A11-14 were not recognised by PQC-specific $CD4^+$ T cells.

Levels of recognition of the remaining peptides were similar to that of natural peptides and therefore the sensitivity of these peptides to lysosomal degradation could be investigated in peptide degradation assays using $CD4^+$ T cells.

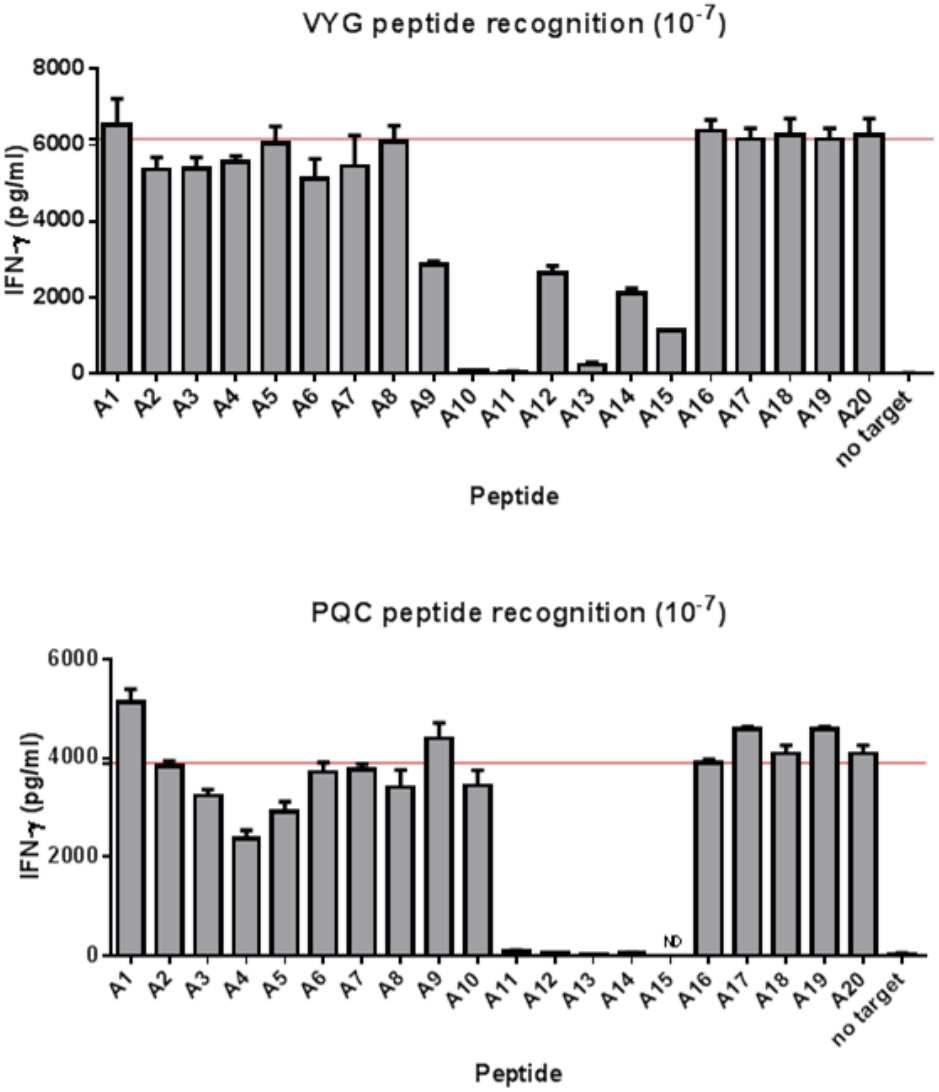


Figure 6.6 T cell recognition of VYG and PQC alanine scan peptide libraries

LCLs were pulsed with individual peptides from VYG and PQC alanine scan peptide libraries (10^{-7}μM, 1 h). T cell recognition of peptide pulsed LCLs was then measured. Red lines shown T cell recognition of natural peptides. VYG peptides A10, 11 and 13 were not recognised by VYG-specific $CD4^+$ T cells. PQC peptides A11-14 were not recognised by PQC-specific $CD4^+$ T cells. Recognition of PQC A15 was not measured (ND).

To test if the remaining peptides from the alanine scan libraries were less susceptible to degradation by lysosomal proteases, the peptides were incubated with lysosomal extracts. Degradation was again measured by loading peptides onto LCLs and measuring T cell recognition of these peptide loaded LCLs.

Figure 6.7 shows T cell recognition of LCLs pulsed with alanine scan peptides following incubation with lysosomal extracts. Levels of recognition are given as a percentage of recognition of the natural peptide incubated on ice. As has been seen previously the natural VYG peptide was degraded and levels of recognition were approximately 30% of that of control, undigested peptide. Levels of degradation of all other alanine scan VYG peptides were similar showing that no mutation was able to protect VYG from degradation.

Degradation of PQC peptides is also shown in Figure 6.7. As has been seen previously the natural PQC peptide was heavily degraded and levels of recognition were approximately 10% of that of control, undigested peptide. Levels of degradation of all other alanine scan PQC peptides were similar. Again this showed that no mutation of the PQC peptide sequence was able to protect VYG from degradation.

As no single mutation was able to alter peptide degradation, experiments to protect epitopes from lysosomal proteases were pursued no further.

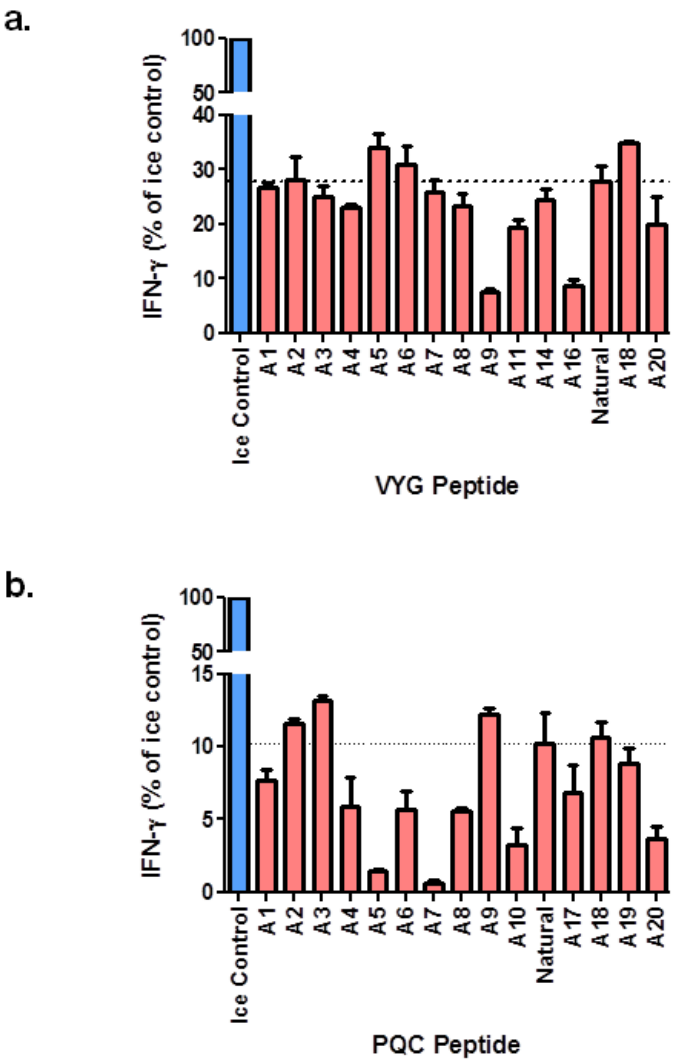


Figure 6.7 Mutation of epitope peptides to protect against degradation by lysosomal peptidases

Limiting doses of alanine scan peptides were incubated with lysosomal extracts for 16h. Peptides were then loaded onto LCLs and T cell recognition of LCLs was assayed. Peptide degradation is observed by decrease in T cell recognition. **A**. Recogntion of natural VYG was reduced to 28% of ice control by incubation with lysosomal extracts. Similar or greater decreases were observed for all other VYG peptides. **B.** Recogntion of natural PQC peptide was reduced to 10% of ice control by incubation with lysosomal extracts. Similar or greater decreases were observed for all other PQC peptides

6.6 Establishing a biochemical assay to measure peptide degradation by lysosomal proteases

Although T cells are very sensitive and can detect very small changes in levels of peptide on the surface of antigen presenting cells, the magnitude of the change in response will differ between T cells depending on a number of different factors such as T cell avidity. A limiting dose of peptide was used in all previous degradation experiments and that dose was different for each peptide, depending on the T cell clone used. A 50% drop in IFN-γ production by T cells may not accurately represent a 50% reduction in epitope levels. Therefore although the system is probably accurate in detecting the large difference in destructive processing, the use of T cells may limit the ability to differentiate between more similarly susceptible epitopes.

To allow a more direct comparison between epitopes a different and original system was devised which would allow direct quantification of peptides before and after incubation with lysosomal extracts. This allowed direct quantification of peptide degradation by lysosomal extracts and removed any errors that may be introduced by differences in T cell clones.

A schematic of this biochemical peptide degradation assay is shown in Figure 6.8. Briefly, peptides representing the core 20 amino acids of epitopes labelled with an N-terminal fluorophore and C-terminal biotin were generated. These peptides were then exposed to lysosomal extracts to allow digestion of peptides. After such time the peptide lysosome mix was incubated with streptavidin-coated beads. Samples were washed over a 1.2μm filter to remover unbound fluorophores. Beads were then analysed by flow cytometry to quantify uncleaved peptide levels.

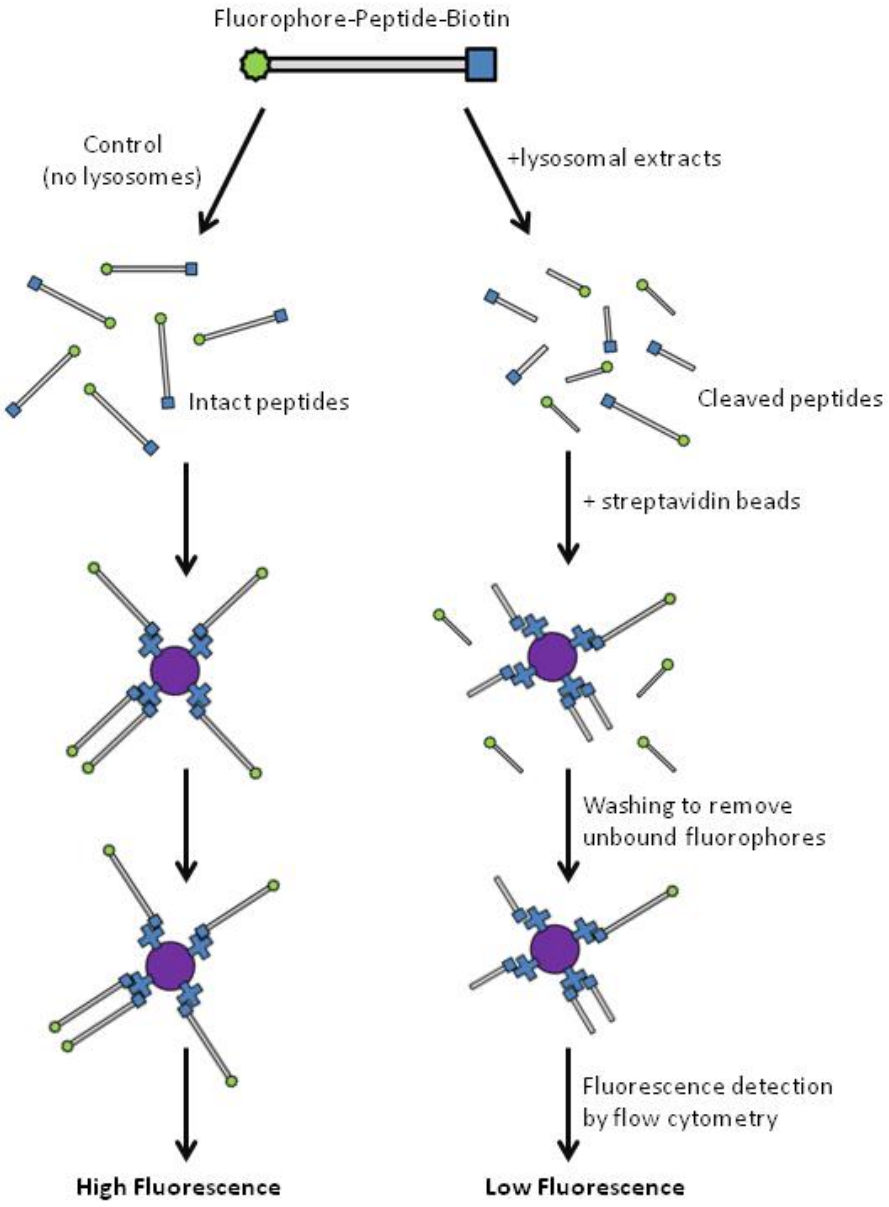


Figure 6.8 Biochemical peptide degradation assay

Target peptides labelled with a fluorophore and a biotin molecule are incubated with lysosomal proteases. Degradation of target peptides cleaves released the fluorophore from the biotin molecule. Peptides are incubated with streptavidin coated beads and unbound fluorophores removed by washing through a 1.2 µm filter. The beads are then analysed by flow cytometry and uncleaved peptides can be quantified by detection of fluorescence.

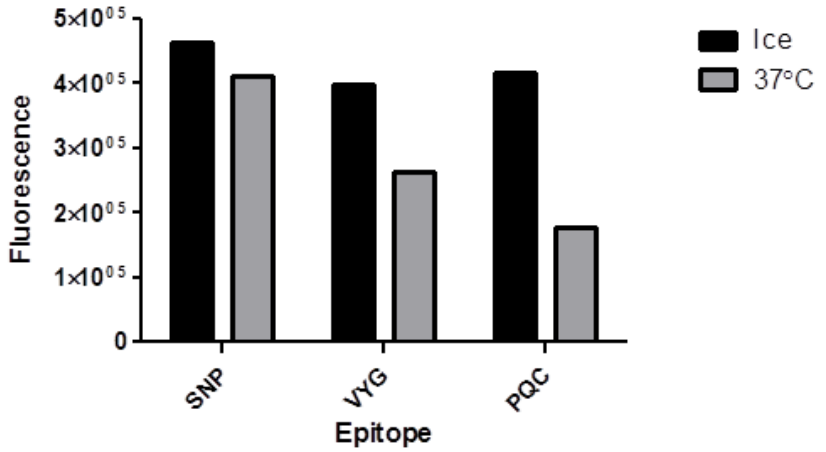
Control assays conducted using decreasing amounts of peptide showed a linear decrease in fluorescence of streptavidin beads (data not shown). This shows that levels of fluorescence directly correlate with the level of uncleaved peptide present in a sample and therefore this assay provides an accurate biochemical method by which to study peptide degradation.

6.7 Measuring degradation of EBNA1 MHC-II epitope peptides by a biochemical method

To confirm the results obtained in previous degradation experiments using T cells, degradation of the three EBNA1 epitope peptides SNP, VYG and PQC was measured using the biochemical peptide degradation assay. The results of this experiment are shown in shown in Figure 6.9.

Peptides were incubated with lysosomal extracts for 16h either at 37°C or as a negative control on ice to inhibit any enzymatic degradation. Peptide degradation was determined by flow cytometry according to the biochemical peptide degradation assay. Figure 6.9a shows the levels of fluorescence detected in each sample representing the amount of intact peptide remaining. Figure 6.9b shows the decrease in fluorescence observed when peptides were incubated at 37°C compared to that of peptides maintained at 0°C on ice. The results show that 11% of the total SNP peptide input into the assay was degraded by incubation at 37°C. Levels of degradation were higher in both VYG and PQC. 34% of VYG peptide was degraded and 58% of PQC peptide was degraded. This assay confirms the data obtained from assays using T cells, in that SNP is most resistant to destructive processing and PQC is most susceptible.

a.



b.

Epitope	Fluorescence decrease (%)
SNP	11.3
VYG	33.5
PQC	57.6

Figure 6.9 Degradation of EBNA1 MHC-II epitope peptides by lysosomal proteases

Peptides were incubated with lysosomal extracts for 16h either at 37°C or on ice. Peptide degradation was determined by flow cytometry according to the biochemical peptide degradation assay. **A**. Levels of fluorescence detected in each sample representing the amount of intact peptide remaining. **B.** . Decrease in fluorescence observed when peptides were incubated at 37°C compared to that of peptides maintained at 0°C on ice. SNP was reduced by 11%, VYG by 34% and PQC 58%.

6.8 Autophagy-dependent epitopes from other proteins are resistant to degradation by lysosomal proteases

This work has shown that presentation of EBNA1 MHC-II epitopes via autophagy correlates with the epitope peptide being resistant to degradation by lysosomal proteases. It is not known if this correlation is specific to EBNA1 MHC-II epitopes or if it will extend to other epitopes presented through autophagy. I hypothesised that all epitopes presented through autophagy will be resistant to degradation by lysosomal proteases.

To test this theory, MHC class II epitope peptides from a range of proteins were tested for susceptibility to lysosomal degradation by the previously described biochemical peptide degradation assay. Five MHC class II epitope peptides from four different proteins were tested in this assay. The SNP peptide was tested as a control. The DRY peptide from NeoR, the SVS peptide from the murine influenza protein HA and the VAN peptide from the mycobacterium tuberculosis protein Ag85b were all tested as they had previously been shown to be generated by autophagy, either naturally or in the case of the SVS peptide, by tagging to LC3. A second peptide from the murine influenza protein HA, CPK was included as this protein had shown not to be generated by autophagy when tagged to LC3 in the same system.

The results of biochemical peptide degradation assays using these peptides are shown in Figure 6.10. The peptides were incubated with lysosomal extracts for 16h either at 37°C or as a negative control on ice to inhibit any enzymatic degradation. Peptide degradation was determined by flow cytometry. Figure 6.10a shows the levels of fluorescence detected in each sample representing the amount of intact peptide remaining. Figure

6.10b shows the decrease in fluorescence observed when peptides were incubated at 37°C compared to that of peptides maintained at 0°C on ice.

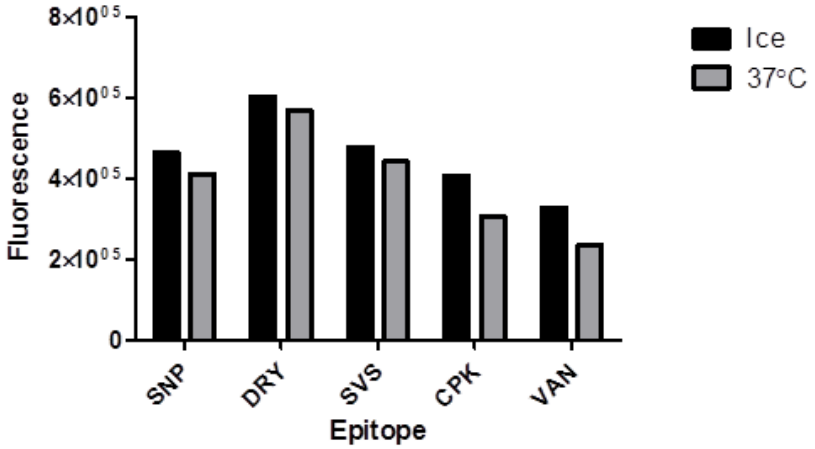
The results in Figure 6.10b show that the DRY peptide was very stable when exposed to lysosomal extracts; degradation of the epitope was less than the degradation of SNP. The DRY epitope has previously been shown to be generated by autophagy in LCLs for presentation to a $CD4^+$ T cell clone and therefore this supports the hypothesis that resistance to degradation is able to confer presentation by autophagy.

Comparison of the two epitopes from the murine influenza virus protein HA showed that the SVS epitope that is able to be presented through autophagy is highly resistant to degradation. The CPK epitope that cannot be generated through autophagy is more labile. 25% of the CPK epitope peptides were degraded in this experiment.

An unexpected result was observed when the degradation of the Ag85b epitope VAN was studied. This epitope is known to be presented through autophagy; however this epitope was the least resistant to degradation in that 29% of the VAN epitope peptides were degraded. This result was unexpected and did not fit with the hypothesis.

However, presentation of the VAN epitope by autophagy was not observed in an LCL but in the murine bone marrow macrophage cell line BMA.A3. It is possible that the cathepsins present in the lysosomal extract from LCLs that are responsible for the degradation of VAN in the previous experiment are not expressed in these macrophage cells. It is therefore possible that VAN is resistant to lysosomal degradation in macrophage cells where it is naturally presented by autophagy.

a.



b.

Epitope	Fluorescence decrease (%)
DRY	5.6
SVS	7.7
SNP	11.3
CPK	25.0
VAN	28.9

Figure 6.10 Degradation of MHC-II epitope peptides by lysosomal proteases

Peptides were incubated with lysosomal extracts for 16h either at 37°C or on ice. Peptide degradation was determined by flow cytometry according to the biochemical peptide degradation assay. **A**. Levels of fluorescence detected in each sample representing the amount of intact peptide remaining. **B.** Decrease in fluorescence observed when peptides were incubated at 37°C compared to that of peptides maintained at 0°C on ice. DRY showed the smallest decrease and VAN the largest.

To test this theory, lysosomal extracts were isolated from BMA.A3 cells and the degradation of the VAN epitope by these macrophage derived lysosomal proteases was measured. VAN and EBNA1 epitope peptides were incubated with increasing concentration of either LCL or macrophage lysosomal extracts and the degradation of each peptide was measured in using the biochemical peptide degradation assay.

As seen previously, the VAN epitope peptide was susceptible to degradation by LCL-derived lysosomal extracts. Levels of degradation were similar to the degradation of the VYG peptide (Figure 6.11a). When peptides were incubated with macrophage lysosomal extracts a very different pattern was observed. All three EBNA1 peptides were efficiently degraded by very low concentrations of macrophage lysosomal extracts (Figure 6.11b). In contrast, degradation of the VAN peptide by macrophage-derived proteases was very low. Indeed, the VAN epitope peptide was more resistant to degradation that all EBNA1 peptides including SNP across all doses of lysosomal extract.

This result confirms that the degradation of the VAN peptide by LCL-derived proteases was an LCL-specific effect and that VAN is resistant to degradation in the cells where it is naturally processed by autophagy. This supports the theory that resistance to degradation is required for autophagic presentation.

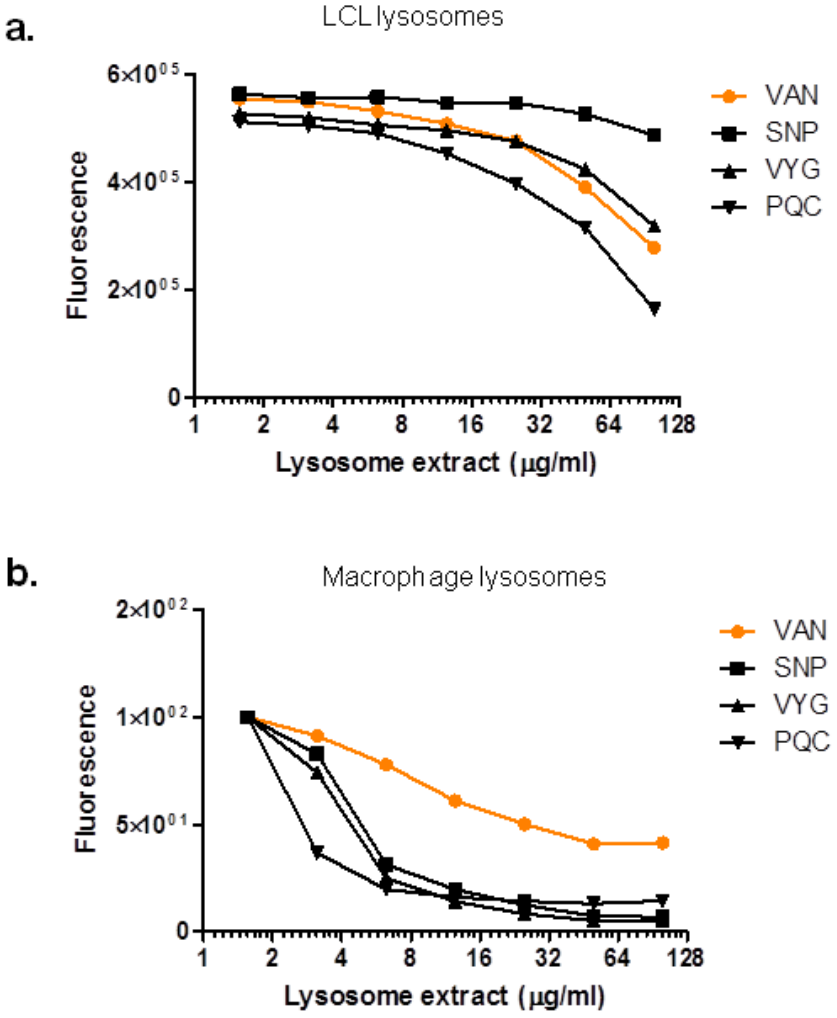


Figure 6.11 Degradation of the VAN epitope by LCL- and macrophage-derived lysosomal proteases

Peptides were incubated with increasing concentrations of lysosomal extracts for 16h at 37°C. Peptide degradation was determined by flow cytometry according to the biochemical peptide degradation assay. **A.** Levels of fluorescence detected in samples incubated with LCL-derived lysosomal extracts. **B.** Levels of fluorescence detected in samples incubated with macrophage-derived lysosomal extracts. VAN showed moderate stability when incubated with LCL-derived lysosomal extracts but was highly stable when incubated with macrophage-derived lysosomal extracts

6.9 Characterising the degradation of EBNA1 MHC-II epitope peptides

As mentioned previously, protecting MHC class II epitope peptides from destructive processing should increase the levels and diversity of MHC class II epitopes displayed on the surface of cells and would therefore increase T cell recognition of such cells.

Previous unsuccessful attempts were made to mutate peptides to make them more resistant to cathepsins. A more effective method of protecting peptides from degradation may be to inhibit cathepsins. This is also more clinically applicable as tumour cells could be treated with cathepsin inhibitors to increase presentation of naturally expressed peptides, thus increasing T cell recognition of tumour cells.

However as discussed in chapter 1, many cathepsins are important for MHC class II presentation and therefore inhibiting all cathepsins would decrease epitope display. If individual cathepsins could be implicated in destructive processing, it may be possible to specifically inhibit these cathepsins, thereby reducing destructive processing without affecting overall MHC class II processing. I therefore aimed to identify if individual cathepsins were responsible for the destructive processing of EBNA1 MHC class II epitopes.

One possible mechanism for differentiating between cathepsins is to measure their activity at different pH. As they are resident in the lysosome, most cathepsins have an optimum pH in the acidic range. However some differences have been observed, for instance cathepsin S has been shown to active across a very broad pH range.

To determine the optimum pH for degradation of EBNA1 MHC class II epitopes, peptides were incubated with LCL lysosomal extracts in a range of pH buffers. Peptide degradation was again studied by biochemical peptide degradation assay.

Figure 6.12 shows the results of this experiment. The optimum pH for peptide degradation was pH6 in all cases. However when degradation at pH5 and pH7 were compared subtle difference can be observed. Both SNP and PQC peptides are degraded more efficiently at pH7 than at pH5, whereas degradation of the VYG peptide is inhibited at pH7. This suggests SNP and PQC may be degraded by cathepsins that are more active at higher pH.

To further elucidate the contribution of individual cathepsins to the degradation of epitope peptides, degradation assays were carried out in the absence and presence of inhibitors. The inhibitors used and their specificities are listed in Table 6.1a. The degradation of peptides is listed in Table 6.1b, results are recorded in terms of percentage of peptide remaining following degradation. The effect of inhibitors is shown also, in terms of inhibition of degradation as a percentage of total degradation.

Levels of degradation in the absence of inhibitors was similar to previous experiments with SNP being more stable than VYG and VYG being more stable than PQC. To monitor the specificity of inhibitors, a control peptide containing a known cathepsin D cleavage site was included and over 80% of this peptide was degraded in the absence of inhibitors, this degradation was inhibited most effectively by inhibitors of aspartyl peptidases, of which cathepsin D is one.

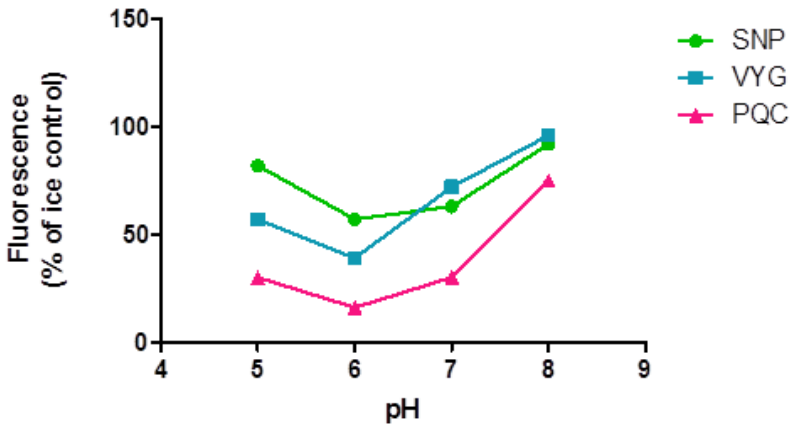


Figure 6.12 Degradation of EBNA1 MHC class II epitope peptides is pH dependent

Peptides were incubated with lysosomal extracts for 16h at 37°C in buffers of different pH. Peptide degradation was determined by flow cytometry according to the biochemical peptide degradation assay. Maximum degradation occurred at pH6 for all peptides. SNP and PQC were degraded more efficiently at pH7 than pH5.

Degradation of VYG was reduced by all inhibitors with the exception of the Cathepsin S inhibitor. This suggests that VYG may be degraded by multiple cathepsins. The degradation of PQC was reduced by fewer inhibitors. The greatest levels of inhibition were observed with inhibitors of cysteine proteases and the combined cathepsin B and S inhibitor. The cathepsin S inhibitor failed to inhibit degradation of PQC. Taken with the result of the combined inhibitor this may suggest just cathepsin B is degrading PQC. The specific cathepsin B inhibitor only caused a small decrease in PQC degradation though this may suggest the inhibitor is not very potent and fails to protect PQC which is a highly labile epitopes.

Taken together these data suggest that VYG is degraded by multiple cathepsins and PQC may be specifically degraded by cathepsin B.

Table 6.1 Inhibition of cathepsin mediated degradation of EBNA1 MHC class II epitope peptides

Peptides were incubated with lysosomal extracts for 16h on ice or at 37°C in the absence and presence of inhibitors. **A.** Inhibitors and their specificities. **B.** Fluorescence is shown as a percentage of 0°C control. Inhibition of degradation is colour coded according to the level of inhibition achieved as a percentage of total degradation.

a.

Inhibitor	Specificity
2µM E64	cysteine proteases
20µM Leupeptin	cysteine and serine proteases
2µM Pepstatin	aspartyl peptidases
2µM CA074	Cathepsin B
2µM ZFLCOCHO	Cathepin B and S
0.2µM ZFLCOCHO	Cathepsin S

b.

Condition	SNP	VYG	PQC	control
0°C	100.0	100.0	100.0	100.0
37°C	88.7	66.5	42.4	17.4
2µM E64	99.2	99.5	95.9	37.8
20µM Leupeptin	96.2	99.2	86.5	22.3
2µM Pepstatin	88.5	87.0	43.1	76.1
2µM CA074	93.4	88.2	57.2	20.0
2µM ZFLCOCHO	97.9	99.0	93.6	35.0
0.2µM ZFLCOCHO	91.0	70.8	52.3	17.0

Inhibition
>80%
>60%
>40%
>20%

6.10 Discussion

In this chapter I aimed to investigate the factors that affect the generation of MHC class II epitopes via non-classical processing of endogenous proteins. As previously discussed, multiple examples of endogenous proteins accessing the endocytic compartment have now been defined. Access can occur via multiple pathways, including macro- and micro autophagy; however access to this compartment alone is not sufficient to generate MHC class II epitopes.

EBNA1 presents an excellent model in which to study MHC class II epitope generation from non-classical processing of endogenous proteins. EBNA1 is able to access the endocytic compartment via autophagy but this pathway only generates a single MHC class II epitope, SNP. Other epitopes are generated in an autophagy-independent manner or in the case of the PQC epitope, not at all.

6.10.1 Destructive processing of EBNA1 MHC class II epitope peptides

Work by Leung *et al.* had previously suggested that PQC may be more susceptible to degradation by cathepsins than other EBNA1 MHC class II epitopes. I therefore hypothesised that destructive processing limits the presentation of two EBNA1 MHC class II epitopes VYG and PQC through autophagy.

Experiments in which a cytoplasmic EBNA1 construct was over-expressed in LCLs showed that high levels of protein expression can force the generation of VYG and PQC through autophagy. This result could suggest that at high levels of protein expression cathepsins become limiting and therefore destructive processing of EBNA1 is impaired. This allows the generation of VYG and PQC.

As PQC presentation required higher expression of EBNA1 than VYG, I suggest that the PQC peptide is more susceptible to destructive processing than VYG. As SNP is naturally generated through autophagy, the SNP peptide should be resistant to destructive processing. From this I predicted a hierarchy in the susceptibility of peptides to degradation. SNP should be most resistant to degradation, VYG should show intermediate levels of resistance and PQC should be least resistant to degradation.

6.10.2 Testing the susceptibility of EBNA1 MHC class II epitope peptides to degradation

To test this predicted hierarchy all three peptides were exposed to lysosomal extracts from LCLs to measure degradation by lysosomal proteases. In early experiments T cells were used to quantify peptide degradation. LCLs were incubated with peptides pre-incubated with lysosomal extracts. T cell recognition of these LCLs was then measured to quantify the amount of peptide remaining after incubation with lysosomal extracts.

These experiments were successful and differences between the levels of degradation of each peptide could be readily detected. The T cell degradation assays confirmed the predicted hierarchy, confirming SNP was most stable and PQC most labile when exposed to lysosomal extracts. It was presumed and later confirmed in inhibiter experiments that this degradation was mediated by lysosomal cathepsins.

Many different cathepsins with different cleavage specificities are expressed in lysosomes of LCLs as was confirmed in assays monitoring cathepsin activity. Different explanations exist for the hierarchy observed. It is possible that PQC contains multiple cathepsin target sites and therefore can be degraded by multiple cathepsins. It is also

possible that PQC is degraded by a cathepsin that is expressed at high levels in LCLs lysosomes and that this cathepsin is unable to degrade SNP or VYG efficiently.

Lysosome extracts from three different LCL were tested for cathepsin activity and differing levels of cathepsins was detected in each. However when peptide degradation assays were repeated with different LCL extracts the same hierarchy was observed. This perhaps suggests that PQC is degraded by multiple cathepsins rather than one highly expressed cathepsin.

6.10.3 Protecting epitopes to increase T cell recognition of target cells

As destructive processing appears to limit the amount and range of MHC class II epitopes presented on the surface of EBV infected cells, I proposed that protecting epitopes from destructive processing may increase epitope display and therefore $CD4^+$ T cell recognition of target cells.

Initial attempts were made to protect epitope peptides from degradation by introducing point mutations into peptides with the intention of disrupting cathepsin target sites. This was unsuccessful in that no single point mutation was able to decrease degradation of either the VYG or PQC peptide.

Although these experiments were unsuccessful they did reveal some interesting data about the two epitope peptides. Initially, T cell recognition of undigested peptides was studied and some point mutations were shown to inhibit T cell recognition of the peptide. These point mutations identify the amino acids within the core 20mers required for either binding to MHC class II molecules, so called anchor residues, or the amino acids that are displayed in the peptide groove to mediate peptide binding to TCR.

A number of explanations for the failure of point mutations to protect epitopes from destruction exist. First, it is possible that VYG and PQC both contain multiple cathepsin recognition sites and therefore a single point mutation would not disrupt cathepsin degradation. Second, it is possible that cathepsin recognition sites are within the key amino acids required for T cell recognition; these mutants were not tested in degradation assays as they were not recognised by T cells. Finally it is possible that flexibility in the cathepsin target sites meant the point mutations introduced were not sufficient to inhibit cathepsin targeting.

6.10.4 Degradation of other autophagy-dependent epitopes

Though the T cell based peptide degradation assays produced interesting data, they do present some limitations. As different T cell clones had to be used to measure degradation of different peptides, it is possible that differences in the T cells could introduce errors into the experiment. Also the use of T cells limited the range of peptides that could be tested in this assay. To test a peptide a matched T cell clone was required.

To counter both of these problems a biochemical assay was developed to monitor peptide degradation by fluorescence based flow cytometry. This assay should remove any variability introduced by T cells and allowes any peptide to be tested. Also this assay was adaptable to high throughput work which would be very useful later for repeating assays in the presence and absence of many different inhibitors.

First the degradation of EBNA1 MHC class II peptides was tested by this assay to confirm the results seen in T cell based assay. The data from the biochemical assay matched the data from the T cell assay again confirming the hierarchy of SNP, VYG, PQC.

Next the assay was used to measure degradation of other non-EBV peptides. The conclusion from this work so far is that resistance to destructive processing is able to confer epitope generation by autophagy in EBNA1. It is not known if this in an phenomenon specific to EBNA1 or if it is applicable to all epitope peptides generated through autophagy.

Four peptides from three different proteins were tested for degradation by LCLs derived cathepsins. Two epitopes previously shown to be presented though autophagy were shown to be resistant to degradation and a third epitope not presented through autophagy was shown to be susceptible to degradation. However one peptide, VAN, known to be presented thorough autophagy was degraded by LCL cathepsins.

VAN has been shown to be presented by autophagy in a macrophage line. It is therefore possible that it will be resistant to degradation by macrophage derived cathepsin. This was indeed shown to be the case. VAN was the most stable peptide tested in degradation assays using macrophages.

Taken all together these data present very strong evidence that the range of autophagy-dependent peptides presented by antigen presenting cells is defined by the susceptibility of each epitope peptide to destructive processing. Peptides that are resistant to destructive processing are presented by autophagy and those that are susceptible to destructive processing are not.

6.10.5 Characterising Peptide degradation

Though previous attempts to protect peptides from destructive processing were unsuccessful, manipulating destructive processing to increase epitope display is still an attractive mechanism for increasing T cell recognition of pathogen infected cells.

Rather than trying to alter peptides, a more therapeutically applicable method might be to inhibit cathepsins. Therefore attempts were made to characterise the degradation of MHC class II epitope peptide degradation with the intention of indentify individual cathepsins that could be targeted in increase epitope presentation.

Inhibitor experiments revealed inhibitors of different classes of cathepsins were all able to inhibit the degradation of VYG. However the same experiments suggest that PQC may be specially degraded by cathepsin B. In the light of this knowledge I returned to the results of cathepsin activity assays. Cathepsin B activity showed the highest activity in all LCLs. It is difficult to compare activity of different cathepsins using different substrates however this may suggest that cathepsin B is highly expressed and active in LCLs. This provides an explanation as to why PQC is so labile and also defines cathepsin B as a possible target for manipulation of destructive processing to increase T cell recognition of EBV infected cells.

7. FINAL DISCUSSION

As discussed throughout this work, increasing evidence suggests that $CD4^+$ T cells have an increasingly important and therapeutically relevant role as direct effectors in immunotherapy.

The main aim of this body of work was therefore to study MHC class II processing of tumour antigens and subsequent presentation to $CD4^+$ T cells. A particular focus was placed on studying the non-classical MHC-II processing pathways involved in processing of endogenous antigens.

A secondary aim was to apply the knowledge of MHC class II processing to develop novel strategies to improve both the range and levels of MHC class II epitopes presented to tumour-specific $CD4^+$ T cells.

7.1 Findings

Originally work was focussed on WT1, a promising tumour antigen recognised by both $CD8^+$ and $CD4^+$ T cells. Experiments were conducted to investigate the expression of WT1 in a number of cell lines. A complex pattern of expression was revealed with many different isoforms, including previously undescribed isoforms of WT1 being expressed. These experiments also revealed that no single method for the detection of WT1 was able to identify all isoforms of WT1.

The frequency and breadth of the WT1 T cell response in the healthy donors and cancer patients was also investigated in this section. Many highly sensitive methods were

applied and all experiments revealed that WT1-specfic T cells are exceptionally rare in both healthy donors and the all tested patients.

Some attempts were made to investigate the MHC class II processing of WT1 but the ultimate failure to generate WT1-specific T cells limited this work.

Later work focussed on investigating the MHC class II processing of the EBV antigen EBNA1. EBNA1 is a better studied tumour antigen and more is known about how endogenous EBNA1 is presented to $CD4^+$ T cells. However, many questions remain about the mechanisms behind EBNA1 MHC class II processing. Also $CD4^+$ T cell recognition of EBNA1 positive cells is relatively poor suggesting there is scope to improve the presentation of EBNA1 with possible important and therapeutic benefits.

Attempts were made to improve the presentation of EBNA1 by relocalisation of the protein using the CDK inhibitor Roscovitine. These experiments were ultimately unsuccessful but did reveal a very interesting finding. Roscovitine was able specifically inhibit the autophagic presentation of EBNA1; inhibition was concurrent with a Roscovitine-mediated downregulation of the multiple autophagy adaptors.

Correlation of reduced autophagy adaptor expression and reduction in autophagic presentation was explored in the subsequent chapter. siRNA experiments proved that three autophagy adaptors, NBR1, CALCOCO2 and OPTN were required for the autophagic presentation of EBNA1 MHC class II epitopes. This was a very interesting result and represents the first known evidence that autophagic degradation and subsequent generation of MHC class II epitopes is mediated through autophagy adaptors.

A final set of experiments were conducted to answer the important question of why only some epitopes are presented through autophagy. Originally this work was conducted using MHC class II epitopes from EBNA1 but was later extended to study autophagy-dependent epitopes from a range of different proteins. The results of these experiments showed that destructive processing by lysosomal cathepsins limits the range of epitopes presented through autophagy. All autophagy-dependent epitopes tested were resistant to degradation, whereas epitopes not presented by autophagy were degraded by cathepsins.

7.2 Conclusions and future work

A number of important conclusions can be drawn from each section completed in this body of work.

Experiments on WT1 suggest that reports in the literature regarding the expression of WT1 may have significantly underestimated the range of cells in which WT1 is expressed. Heterogeneity in WT1 expression means no single method can detect all WT1 isoforms. This suggests WT1 may be expressed in even more tumours than is currently thought. The second main conclusion from the work on WT1 is that WT1-specific T cells are incredibly rare; this rarity is not apparent from the published literature.

The work on Roscovitine shows that this CDK inhibitor is not able to relocalise endogenous EBNA1. It also proves that any inhibition of cellular proliferation caused by Roscovitine is independent of any effect Roscovitine may be having on EBNA1. The important finding from the work on Roscovitine was the preliminary data that suggested a possible link between autophagy adaptors and EBNA1 degradation.

The work on autophagy adaptors and destructive processing can be analysed together to create a better understanding of how EBNA1 is processed and presented. From this work I suggest the following model to explain the presentation of EBNA1 MHC class II epitopes. I propose that autophagy adaptors are required to target EBNA1 for degradation by autophagy. Once in the endocytic pathway lysosomal proteases degrade EBNA1 and generate MHC class II epitopes. However, with the exception of the SNP epitope, all EBNA1 MHC class II epitope peptides are susceptible to destructive processing by cathepsins and are therefore destroyed. As the only epitope peptide to survive within the lysosomal compartment, SNP alone is presented by autophagy.

I believe this work has developed a clearer understanding of EBNA1 MHC class II processing. However, many unanswered questions remain. Nuclear EBNA1 is targeted for autophagic degradation in the cytoplasm by autophagy adaptors but the mechanism of this interaction has not been elucidated. Future work to identify how EBNA1 enters the cytoplasm and how EBNA1 and autophagy adaptors interact should be carried out. One possible mechanism for EBNA1 entering the cytoplasm is that newly synthesised EBNA1 is sequestered in the cytoplasm by autophagy adaptors prior to import into the nucleus. Experiments using protein synthesis inhibitors or the dox inducible over-expression system could be used to study this possibility. At the time this thesis was submitted no direct interaction between EBNA1 and autophagy adaptors has been discovered, further EBNA1 deletion experiments as well as co-localisation experiments may reveal a direct interaction between EBNA1 and autophagy adaptors.

Finally the work on destructive processing revealed a possible role for cathepsin B in the degradation of the PQC epitope. Further work is required to confirm this result. Purified

cathepsins could be used to study the contributions of individual cathepsins to degradation. Also further inhibitor studies including attempts to study destructive processing within cells should be attempted. Cell permeable cathepsin inhibitors could be tested for their ability to inhibit destructive processing. This may increase the presentation of many MHC class II epitopes, a finding that could be very therapeutically relevant.

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