UNIVERSITY OF SOUTHAMPTON
School of Medicine

THE EFFECT OF MANIPULATING APOPTOTIC CELL UPTAKE ON THEIR IMMUNOGENICITY

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ABSTRACT
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By Kathrine Elizabeth Attfield

Failure of the immune system to differentiate between apoptotic tumour cells and cells rendered apoptotic as part of homeostasis prevents a successful response being mounted against tumours. Apoptotic cells are cleared rapidly by professional phagocytes thus preventing the release of potentially inflammatory or immunogenic material into the surrounding environment. In addition, macrophages release immuno-suppressive cytokines such as tumour growth factor β (TGF-β) and interleukin-10 (IL-10) that dampen the initiation of cytotoxic T cell (CTL) immunity and render T cells tolerant. It was hypothesised that manipulation of this clearance pathway, which is normally employed to prevent autoimmunity, may alleviate immuno-suppression of apoptotic tumour cells and promote the expansion of a tumour antigen-specific T cell response.

Milk fat globule – epidermal growth factor 8 (MFG-E8) is a glycoprotein secreted by activated macrophages and immature dendritic cells. It facilitates the uptake of apoptotic cells by acting as a bridging molecule between integrins on the phagocyte and phosphatidylserine (PS) on the apoptotic cell surface. Here, two recombinant dominant-negative MFG-E8 proteins were generated: one which is shown to inhibit PS-dependent apoptotic cell uptake by macrophages by over 40% (DN-MFG-E8), and a second which re-directed apoptotic cells through Fcγ receptors and conferred enhanced phagocytosis by both macrophages and immature dendritic cells (DN-MFG-E8-Fc) in a dose-dependent manner.

Cross-presentation of cell-associated antigen by bone marrow-derived dendritic cells (BMDCs) was determined by CD8+ T cell proliferation assays *in vitro* and *in vivo*. Loading BMDCs with apoptotic cells via a PS-independent pathway or through Fcγ receptors (FcγRs) reduced their ability to induce CD8+ T cell proliferation *in vivo*, suggesting the blockade of a mechanism which is intrinsic for DC maturation or migration. Similarly, the balance between activating and inhibitory FcγRs proved essential for effective DC maturation. Apoptotic cells treated with DN-MFG-E8-Fc protein resulted in upregulation of costimulatory molecules, CD86 and CD70, when BMDCs were deficient for the inhibitory FcγR, FcγRIIb.

Additionally, the immunosuppressive effect of apoptotic cells on antibody production proved dependent on the exposure of PS; whereby both DN-MFG-E8 and DN-MFG-E8-Fc proteins were shown to alleviate this suppression.

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I, KATHRINE ELIZABETH ATTFIELD……., declare that the thesis entitled:

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ABBREVIATIONS

APC	Allophycocyanin
BCL	B cell lymphoma
BMDC	Bone marrow-derived dendritic cell
BSA	Bovine serum albumin
CHO	Chinese hamster ovary
CFSE	Carboxyfluoroscein Succinimidyl Ester
CTL	Cytotoxic T lymphocyte
DMSO	Dimethyl Sulphoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACs	Fluorescent-activated cell sorting
FBS	Foetal bovine serum
FITC	Fluoroscein (isothiocyanate)
HEK	Human Embryonic Kidney
mAb	Monoclonal antibody
MFG-E8	Milk fat globule – epidermal growth factor 8
OVA	Ovalbumin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLL	Poly levo-lysin
PS	Phosphatidylserine
RBC	Red blood cell
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TUNEL	TdT-mediated dUTP nick end labelling
UVC	Ultraviolet C

Chapter 1

Introduction

1.1 Overview

With the ever aging population in the Western world, cancer has inevitably become one of the most common causes of death, second only to cardiovascular disease. Environmental and genetic factors contribute to DNA damage or mutations; consequently there is elevated survival and proliferation of abnormal cell populations caused by their ability to evade or over-ride normal cell growth-regulating mechanisms. Thus far, treatments for cancer include surgery, chemotherapy, radiation therapy and immunotherapy. The success of each is highly variable and as yet there is no known universal cure.

One of the key aims in current cancer research is to understand the manner in which cancer cells evade recognition by the immune system. The fundamental problem when treating cancer is its inability to be distinguished by the immune system as a component other than self. Current antibody therapies exist to target tumour cells specific for certain markers (antigens) [1]. The results to date are encouraging but far from absolute. Further understanding of the immune response to cancer and the manners in which the immune system can be manipulated is therefore imperative.

1.2 The immune system

The environment challenges our immune system on a continuous basis with an endless array of infectious microbes – viruses, fungi, protozoa, and multi-cellular parasites. Their successful infection into a host organism will in many cases lead to disease, some of which may prove fatal. Fortunately however, the immune system is highly effective in ensuring that most infections are short-lived and that any permanent damage is minimal. An immune response can be defined in two steps; firstly, the immune system must recognise the pathogen or foreign material and secondly it must then initiate an immune response in which to eliminate it [2].

1.2.1 Innate immunity

Innate immunity is regarded as a defence barrier which comprises of 'basic' mechanisms aimed non-specifically at invading pathogens. The innate immune response is short lived and does not generate long-lasting protective immunity against foreign antigen; it serves to limit infection and to facilitate activation of the adaptive immune response (see section 1.2.2). Innate immunity is considered to comprise of four general defence barriers; anatomic (skin and mucous membranes), physiologic (temperature, low pH, and chemical mediators), phagocytic (ingestion of extracellular particulate material through phagocytosis by blood monocytes, neutrophils and macrophages) and inflammation (which allows an influx of phagocytes into the infected site). If an infectious organism eludes innate mechanisms or is not cleared by them, the host is equipped to surmount the infection through successive activation of the adaptive immune system.

1.2.2 Adaptive immunity

The adaptive immune system is found exclusively in vertebrates and is capable of mounting a reaction against a specific antigenic challenge. The success and specificity of adaptive immunity is owing to its four attributes; antigenic specificity, diversity, immunologic memory, and self/non-self recognition.

An effective immune response against a specific antigenic challenge is mediated by two major groups of cells: lymphocytes and antigen presenting cells (APCs). Both B and T lymphocytes are the key mediators of the immune system; however their function is dictated largely by antigen presenting cells, namely dendritic cells (DCs). Innate signalling precedes and is essential for the generation of both T and B cell responses and it is acknowledged that dendritic cells provide the crucial connection between innate and adaptive immunity [3]. There are multiple innate pathways that activate DCs, most likely so that infection can be detected at an early stage, ensuring that the pathogen has limited time in which to become established [4].

B cells are the precursors of antibody-secreting cells that can directly recognise native antigen through their B cell receptors. T lymphocytes however will only recognise antigen via its T cell receptor when it is presented to them by an antigen presenting cell through a major histocompatibilty complex [5].

1.2.3 Role of the immune system in cancer

Cancer immunosurveillance: the concept that the immune system can recognise and eliminate the development of tumours in the absence of external intervention; a hypothesis which has been in the scientific community since its first proposal by Paul Ehrlich in 1909 (reviewed in [6]). His work was then later revised by Burnett and Thomas ([7] and reviewed in [6]), 50 years later, who suggested that the immune system continuously surveys the body for the presence of abnormal cells, and can eradicate precancerous or cancerous cells through a "surveillance" mechanism.

Only until recently has the technology been available to test this theory. Through studying mice with targeted deletion of specific genes and the use of monoclonal antibodies (mAbs) against immune components, it has

become clear that the immune system has the capacity to both protect from and promote tumour growth; re-classifying this hypothesis as cancer immunoediting.

The idea of cancer immunoediting states that there are three phases in the evolution of a tumour; elimination, equilibrium, and escape [8]. This theory suggests that most tumours have evolved the ability to evade the immune system by the time that they become clinically detectable.

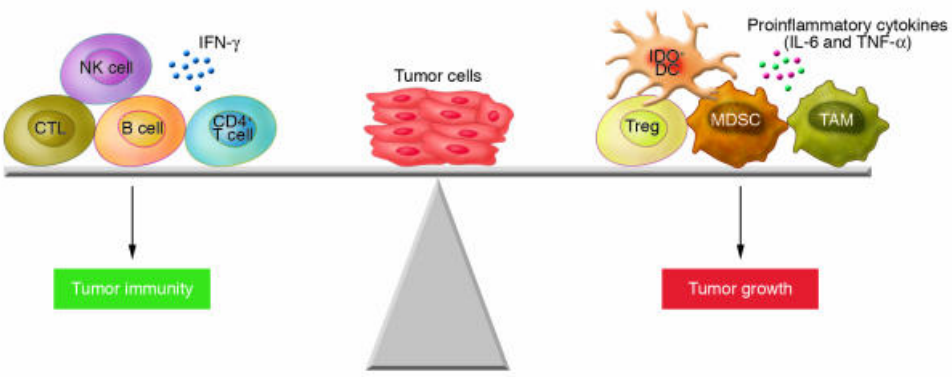


Figure 1.1 Some of the immunological factors that affect tumour development.
(diagram from N. Bhardwaj. Harnessing the immune system to treat cancer. Journal of Clinical Investigation. 2007 [6])

Elimination - The involvement of both the innate and adaptive arms of the immune system promote the elimination of cancerous cells. Studies to support this have shown that RAG2-deficient mice (which do not have T or B cells) spontaneously develop adenomas of the intestine and lung. Additionally, interferon-γ (IFN-γ)-deficient mice also have a higher incidence of tumour development [9]. Additionally, the role of NK and NKT cells as effectors of immune surveillance was originally established when C57BL/6 mice were depleted of both cell types by using an anti-NK1.1 antibody. These mice were 2 to 3 times more susceptible to developing tumours compared to non-treated mice [10]. Another study to support this showed that mice treated with the NKT cell-activating ligand α-galactosylceramide after treatment with tumour-inducing agents, showed

a reduced incidence of tumours and had a longer latency period of tumour formation compared to the control [11].

Equilibrium - As a result of tumour elimination, the selection and sculpting of tumour cells around the immune response promotes the generation of tumour cell variants which decrease their immunogenicity and thus are able to become resistant to immune effector cells. Again, evidence to support this originates from studies where the transfer of tumour cells into wild-type mice that re-emerge during the equilibrium phase results in their rejection, however grow progressively in syngeneic immunodeficient mice [12].

Escape - When this balance is finally tipped in favour of the tumour, its ability to escape immune recognition is accomplished by the induction of tumour-derived soluble factors into the tumour microenvironment. Firstly, tumours are able to inhibit antigen presenting cell (APC) maturation, thus rendering T cells tolerant due to the absence of the necessary costimulatory molecules (described further in section 1.3). T cells are also able to be rendered functionally anergic by factors secreted by the tumour. An example of this is indolemine-2,3, dioxygenase (IDO); an enzyme involved in tryptophan catabolism that can be over-expressed by tumours [13]. IDO is capable of blocking the activation and expansion of T cells, which are dependent on tryptophan for cell cycle progression [14]. Additionally, tumour infiltrating macrophages (TIMs) can secrete immunosuppressive cytokines such as interleukin10 (IL-10) and transforming growth factor β (TGF-β). IL-10 down regulates antigen presentation and T cell expansion [15] and TGF-β can directly inhibit T cell activation and proliferation [16].

As an alternative to the model of active immunosuppression, it has been suggested that immunogenic tumours do not escape from tumour recognition but instead induce a state of immune tolerance by the expansion of non-functional T cells [17]. One particular study has shown that tumour-specific T cells still have immunogenic potential, however the tumour microenvironment induces a state of T cell tolerance [18].

1.3 Antigen processing and presentation

1.3.1 Introduction

The immune system is centred on two major types of lymphoid tissues; primary lymphoid organs such as the thymus provide the population of T cells from precursors, and secondary lymphoid organs such as the spleen, lymph nodes and Peyer's patches represent the sites of immune induction. The activation of naïve T cells by their cognate ligand occurs within the secondary lymphoid organs. Each of these organs is responsible for surveying different components of the body. The spleen monitors antigens which are derived from the blood, while the lymph nodes screen their local regions. Activated T cells (effector cells) are then able to exit the secondary lymphoid organs to the peripheral tissues where they can then target the source of immune activation [2].

1.3.2 Role of antigen presenting cells

1.3.2.1 Overview

To elicit a humoral or cell-mediated immune response to an antigen, T cells must recognise and interact with antigen that has been degraded into peptides and then processed and presented within the cleft of an MHC molecule on the cell membrane.

The majority of cells express MHC class I molecules on their surface, therefore are able to present peptide to T cells. Cells which express peptides associated with MHC class I molecules to CD8+ T cells, which generally function as cytotoxic T cells (CTLs), are referred to as target cells. A subset of cells, namely antigen presenting cells, in addition to expressing MHC class I molecules, also express MHC class II molecules on their surface and can present peptides associated with the MHC class II molecules to CD4+ T cells, which are typically referred to as T helper (T_H) cells. Peptides which are derived from endogenous antigens and processed within the cytoplasm; such as self antigens, tumour-associated antigens, viral or bacterial antigens are presented with MHC Class I molecules.

Exogenous antigens which are internalised by either phagocytosis or endocytosis are displayed with MHC Class II molecules.

1.3.2.2 Professional antigen presenting cells

All professional antigen presenting cells are able to take up antigen and present it to CD4+ T cells via MHC class II molecules, causing their proliferation and differentiation into effector T cells. Some antigen presenting cells, specifically dendritic cells, are also able to present exogenous antigens via MHC class I molecules to CTLs, a process known as cross presentation, discussed in section 1.3.5.

Dendritic cells are heterogeneous and can be divided into two subsets; plasmacytoid and conventional. Upon encounter with toll-like receptor ligands such as CpG, plasmacytoid DCs produce high levels of type-I interferon, they promote activation of other DCs and can block viral replication. They characteristically express CD45RA and only express low levels of the integrin CD11c. Conventional DCs are sub-divided further into two groups; blood-derived DCs and tissue-derived DCs. Blood-derived DCs are found in the spleen and lymph nodes, whereas tissue-derived DCs (langerhan's cells and dermal or 'interstitial' DCs) migrate only to the lymph nodes. [19].

Macrophages are only able to express MHC class II molecules and the B7 costimulatory membrane molecules once they have phagocytosed material, either of self or non-self origin. B cells constitutively express MHC class II molecules however must undergo activation before they express B7 [2].

1.3.2.3 Non-professional antigen presenting cells

Fibroblasts, glial cells, pancreatic beta cells, endothelial cells and epithelial cells can all be induced to express MHC class II. In these cases however the period of peptide-MHC class II presentation is limited to serve for the duration of an inflammatory response [2].

1.3.3 Presentation of endogenous antigens : The cytosolic pathway

Viruses which infect and then replicate within a cell provide a source of endogenous antigens. Similarly altered self cells such as tumour cells, aging body cells or allogeneic cells from a graft also provide a source of intracellular antigen for presentation to CD8+ T cells.

1.3.3.1 The Ubiquitin-proteosome system

The degradation of approximately 80% of both normal and abnormal cytosolic proteins is mediated through their ubiquitination and subsequent degradation by the 26S proteosome; a 700kD multi-subunit proteolytic complex.

Ubiquitin is a 76 residue protein which selectively attaches to proteins; causing their remodelling in a manner which affects their stability, activity, interactions with other proteins, and their sub-cellular localisation. Proteins that have been ubiquitinated are then targeted for proteolysis through their recognition by the proteosome. The proteosome removes denatured, misfolded, damaged and improperly translated proteins. Additionally, it also regulates the levels of proteins such as cyclins and transcription factors. It is made up of a core protease – barrel shaped 20S complex (referred to as the 20S proteosome) which is responsible for the proteolytic activity of the proteosome. It consists of four stacked rings; two outer α-rings and two inner β-rings. Each ring is comprised of 7 subunits, one stacked on top of the next. In addition to the 20S proteosome, the 26S proteosome also consists of two 19S regulatory particles which cap the ends of the 20S proteosome. These subunits control the recognition of the ubiquitinated proteins, the ATP-dependent unfolding and the opening of the channel in the 20S proteosome that allows entry into the proteolytic chamber.

1.3.3.2 Immunoproteosome

The degradation of endogenous proteins into peptides for presentation by MHC class I molecules also utilises this proteosomal pathway, however, the activation of the 20S proteosome is not regulated by the 19S regulatory

particles but through the binding of PA28. PA28 is a ring-shaped, 11S multimeric complex consisting of two homologous subunits, α and β, and the Ki antigen. PA28, the expression of which is inducible by IFN-γ, binds to 20S along with a 19S regulatory particle to form the immunoproteosome. The immunoproteosome is capable of generating MHC class I-binding peptides due to its ability to dramatically enhance its capability to hydrolyse oligopeptides which leads to the generation of small oligopeptides suitable for MHC class I presentation [20].

Peptides generated through this immunoproteosomal pathway are transported to the rough endoplasmic reticulum (RER) via a protein, TAP (transporter associated with antigen processing). TAP belongs to the family of ATP-binding cassette proteins and is a membrane spanning heterodimer that consists of two subunits, TAP1 and TAP2.

The α-chain and β_2-microglobulin components of the class I molecule are synthesized on polysomes along the rough endoplasmic reticulum. The MHC class I complex can only assemble and exit the RER when there is a peptide within the binding groove. Calnexin is a resident membrane chaperone protein in the ER which associates with free class I α chains and promotes its folding. When the β_2-microglobulin chain binds to the α chain, calnexin is released and the class I molecule can then associate with the chaperone calreticulin and tapasin. Tapasin (TAP-associated protein) facilitates the TAP transporter into proximity with the MHC class I molecule and allows it to acquire the antigenic peptide. Once the MHC class I-peptide complex is generated, it confers increased stability allowing its release from the calreticulin and tapasin and its exit from the RER; after which it proceeds to the cell surface via the Golgi [2].

1.3.4 Presentation of exogenous antigens : The endocytic pathway

APCs are able to ingest material via either phagocytosis or endocytosis. Once the material has been internalised the antigens are degraded into peptides within increasingly acidic compartments of the endocytic pathway; early endosome (pH6 – 6.6) to the late endosome (pH5 – 6) to the

lysosome (pH 4.5-5). In each compartment, the peptides encounter hydrolytic enzymes which promote their gradual degradation into 13-18 amino acids in length, which is the optimal length for MHC class II binding.

When the MHC class II molecules are generated in the RER, the molecule associates with another protein called the invariant (Ii) chain which prevents it from binding to the same peptides as MHC class I molecules. Three pairs of class II αβ chains associate with a preassembled invariant chain trimer. This trimeric protein interacts with the peptide binding cleft of the class II molecules, preventing any endogenously derived peptides from binding to the cleft while the MHC class II molecule is within the RER.

The MHC class II-invariant chain complex is then processed through the endocytic pathway used to degrade antigenic peptides. This movement through increasingly acidic compartments causes gradual degradation of the invariant chain. The only part of the chain which remains is called CLIP (class II-associated invariant chain peptide). CLIP prevents premature binding of the peptide. The protein HLA-DM then removes CLIP and subsequently loads the MHC class II molecules with antigenic peptides. The MHC class II-peptide complex can then translocate to the plasma membrane where the pH is neutral, therefore conferring its stability [2].

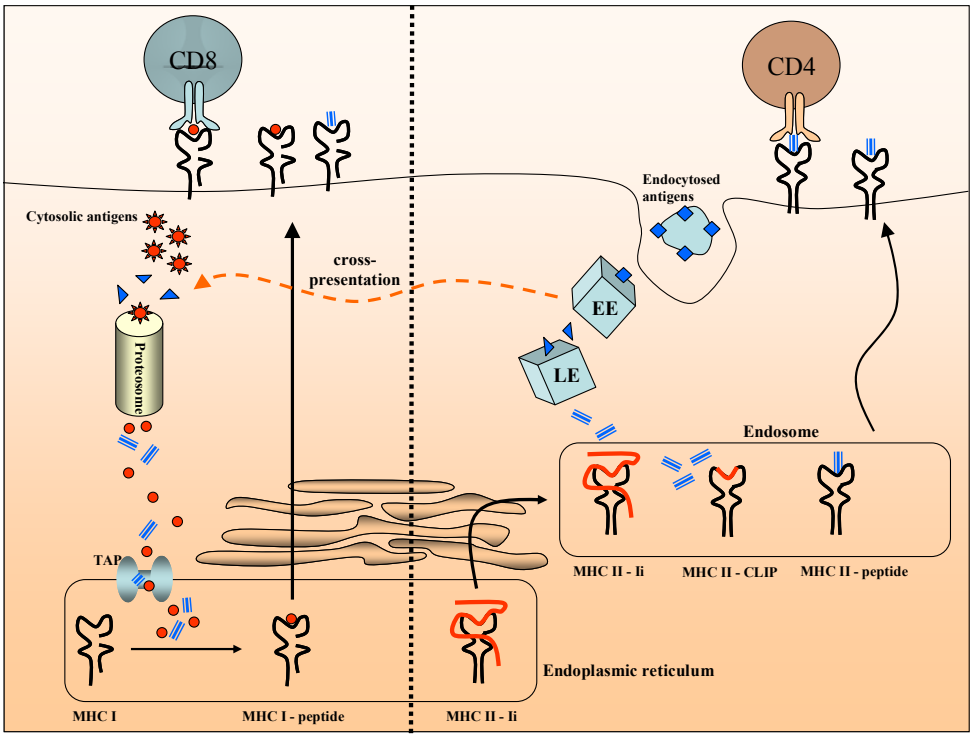


Figure 1.2 Antigen processing and presentation. Schematic representation of antigen processing via the MHC class I, MHC class II, and cross-presentation pathways. Exogenous antigen is internalised by antigen presenting cells and processed within the lysosomal compartments where it associates with MHC class II molecules before translocating to the cell surface as an MHC class II –peptide complex for presentation to CD4+ T cells. Endogenous antigen is processed through the proteosome, passes into the endoplasmic reticulum through TAP where is associates with MHC class I molecules before translocating to the cell surface as an MHC class I – peptide complex for presentation to CD8+ T cells.

1.3.5 Cross-presentation

1.3.5.1 Overview

The limitation of MHC class I- and MHC class II-peptide complexes to stimulate CD8+ and CD4+ T cells respectively is referred to as self-MHC restriction. Self-MHC restriction is designed to ensure that only infected or self-altered cells are killed, meaning that bystander cells which have endocytosed infected debris cannot process these antigens and present them with MHC class I molecules and therefore become targets themselves.

Some cell types, in particular dendritic cells are able to use distinct endocytosis mechanisms to simultaneously introduce antigen into separate intracellular compartments dedicated to either presentation with MHC class I or MHC class II molecules [21]. The access of exogenous peptides into the MHC class I presentation pathway is referred to as cross-presentation (as shown in figure 1.2). Cross-presentation of exogenous antigen is required for effective immunity against viruses that do not infect professional APCs directly or against tumour antigens that are not endogenously expressed by them [19]. As discussed previously, there are many DC subsets; only some of these are able to efficiently cross present exogenous antigen, some do this constitutively such as murine $CD8\alpha^+$ DC's [22] and others cross-present upon activation by stimuli such as immune complexes (discussed further in section 1.4), T cell help, and toll-like receptor ligands. Other cell types such as B cells, endothelial cells and macrophages have also been reported to be able to cross-present [23, 24].

Recent evidence suggests that it is the manner in which exogenous antigen is taken up by APCs which controls their ability to cross present antigen to CD8+ T cells [21]. The mannose-receptor for example introduces exogenous antigen through endocytosis. This method of uptake allows antigen to localise in early endosomes but is excluded from late endosomes or lysosomes, thus permitting access to the MHC class I presentation pathway. The constitutive pinocytosis of antigen by APCs is however transported exclusively towards lysosomes where it can co-localise with MHC class II molecules. This apparent receptor dependence for cross-presentation may allow APCs to restrict their ability to cross-present antigen depending on their stages or maturation or to limit cross-presentation of specific classes of antigen. It is also plausible for it to be subsequently limited to particular APC subpopulations; such as the murine $CD8\alpha^+$ DC subset which expresses the mannose receptor [25].

The constitutive pinocytosis observed in all DC subsets ensures that DCs which cross-present are also able to acquire antigen for the induction of

cognate CD4+ T cell help; which requires that the same DC stimulates both specific CD4+ and CD8+ T cells (see section 1.3.5.2).

The resulting T cell activation after presentation of exogenous antigen with class I molecules leads to two fundamentally different immune outcomes. The first is termed cross priming. In this instance, T cells become stimulated and expand into peptide-specific CTLs after which they can then travel into the peripheral tissues and target cells expressing their cognate ligand [26]. Conversely, cross-presentation of antigen can also lead to cross-tolerance where by T cells which are specific for the peptide being presented are either deleted or rendered anergic; a process which is essential for the maintenance of peripheral tolerance and the prevention of autoimmunity [27].

1.3.5.2 DC maturation state dictates immune outcome

The ability of exogenous antigen to become internalised and transported to the cytosol for presentation within the MHC class I complex is restricted to the early immature state of dendritic cells [28]. In the peripheral tissues, DCs are immature and characteristically express low levels of MHC class I and class II molecules, CD86, CD40 and CD54. They are however highly efficient at antigen capture either by taking up particles or microbes by phagocytosis, forming large pinocytic vesicles in which extracellular fluid is sampled (macropinocytosis) or by receptor mediated endocytosis in which they utilise numerous surface receptors including $\alpha_v\beta_5$ integrin, CD36 and Fcγ-receptors [29] (discussed further in section 1.5.3). During the course of an infection, DCs will mature either as a result of a pathogen such as the binding of lipopolysaccharide (LPS) or whole bacteria to toll-like receptors (TLRs) or indirectly through inflammatory stimuli such as interleukin-1 (IL-1), granulocyte-macrophage colony stimulating factor (GM-CSF), or tumour necrosis factor α (TNF-α). In the first stages of DC maturation, TLRs enhance the ability of DCs to capture antigen through up-regulating macropinocytosis. This results in their improved capability of antigen presentation on MHC class I and class II molecules [30]. Further DC maturation leads to their eventual down-regulation of endocytic

ability followed by their subsequent migration to the draining lymph nodes where they can then terminally differentiate [29].

The two signal model of T cell activation is typically used to explain how cross-presentation of exogenous antigen can either lead to cross-priming or cross-tolerance. The first signal of T cell activation is provided for by the expression of MHC-peptide complexes which permits T cell receptor signalling. The second signal is provided for upon DC maturation when co stimulatory molecules are up-regulated. Evidence suggests that only the two signals working simultaneously ensure efficient T cell activation. Signal 1 in the absence of signal 2 causes the T cell to become deleted or alternatively induces a state of antigen-specific T cell unresponsiveness or anergy, referred to typically as tolerance [31].

In the absence of inflammation, effective CTL immunity can also be generated by a process referred to as DC licensing [32]. DC licensing describes the interaction between CD154 on activated CD4+ T helper cells and CD40 on DCs which promotes DC maturation, enhancing their expression of the costimulatory molecules CD80, CD86, CD70, 4-1BB ligand, and OX40 ligand on their surface and subsequently allows them to become fully competent at stimulating naïve CD8+ T cells [33]. DC licensing can also be induced without CD4+ T cell help by substitution with artificial stimuli such as the addition of the CD40 ligand, IL-1β or IL-12 [33].

1.3.5.3 Apoptotic cells provide a source of antigen for cross-presentation

The engulfment of apoptotic cells by dendritic cells provides a source of antigen which can be cross-presented for the generation of both MHC class I and MHC class II complexes [34]. Cross-presentation refers to the ability of antigens to "cross" classically defined restrictions for MHC class I presentation [35] and accounts for both the cross-priming and cross-tolerance of apoptotic cell associated antigens. Both macrophages [36] and dendritic cells [37] are able to cross-present antigens from apoptotic cells

in vitro however it is the migration of dendritic cells to secondary lymphoid organs after antigen capture which gives them their unique ability of presenting cell-associated antigen to CD8+ T cells *in vivo*.

One of the first reports showing that antigen derived from apoptotic cells could be cross-presented to T cells by antigen presenting cells was by Albert and colleagues in 1998. They showed that uninfected dendritic cells but not macrophages could acquire antigen from Influenza A virus-infected monocytes and cause proliferation of virus-specific CTLs *in vitro* [38]. Continuous studies thereafter have supported this evidence, showing that dendritic cells can process antigen from apoptotic cells that contain whole or partially processed protein. In addition to this, studies have also been completed which highlight the ability of the apoptotic cell to process antigens before being taken up by the DC through utilising its own proteosome and transporter machinery; the process of which does not then have to rely on antigens having to access the endocytic pathway of the DC. Apoptotic cells are therefore likely to play an active role in antigen presentation through the delivery of processed antigen which would allow for efficient generation of MHC class I – peptide complexes by the DC [34].

In the mouse, cross-presentation of cell-associated antigens is observed exclusively by a subset of dendritic cells which express high levels of CD11c, CD8α, DEC-205 and CD36. Studies have shown that they are highly efficient at internalising apoptotic cells in contrast to other murine DC subsets [39].

DCs are also able to ingest apoptotic tumour cells and present tumour associated antigens to immune cells [40]. The differences in tumour associated antigen processing and presentation by DCs depends on the source or form of the tumour-derived materials, the maturation stage of the DCs, and the responsiveness of the T cell populations available for stimulation by DCs [41]. Recent data has shown that initial priming of tumour specific T cells does not necessarily lead to long lasting protective

immunity against the tumour. DCs pulsed with peptide and matured *ex vivo* are able to stimulate naïve tumour-specific T cells causing reduction in tumour size. T cells which were however persisting within the tumour environment gradually lost their antigen reactivity and became tolerant. To elicit an effective anti-tumour response, T cells must be efficiently primed but must also be protected from the suppressive mechanisms of the tumour microenvironment to avoid tolerance [42].

1.3.5.4 Dendritic cells play a vital role in peripheral T cell tolerance

To prevent autoimmunity, mature T cells do not respond to self-peptides that are presented to them. The development of both the B and T cell repertoire involves the rearrangement of immunoglobulin and T cell receptor genes to produce clones of lymphocytes which display unique receptors. This rearrangement is random and therefore clones which respond to self are also generated. During T cell development, only those T cells that have no, or low affinity towards the peptide antigens which are present in the thymus are allowed to mature and enter the circulation. Any T cells that react with self-peptides which are presented to them by MHC molecules on murine thymic epithelial cells or dendritic cells are deleted, a process referred to as central tolerance. Not all self antigens are represented in the thymus and consequently further deletion or silencing of self-reactive T cells in the peripheral tissues is imperative for the prevention of an autoimmune response; a process which is referred to as peripheral tolerance [43].

Under steady state conditions (i.e. in the absence of infectious or inflammatory stimuli), DCs ingest exogenous antigen and migrate from the peripheral tissues to the lymphoid tissues. Dendritic cells which present self-antigens in these conditions will not have received any maturation stimuli. Their remaining immature state means that they will express low levels of co stimulatory molecules and thus will tolerize both self reactive CD4+ and CD8+ T cells (cross-tolerance) [44].

To the most part, peripheral tolerance to self antigens prevents the onset of autoimmunity so must therefore be considered as advantageous to the host. There are, as would be expected, exceptions to this rule where by cross-tolerance is detrimental to the elimination of persistent infections such as HIV-1 or tumour cells, where the antigenic load is continuously high, resulting in the deletion or silencing of HIV-1 or tumour-specific T cells.

As an example, the induction of tumour-antigen specific T cell tolerance by B cell lymphomas *in vivo* is attributed to bone marrow derived antigen presenting cells. These APCs take up antigens shed from tumours and then present processed antigenic peptides to antigen-specific T cells. The T cells, upon failure of receiving co-stimulation, are then either deleted or rendered anergic [45].

The manner in which tumour cells are cleared by dendritic cells greatly influences the efficiency in which the tumour antigen is processed and presented. The targeting of antigen to Fcγ receptors expressed on the dendritic cell surface has proven to enhance cross-presentation [46], a strategy which is currently of great interest for the immunotherapy of tumours.

1.3.6 Role of Fcγ receptors in controlling immune outcome

1.3.6.1 Overview

As part of the immune response to bacterial infection, antibodies are secreted by plasma cells. Upon their encounter with microbes, they opsonise their surface and stimulate their ingestion by phagocytes through the interaction of Fc receptors, expressed constitutively on the phagocyte surface. There are five classes of antibody – IgG, IgA, IgM, IgD and IgE all of which have a basic unit of two light chains and two heavy chains, the chains of which are folded into regions called domains. Immunoglobulins are bifunctional in which one region of the molecule binds to its cognate antigen, while another region mediates their effector functions depending on the class of antibody. Immunoglobulin G (IgG) is the most abundant class in serum, typically consisting of approximately 80% of the total

serum immunoglobulin. The IgG molecule is made up of two γ heavy chains and two κ or two λ light chains.

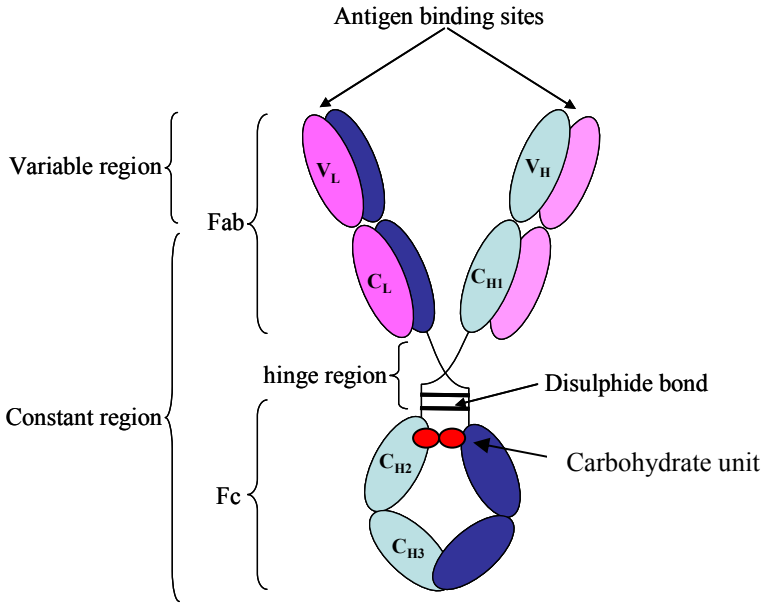


Figure 1.3 General structure of the IgG molecule. The N-terminal end of IgG is characterised by its variable region in both the heavy and light polypeptide chains, referred to as the V_H and V_L regions, respectively. The constant region of the light chain is termed C_L. The constant region of the heavy chain is divided into three structurally discrete regions: C_H1, C_H2, and C_H3. These domains are stabilised by intrachain disulphide bonds. The hinge region allows flexibility permitting the two antigen-binding sites to operate independently. Carbohydrate units are attached to the C_H2 domains.

The four subclasses of IgG in mammals are distinguishable by their differences in γ-chain sequences and in mice are numbered IgG1, IgG2a, IgG2b and IgG3 (see figure 1.3 for basic IgG structure) [2]. Each subclass has evolved to drive different effector immune responses such as cytotoxicity, phagocytosis, and the release of inflammatory mediators. Their expression is largely dictated by a number of factors, such as the cytokines released during the start of an immune response and also the type of stimulating antigen. Among the different subclasses, IgG2a and IgG2b are considered the most potent for the activation of effector responses and dominate antiviral and autoimmune responses. The different level of potencies observed by the different IgG subclasses is attributable to their differential binding to Fcγ receptors [47].

1.3.6.2 Fcγ receptors

The interaction of Fc receptors for IgG provide a good example of how simultaneous triggering of activating and inhibitory signalling pathways set thresholds for cell activation and generate a well-balanced immune response. The murine Fcγ receptor family includes the high affinity FcγRI, low affinity FcγRIIb and FcγRIII, and the intermediate affinity FcγRIV.

1.3.6.3 Activating Fcγ receptors

Activating Fcγ receptors (FcγRI, FcγRIII and FcγRIV) are expressed on all myeloid cells (neutrophils, monocytes, macrophages, and dendritic cells) and are not expressed on lymphoid cells. They associate with the gamma chain which transmits the activation signal. The intracellular region of the gamma chain contains an Immunoreceptor Tyrosine-based Activation Motif (ITAM) in their cytoplasmic tail that binds IgG either with relative high or low affinity. Signalling through this ITAM occurs by cross-linking of the ligand binding extracellular domain. This then results in the phosphorylation of the tyrosine of the ITAM by members of the src kinase family, following subsequent recruitment of the SH2 containing signalling molecules. The cellular phenotypes associated with activation through these Fcγ receptors include degranulation, phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), the transcription of cytokine genes, and the release of inflammatory mediators. Mice which do not express these activation receptors are resistant to various autoimmune diseases such as collagen-induced arthritis (CIA) and glomerulonephritis [48, 49].

1.3.6.4 Inhibitory Fcγ receptor IIB

The activation responses observed by FcγRI, III and IV are modulated in the presence of the inhibitory Fcγ receptor IIB (FcγRIIB). FcγRIIb is the most widely expressed FcR and is the only one expressed on B cells and the only inhibitory Fcγ receptor in the mouse. It is expressed on effector cells such as macrophages, dendritic cells, neutrophils, and mast cells, however is not expressed on T and NK cells. FcγRIIb exists in a monomeric form that encodes an Immunoreceptor Tyrosine-based

Inhibition Motif (ITIM), the signalling of which promotes an inhibitory response, functioning to suppress B cell, mast cell and macrophage activation by cross-linking the B cell receptor (BCR) or FcRs [49]. It is subsequently able to suppress the development of autoimmune diseases such as CIA. Macrophages from FcγRIIb $^{-/-}$ mice are hyper-responsive to stimulation with IgG immune complexes (antibody:antigen complexes) which leads to the augmented release of pro-inflammatory cytokines such as interleukin-1α (IL-1α); promoting the up-regulation of integrins and adhesion molecules [50]. Similarly on dendritic cells, the expression of FcγRIIb enforces peripheral tolerance by inhibiting effector T cell responses. The coengagement of activating Fcγ receptors with the inhibitory receptor is able to suppress both human and mouse ITAM-induced DC maturation [51].

1.3.7 Fcγ receptor-mediated maturation and cross-priming potential of dendritic cells

1.3.7.1 Overview

In addition to mediating the internalisation of antigen-IgG complexes and promoting efficient MHC class II-restricted antigen processing and presentation, Fcγ receptors are also able to induce the maturation of dendritic cells upon antigen capture; and additionally sanction their processing of exogenous antigen into the MHC class I-restricted presentation pathway (cross-presentation) [52].

1.3.7.2 IgG-complexed antigens are transported into specific compartments for cross-presentation

Early studies observing the internalisation by phagocytes, such as macrophages, of IgG-opsonised particles through Fcγ receptors provided evidence that distinct cytoskeletal structures are involved in the phagocytosis compared to other receptors such as those mediated through complement [53]. This supports much more recent data in which IgG-complexed antigens are transferred from the endocytic or lysosomal

compartments to the cytosol via the TAP transporter where they reach the conventional MHC class-I presentation pathway.

FcγR-mediated internalisation of IgG-opsonised particles is only able to promote cross-presentation of antigen into the MHC class I pathway by dendritic cells. FcγR internalisation by macrophages or transfected B lymphoma cells does not result in MHC class I presentation, even though both are efficient in mediating FcγR-mediated IC internalisation.

1.3.7.3 FcγR-induced DC maturation and MHC class-restricted antigen presentation

The engagement of Fcγ receptors induces dendritic cell maturation, as determined by their increased surface expression of MHC, CD40, CD80 and CD86 upon internalisation of IgG-opsonised material as comparable to that observed with LPS or inflammatory cytokines. FcγR-mediated cross-priming of antigen is, as well as being specific to dendritic cells, only achievable by immature dendritic cells [52], suggesting that access to the cross-presentation pathway is limited to the time prior to their maturation.

The ability of dendritic cells to capture antigen via Fcγ receptors and induce both MHC class I and MHC class II antigen presentation whilst undergoing maturation, permits not only the role of CD4+ T cells in providing cognate help for CD8+ T cells responses, but also allows the bypassing of cognate help and the priming of CD8+ T cells directly. This role, specifically held by dendritic cells would implicate immune advantages in both anti-viral and anti-tumour immune responses. Conversely, it could also lead to the onset of a cytotoxic CD8+ T cell response against a self-antigen [52].

1.3.8 Inducing tumour immunity through engagement of activating Fcγ receptors on dendritic cells.

The vaccination of patients with defined tumour-specific peptides restricts the T cell repertoire and does not allow for CD4+ T cell help. The

advantage of using full length protein is that is has the potential to induce both CD8+ and CD4+ T cell responses against multiple epitopes. Numerous studies have shown that coating whole tumour cells with anti-tumour monoclonal antibodies (mAbs) leads to enhanced cross-presentation by dendritic cells to tumour-specific CD8+ T cells. Moreover, research has shown that this is dependent of Fcγ receptor-mediated tumour cell clearance [54].

Studies to determine whether DCs loaded with tumour cells via Fcγ receptors could be effective in cancer therapies started by firstly determining which DC population is best for tumour antigen cross-presentation, their required maturation state and also how or if they need to be activated prior to vaccination. In one such study, primary human DCs were isolated directly from the blood (PBDCs) or generated *in vitro* from monocytes or from CD34+ progenitors. Only CD1+ PBDCs and monocytes-derived DCs were capable of cross-presentation. Plasmacytoid DCs as well as B cells, monocytes, and macrophages were unable to cross-present tumour-associated antigen, irrespective or their maturation status. Targeting tumour antigen to Fcγ receptors however enhanced the cross-presentation ability of plasmacytoid DCs [55].

Human dendritic cells which are positively selected 4 h post culture with live mAb-treated tumour cells were shown to present tumour antigen more efficiently than DCs loaded with apoptotic tumour cells. This was not, however attributable to the increased phagocytosis observed, as control mAbs did not cause this enhanced efficiency. Additionally, treatment of the DCs with antibodies against FcγR is able to abrogate the increased cross-presentation by the DCs suggesting that this mechanism is clearly FcγR mediated [56]. This would suggest Fcγ receptor engagement increases the activation state of the dendritic cells rather than their phagocytic ability.

1.4 Apoptotic cell clearance

1.4.1 Programmed cell death

1.4.1.1 Apoptosis

Apoptosis is commonly construed as a non-inflammatory form of programmed cell death where by cells are able to activate an intracellular pathway which orchestrates their demise [57]. Characterised by cell shrinkage, chromatin condensation, nuclear fragmentation, preservation of organelles and cell surface changes [58, 59], apoptotic cell clearance *in vivo* occurs rapidly and efficiently. This prevents lysis and consequently affords the protection of surrounding tissues from the release of potentially noxious or immunogenic substances [59, 60]. In some cell lineages, apoptosis is distinguished by blebbing of the plasma membrane which often leads to the detachment of membrane-bound bodies that can contain organelles or nuclear fragments. (Reviewed in [61]).

The treatment of cells with apoptotic stimuli such as ultraviolet radiation has been shown to promote the release of uric acid, an endogenous immunological danger signal which promotes dendritic cell maturation upon antigen capture in its presence. The release of uric acid is thought to have an important role in immune surveillance by alerting the immune system to the presence of dying cells [62].

1.4.1.2 Necrosis

Necrosis is traditionally referred to as a passive form of cell death in which cells die when subjected to high levels of stress caused by numerous toxins, hypoxia or extremes of temperature. The resulting depletion in ATP prevents cell survival causing vacuolation of the cytoplasm and breakdown of the plasma membrane; the result of which is subsequent release of cellular contents such as heat shock proteins (HSPs) and high mobility group box protein 1 (HMGB1) into the local environment. This in most

instances leads to inflammation and results in damage to neighbouring cells [59].

Heat shock proteins (HSP) including gp96, calreticulin, hsp90 and hsp70 are intracellular, soluble proteins that are conserved from bacteria to mammals. Their release into the extracellular plasma induces macrophage stimulation and the subsequent release of cytokines such as tumour necrosis factor-α (TNF-α) [63]. Heat shock proteins bind to heat shock protein receptors on antigen presenting cells such as macrophages and dendritic cells, allowing the production of HSP-peptide complexes which are capable of inducing a partial maturation signal to dendritic cells [64]. Studies have shown that only microgram quantities of HSP-peptide complexes are required to immunise rats or mice to elicit antigen-specific T cell responses [65].

A key characteristic of cells which have undergone necrosis is their passive release of the ubiquitous nuclear protein, high mobility group box protein 1 (HMGB1). HMGB1 is secreted by endotoxin- or pro-inflammatory cytokine-activated macrophages and acts as a late mediator of endotoxaemia and sepsis [66]. Passive immunisation with neutralising antibodies is able to prevent organ damage and epithelial dysfunction in mice subjected to severe sepsis [67] Apoptotic cells in clear contrast retain HMGB1 bound to their chromatin. The presence of HMGB1 in extracellular tissue can therefore be used as a necrotic marker [68]. Extracellular HMGB1 acts as an endogenous adjuvant to promote DC maturation. In tissue repair, HMGB1 acts as a chemoattractant for fibroblasts and induces the expression of surface molecules on endothelial cells involved in the recruitment of leukocytes [69].

If the load of apoptotic cells exceeds the local capacity for their phagocyte mediated clearance or there is a defect within in the clearance pathway, apoptotic cells which are not ingested will ultimately disintegrate and release their intracellular contents. This form of cell death is referred to as secondary necrosis. Secondary necrosis is physiologically identical to

primary necrosis and leads to the same inflammatory response caused by the release of immune stimulating material such as proteolytic enzymes, lytic enzymes, cationic proteins, oxidising molecules and self antigens [70].

1.4.1.3 Autophagy

In recent years, it has become apparent that cells are able to undergo programmed cell death by a mechanism other than apoptosis. Autophagy, literally meaning, 'to eat oneself', refers to an alternative form of cell death. Autophagy can be initiated as a response to extracellular stimuli such as nutrient starvation, hypoxia, overcrowding, and high temperatures. It can also be caused by intracellular stimuli such as damaged or redundant organelles. Autophagy provides an intracellular mechanism for degrading and recycling long-lived proteins and organelles and is recognised as playing a vital role in time of starvation, particularly in *C.elegans.* (Reviewed in [71]).

The manner in which a cell undergoes its demise is able to determine the outcome of the inflammatory or immunological response. As a prevention of auto-immunity and inflammation, apoptosis depicts the form of cell death utilised as part of embryogenesis, normal cell turnover and auto-reactive lymphocyte deletion. In addition, apoptosis is also important to induce peripheral tolerance and the killing of target cells by CTLs or natural killer (NK) cells [2].

1.4.2 Caspase-dependent apoptosis

The mechanisms that drive apoptosis are conserved as a feature of all eukaryotic cells from the worm, *Caenorhabditis elegans* to mammals. This fact has enabled research across species to enhance our understanding of apoptosis in humans.

The genes that drive apoptotic cell recognition and clearance were initially characterised by observing the consequences of genetic mutations during the development of the nematode, *C. elegans*. Studies have identified the

genes involved in the initiation of apoptosis, *ced*-3 and *ced-4*, genes that promote engulfment, *ced-1,-2,-5,-6,-7,-8, and ced-10* and a gene, *nuc-1*, that encodes a nuclease that is required for the digestion of DNA in the apoptotic cell once it has been engulfed [72]. The use of *C.elegans* as a model organism to understand apoptotic cell clearance has aided the search for potential homologous genes within higher organisms. Interleukin-1-converting enzyme (ICE) was the first human gene to be identified that is homologous to a gene involved in nematode development, *ced*-3. ICE is an intracellular protease that cleaves IL-1 (a signalling protein that induces inflammation). The link between ICE and *ced-3* provided one of the first indications that the programmed cell death pathway was dependent on proteolysis, to which the family of caspases were identified [73].

Caspases are a unique class of cysteine proteases that control the majority of developmental cell death within nematodes, insects and mammals. Humans encode 13 caspases (caspase 12 is a pseudogene in all populations, with the exception of a small subset of individuals of African decent). Of the identified caspases, 7 of them (caspases 2, 3, 6-10) contribute directly to apoptosis. These can be divided further into two groups: initiator caspases, (caspases 2, 8, 9, and 10) and effector caspases (caspases 3, 6, and 7).

Caspases are synthesized in an inactive form called procaspases and require activation either by proteolytic maturation or an allosteric activator. Some caspases, such as caspase 3, are also able to autoactivate [74]. There are two well defined pathways for caspase-dependent apoptosis; the extrinsic pathway and the intrinsic pathway. The extrinsic pathway is activated upon ligation of death receptors such as CD95-Fas. This promotes the generation of the death-inducing signalling complex (DISC) which causes proteolytic maturation of pro-caspase 8 followed by activation of the effector caspases. The intrinsic pathway is activated in response to DNA damage, which can be caused by radiation and a number of anti-cancer drugs [75], and is initiated by the piddosome, a death domain-containing protein complex that catalyses the proteolytic

maturation of pro-caspase 2 (figure 1.1) [76]. Active caspase 2 leads to mitochondrial outer membrane permeabilisation (MOMP) causing the release of cytochrome c and subsequent generation of the apoptosome in which Apaf-1 mediated activation of caspase 9 occurs (figure 1.1) [77].

One of the key enzymes required for DNA degradation (a classic hallmark of apoptosis) is caspase-activated DNAse 1. Treatment with caspase inhibitors prevents this DNA degradation and subsequent nuclear condensation. When the cell death program is prevented in this manner, there is a phenotypic shift in the morphological manifestation of cell death. As described previously, a number of caspases are involved in signal transduction and act as catabolic enzymes in apoptosis. A small subset of caspases however, (caspases 1, 4, and 5 in humans and caspases 1 and 11 in mice), are involved primarily in inflammatory cytokine production such as IL-1β and IL-18; neither or which are actively involved in apoptosis. Evidence suggests in mammalian models that activation of caspases does not necessarily initiate apoptosis. The permeabilisation of the mitochondrial outer membrane causes the release of mitochondrial proteins that are in most instances toxic within the cytosol. This leads to a rapid decrease in ATP levels and is considered as the 'point of no return' in apoptosis – caspase inhibition will no longer rescue the cell from undergoing programmed cell death thereafter. In this instance, apoptosis is considered to be independent of caspases thereby promoting a death more comparable to that seen in necrosis or autophagy [74].

1.4.3 Apoptotic cell removal

Apoptotic cells are highly proficient at being recognised by the innate immune system resulting in their rapid clearance by both 'professional' phagocytes such as macrophages, and 'non-professional' phagocytes such as fibroblasts, epithelial cells, muscle cells and immature dendritic cells. There is much evidence which shows that the persistence of apoptotic cells contributes to the pathogenesis of inflammatory and autoimmune diseases [78].

A number of mechanisms are therefore in place to ensure that this does not occur; cell surface changes on the apoptotic cell provide 'eat me' signals to the phagocytes, in addition they also secrete 'find me' signals into the plasma which are detected by receptors on the phagocyte surface. Additionally, ligands which are expressed on viable cells to prevent accidental removal are actively down regulated upon apoptosis. The numerous receptor-ligand interactions which promote apoptotic cell clearance in a non-inflammatory setting again reinforce the importance of maintaining tissue homeostasis.

1.4.4 Apoptotic cell surface changes

1.4.4.1 Phosphatidylserine exposure

The removal of apoptotic cells is dictated by a vast array of molecules that are either derived from the dying cell or the plasma and are collectively referred to as "eat me" signals [79]. The exposure of phosphatidylserine (PS) on the surface of cells which have become apoptotic is a fundamental 'eat me' signal throughout cell types [80] with the only known exceptions being specific tumour cell lines such as PLB-985, a human acute myeloid leukaemic cell line [81, 82]. In resting cells, the plasma membrane consists of an asymmetric bilayer of phospholipids. Phosphatidylserine and phosphatidyletholamine (PE) reside to the most part in the inner membrane and phosphatidylcholine (PC) and sphingomyelin are found abundantly in the outer membrane [83]. The distribution of lipids throughout the membrane in resting cells remains relatively stable, with only a small exchange of lipids between bilayers. The stability of the plasma membrane is maintained by the activity of an enzyme called adenosine triphosphate (ATP)- aminophospholipid translocase, which becomes rapidly down-regulated when cells become apoptotic [83, 84]. In contrast, a calcium-dependent bidirectional movement of phospholipids across the membrane termed, 'scrambling', occurs which shows no preference for the location of PS throughout the membrane. During apoptosis, the enzyme which controls this movement, phospholipid scramblase, becomes activated through phosphorylation by protein kinase C-δ, and as a consequence, is

able to enhance the exchange of PS from the inner to the outer membrane (Figure 1.4).

Quite unexpectedly, plasma membrane lipid re-distribution is also required on the cell surface of the phagocyte initiated to engulf the apoptotic cell. The ATP transporter protein, ABC1 (ATP- binding cassette transporter 1), involved in the transport of substrates across membranes, is suggested to be involved in this process as it is expressed by macrophages in localised areas during developmental cell death. Disruption of the ABC1 transporter protein greatly reduces the efficiency of phagocytosis [60, 85].

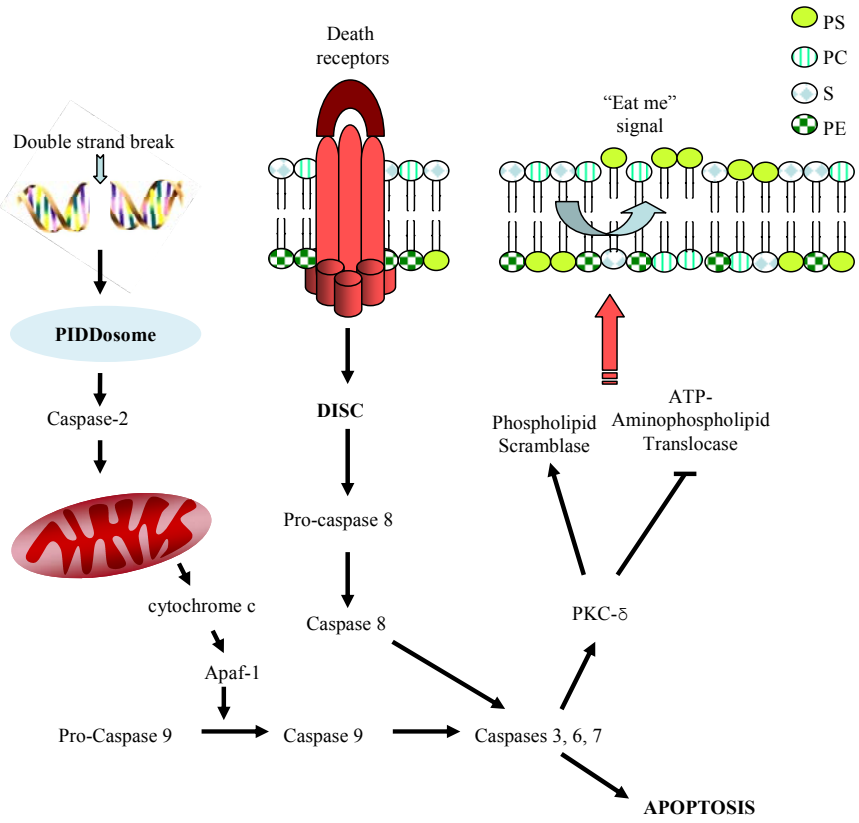


Figure 1.4 Intracellular signalling cascades which promote the exposure of PS on the apoptotic cell surface. The exposure of apoptotic stimuli such as the binding of death domain receptors, UV or gamma radiation causes an intracellular signalling cascade which causes the eventual movement of phospholipids in the plasma membrane; namely that of the exposure of phosphatidylserine which serves to act as an 'eat me' signal to promote apoptotic cell uptake. PS = Phosphatidylserine, PC = phosphatidylcholine, S = Sphingosine, PE = Phosphatidyletholamine [76, 80].

1.4.4.2 Additional cell surface 'eat me' markers.

In addition to the exposure of PS on the apoptotic cell surface, there are a number of other cell surface changes which act as 'eat me' signals to promote tethering to phagocytes. These include changes to surface carbohydrates such as the loss of sialic-acid residues on complex sugars [86]. Apoptotic cells are also able to induce partial activation of both the classical and alternative complement pathways, which results in the formation of intermediates such as C1q, iC3b, mannose-binding lectin (MBL) and collectins such as surfactant binding protein A [87].

Further still, there are also molecules that promote apoptotic cell uptake that on viable cells function normally to promote adhesion and movement. Intercellular adhesion molecule 3 (ICAM-3) is expressed on both the surface of apoptotic and healthy leukocytes, but will only promote phagocytosis of the former. Similarly, thrombospondin 1 (TSP-1), a calcium-binding protein which is secreted from apoptotic cells participates in cellular responses to growth factors, cytokines and injury and regulates cell proliferation, migration and apoptosis in a variety of physiological and pathological settings, including wound healing, inflammation, angiogenesis, and neoplasia [88]. The dual function of these molecules is only however possible due to further distinguishable features between viable and apoptotic cells.

1.4.4.3 Viable cells actively protect themselves from accidental uptake

The ability of macrophages to discriminate between viable and apoptotic cells is supported by studies that show the ability of viable cells to transmit detachment signals after 'accidental' ligation to macrophages. CD31 (also known as platelet-endothelial cell adhesion molecule-1, (PECAM-1)) is a member of the immunoglobulin superfamily of transmembrane cell-surface adhesion and signalling molecules that is expressed on the surface of both leukocytes and macrophages. The ligation of macrophages to CD31 on the viable cell surface promotes their active, temperature-dependent detachment. When cells become apoptotic, CD31 on the apoptotic cell

surface becomes disabled, allowing tethering to phagocytes which can then engage with ‘eat me’ signals such as PS [89].

1.4.4.4 Apoptotic cells release soluble ‘find me’ markers

Unlike in lower eukaryotes such as *C.elegans*, it is probable in higher organisms such as humans that when cells are rendered apoptotic, they are not within close proximity of a phagocyte. Apoptotic cells are also able to secrete chemotactic factors that act as ‘find me’ markers for the phagocyte. The phospholipid, lysophosphatidylcholine (LPC) is one such marker. Studies have shown that upon apoptosis, caspase 3-mediated activation of phospholipase A2, leads to hydrolysis of the membrane lipid phosphatidylcholine into LPC which is then released into the plasma. Phagocytes which then express a receptor for LPC are then recruited to the site of apoptotic cell death to mediate their clearance [90]. Further studies by Kim *et al* in 2002 have also suggested that in addition to LPC being secreted by apoptotic cells, it also becomes localised on the plasma membrane and is integrated as an additional ‘eat me’ signal [91].

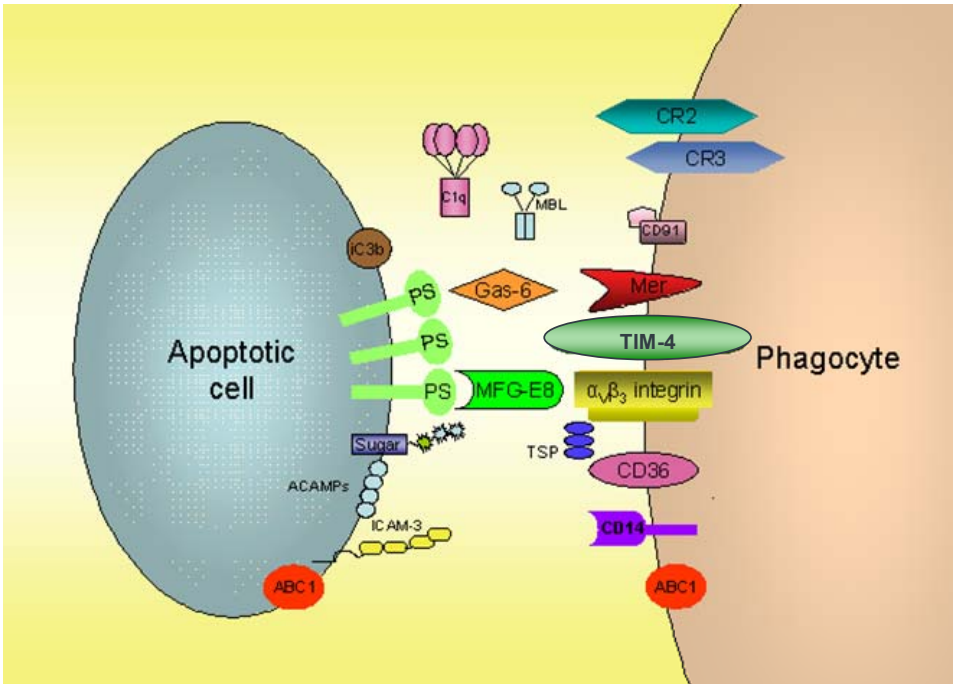


Figure 1.5 Diagram illustrating the receptors, ligands and bridging molecules required for phagocyte recognition of apoptotic cells. Cells which are apoptotic up regulate a number of markers on their cell surface such as phosphatidylserine (PS), Intracellular adhesion molecule-3 (ICAM-3), sugars, and sites which bind components of complement such as C1q and iC3b. The recognition of apoptotic cells is mediated by a plethora of receptors on the cell surface which include the $\alpha_v\beta_3$ integrin on macrophages, the class B scavenger receptor, CD36, T cell immunoglobulin and mucin domain containing molecule 4 (TIM-4), the LPS receptor, CD14 and complement receptors CR3 and CR4. A number of opsonizing molecules serve as a bridge between these markers; which include the milk fat globule-epidermal growth factor 8 (MFG-E8) and Gene arrest specific gene-6 (Gas-6) which provide a bridge between PS and $\alpha_v\beta_3$ integrin, CD36 associates with the $\alpha_v\beta_3$ integrin via the bridging molecule thrombospondin (TSP) to PS as well as surface sugars.

1.4.5 Phagocyte recognition of apoptotic cells.

1.4.5.1 Phosphatidylserine recognition

A myriad of receptors expressed on the surface of phagocytes exist which recognise apoptotic cell 'eat me' markers. As discussed previously, the exposure of PS on the apoptotic cell surface is an integral part of apoptotic cell recognition. It is therefore not unforeseen that there are numerous receptors which recognise and bind to it either directly or via bridging

molecules. The search for a receptor which binds directly to PS on apoptotic cells has taken much longer than was first expected, owing to the original PS receptor being incorrectly identified in 2000 by Fadok *et al*. This group showed that the use of antibodies against this potential PS receptor greatly reduced the clearance of apoptotic cells [92]. A number of studies using mice as a model organism also showed that phagocytes that lack this molecule were also defective in removing apoptotic cells causing abnormal development and eventual neonatal lethality [93]. Initially Fadok *et al* suggested that the PSR bound directly to PS on apoptotic cells. Hoffman *et al* then demonstrated that this may not be the case and that the PSR binds via a bridging molecule, Annexin I. Annexin I is expressed in the cytosol and upon apoptosis it becomes translocated to the outer cell membrane where it associates with phosphatidylserine plaques [94]. Careful analysis of this PSR receptor by Bose et al [95] however indicated that PSR is not involved in the engulfment of apoptotic cells, and it is unlikely to work as a receptor for PS due to it not localising to the cell surface.

The identity of a direct PS receptor was finally discovered in 2007 by Nagata's group [96], who identified the T cell immunoglobulin- and mucin domain-containing protein, (TIM-4) as the phosphatidylserine receptor. Tim-4 is a member of the *TIM* gene family, some of which are differentially expressed by T_H1 and T_H2 cells. This is however the first TIM family member to be expressed in APC populations and not T cells [97]. Mouse Tim-4 is a type I membrane protein, consisting of a signal sequence and extracellular transmembrane and cytosolic regions. Tim-4 contains and RGD motif in its IgV domain, a motif also found in integrin binding adhesion molecules such as fibronectin, thrombospondin, and Milk fat globule – epidermal growth factor 8 (MFG-E8). Tim-4 mediates the engulfment of apoptotic cells by recognising exposed PS. It is expressed by resident peritoneal macrophages, and within tissues such as the spleen, lymph nodes, and fetal liver [96]. Additionally, Tim-1 has also shown to enhance the PS-dependent engulfment of apoptotic cells within the kidney tubule post ischaemia, suggesting that is may have a role in removing

apoptotic or injured cells during the restoration of the kidney. As yet there are no publications providing functional evidence for this molecule such as mouse knock out models, so its physiological role is yet to be disclosed. The TIM family genes of human and mouse are however located on a genetic interval that is linked to a number of autoimmune diseases, such as asthma and allergy, making the role of Tim-1 and Tim-4 of key interest.

In addition to TIM-4 binding directly to PS on apoptotic cells, there are also a number of bridging molecules and opsonising proteins which facilitate apoptotic cell removal by PS. On macrophages, $\alpha_v\beta_3$ integrin binds via the bridging molecule MFG-E8 (Milk Fat Globule Epidermal Growth factor 8) to PS and mediates apoptotic cell clearance [98]. Immature dendritic cells have also been shown to utilise MFG-E8 as a bridging molecule to PS via binding of the $\alpha_v\beta_5$ integrin receptor [37]. A more detailed role of MFG-E8 is described in more detail in section 1.6. Additionally the tyrosine kinase receptor, Mer on phagocytes, binds via its bridging molecule, Gas-6 (Growth Arrest Specific Gene-6) [99], and the β2-glycoprotein-1 receptor via β2-glycoprotein [100]. Savill *et al* have shown that the $\alpha_v\beta_3$ integrin also associates with the class B scavenger receptor, CD36, via thrombospondin, promoting apoptotic cell clearance [101].

1.4.5.2 CD14 receptor mediated apoptotic cell uptake

CD14 is a glycosylphosphatidylinositol-linked plasma membrane protein which is expressed on the surface of monocytes, macrophages and granulocytes as well as being found in soluble form (sCD14) in plasma. Until recently, the function of CD14 was thought to be related only to innate immune responses to bacterial and microbial structures such as LPS, muramyldipeptide and soluble peptidoglycan. [102, 103]. It is now well established however that CD14 also plays a vital role in the tethering and clearance of apoptotic cells in a non-inflammatory setting. Antibody-inhibition studies have shown that CD14 binding to apoptotic cells occurs through a domain identical to, or overlapping with the binding site for LPS [104]. It is most likely therefore that CD14 associates with apoptotic-cell

associated molecular patterns (ACAMPs); which are similar to but clearly distinct from pathogen-associated molecular patterns (PAMPs). Studies have shown that CD14 binds to phospholipids [105], including, but not exclusively phosphatidylserine [106]. This would suggest that it is not a receptor for PS-mediated apoptotic cell recognition. Additionally, CD14 interacts with ICAM-3 which is expressed on the surface of leukocytes [103], however its ability to recognise apoptotic cells is not specific to leukocytes which again supports the theory that it also binds to another apoptotic cell marker, again the likelihood of which are apoptotic cell-associated molecular patterns [104].

$CD14^{-/-}$ macrophages *in vivo* are defective in clearing apoptotic cells, confirming its role in apoptotic cell removal. Notably however, the resulting persistence of apoptotic cells does not cause inflammation or increased auto-antibody production. A possible explanation for this is, while $CD14^{-/-}$ macrophages are unable to promote apoptotic cell clearance, they retain the ability to generate anti-inflammatory signals in response to the apoptotic cells thus impeding maturation stimuli to dendritic cells and consequently rendering subsequently activated CD8+ T cells tolerant [106].

1.4.6 Mechanisms of apoptotic cell uptake

1.4.6.1 'Tether and tickle'

The exposure of phosphatidylserine on the cell surface does not only occur as a result of apoptosis. When cells become activated, such as neutrophils which are exposed to the chemotactic factor, fMLP [107], phospholipid scramblase can become up-regulated causing PS movement to the outer membrane leaflet. The first line of defence against accidental cell removal by PS exposure is provided by ATP-aminophospholipid translocase which remains unaffected during cell activation therefore is able to resolve any lost phospholipid asymmetry [81]. An additional protective measure is conferred by the classically defined two step process required for successful apoptotic cell clearance termed 'tether' and tickle' [108] in which the 'tether' step refers to the interaction of apoptotic cells with

receptors such as CD36, integrins, and CD14 and the 'tickle' step that refers to the interaction of phosphatidylserine with its receptors or bridging molecules such as MFG-E8. Without both mechanisms in tact, the removal of apoptotic cells through macrophage mediated phagocytosis is not achieved [109].

1.4.6.2 Two partially redundant pathways mediate engulfment of apoptotic cells

The removal of apoptotic cells by phagocytosis is coordinated by ligands on apoptotic cells and receptors and other signalling proteins on the phagocyte. Genetic screens in *C.elegans* have identified over a dozen genes that function in the apoptosis program, 7 of these of which are required for the efficient engulfment of apoptotic cells; *ced-1, ced-2, ced-5, ced-6, ced-7, ced-10,* and *ced-12*. Using double mutant analyses of these genes, 6 of them have been placed into two partially redundant pathways; *ced-1, ced-6* and *ced-7* in one, and *ced-2, ced-5* and *ced-10* in the other [110]. Their mammalian homologues have since been identified.

Within the first group, *ced-1* encodes a transmembrane protein which shows homology to mammalian scavenger receptors, namely the class B scavenger receptor, CD36 [111]. *ced-7* encodes an ATP binding cassette receptor which is homologous to the mammalian ABC1 transporter protein [85] and *ced-6* encodes an adaptor protein [112]. In the second group of genes, *ced-2, ced-5* and *ced-10* encode homologues of the mammalian CrkII, Dock180 and Rac1 GTPase proteins, respectively [113], all of which mediate reorganisation of the cytoskeleton during phagocytosis of apoptotic cells [114]. CrkII was originally identified as part of a viral oncogene however is now also implicated in a variety of cell shape and cytoskeletal changes. DOCK180 binds to the first SH3 domain of CrkII. Genetic studies have shown that it functions upstream of Rac activation [115]. The activation of Rac GTPase leads to actin polymerisation at the edges of the cells, the formation of lamallipodia, cell motility and phagocytosis [116, 117]. *ced-12* and its mammalian homologues *elmo-1* and *elmo-2* are also required for the engulfment process. ELMO-1

functionally cooperates with CrkII and Dock180 to promote phagocytosis and cell surface changes. It binds directly to Dock180 which then stimulates Rac1 activation [118].

As discussed previously, integrin receptors are important in the phagocytosis of apoptotic cells. Studies have shown that the activation of $\alpha_v\beta_5$ integrin receptor by the opsonising bridging molecule, MFG-E8 [119] mediates both the binding but also the internalisation of apoptotic cells. The internalisation is dependent upon signalling through the β_5 cytoplasmic tail, resulting in recruitment of the p130cas-CrkII-Dock180 complex [120]. Genetic and biochemical studies also show that the *C.elegans* homologue to the PS receptor, psr-1 directly binds to CED12 (ELMO) and CED-5 (Dock180) to promote engulfment [121]. In 2007, a new type of engulfment receptor belonging to the adhesion-type G-protein coupled receptor (GPCR) family, BAI1 was identified. BAI1 (brain-specific angiogenesis inhibitor 1) – named because of an extra cellular fragment which inhibited neovascularisation in experimental angiogenesis binds to PS and forms a trimeric complex with ELMO and Dock180 [122]. The natural function of BAI1 in tissues is still unknown; however loss of BAI1 from gliomas promotes the formation of more aggressive glioblastomas, implicating a potential role in its treatment.

1.5 Milk Fat Globule-Epidermal Growth Factor 8 (MFG-E8)

1.5.1 Structure and function

Milk Fat Globule – Epidermal Growth Factor 8 (MFG-E8) was first identified a component of milk fat globules. It is a secreted glycoprotein that was characterised in 1990 following the isolation of a 2.1 kb cDNA that encoded for a secreted protein from a murine mammary gland λgt11 cDNA library. MFG-E8 encodes 1389 bp and has a mass of 51.5kDa [123]. It consists of two epidermal growth factor (EGF)-like domains, E1 and E2, at the N-terminus and two discoidin-like C domains, C1 and C2, at the C-terminus which show homology to the human blood clotting factors VIII and V respectively.

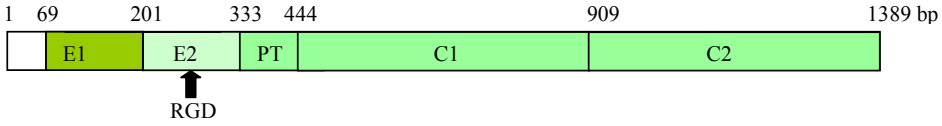


Figure 1.6 Schematic representation of the primary structure of the long variant of murine MFG-E8. The murine MFG-E8 gene is encoded by 10 exons within 16kb of genomic DNA on chromosome 7. Structural analysis of this protein found two functional domains. E1 and E2 are two N-terminal cysteine-rich domains which have homology to the epidermal growth factor domains of *Drosophila* Notch-1 protein. The EGF-domain binds to $\alpha_v\beta_3$ integrin expressed on professional phagocytes via an RGD (arginine-glycine-aspartate) motif located in the E2 domain of the gene. C1 and C2 domains are 54.4% homologous with the C-terminal domains of human clotting factors VIII and V. The two carboxyl-terminal domains are able to bind to PS. PT is a proline/threonine rich domain which is only found in the longer form of MFG-E8 [98, 124].

A monoclonal antibody raised against the protein lead to the discovery of two isoforms of MFG-E8 which originate from two spliced variants of the same pre-messenger RNA [124]. The longer variant of 57kDa (also known as MFG-E8-L) encodes an additional proline/threonine rich domain whilst the shorter variant at 53kDa (also known as MFG-E8-S) does not encode this additional domain. MFG-E8-L is produced predominantly by the lactating mammary gland, whilst the shorter isoform, MFG-E8-S, is

expressed ubiquitously in other tissues including the brain, lung, heart, kidney, skin and germinal centres in the spleen [125]. Expression of MFG-E8-S can also be induced by peritoneal macrophages through stimulation with thioglycollate [98].

MFG-E8 is strongly expressed in the germinal centres of the spleen and lymph nodes by "tingible body" macrophages which express MFG-E8 and CD68 [126]. MFG-E8 binds via an Arg-Gly-Asp (RGD) sequence motif located in the EGF-like domain, E2, to integrins, $\alpha_v\beta_3$ and $\alpha_v\beta_5$, which are expressed on the surface of macrophages and dendritic cells, respectively [127]. The C-terminus of MFG-E8, specifically the C2 domain, enables binding of phospholipids, with high specificity to phosphatidylserine. MFG-E8 provides a bridge between apoptotic cells and phagocytes to facilitate their rapid and successful clearance both *in vitro* and *in vivo* [98].

1.5.2 MFG-E8 protein mediates apoptotic cell clearance

The observation that MFG-E8 serves as a bridging molecule between apoptotic cells and phagocytes to enhance their engulfment was originally determined using a model system that could detect apoptotic cell uptake by macrophages [98].

The *in vitro* system uses thymocytes from CAD (caspase-activated DNase)- deficient mice, (CAD-deficient thymocytes do not undergo DNA degradation when exposed to apoptotic stimuli [128]) which were induced into apoptosis by treatment with dexamethasone. Their ingestion by macrophages and consequent DNA degradation in the phagosome could then be detected by TUNEL (TdT-mediated dUTP nick end labelling) staining.

Hanayama *et al* (2004) [126] studied the role of MFG-E8 in macrophage mediated apoptotic cell clearance by generating MFG-E8$^{-/-}$ mice through gene targeting. When compared with MFG-E8$^{+/+}$ macrophages, MFG-E8$^{-/-}$ macrophages were substantially defective in their ability to phagocytose apoptotic CAD$^{-/-}$ thymocytes. The phagocytic index (average number of

apoptotic cells engulfed per macrophage) obtained using MFG-E8$^{+/+}$ and MFG-E8$^{-/-}$ macrophages was 1.20 and 0.31, respectively. The phagocytic index could however be restored by the addition of recombinant MFG-E8 protein, in a dose-dependent manner.

MFG-E8$^{-/-}$ mice are fertile, however they develop splenomegaly in an age-dependent manner. At the age of 40 weeks, the spleen is approximately 3 times the weight of a wild type spleen. Histological analysis revealed that the white pulp was greatly enlarged and carried numerous germinal centres. There were also 2-3 times more lymphocytes in the spleen when compared to wild type mice. Further examination unveiled that the apoptotic cells had become localised to the tingible body macrophages, but they had intact membranes indicating that the apoptotic cells were located outside the macrophages. The macrophages had however wrapped around the apoptotic cells without engulfing them. This suggests that MFG-E8 serves to facilitate apoptotic cell uptake, but is not required for apoptotic cell tethering to macrophages [126].

As discussed in section 1.4.6.2, the $\alpha_v\beta_5$ integrin expressed on the surface of dendritic cells is able to recruit the CrkII/Dock180/Rac1 complex for the initiation of phagocytosis of apoptotic cells. Further studies have now shown that MFG-E8 is functionally linked to this $\alpha_v\beta_5$ signalling pathway. Both MFG-E8 and Dock180 are expressed by immature dendritic cells and their expression is greatly down-modulated upon DC maturation indicating that MFG-E8 is able to potentiate $\alpha_v\beta_5$ integrin-mediated Rac activation, which is dependent on the maturation state of the DC [119].

1.5.3 MFG-E8 plays an essential role in the prevention of autoimmunity

1.5.3.1 MFG-E8 deficient mice develop autoimmune disease

Previous studies have shown that repeated systemic injections of apoptotic cells leads to the production of autoantibodies [129]. MFG-E8$^{-/-}$ mice spontaneously develop autoantibodies in an age-dependent manner where

at 40 weeks the concentration of double stranded DNA (dsDNA) antibodies and anti-nuclear antibodies (ANA) in the serum is significantly higher (by up to 1000 times in half the mice) when compared with wild type mice [126].

In the germinal centres, B cells which have a low affinity for their antigen are rendered apoptotic. Tingible body macrophages are responsible for the clearance of these cells [130]. Impairment of apoptotic B cell clearance in the germinal centres by MFG-E8$^{-/-}$ macrophages would therefore suggest that their accumulation lead to the onset of autoimmunity; an observation made by Hanayama *et al* [126]. A recent study in 2008 supports this evidence however it has also revealed that although tingible-body macrophages removed the apoptotic B cells, the source of MFG-E8 which facilitates apoptotic cell clearance within the germinal centres and primary B cell follicles is follicular dendritic cells. The discovery was made simply by chance, when a marker used to detect follicular dendritic cells in the mouse (FDC-M1), was in fact identical to murine MFG-E8 [131].

As a consequence of circulating autoantibodies, immune complexes become deposited in the kidneys, which leads to glomerulonephritis; resulting in high concentrations of protein in the urine [132]. Human patients with system lupus erythamatosus (SLE) often show defects in apoptotic cell clearance by tingible body macrophages in germinal centres. The possibility of a defect in MFG-E8 would be a plausible explanation for such a phenotype [133].

1.5.3.2 Masking phosphatidylserine induces autoantibody production in mice

A dominant-negative recombinant MFG-E8 protein has also been used to examine the requirement for the protein in apoptotic cell clearance. Site-directed mutagenesis was used to make an amino acid change (D89E) in the RGD motif of the E2 domain which is required for the binding to the integrin receptor. The recombinant protein was subsequently only able to bind to PS on apoptotic cells and no longer had the ability to act as a bridge

between apoptotic cells and phagocytes to facilitate their uptake [98]. Administration of D89E intravenously into wild type mice led to inhibition of apoptotic cell clearance in certain organs by binding to exposed PS and blocking endogenous wild type receptors from binding and mediating their uptake; causing autoantibody production. The level of autoantibodies in the serum could be further enhanced by injecting apoptotic cells [134].

Injected apoptotic cells are also phagocytosed by CD8+ DCs. This normally leads to cross-tolerance in the absence of maturation signals [62]. The administration of D89E intravenously into wild type mice inhibited the production of the anti-inflammatory cytokine, IL-10 in LPS-stimulated macrophages which had been co-incubated with apoptotic cells. It is therefore feasible that inhibiting PS-mediated apoptotic cell clearances induces inflammation and consequently leads to the break down of self-tolerance [134].

1.5.4 Developmental endothelial locus-1 (Del-1) is structurally and functionally homologous to MFG-E8

As discussed, MFG-E8 is secreted by only a limited subset of phagocytes. Apoptotic cell clearance is therefore dependent on other mechanisms and molecules, such as Mer for apoptotic cell removal in the thymus, for example [135]. Studies have also shown that developmental endothelial locus-1 (Del-1) also provides a bridge between PS on apoptotic cells and $\alpha_v\beta_3$ integrin on macrophages to facilitate uptake [136].

Del-1 was originally identified as a matrix protein expressed in endothelial cells of mouse embryos is both structurally and functionally homologous to MFG-E8 [137]. Del-1 encodes two epidermal growth factor-like domains which bind $\alpha_v\beta_3$ integrin with the same affinity as MFG-E8, and two blood clotting factor VIII-like domains (C1 and C2) which bind to PS. Studies have shown that Del-1 is expressed by a different subset of macrophages than MFG-E8, namely fetal liver and thymic macrophages [136]. This again supports the previous evidence that different tissues use different

subsets of macrophages, secreting different molecules to facilitate apoptotic cell removal.

1.5.5 Homologues of MFG-E8 are expressed in various mammalian species

Orthologous proteins to murine MFG-E8 have been identified in other mammalian species including bovine (bovine-associated mucoprotein (PAS-6/7)) [138], human (Lactadherin) [139] and rat (rAGS) [140].

The human homologue, Lactadherin, was first identified from a human infant brain cDNA library after a database search [141] and is 68% identical to murine MFG-E8 at the protein level [142]. In human breast carcinoma's, antibodies are naturally produced against surface components of breast epithelial cells. After screening these antibodies against cDNA's from a lambda gt11 expression library from lactating breast, a sequence of 1270 base pairs, encoding 217 amino acids was identified. The cDNA encoded the partial sequence of Lactadherin from nucleotide 514 to 1190 and then extends an additional 600 bases into an un-translated region. The sequence analysis of the 46kDa glycoprotein showed strong homology to serum factors VII and V, which are involved in phospholipid binding. When this cDNA was used as a probe, it was found that a 2.2kb RNA was specifically over expressed in two human breast tissue carcinoma cell lines and a human ovary carcinoma cell line. As a result, the probe is now used for both breast cancer diagnosis [139].

1.5.6 MFG-E8 mediates apoptotic cell clearance in mammary gland involution

Mammary glands are organs which develop during pregnancy to nurse offspring under the control of lactogenic hormones such as prolactin. In each cycle of pregnancy mammary glands undergo lactation and then involution. Initially mammalian epithelial cells proliferate and differentiate and then undergo apoptosis upon weaning, causing subsequent involution. Apoptotic epithelial cells are initially cleared by neighbouring epithelial

cells however macrophages soon migrate into the glands to facilitate further clearance [143].

Studies have shown that MFG-E8 is secreted by both mammary epithelial cells and macrophages during involution and its involvement in apoptotic cell clearance is highlighted by observing MFG-E8 deficiency, in which there is a large accumulation of milk fat globules in the mammary ducts during involution [144].

Milk fat is composed mainly of triglycerides and is secreted as droplet of variable sizes. Approximately 99% of the total lipid is found as milk fat globules. These are initially released from the endoplasmic reticulum into the cytosol as microlipid droplets coated by proteins and polar lipids. The lipid droplets are then secreted from the mammary epithelial cells during lactation [145]. The biological functions of the major components of milk fat globule membrane such as MFG-E8, butyrophilin and CD36 have been assessed during the gestation and lactation periods.

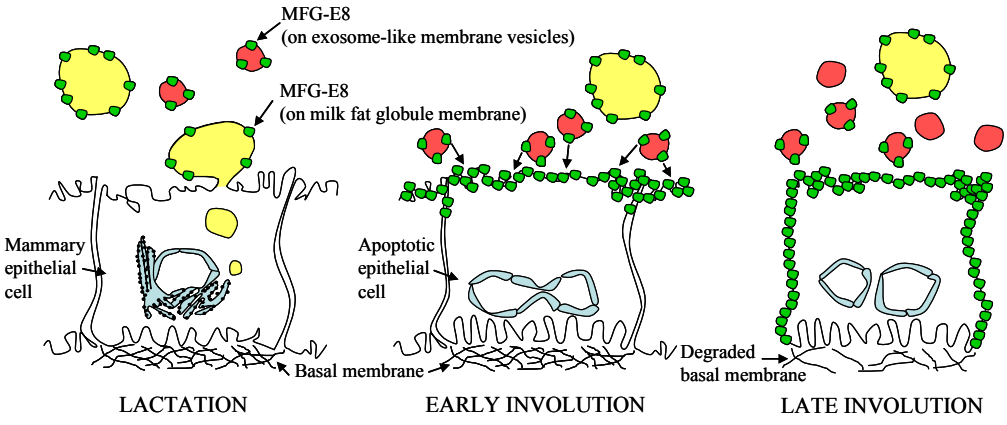


Figure 1.7 MFG-E8 mediated elimination of apoptotic mammary epithelial cells during mammary gland involution. During lactation, MFG-E8 is released in association with exosome-like vesicles and milk fat globules. MFG-E8 then binds to apoptotic epithelial cells where they can then be ingested by either macrophages or neighbouring epithelial cells. (Adapted from [146]).

MFG-E8 plays an important role in the removing apoptotic epithelial cells and the removal of milk fat globules during mammary gland involution. Impairment of this process causes periductal mastitis and inhibits re-development of the glands [144]. The gene expression in the mouse mammary gland during involution indicated that MFG-E8 transcripts gradually increase to about 1.5 times. MFG-E8 also increases markedly in mammary gland induction, detectable at both the mRNA and protein levels. This suggests therefore that the presence of MFG-E8 in facilitating macrophage mediated clearance of apoptotic mammary epithelial cells during mammary gland involution is essential to prevent inflammation and autoimmune responses to intracellular antigens [146].

1.6 Tumour Immunology

1.6.1 Overview

Despite the expression of cancer-specific antigens and the generation of lymphocytes that can recognise these antigens, tumours are nonetheless able to escape immune attack and successfully become established within the host. This is largely owing to the fact that anti-tumour T cells which both migrate to and accumulate within the tumour become tolerized within the tumour microenvironment and therefore lose their effector functions [147, 148]. This theory is supported strongly by research showing that the tumour-specific T cells, although ineffective within the tumour, can have profound effector functions at other sites within the host [147]. The development of effective immunotherapies to cancer has focused strongly on converting the immune unresponsiveness of these tumour-specific T cells into pathogenic cells capable of destroying the tumour.

1.6.2 Tumour-associated antigens

One of the overwhelming challenges when trying to generate a tumour-specific immune response is that the majority of tumour associated antigens (TAA's) that have been described are self antigens that are either selectively or over expressed by the tumour [149]. As a result of thymic negative selection, T cells which have high affinity for their cognate antigen are deleted. Only low affinity autoreactive T cells escape thymic selection, unarmed with the capacity to generate effector functions in the immunosuppressive microenvironment of the tumour.

The molecular identification of tumour specific antigens, their immunodominant peptides and TCR that recognise them have been focused on heavily in the development of cancer immunotherapy. Point mutations within tumour cells can generate peptides that bind to MHC molecules or can generate new peptide epitopes. Alternatively, a gene can be expressed by a tumour cell that is silent in normal cells (reviewed in [150]). Antigens unique to that tumour or tissue, such as the prostate, encode prostate specific antigen (PSA), prostatic acid phosphatase (PAP), prostate specific

membrane antigen (PSMA), and prostate stem cell antigen (PSCA), have all been used to generate cytotoxic T cells responses [151]. A family of antigens (MAGE) which is restricted to melanomas has also been identified using T cells derived from tumour infiltrating lymphocytes which were obtained from patients [149].

1.6.3 The tumour microenvironment – what makes it so immunosuppressive?

It is widely accepted that the tumour microenvironment can protect the tumour from immune attack through its production of soluble and cell-associated factors such as TGFβ, IL-10, prostaglandin E2, CTLA-4 ligands and PD-L1. Two dominant cell types infiltrate solid tumours and orchestrate immune suppression; FoxP3+ regulatory T cells (T regs) and tumour associated macrophages (TAMs). Both FoxP3+ T regs and tumour associated macrophages are found in high numbers in a number of human tumours [152, 153] as well as the peripheral blood [154]. They have been shown to suppress anti-tumour CTL immunity in experimental mouse models [155] through either direct interaction or through secretion of IL-10 and TGFβ [156]. The depletion of CD25+ T regs in mice has shown to significantly reduce growth of a B cell lymphoma and conferred increased survival, owing as a result of an increased infiltration of CD8+ T cells and higher serum antibody levels. Additionally however, 40% of mice depleted of T regs developed clinically detectable signs of autoimmunity [157].

Sakaguchi *et al* [158] first coined the term 'regulatory T cells' through the identification of a population of CD4+,CD25+ T cells which prevented autoimmunity in a murine model. Naturally occurring T regs represent approximately 5-6% of the overall CD4+ T cell population and are paramount in ensuring suppression to immune responses generated against self [159]. They phenotypically express CD4, CD25, FoxP3, glucocorticoid-induced TNFR family related gene (GITR) and Cytotoxic T lymphocyte antigen 4 (CTLA-4). Although T regs are functionally

anergic, they have the ability to actively inhibit CD4+,CD25- T cells, B cells [160], NKT cells [161], dendritic cells [162] and CD8+ T cells [155].

Coinhibitory molecules such as CTLA-4 are essential in the prevention of autoimmunity, highlighted by CTLA-4-deficient mice developing lethal autoimmunity and lymphoproliferation. In the tumour however, CTLA-4 expression on T regs results in their binding to CD80 and CD86 on dendritic cells, thereby suppressing their ability to activate other T cells [163]. Additionally, the expression of PD-L1 by tumours strongly correlates with the survival of cancer patients (reviewed in [164]). Interaction of PD-L1 on T cells with PD-1 on dendritic cells again, induces tolerance [163]. The targeting of either CTLA-4 or PD-1 interactions may therefore prove effective as a cancer therapy.

1.6.4 Enhancing the immunogenicity of tumour cell death

Most chemotherapy and radiation therapies used to treat cancer patients have immunosuppressive side effects and thus eliciting an anti-tumour response thereafter is the focus of much research. Upon exposure to cytotoxic drugs or radiation, tumour cells will die by apoptosis; an immunologically silent or even tolerogenic process. Some studies in mice have however shown that the type of apoptosis induced with a specific drug largely dictates the tumour cell's immunogenicity. The treatment with the anthracyclin, doxorubicin, enhances the immunogenicity of dying tumour cells in a colon carcinoma model and as a consequence is able to elicit a CD8+ specific T cell response against the tumour. This type of immunogenic tumour cell death is dependent on caspases, and requires that the cell be intact upon uptake by DCs [165]. Further work since has shown that the treatment of tumours with anthacyclins, as opposed to other treatments such as other DNA damaging agents such as etoposide or mitomycin C, confers enhanced immunogenic cell death due to the rapid upregulation of calreticulin on the cell surface. Calreticulin (CRT) is an 'eat me' signal exposed by pre-apoptotic cells. i.e. before the exposure of phosphatidylserine. Unlike PS however, the exposure of CRT on the cell surface dictates an immunogenic tumour cell death. The treatment of

soluble CRT is alone however, unable to confer enhanced tumour immunity. It is therefore likely that the signalling events that occur as part of its exposure determines its immunogenic effect [166].

As a bid to overcome the tolerizing effect of PS exposure of apoptotic tumour cells, the immunogenicity of apoptotic cells can be enhanced by the blockade of PS using a PS binding protein, annexin V. Annexin V binds to PS on apoptotic cells with high affinity and is able to inhibit apoptotic cell clearance by macrophages. Upon injection of apoptotic cells treated with annexin V, one particular study revealed that PS blockade alleviated the immunosuppression observed with the administration of apoptotic cells alone and enhanced secondary humoral immune responses. [167]. (This technique is explored further in chapter 5).

1.6.5 Adoptive T cell immunotherapy

The adoptive transfer of tumour antigen-specific T cells has re-confirmed the important role of T cell immunity in the control of cancer growth and metastasis. The challenges when trying to achieve this particular therapy include the generation of large amounts of T cells for adoptive transfer which need to retain their proliferative state as well as have the ability to home to the site of the tumour and retain their effector functions. Additionally, they also need to be able to confer long-term memory within the host.

In addition to dendritic cells being administered in isolation (discussed in section 1.6.6), studies using mouse models have also shown that peptide-pulsed dendritic cells are able to boost the anti-tumour activity of adoptively transferred T cells *in vivo*. Mice which had B16 melanomas expressing the gp100 tumour antigen were treated with T cells encoding a transgenic T cell receptor which recognises the gp100 peptide in association with MHC class I. In addition to this, they were also treated with or without peptide-pulsed dendritic cells. The results of this particular study revealed that the dendritic cells produced cytokines and permitted effective T cell proliferation and increased tumour infiltration of the

adoptively transferred T cells. This combined therapy was shown to be more effective than either of the therapies given separately [168] ; another potential route of vaccination needed to be tested for effectiveness in patients.

A clinical trial in 2005 showed that lymphopenic patient immunity could be restored by vaccination and adoptive T cell transfer after they had received high dose chemotherapy for myeloma. After treatment of myeloma with high-dose chemotherapy, the immune system is significantly impaired, even after autologous stem cell transplant. This is partly owing to the limited T cell receptor repertoire. A single infusion of antigen-primed *ex vivo* costimulated autologous T cells however leads to accelerated immune reconstitution and enhanced antigen-specific CD4+ and CD8+ T cell functions *in vivo* [169].

1.6.6 Dendritic cell vaccines

To date, it has been difficult to generate tumour-specific immune responses that correlate to good clinical outcome. Current vaccines require that antigen is administered with exogenous adjuvants so as to generate an effective immune response. The use of dendritic cells tries to overcome the requirement of exogenous adjuvants, providing a potentially safer and more specific immune response to tumours.

Inaba *et al* were the first group to demonstrate that the injection of dendritic cells loaded ex vivo with antigen could sensitize normal mice to protein antigens [170]. The potential therapeutic benefit of dendritic cell based vaccines is currently one of the new vaccination methods being investigated. A recent study has focused on a cancer testis antigen, NY-ESO-1, a highly immunogenic antigen, capable of eliciting both a humoral and cell-mediated immune response in many patients. Initial trials using this as a peptide vaccine alone showed a CD8+ T cell response, however it had no clinically beneficial output. This was potentially due to the peptide-specific CD8+ T cells being low affinity or did not recognise naturally processed NY-ESO-1 antigen. To improve the immunogenicity of the

peptide, one study [171] pulsed monocytes-derived dendritic cells with the MHC class I-restricted NY-ES0-1 peptide (p157-165) sequence that had been fused to the gene encoding heat shock protein 70 (hsp70). HSP70 is an intracellular protein which acts as a transporter to chaperone processed peptides for loading onto MHC class I molecules in the endoplasmic reticulum. The aim of the study was to determine whether the hsp70-NY-ESO-1 fusion-pulsed DCs were able to present endogenously processed peptide to NY-ESO-1-specific CD8+ T cells. The receptor-mediated internalisation of HSP-peptide complexes via toll-like receptors or CD91 is hypothesised to generate efficient antigen delivery to APCs *in vivo*. This study however did not demonstrate that DC maturation can be achieved by internalisation of HSP70, therefore could not induce effective cross-presentation of NY-ESO-1 peptide to CTLs.

Thus far, a number of groups have successfully loaded both human and mouse dendritic cells with synthetic peptides, viral vectors, apoptotic or necrotic tumour cells, or RNA. No consensus has yet emerged as to the most effective mode of antigen delivery to use for immunisation. More research is therefore needed into which type of dendritic cells to use, their ideal maturation stage at immunisation, the optimal maturation stimuli to use, and at what stage of metastatic disease should they be used to provide the best clinical outcome.

1.6.7 Current immunotherapy-based clinical trials

Cancer Immunotherapy	Agent	Target	Main Clinical Outcomes
Monoclonal Antibodies	Metelimumab (AP-12009: antisense oligodeoxynucleotide)	TGFβ	Suggested survival advantage in patients with glioma and non small cell lung cancer.
	Bevacizumab (blocking mAb)	VEGF	Approved for treatment of cancer (plus chemotherapy).
	Ipilimumab (MDX-010) ticilimumab (CP-675) blocking mAb	CTLA-4	Active against melanoma (tumour response rate: 14%), frequent autoimmunity (enterocolitis : 21%)
	MDX-1106 (mAb)	PD-1	Phase I clinical trial ongoing.
	Anti-CD40	CD40	Objective tumour regression and tumour-specific immune responses.
Fusion protein	Denileukin diflitox (anti-CD25 fusion protein)	CD25	Approved for CD25+ tumours. Conflicting results with T reg depletion and anti cancer immune responses.
Dendritic cell	Blood DCs	Tumour-specific idiotype	B cell lymphoma
	Mo-DCs	Tumour-lysate/peptide + KLH	Melanoma
	Mo-DCs	Tumour lysate	Solid tumours
	Blood DCs	Recom. mouse prostate Ag	Prostate cancer
	Mature Mo-DCs	MHC class I/ II peptides	Melanoma

Figure 1.8 Table showing examples of current immunotherapy-based cancer therapies. Examples are explained in further detail in reviews [6, 172-174].

1.7 Aims of the project

Manipulating the mechanisms which control the protective immune response against tumours is a prerequisite to the development of tumour-specific vaccines and immunotherapies. One of the fundamental reasons which explain how tumours evade recognition by the immune system is the manner in which tumour cells die by apoptosis and are cleared through the same mechanisms that facilitate the removal of cells rendered apoptotic as part of normal cell turnover.

Hypothesis: Re-directing tumour cells rendered apoptotic by artificial stimuli via a pathway independent of phosphatidylserine will break tolerance to tumour-associated antigens and confer protection against subsequent tumour challenge.

The specific aims of this study are to:

- Generate recombinant MFG-E8 proteins which bind to phosphatidylserine on apoptotic cells which can either a.) Steer apoptotic cell uptake via a pathway other than PS, or b.) Specifically block PS-mediated apoptotic cell clearance and re-direct their uptake through activating Fcγ receptors on antigen presenting cells.
- Determine the ability of the recombinant MFG-E8 proteins to bind to apoptotic cells and subsequently examine their ability to manipulate their clearance by professional phagocytes.
- Establish a model to examine the effect of loading apoptotic cells via different receptors on the ability of antigen presenting cells to present antigen to cytotoxic CD8+ T cells.
- Investigate the effect of different apoptotic cell clearance mechanisms on the humoral immune response.
- Ascertain whether the manipulation of the apoptotic tumour cell clearance pathway is alone, able to confer enhanced tumour immunity.

Chapter 2

Materials and Methods

2.1 Cell Culture

2.1.1 Cells lines and culture

A20 mouse B cell lymphoma cells were grown in RPMI 1640 medium supplemented with 10% foetal calf serum, 50μM 2-Mercaptoethanol, 2mM L-glutamine, 1mM pyruvate, 100U/ml penicillin, and 100μg/ml streptomycin at 37^0C, in a humidified atmosphere containing 5% CO_2.

π-BCL.1 mouse B cell lymphoma cells were grown in RPMI 1640 medium supplemented with 10% foetal calf serum, 50μM 2-Mercaptoethanol, 2mM L-glutamine, 1mM pyruvate, 100U/ml penicillin, and 100μg/ml streptomycin at 37^0C, in a humidified atmosphere containing 5% CO_2. Cells were subcultured when the density reached approximately 2.0 x 10^7 cells / 75cm^2. Cells were re-suspended into medium using a cell scraper.

CHO-K1 (Chinese Hamster Ovary-epithelial like) cells were grown in RPMI 1640 medium supplemented with 10% foetal calf serum, 2mM L-glutamine, 1mM pyruvate, 100U/ml penicillin, and 100Ug/ml streptomycin, at 37^0C, in a humidified atmosphere containing 5% $C0_2$. Cells were subcultured when the density reached approximately 2.5 x 10^6 cells / 75cm^2. Cells were re-suspended into medium by adding trypsin and incubating them for 5 min at 37^0C.

CT26 (Murine colon carcinoma cell line) cells were grown in RPMI 1640 medium supplemented with 10% foetal calf serum, 2mM L-glutamine, 1mM pyruvate, 100U/ml penicillin, and 100Ug/ml streptomycin, at 37^0C, in a humidified atmosphere containing 5% $C0_2$. Cells were subcultured when the density reached approximately 2.5 x 10^6 cells / 75cm^2. Cells were re-suspended into medium by adding trypsin and incubating them for 5 min at 37^0C.

E.G7 cells ((EL-4 cells (C57BL/6, H-2b, thymoma) transfected with OVA) were grown in RPMI 1640 supplemented with 10% foetal calf serum, 50µM 2-Mercaptoethanol, 2mM L-glutamine, 1mM pyruvate, 100U/ml penicillin, 100µg/ml streptomycin, and 400µg/ml geneticin at 37^0C, in a humidified atmosphere containing 5% CO_2.

Human Embryonic Kidney 293-F cells (supplied by Invitrogen) were grown in Freestyle 293 Expression medium and incubated in a 37^0C incubator that has a humidified atmosphere containing 8% CO_2 on an orbital shaker platform rotating at 125rpm. Cells were subcultured when the density was between 2-3 x 10^6 cells/ml.

Jurkat leukemic T cells were grown in RPMI 1640 medium supplemented with 10% foetal calf serum, 2mM L-glutamine, 1mM pyruvate, 100U/ml penicillin, and 100µg/ml streptomycin at 37^0C, in a humidified atmosphere containing 5% CO_2.

J558L mouse BALB/c myeloma cells (supplied by European Collection of Cell Cultures) were grown in Dulbecco's Modified Eagle Medium supplemented with 10% foetal calf serum, 50µM 2-Mercaptoethanol, 2mM L-glutamine, 1mM pyruvate, 100U/ml penicillin, and 100µg/ml streptomycin at 37^0C, in a humidified atmosphere containing 5% CO_2.

J774A.1 mouse BALB/c monocyte macrophages (supplied by European Collection of Cell Cultures) were grown in Dulbecco's Modified Eagle Medium supplement with 10% foetal calf serum, 2Mm L-glutamine, 1mM pyruvate, 1000U/ml penicillin, and 100µg/ml streptomycin at 37^0C, in a humidified atmosphere containing 5% CO_2.

P388D.1 mouse macrophages (supplied by European Collection of Cell Cultures) were grown in RPMI 1640 medium supplemented with 10% foetal calf serum, 2mM L-glutamine, 1mM pyruvate, 100U/ml penicillin, and 100µg/ml streptomycin at 37^0C, in a humidified atmosphere containing 5% CO_2.

2.1.2 Primary cell culture

Resident peritoneal macrophages were obtained from 8 – 10 week old BALB/c or C57BL/6 mice. Initially, mice were sacrificed and 10 ml, sterile PBS at 4^0C was used to lavage the peritoneal cavity. Cells obtained were centrifuged at 400 g for 5 min at 4^0C. The percentage of macrophages was determined using APC labelled CD11b antibody (B Pharmingen). Typically approximately 20% peritoneal cells were CD11b positive. Cells were then centrifuged again at 400 g for 5 min at 4^0C and were re-suspended into Dulbecco's Modified Eagle Medium supplemented with 10% foetal calf serum, 50μM 2-Mercaptoethanol, 2mM L-glutamine, 1mM pyruvate, 100U/ml penicillin, and 100μg/ml streptomycin. Cells were plated into either 24 or 48 well plates and were incubated at 37^0C, in a humidified atmosphere containing 5% CO_2 for 1.5 h to allow macrophages to adhere. Non-adherent cells were then washed using fresh supplemented DMEM medium and macrophages were incubated for a further 1.5 h, after which they were ready for use in assays.

Thymocytes were obtained from 4 – 8 week old BALB/c mice. After removing the thymus, a single cell suspension was made using a nylon filter. Thymocytes were then washed twice with sterile PBS and then counted. Thymocytes were then incubated in RPMI medium supplemented with 10% foetal calf serum, 50μM 2-Mercaptoethanol, 2mM L-glutamine, 1mM pyruvate, 100U/ml penicillin, and 100μg/ml streptomycin at 37^0C, in a humidified atmosphere containing 5% CO_2.

Bone marrow-derived dendritic cells (BMDCs) were obtained from the femurs and tibias from 12 week old male C57BL/6 mice which were removed and placed in RPMI-1640 medium (supplemented with PS) on ice until all bones were prepared. The ends of the bones (epiphyses) were cut with scissors and the bone marrow was obtained by flushing out each of the shafts with a 27 gauge myjector syringe. The marrow was passed through a nylon filter to make into a single cell suspension. Cells were then centrifuged at 300 g for 10 min. Cells were re-suspended in 5 ml red cell

lysis buffer and incubated at room temperature for 3 min. Cells were then centrifuged at 300 g for 10 min. Cells were washed once with RPMI-1640 medium (supplemented with PS) and then re-suspended into RPMI-1640 medium supplemented with 5% FBS, PS, GP and 20 ng/ml GM-CSF at 1 x 10^6 cells / ml. Cells were then plated into 24 well tissue culture plates. On day 2 and 4, non-adherent cells were removed by washing twice with RPMI-1640 medium (supplemented with PS) and fully supplemented medium was replaced. On day 6, bone marrow-derived dendritic cells were collected (which included both non-adherent and slightly adherent cells). Staining cells with anti-CD11c antibody determined that the cells were approximately 90% CD11c+.

2.1.3 Cell storage

Cell lines were stored in freeze media (90% FCS and 10% dimethylsulphoxide) at -80° C or -196° C (liquid nitrogen). After storage, cells were washed and re-suspended in media as required.

2.1.4 Cell quantitation

Cell concentrations were determined using a Coulter Industrial D cell counter (Coulter Electronics), or by counting manually using a haemocytometer (Improved Neubauer). Cell viability was measured on the haemocytometer by diluting 1:1 with trypan blue (Sigma), and observing dye exclusion from viable cells.

2.1.4 CFSE cell labelling

5 x 10^7 cells were washed twice with sterile PBS and then re-suspended into 1 ml PBS / 0.1% BSA containing 10μM CFSE (in DMSO). The cells were then incubated at 37^0C for 10 min. Cells were then washed twice with 10 ml of complete growth medium.

2.2 Molecular Biology

2.2.1 Generation of recombinant mCD4-DN-MFG-E8 fusion protein.

2.2.1.1 Cloning the full-length cDNA of murine MFG-E8.

The full length murine MFG-E8 cDNA was isolated from a mouse macrophage cell line, P388D.1. 1 x 10^7 cells were isolated from cell culture and total RNA was made by following the protocol described using an RNeasy kit, (Qiagen). The sample was eluted in 50µl dH_2O. 5µg total RNA was then used for 1st strand cDNA synthesis using the $d(T_{18})$ primer and M-MuLV reverse transcriptase (Qiagen). The MFG-E8 cDNA was then isolated by PCR using the 1st strand cDNA template using primers based on its published sequence [124] (PubMed accession number: AB021130) and *Pfu* polymerase.

2.2.1.2 Sub-cloning the PT, C1 and C2 domains of murine MFG-E8.

The PT, C1, and C2 domains of full length murine MFG-E8 were amplified using PCR (*Pfu* polymerase) using the following primers; primer 1: 5'-CGAATTCCGAAA CCGAGACCAACTACTAC-3' which encodes an *EcoR1* restriction site upstream from the PT domain. Primer 2: 5'-CAGATCTTTAACAGCC CAGCAGCTC-3' which encodes a *BglII* restriction site downstream of the C2 domain, (PCR product = 1086 base pairs). In a total volume of 20 µl, 1 ng full length murine MFG-E8 was used as a template and added to 0.5 µM primer 1, 0.5 µM primer 2, 0.2 mM dNTP's, 1 x *Pfu* Buffer and 1.25 µl *Pfu* polymerase. The PCR reaction was as follows: Step 1 = 95^0C for 6 min, step 2 = 95^0C for 1 min, step 3 = 62^0C for 1 min, step 4 = 72^0C for 3 min, step 5 = go to step 2, 24 more times, step 6 = 72^0C for 20 min, step 7 = 4^0C. The PCR product was visualised by running 10µl of the reaction on a 1% Agarose gel stained with Ethidium Bromide. 0.5 ng of the PCR product was then cloned into a pCR-BluntII-TOPO vector (supplied by Invitrogen) by following the manufacturer's protocol. In brief, 0.5 ng PCR product was incubated with sodium chloride and magnesium chloride (200 mM NaCl, 10 mM $MgCl_2$) and 1 µl TOPO vector to a final volume of 6 µl at room temperature for 5 min. 2µl of the

TOPO cloning reaction was then added to 50 µl competent TOP10 cells and then incubated on ice for 15 min. The cells were then heated shocked for 45 sec at 42^0C and then immediately returned to ice. 250 µl S.O.C. (Super Optimal Broth (with Catabolite repression)) medium (Invitrogen) was then added and the cells were incubated at 37^0C at 200 g for 1 h. 100 µl of cells were then plated onto a agar plate supplemented with 50 µg/ml Kanamycin and incubated over night at 37^0C. Colonies were selected at random from the plate and 100 µl dH_2O was added per colony. 1 µl of sample was then used per PCR reaction with M13 primers (supplied by Invitrogen) to confirm successful cloning into the TOPO vector. 10µl of the PCR product was then visualised on a 1% Agarose gel stained with Ethidium Bromide. The dominant-negative MFG-E8 (DN-MFG-E8) was then sequenced by following the protocol described in section 2.2.3.7.

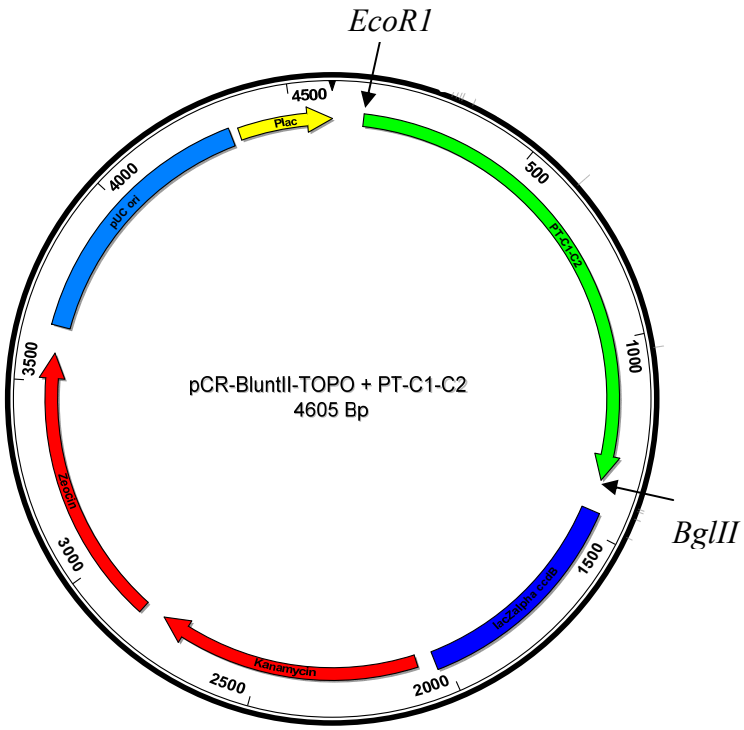


Figure 2.1 Plasmid map showing PT, C1 and C2 domains ligated into the blunt II-TOPO vector. (diagram generated using Lasergene 6 – Seqbuilder).

2.2.2 Generation of recombinant DN-MFG-E8-IgG2A-Fc fusion protein.

2.2.2.1 Sub-cloning of the PT, C1, and C2 domains of murine MFG-E8 into a murine IgG2A Fc vector.

The PT, C1 and C2 domains were amplified by PCR (using *Pfu* polymerase) from the pFLAG-PT-C1-C2 DNA template using the following primers; Primer 1 – $^{5'}$ – CGAATTCCGAAACCGAGACCAACTACTAC – $^{3'}$ – which encodes an *EcoR1* restriction site upstream from the PT domain. Primer 2 – $^{5'}$- CACATCTACATCC TCCTCCGCCACAGCCCAGCAGC TGCTCCAG-$^{3'}$ – encodes a $(Gly)_4Ser$ linker and then a Bgl II restriction site downstream from the C2 domain. (PCR product = 1098 base pairs). 1ng DNA template was added to 5µM Primer 1, 5µM Primer 2, 0.2Mm dNTP's, 1 x *Pfu* polymerase buffer and 1.25µl *Pfu* polymerase in a total volume of 50µl with dH_2O. PCR reaction was as follows: Step 1 = 95^0C for 6 min. Step 2 = 95^0C for 1 min. Step 3 = 61^0C for 1 min. Step 4 = 72^0C for 3 min. Step 5 = Go to step 2, 24 more times. Step 6 = 72^0C for 20 min. Step 7 = 4^0C. The PCR product was visualised by running 10µl of the reaction on a 1% Agarose gel stained with Ethidium Bromide. 0.5ng of the PCR product was then cloned into a pCR-BluntII-TOPO vector (supplied by Invitrogen) by following the manufacturer's protocol. In brief, 1ng PCR product was incubated with sodium chloride and magnesium chloride (200mM NaCl, 10mM $MgCl_2$) and 1µl TOPO vector to a final volume of 6µl at room temperature for 5 min. 2µl of the TOPO cloning reaction was then added to 50µl competent TOP10 cells and then incubated on ice for 15 min. The cells were then heated shocked for 45 sec at 42^0C and then immediately returned to ice. 250µl S.O.C. medium was then added and the cells were incubated at 37^0C, rotating at 200 g for 1 h. 100µl of cells were then plated onto a agar plate supplemented with 50µg/ml Kanamycin and incubated over night at 37^0C. Colonies were selected at random from the plate and 100µl dH_2O was added per colony. 1µl of sample was then used per PCR reaction with M13 primers (supplied by Invitrogen) to confirm successful cloning. 10µl of the PCR product was then visualised on a 1% Agarose gel stained with Ethidium Bromide. The DN-MFG-E8-$(Gly)_4Ser$

construct was then sequenced by following the protocol described in section 2.2.3.7. The construct was then excised using *EcoR1* and Bgl II restriction enzymes and cloned into pFUSE-mFc2 (IL2ss) shown in figure 2.2.

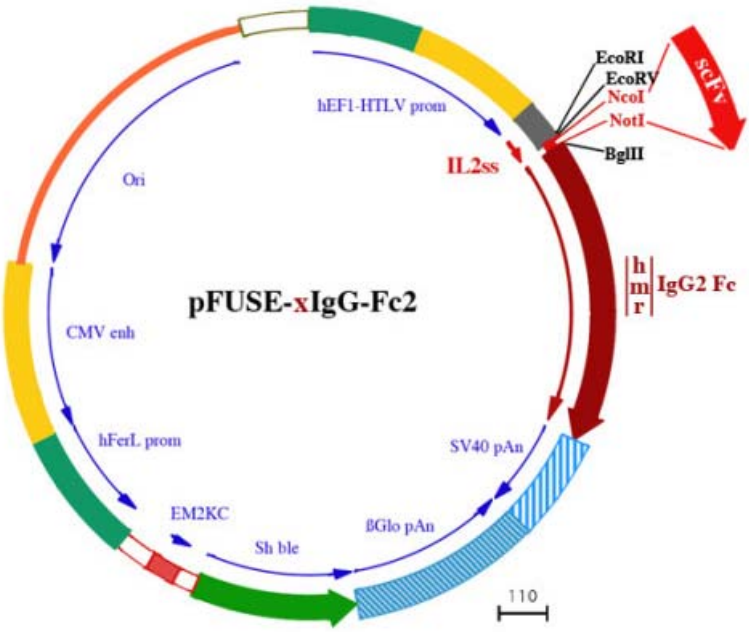


Figure 2.2 pFUSE-mFc2 (IL2ss) – mouse IgG2A plasmid designed for the construction of Fc-Fusion proteins. (supplied by Invivogen)

2.2.3 Molecular Biology techniques

2.2.3.1. Mini and Maxi preparation of DNA

Miniprep: A single colony of *E.coli* was picked from a freshly streaked selective plate which was then used to inoculate a culture of 5 ml Luria-Bertani (LB) medium containing the relevant antibiotic at 100µg/ml. This was then incubated over night at 37^0C on a rotating platform, rotating at 150 rev / min. The bacteria were harvested by centrifuging at 1500 g for 5 min at room temperature. The following steps were then taken as instructed by the Qiagen miniprep handbook. In brief: The pelleted bacterial cells were re-suspended in 250µl buffer containing RNase A. Cells were then lysed with the addition of 250µl lysis buffer containing NaOH / SDS. To neutralise the reaction, 350µl of high salt buffer was added which causes

precipitation of the denatured proteins, chromosomal DNA, cellular debris, and SDS, whilst the smaller plasmid DNA re-natures and remains in solution. The precipitated material is then collected by centrifugation for 10 min at 30,000 g in a table-top centrifuge. The supernatant was then collected and placed onto a spin column provided in the Qiagen kit and centrifuged for 1 min at 30,000 g. The flow-through was discarded. The column was then washed with 0.5 ml PB buffer and again centrifuged for 1 min at 30,000 g. The flow-through is discarded. The column was then washed with 0.75ml buffer PE containing ethanol and then centrifuged at 30,000 g. The flow-through was discarded and the column is centrifuged again. The Qiaprep column was then placed in a clean 1.5 ml microcentrifuge tube. The DNA was then eluted with 50µl 10Mm Tris.Cl, pH8.5. The column was left to stand at room temperature for 1 min before being centrifuged at 13,000 g for 1 min. The concentration of DNA was then determined by measuring the absorbance at 260nm.

Maxiprep: To produce larger quantities of plasmid DNA for use in transient and stable cell transfections, the protocol for HiSpeed Plasmid Maxi purification provided by Qiagen was followed. In brief: A single colony of *E.coli* was picked from a freshly streaked selective plate which was then used to inoculate a culture of 5 ml Luria-Bertani (LB) medium containing the relevant antibiotic at 100µg/ml. This was then incubated for 7 h at 37^0C on a rotating platform, rotating at 150 rev / min. 2 ml of this growth medium was then added to 150 ml LB medium containing the relevant antibiotic at 100µg/ml and was then incubated over night at 37^0C on a rotating platform, rotating at 150 rev / min. The bacteria were harvested by centrifuging at 1500 g for 15 min at 4^0C and the protocol in the handbook was followed to the point at which the DNA is eluted from the column. To precipitate the DNA, 10.8 ml isopropanol was added to the eluate and then centrifuged at 8,000 g for 30 min at 4^0C. The supernatant was then removed and the pelleted DNA was washed with 5 ml 70% Ethanol. The sample was then centrifuged at 8,000 g for 10 min at 4^0C. The supernatant was removed and the DNA pellet was allowed to dry at room temperature for 90 min. The pellet was then dissolved in 400µl of 10mM Tris, 0.1M EDTA. The

concentration of DNA was then determined by measuring the absorbance at 260nm.

2.2.3.2 Restriction Enzyme Digests

To determine the successful cloning and amplification of DNA, known restriction sites were cleaved with specific restriction enzymes and visualised by agarose gel / ethidium bromide electrophoresis. Protocol: 300ng DNA were added to 5-7 units of restriction enzyme per µg DNA, 1 x Enzyme buffer, then made up to a final volume of 20µl with dH_2O. (n.b. Glycerol content should not exceed more than 10% of total reaction volume.) Digest reactions were incubated at 37^0C for 1.5 h.

2.2.3.3. Agarose Gel Electrophoresis

DNA fragments were analysed on 1.0 % agarose gels made in TBE buffer (89 mM tris, 89 mM boric acid, 2mM EDTA, 0.5 µg/ml ethidium bromide), and compared to standard 1kB markers (Gene-ruler, MBI-Fermentas, UK) to allow estimation of fragment size. DNA was visualised under UV light and a photograph record taken. DNA samples were mixed 10:1 with 10 x loading buffer (20 % (w/v) Ficoll 400, 0.1 M EDTA, 0.25 % (w/v) bromophenol blue) before loading. Gels were run in TBE buffer at 70 mA in a BioRad electrophoresis system for the appropriate time and visualised on a UV illuminator.

2.2.3.4 Polymerase Chain Reaction

DNA amplification was performed using either Taq polymerase (Promega, Southampton) or *Pfu* polymerase (Stratagene). Each reaction contained approximately 100 ng of cDNA, 0.5 µM forward and reverse oligonucleotide primers, 0.2 mM dNTPs (Promega), 1x appropriate reaction buffer (Promega/Statagene) and 2.5 U Taq or *Pfu* polymerase made up with sterile deionised water. Amplification was performed in a thermocycler (PTC-100, MJ Research Inc.). DNA was denatured at 95^oC for 5 minutes, followed by 25 cycles of denaturing at 95^oC for 30 seconds, annealing at 62^oC for one minute, and elongation for two minutes at 72^oC. A final

elongation reaction of 10 minutes was performed to ensure formation of full-length transcripts.

2.2.3.5 DNA ligation

The required vector and insert DNA fragments from restriction digests were isolated using agarose gel electrophoresis and purified from the gel using a QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. Vector and insert were ligated at 1:1 or 1:3 ratios with a total of 100 ng DNA in the presence of 1.5 U T4 DNA ligase (Promega) and 1 x T4 ligase buffer (Promega) for 4 h at room temperature.

2.2.3.6 Bacterial transformation and amplification

Ligated DNA was transformed into chemically competent *E.coli* cells (Promega). Approximately 100 ng of DNA was mixed with 50 µl of *E.coli* cells (Promega) and incubated on ice for 30 minutes, before heat shocking at 42 °C for 45 seconds. Cells were then returned to ice for 2 minutes before addition of 250 µl SOC media (Promega) and incubation at 37 °C in an orbital incubator for 1 hour. Cultures were spread on agar plates (1.5 % (w/v) Bacto-Agar (Difco), 10 % (w/v) tryptone (Sigma), 5 % yeast extract (Sigma), 5 % NaCl) containing the appropriate selection antibiotic (ampicillin 100 µg/ml or kanamycin 50 µg/ml) and incubated at 37 °C overnight. Individual colonies were selected and grown up by overnight culture in 10 mls LB-Broth (10 % (w/v) tryptone (Sigma), 5 % yeast extract (Sigma), 5 % NaCl, plus selection antibiotic), before DNA preparation.

2.2.3.7 DNA sequencing

Sequencing reactions were set up containing 500 ng of the DNA of interest, 4 µl of BigDye terminator ready reaction mix (ABI-PRISM BigDye Terminator Cycle Sequencing Kit, PE Applied Biosystems) and 1.6 pmoles of T7 or SP6 oligonucleotide primer, made up to 10 µl final volume with sterile deionised water in 0.2 ml thin-walled PCR tubes (PE Applied Biosystems). Reactions were run on a thermocycler (GeneAmp 9700) for 25 cycles of: 96 °C for 10 seconds, 50 °C for 5 seconds, 60 °C for 2 min.

DNA was precipitated on ice for 10 minutes by addition of 10 % (v/v) 3 M sodium acetate and 3 volumes of cold 100% ethanol and pelleted in a microfuge at 11500g for 30 minutes. The DNA was rinsed with 70 % (v/v) cold ethanol, re-pelleted briefly and air-dried. DNA was resuspended in 1.5 µl loading buffer (20 % (v/v) dextran blue loading buffer (PE Applied Biosystems), 80 % (v/v) formamide) and denatured at 95 °C for 2 minutes. Samples were run on a 5.25 % denaturing polyacrylamide gel on an ABI-PRISM 3000 sequencer (PE Applied Biosystems) by Gavin Baggage (Tenovus Research Laboratory, Southampton) and analysed using DNAstar software.

2.3 Protein expression; purification; and detection.

2.3.1 Transient cell transfection

2.3.1.1 Transient transfection of HEK 293 F cells

One day prior to transfection, HEK 293 F cells (Invitrogen) were seeded at 8.0 x 10^5 cells/ml. On the day of transfection, cells were counted and diluted to a concentration of 1.0 x 10^6 cells/ml and made into a single cell suspension by vortexing. The DNA-lipid complex was then prepared as follows; 100µg DNA (for a 100ml transfection) was added to 5ml OPTI-MEM I reduced serum (supplied by Invitrogen) and incubated at room temperature for 5 min. At the same time, 130µl 293fectin (supplied by invitrogen) was added to 5ml OPTI-MEM I reduced serum and incubated at room temperature for 5 min. The two samples were then added together and incubated at room temperature for a further 30 min. The DNA-lipid complex was then added to the 293 F cells and then incubated at 37^0C in a humidified atmosphere containing 8% CO_2 in air on an orbital shaker platform rotating at 125 rpm for 72 hours. The cells were then centrifuged for 5 min at 400 g and the supernatant was collected. The supernatant was again centrifuged for 5 min at 400 g. The supernatant was then ready for purification.

2.3.2 Stable cell transfection

2.3.2.1 Stable transfection of CHO-K1 cells with DN-MFG-E8 or DN-MFG-E8-Fc DNA constructs.

One day prior to transfection, CHO-K1 cells were seeded at 8.0 x 10^5 cells / 75cm^2 in GMEM-supplemented medium containing 10% foetal calf serum, 2mM L-glutamine, 1mM pyruvate, 100U/ml penicillin, and 100U/ml streptomycin. The $CaC1_2$-DNA precipitate was prepared as follows; sample 1: 186µl 1M $CaCl_2$, 15µg DNA, made up to a total volume of 750µl with dH_2O. Sample 2: 750µl 2 x HBS, 5.2µl 1M HCl. Sample 1 was then added over a period of 1 min to sample 2, whilst vortexing. The solution was then incubated for 20 min at room temperature to allow a precipitate to develop.

The precipitated solution was then added to the CHO-K1 cells and incubated for 5 h at 37^0C, in a humidified atmosphere containing 5% CO_2.

The supernatant was then removed and the cells were washed twice with 15ml GMEM-supplemented medium. The cells were coated with 2 ml 15% w/v glycerol / 1 x HEPES-buffered saline (HBS) and incubated at 37^0C for 2 min. They were then washed twice with 15 ml GMEM-supplemented medium. The cells were then incubated with 15ml GMEM-supplemented medium containing 10% dialysed foetal calf serum, 100U/ml penicillin, and 100U/ml streptomycin over night at 37^0C, in a humidified atmosphere containing 5% CO_2. The following day, the medium was removed and 5 ml Trypsin-EDTA was added for 4 min at 37^0C. The cells were re-suspended with GMEM-supplemented medium containing 10% dialysed foetal calf serum, 100U/ml penicillin, and 100U/ml streptomycin and 25μM MSX at a concentration of 5.0 x 10^4 cells/ml. The cells were then plated into 96 well flat bottomed micro titre plates at 1.0 x 10^4 cells/well and incubated at 37^0C, in a humidified atmosphere containing 5% CO_2. After two weeks, colonies appeared in approximately 10% of wells per plate. When the wells were fully confluent, the supernatant was analysed by ELISA and the cells were placed into increasingly larger flasks. The clone that produced the highest concentration of secreted protein was then placed into a 3 layer flask and the supernatant was collected and purified by immunoaffinity column chromatography.

2.3.3 Protein purification

2.3.3.1 Anti-murine CD4 Immunoaffinity Column Chromatography

Rat anti-mouse CD4 YTA3.1.2 antibody was concentrated to 2mg/ml. 30mg of antibody were injected into a 12ml dialysis cassette and then dialysed with 5 litre 0.2M citrate buffer (0.2M citric acid, 0.58M NaOH, pH6.5), over a period of 48 h at 4^0C.The antibody was then diluted to a final concentration of 2mg/ml. 200ml, 10mM HCl were added to 1.2g CNBr activated sepharose 4B beads (supplied by Amersham Biosciences) and left at room temperature for 20 min. The beads were then poured over a

sintered glass filter and washed with 500ml 10mM HCl followed by 500ml 0.2M citrate buffer. The beads were placed into a universal tube and 26 mg anti-mouse CD4 (YTA3.1.2) antibody was added. The coupling reaction was incubated, rotating for 4 h at room temperature. The beads were then poured onto a sintered filter and were then washed with 20 ml, 1M Ethanolamine.HCl, pH9.5. The beads were rotated for 1 h at room temperature. The beads were again poured onto the sintered filter and were washed with 1 litre 0.1M Tris, 0.5M NaCl, 5mM Na_2EDTA followed by ammonium thiocyanate, followed again by 1 litre 0.1M Tris, 0.5M NaCl, 5mM Na_2EDTA.

To make the column, a small amount of glass wool was added to the bottom of a 10 ml syringe. 5 ml of beads were added to the column and the column was washed with 0.03M Tris, 0.14M NaCl, 1.43mM Na_2EDTA, and 0.7M HCl. The column was then washed with 0.1M Glycine.HCl, pH2.5 and then then neutralised with 5 column volumes of 0.1M Tris, 0.5M NaCl, 5mM Na_2EDTA and 5mM HCl followed by 5 column volumes of 0.03M Tris, 0.14M NaCl, 1.43mM Na_2EDTA, and 0.7M HCl. The recombinant DN-MFG-E8 protein was then purified from the transfected supernatant by being passed through the column at a rate of approximately 1 ml / min at 4^0C. The column was then washed with 5 column volumes of 0.03M Tris, 0.14M NaCl, 1.43mM Na_2EDTA, and 0.7M HCl followed by 5 column volumes of 0.1M Tris, 0.5M NaCl, 5mM Na_2EDTA and 5mM HCl, followed again by a further 5 column volumes of 0.03M Tris, 0.14M NaCl, 1.43mM Na_2EDTA, and 0.7M HCl. The protein was then eluted from the column using 0.1M Glycine.HCl, pH 2.5 and the eluate was collected in 1 ml fractions containing 100µl, 2M Tris, pH 8.5. The absorbance of each fraction was measured at 280nm and all fractions containing protein were pooled and concentrated to between 0.5 – 1mg/ml and stored at -20^0C.

2.3.4 Enzyme-linked Immunosorbent Assay (ELISA)

2.3.4.1 Inhibition ELISA

Capture reagent (CD30-CD4) was diluted to 5 µg/ml in coating buffer (15 mM Na_2C0_3, 28.5 mM $NaHC0_3$, pH 9.6) and 100 µl was added to 96-well ELISA plates (Nunc Immunoplate; Invitrogen) which were incubated for 1 hour at 37°C and then overnight at 4°C. Coating solution was removed and 100 µl/well blocking solution (1 % (w/v) BSA in PBS) added and incubated for 1 hour at 37°C. The inhibition reaction was also incubated during this time for 1 h at 37°C which was set up according to the following protocol: Standard curve: 0.02µg/ml anti mCD4 antibody (YTA 3.1.2) was added to a titration of mCD4-DN-MFG-E8 protein or mCD4-CD30 protein at concentrations of 0, 0.1, 0.2, 0.3, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8 µg/ml which were diluted with 0.25%BSA/PBS. To determine the level of mCD4-DN-MFG-E8 protein present in the supernatant, 0.02µg/ml anti mCD4 antibody was added to fold dilutions of the supernatant; again this was diluted with 0.25% BSA/PBS. The microtitre plate was then washed 3 times with 200µl PBS/0.1% Tween and 100µl of the samples were added to each well then incubated for 1 h at room temperature. Again the wells were washed 3 times with 200µl PBS/0.1% Tween and 100µl of rabbit anti rat HRP labelled antibody (1 in 40,000 dilution) was added to each well and incubated for 1 h at room temperature. The wells were washed 3 times with 200µl PBS/0.1% Tween and then 100µl substrate (1 tablet of 0-Phenylenediamine, 25ml Citrate (19.5g/l citric acid), 25ml Phosphate (28.4g Na_2HPO_4/l), 20µl H_20_2, and 49.98µl dH_2O) was added to each well and incubated at room temperature in the dark for 15-20 min. To neutralise the reaction, 50µl H_2SO_4 was added. The $absorbance_{490nm}$ of each well was determined using a Dynatech MR4000 plate reader (Dynatech laboratories).

2.3.4.2 Sandwich ELISA

Capture reagent (polyclonal rabbit anti-mouse IgG) was diluted to 25 µg/ml in coating buffer (15 mM Na_2C0_3, 28.5 mM $NaHC0_3$, pH 9.6) and 100 µl was added to 96-well ELISA plates (Nunc Immunoplate; Invitrogen) which were incubated for 1 h at 37°C and then overnight at 4°C. Coating solution

was removed and 100 µl/well blocking solution (1 % (w/v) BSA in PBS) added and incubated for 1 h at 37°C. The plate was then washed three times with wash solution (PBS + 0.05 % Tween-20) and the test supernatants and standards added to the wells. Following incubation for 60 minutes at 37°C, the plate was washed again (x 5 in wash solution). Horse-radish peroxidase (HRP)-conjugated detection reagent (diluted 1:2000 to 1: 10000) was added to the plate (100 µl/well) for 60 minutes. Following washing (x 5 in wash solution), 100 µl of HRP substrate (0.375 M citric acid, 0.8 M Na_2HPO_4, 0.2 M o-phenylenediamine, 0.006% (v/v) H_2O_2) was added and incubated in the dark at room temperature for 15-20 min. The reaction was terminated by the addition of 50 µl/well 2.5 M H_2S0_4 and the subsequent colour change quantified by measurement of absorbance at 495 nm on a micro plate photometer (Dynatec MR4000, Dynatec). Colour change was proportional to protein concentration and unknowns were determined using standard calibration curves of known concentration.

2.3.4.3 ELISA to determine saturation concentration of DN-MFG-E8-Fc onto tissue culture plate

A titration of DN-MFG-E8-Fc protein (0 – 150 µg/ml) was coated onto a 24 well tissue culture plate in 250µl PBS per well for 2 h at room temperature, followed by incubation at 4°C over night. Each well was then blocked with 2 ml PBS/1% BSA for 1 h at 37°C. Well were then washed 3 times with 3 ml PBS / 0.05% tween. Anti-mouse IgG-HRP was then diluted 1 in 40,000 with PBS / 0.05% BSA and 500 µl was added per well for 1 h at room temperature. Wells were again washed 3 x with 3 ml PBS / 0.05% tween. 500 µl HRP substrate buffer was added and incubated in the dark at room temperature for 15-20 min. The reaction was terminated by the addition of 50 µl/well 2.5 M H_2S0_4 and the subsequent colour change quantified by measurement of absorbance at 495 nm on a micro plate photometer (Dynatec MR4000, Dynatec). Colour change was proportional to protein concentration and unknowns were determined using standard calibration curves of known concentration.

2.4 Induction of apoptosis

2.4.1 γ-irradiation

All cell lines (E.G7, J558L, A20, πBCL.1 and thymocytes) were re-suspended into 1 x 10^7cells / 10ml medium (specific to individual cell line) without foetal bovine serum and placed into a universal tube. Cells were then exposed to 25 Gray of caesium radiation from a closed source. Irradiated cells were then plated back into sterile flasks and incubated for 24 h at 37^0C in a humidified atmosphere containing 5% CO_2. The percentage of apoptotic cells was determined either by trypan or using Annexin V-FITC staining.

2.4.2 UVC-irradiation

E.G7, J558L, A20, πBCL.1 cell lines and thymocytes were centrifuged at 300 g for 5 min. Cells were then washed twice with sterile PBS and were resuspened into 2 x 10^6 cells/ml in PBS. 5 ml (1 x 10^7 cells) were then placed into a sterile petri dish (without the lid) and were exposed to either 30 J/m^2 (Thymocytes) or 50 J/m^2 (all other cell lines) UVC radiation (Stratalinker). Cells were then placed back into a universal tube and were centrifuged at 300 g for 5 min. They were then placed into culture medium (without feotal bovine serum). Cells were then incubated over night at 37^0C in a humidified atmosphere containing 5% CO_2.

2.5 Phagocytosis Assay

2.5.1 Evaluation of tumour cell uptake by resident peritoneal macrophages *in vitro*.

5 x 10^7 E.G7 cells were labelled with CFSE as described in section 2.1.4 and were washed with sterile PBS. They were then placed in a petri dish and exposed to 50 J/m^2 UVC radiation. The cells were then placed in RPMI medium supplemented with 10% foetal calf serum, 50 µM 2-Mercaptoethanol, 2 mM L-glutamine, 1 mM pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin) for 48 h at 37^0C in a humidified atmosphere containing 5% CO_2 after which time they were 100% apoptotic as determined by Annexin V staining. Resident peritoneal macrophages from 8 week old C57BL/6 mice were prepared as described previously. 3.0 x 10^5 CD11b positive macrophages were plated per well in a 48 well plate and incubated in DMEM medium supplemented with 10% foetal calf serum, 2mM L-glutamine, 1mM pyruvate, 100U/ml penicillin, and 100µg/ml streptomycin) at 37^0C in a humidified atmosphere containing 5% CO_2 for 1.5 h. All non-adherent cells were then washed off and macrophages were incubated for a further 1.5 h. 1 x 10^6 CFSE labelled apoptotic E.G7's in 100µl Annexin V buffer were added to each well. Control cells were left untreated and added directly to macrophages. Blocking phosphatidylserine with CD4-DN-MFG-E8 on apoptotic E.G7's : 1 h prior to adding E.G7's to the macrophages, cells were washed once with 1 x Annexin V Binding Buffer and were treated with a titration of CD4-DN-MFG-E8 protein (0.75µg/ml – 60µg/ml), for 1 h at room temperature. Control : E.G7's were washed once with 1 x Annexin V Binding Buffer (0.01M HEPES/NaOH, pH 7.4, 0.14M NaCl, 2.5Mm $CaCl_2$) and incubated for 1 h at room temperature prior to being added to the macrophages. Positive control : 30 min prior to adding control E.G7's to adhered macrophages, 10 mM Sodium Azide was added to the medium.

E.G7's were incubated with the adhered resident peritoneal macrophages for 75 min at 37^0C in a humidified atmosphere containing 5% CO_2. The wells were then washed with DMEM medium without supplements and cells were

then treated with Trypsin-EDTA for 10 min at 37^0C in a humidified atmosphere containing 5% CO_2. Cells were then scraped from each well and placed into FACs tubes. Cells were resuspended in 100 µl PBS / 0.2% BSA and were treated with 1 µl CD11b-APC (BD Pharmingen) and incubated at 4^0C for 20 min. Cells were then washed once with PBS / 0.2% BSA. FACs analysis was then used to determine the percentage of double positive (CFSE + CD11b) cells.

2.5.2 Evaluation of apoptotic cell uptake by resident peritoneal marophages *in vitro* using confocal microscopy.

Thymocytes from a 4 week old BALB/c mouse were prepared as described previously (section 2.1.1), labelled with 1 µM CarboxyFluorescein Succinimidyl Ester (CFSE), (section 2.1.4), and exposed to 2500 RAD caesium γ-radiation. They were then incubated with RPMI medium (supplemented with 1 mM pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin) over night at 37^0C in a humidified atmosphere containing 5% CO_2. Resident peritoneal macrophages from 8 week old BALB/c mice were prepared as described previously (section 2.1.1). 2.5 x 10^5 CD11b positive macrophages were plated onto cover slips coated with Poly-L-Lysine (PLL) that were placed into the bottom of wells in a 24 well plate and were then incubated in DMEM medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 1 mM pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin) at 37^0C in a humidified atmosphere containing 5% CO_2 for 1.5 h. All non-adherent cells were then washed off and macrophages were incubated for a further 1.5 h. 1 x 10^6 CFSE labelled apoptotic thymocytes in 100 µl Annexin V buffer were added to each well. Control cells were left untreated and added directly to macrophages. Blocking phosphatidylserine with recombinant CD4-DN-MFG-E8 protein on apoptotic thymocytes : 1 h prior to adding thymocytes to the macrophages, cells were washed once with 1 x Annexin V Binding Buffer and were treated with a titration of CD4-DN-MFG-E8 protein (30 µg/ml and 60 µg/ml), for 1 h at room temperature. Control : Thymocytes were washed once with 1 x Annexin V Binding Buffer and incubated for 1 h at room temperature prior to being added to the macrophages. Positive control : 30 min prior to adding control

thymocytes to adhered macrophages, 10 mM Sodium Azide was added to the medium. Non-phagocytosed thymocytes were then removed by washing the cover slips with PBS / 0.2% BSA followed by 200 µl Trypsin-EDTA followed by PBS / 0.2% BSA. Each sample was then treated with 10 µg/ml biotinylated F4/80 (BD Pharmingen) in 1 ml PBS / 0.2% BSA for 30 min at 4^0C. Cells were then washed with PBS / 0.2% BSA. Cells were then treated with 10 µg/ml streptavidin – Alexa546 (Invitrogen) in 1 ml PBS / 0.2% BSA for 30 min at 4^0C. Cells were again washed with PSB / 0.2% BSA. Cells were fixed with 2 ml 4% Paraformaldehyde / PBS for 20 min at 4^0C. Cells were washed with PBS. Cells were washed with 2 ml 20 mM Glycine / PBS. Cells were washed with PBS. Cells were visualised using a Leica SP2 confocal laser scanning microscope using an Argon (488) and GreNe (543) laser and a pinhole equivalent to 1 Airy Disc.

2.5.3 Evaluation of apoptotic cell uptake by resident peritoneal marophages *in vivo*

Thymocytes were prepared as described in section 2.1.1 and labelled with 1 µM CFSE as described in section 2.1.4. They were then exposed to 2500 RAD caesium γ-radiation and incubated with RPMI medium (supplemented with 1 mM pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin) over night at 37^0C in a humidified atmosphere containing 5% CO_2. Thymocytes were washed with PBS and then incubated for 45 min at room temperature with or without 100 µg/ml recombinant CD4-DN-MFG-E8. 1 x 10^7 thymocytes were then injected (200 µl) into the peritoneal cavity of 8 week old BALB/c mice. 30 min later, mice were sacrificed and the peritoneal cavity was lavaged with 10 ml cold PBS. Cells were centrifuged at 300g for 5 min at 4^0C. Cells washed in 2 ml PBS / 0.2% BSA and then treated with 10 µg/ml biotinylated F4/80 (BD Pharmingen) in PBS / 0.2% BSA for 30 min at 4^0C. Cells were then washed with PBS / 0.2% BSA and were then treated with 5 µg/ml streptavidin – APC (BD Pharmingen) for 30 min at 4^0C. Cells were washed again with PBS / 0.2% BSA and were then analysed by FACs.

2.5.4 OVA-loading apoptotic bm1 splenocytes

Splenocytes from mice which are unable to bind the OVA peptide, SIINFEKL, to their MHC class I (b haplotype mutation 1 -H2-K^{bm1}) were obtained from 8-10 week old mice. After red blood cell lysis, splenocytes were re-suspended in PBS and exposed to 50 J/m^2 UVC radiation to induce apoptosis. 5 x 10^7 UVC-radiated splenocytes / ml were re-suspended in PBS containing 10 mg/ml soluble OVA. Cells were incubated for 10 min at 37^0C. They were then added to 9ml RPMI 1640 medium without washing the cells (concentration of OVA was then 1 mg/ml). Cells were then cultured over night at 37^0C, in a humidified atmosphere containing 5% CO_2. Prior to use, splenocytes were harvested and washed to remove non-internalised OVA. They were then used for subsequent phagocytosis assays.

2.5.5 Generation and loading of BMDC with apoptotic cells

Bone marrow derived DCs were prepared as described previously. On day 6 of DC culture, non-adherent and loosely adherent cells were harvested by gentle pipetting and replated at 2.5 x 10^5 CD11c+ cells/ml in RPMI supplemented with PS and 20ng/ml GM-CSF (supplied by Peprotech) in a 48 well tissue culture plate. Thymocytes or splenocytes were prepared for DC co-culture by CFSE labelling as described previously and then either treating with 25Gy caesium gamma radiation or 50J/m^2 UVC radiation, respectively, then incubating over night at 37^0C to promote apoptosis. The apoptotic cells were then incubated for 1 hour at 37^0C with recombinant CD4-DN-MFG-E8 protein, DN-MFG-E8-Fc protein, or without protein. DCs were harvested over a time course (1 h, 2 h, 3 h, and over night), washed and determined for apoptotic cell uptake. See below.

2.5.6 Evaluation of apoptotic cell uptake by DC

Cells were labelled with 10μM CFSE before radiation and treatment with recombinant proteins. DCs were harvested after assay and labelled with 10μg/ml biotinylated anti-CD11c antibody (supplied by BD Pharmingen) for 20 min at 4^0C, washed with PBS/0.2%BSA and then stained with

10µg/ml Streptavidin – APC (supplied by BD Pharmingen) for 20 min at 4^0C. Cells were then washed again with PBS/0.2%BSA. Samples were analysed using a FACScaliber flow cytometer and CELLQuest pro software. DCs phagocytosing apoptotic cells were visualised as double positive cells.

2.6 T cell proliferation assays

2.6.1 Thymidine incorporation *in vitro* proliferation assay

Transgenic OVA-specific CD4+ or CD8+ T cells (OT-I and OT-II respectively) were obtained from isolating the spleen and lymph nodes from 8-10 week old transgenic mice. Cells were treated with 5 ml red blood cell lysis buffer for 2 min and then centrifuged for 5 min at 300g. Cells were then washed in PBS and then re-suspended at 2 x 10^5 cells / 100 µl in RPMI 1640 medium supplemented with 10% foetal calf serum, 50µM 2-Mercaptoethanol, 2mM L-glutamine, 1mM pyruvate, 100 U/ml penicillin, and 100µg/ml streptomycin. 100 µl cells were then added per well to a 96 well U-shaped tissue culture plate. OVA-positive cells or soluble OVA protein, OT-I peptide or OT-II peptide were then added to the cells. After 48 h, 1 µCi ^{3}H thymidine was then added per well. Thymidine incorporation was then determined 18 h later by washing the cells extensively using a cell harvester and plating them onto a 96 well unifilter plate (PerkinElmer). The plate was then dried at 37^0C for 2 h and the level of thymidine (cpm) per well was detected using a Topcount -5.00.

Chapter 3 - Results

Expression and characterisation of recombinant MFG-E8 proteins

3.1 Introduction

MFG-E8 is a murine glycoprotein secreted by macrophages and immature dendritic cells that provides a bridge between apoptotic cells and phagocytes to facilitate their uptake. The two carboxyl-terminal domains, C1 and C2, bind to phosphatidylserine upon its exposure on the apoptotic cell surface; and the N-terminal epidermal growth factor-like domains, E1 and E2, bind via an amino acid RGD motif in domain E2 to $\alpha_v\beta_3$ integrin and $\alpha_v\beta_5$ integrin expressed on the surface of macrophages and dendritic cells, respectively. There are two isoforms of MFG-E8 generated by spliced variants from the same mRNA. MFG-E8 L and MFG-E8 S differ only by an additional proline/threonine (PT) rich domain encoded by the longer variant, shown to increase the affinity for PS *in vitro* by up to 8 fold when compared to MFG-E8 S [98].

To elucidate the potential use of MFG-E8 in enhancing the immunogenicity of apoptotic tumour cells; either by blocking PS or additionally, through re-directing their uptake though Fcγ receptors, the recombinant proteins were first expressed and their functions characterised.

Previous studies have shown that mutating the RGD motif required for integrin recognition generates a dominant-negative MFG-E8 protein which no longer has the ability to bind to integrins on phagocytes, and thus inhibits apoptotic cell clearance by up to 40% [98]. In this study, a dominant negative form of the protein has been produced which encodes only the domains required for PS binding (PT, C1 and C2) and no longer encodes the N-terminal domains necessary for integrin recognition (E1 and

E2). The first aim was to investigate the ability of DN-MFG-E8 protein to bind to an array of apoptotic tumour cells and to ensure that the binding was specific to PS.

In addition to blocking PS-mediated apoptotic cell clearance, the effect of re-directing the apoptotic cells through Fcγ receptors was also examined. Previous studies have shown that material opsonised with IgG is phagocytosed through Fc gamma receptors expressed on the surface of dendritic cells, promoting effective cross-priming to cytotoxic T cells [52]. Evidence to support this hypothesis is provided in studies showing that Fc-mediated phagocytosis of apoptotic tumour cells induces tumour immunity by promoting DC maturation in a manner that allows cross-presentation of tumour associate antigens to CD8+ T cells [46]. To utilise this pathway, a second recombinant dominant-negative protein (DN-MFG-E8) was generated which was ligated to the Fc region of mouse IgG2a comprising the CH2 and CH3 domains of the IgG heavy chain and the hinge region. Again, this recombinant protein expression and function has been characterised.

This chapter describes the manner in which both recombinant MFG-E8 proteins were expressed in mammalian cells; their biochemical characterisation; and their ability to bind specifically to apoptotic cells.

3.2 Generation of a dominant-negative MFG-E8 protein.

3.2.1 Cloning and expression of recombinant DN-MFG-E8 protein

Total RNA was extracted from 10 x 10^6 P338D.1 mouse macrophage cells. 5 µg of RNA was then used to generate 1st strand cDNA using a d(T_{18}) primer and M-MuLV reverse transcriptase. 1/20 of the cDNA produced was used to amplify MFG-E8 cDNA by PCR using specific primers (see section 2.2.1.2) and *Pfu* polymerase. The PCR product was then cloned into a TOPO Blunt II plasmid, after which the plasmid was sequenced using M13 primers. The PT, C1, and C2 domains were then ligated to a rat CD4 leader and murine CD4 sequence (domains 3 and 4 only) so that the protein could be detected and purified by anti-CD4 mAb. Figure 3.1 shows a schematic representation of the primary structure of the fusion protein which was cloned into two separate mammalian expression vectors; pEF1/V5-His A and pEE14, for use in both transient and stable cell transfections, respectively.

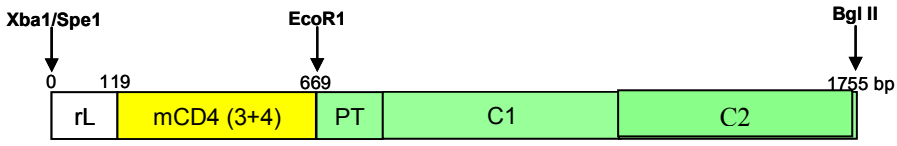


Figure 3.1 Schematic representation of the primary structure of DN-MFG-E8.
Rat CD4 leader and murine CD4 (domains 3 and 4) were isolated and ligated into a pEF1/V5-His A vector at restriction sites, Spe1/Xba1 and *EcoR1*. The PT, C1 and C2 domains of MFG-E8 were then isolated and cloned downstream of the CD4 (3 and 4) domain at restriction sites, *EcoR1* and Xba1.

Initially, the recombinant protein was expressed transiently in 293F cells following the protocol described in section 2.3.1.1. The secreted protein resolved on SDS-PAGE at approximately 70kDa (Figure 3.2) which could be determined after purifying the protein from the cell culture supernatant using anti-CD4 immunoaffinity column chromatography, where a single band was observed (Figure 3.3). Both reduced and non-reduced protein resolved at the same molecular weight which suggests that there are no inter-protein disulphide bonds (Figure 3.4). A schematic representation of the predicted folded protein structure is shown in figure 3.5.

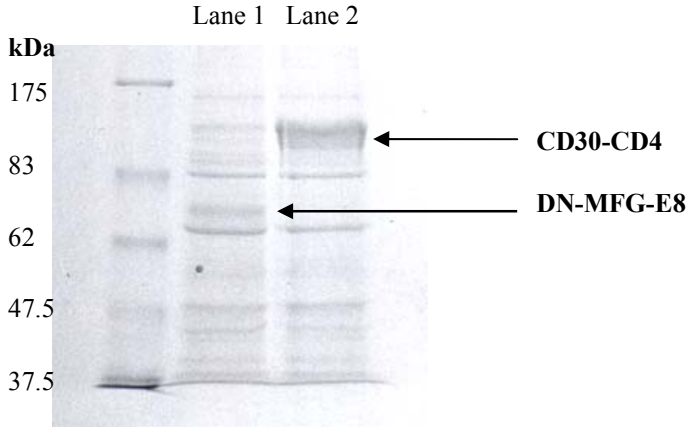


Figure 3.2 T**ransiently expressed recombinant DN-MFG-E8 protein**. 8.5% SDS-PAGE gel. Lane 1, 25µl concentrated (50-fold) supernatant from HEK 293 F cells transfected 72 hours previously with 100µg pEF-1/V5-His A-CD4-DN-MFG-E8. Lane 2, 25µl 200 fold concentrated supernatant from HEK 293 F cells transfected 72 hours previously with 100µg PigPlus-mCD4-CD30 (control).

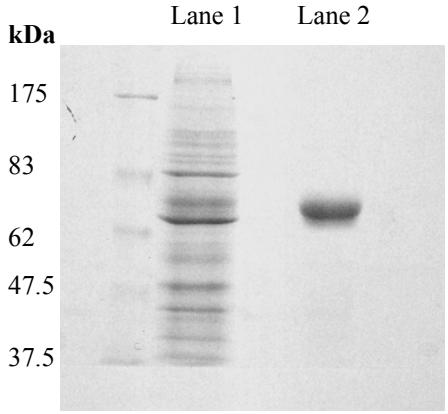


Figure 3.3 Purified transiently expressed recombinant DN-MFG-E8 protein. 8.5% SDS-PAGE gel. Lane 1, 25µl concentrated (50 fold) supernatant from HEK 293 F cells transfected 72 hours previously with 100µg pEF-1/V5-His A-CD4-DN-MFG-E8. Lane 2, 6.5µg DN-MFG-E8 protein eluate from 100ml supernatant from HEK 293 F cells transfected 72 hours previously with 100µg pEF-1/V5-His A-CD4-DN-MFG-E8 and then purified using anti-mCD4 YTA3.1.2 immunoaffinity column chromatography.

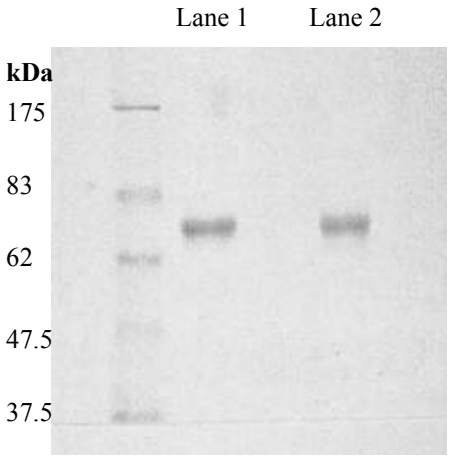


Figure 3.4 Reduced and non-reduced purified recombinant DN-MFG-E8 protein. 8.5% SDS-PAGE gel. Lane 1, 6.5µg DN-MFG-E8 protein reduced with 40µM DTT. Lane 2, 6.5µg non-reduced DN-MFG-E8 protein.

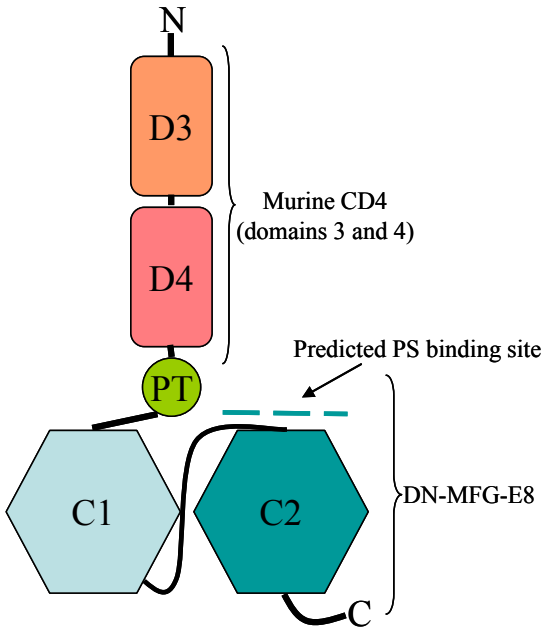


Figure 3.5 Schematic representation of the predicted protein structure of DN-MFG-E8 protein. Domains 3 and 4 of murine CD4 are encoded upstream of the PT,C1 and C2 domains of murine MFG-E8. Domains C1 and C2 show homology to human clotting factors VIII and V, respectively; the PS binding domain of which has been identified as a site within the C2 domain [175].

3.2.2 Stable transfection of mammalian cells to constitutively secrete DN-MFG-E8 protein.

To produce larger amounts of recombinant DN-MFG-E8 protein, the CD4-PT-C1-C2 construct was cloned into a pEE14 vector and was used to stably transfect CHO-K1 cells using the transfection protocol described in section 2.3.2.1. After transfecting CHO-K1 cells with pEE14-CD4-DN-MFG-E8, they were then grown in selection GMEM-S medium, containing 25μM MSX. All colonies were then screened by an inhibition ELISA to detect secreted DN-MFG-E8 protein (Figure 3.6). The most viable and highest secreting clone was then selected for expansion. At each stage of expansion, the supernatant was again tested for protein expressed by inhibition ELISA. The supernatant was collected after the cells were established.

The protein was purified from the supernatant using anti-CD4 immunoaffinity column chromatography (described in section 2.3.3.1) and then dialysed with PBS for use in functional assays. The size of the protein was confirmed using SDS-PAGE and resolved at the same molecular weight (approximately 70kDa) as the transiently transfected construct.

To determine the structure of the protein when it was in solution, 100μg of protein from both a stable and transient transfection was analysed by size-exclusion chromatography using a Superdex 200 column. As figure 3.6 shows, the protein produced transiently resolved as two peaks, corresponding to molecular masses of approximately 50kDa and over 900kDa. The large molecular mass is likely to be protein which has formed as multimers. All of the protein produced by stably transfected CHO-K1 cells appeared to also have formed multimers. Section 3.2.4 (figure 3.10), would suggest however that this does not affect the ability of it to bind to PS on apoptotic cells.

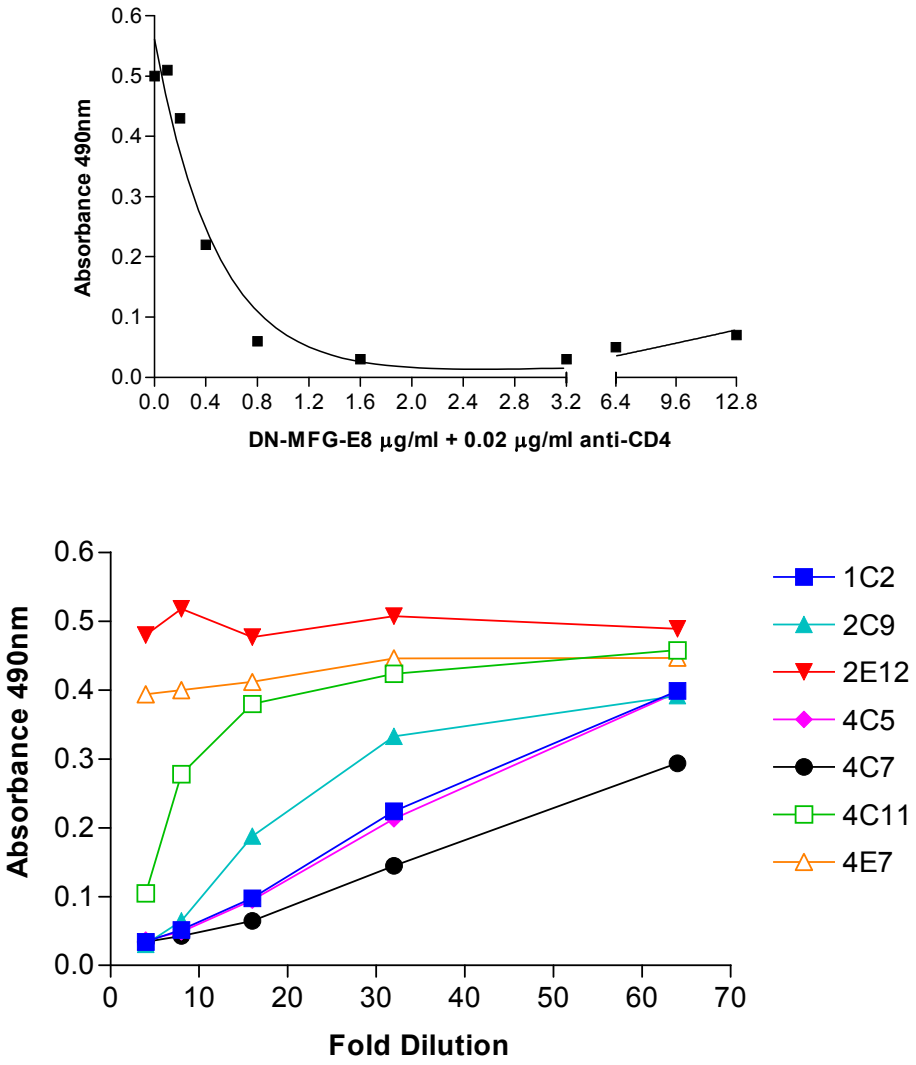


Figure 3.6 Inhibition ELISA to determine secretion levels of DN-MFG-E8 protein produced by stably transfected CHO-K1 cell clones. A: DN-MFG-E8 standard curve. **B:** Dilution curve; Supernatant was removed from each clone that had been stably transfected with pEE14-CD4-DN-MFG-E8 and then had successfully grown in selection medium (+ 25µM MSX). Graph B alone determines the cell lines which are expressing the DN-MFG-E8 protein, whilst graph A can be used to then calculate the final concentration of protein in the supernatant.

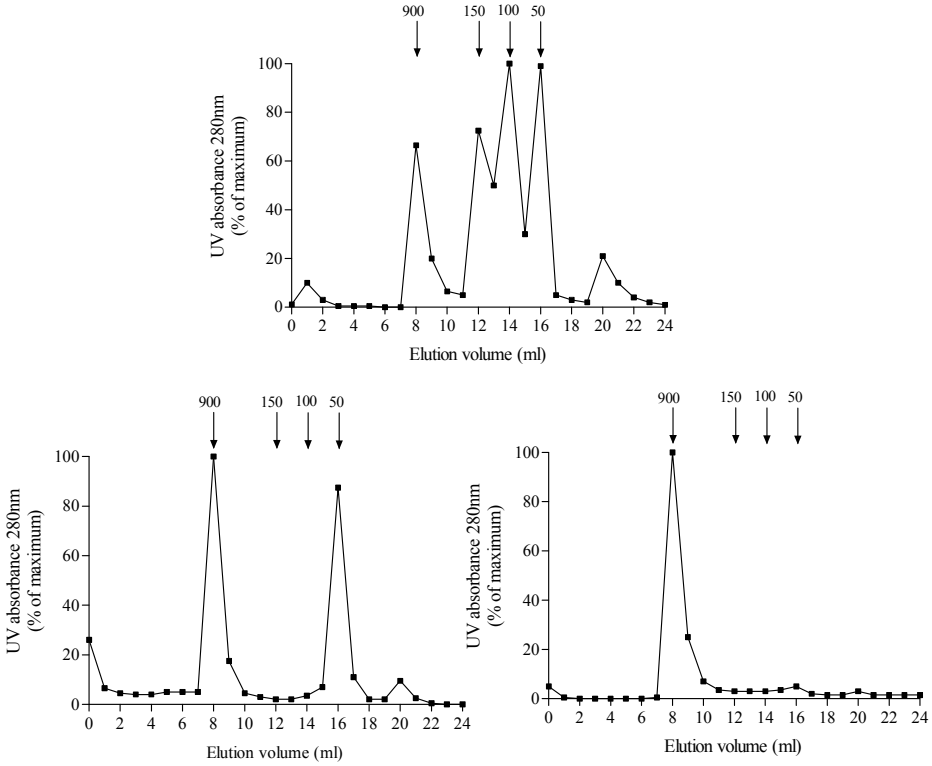


Figure 3.7 High Performance Liquid Chromatography (HPLC) to determine the molecular weight of recombinant DN-MFG-E8. **A:** Molecular weight markers at 1mg/ml (total of 133µg) were loaded onto a Superdex column and eluted in a total of 24ml. 900 = 900 kDa Human IgM, 150 = 150 kDa Human IgG, 100 = 100 kDa OKT3 $F(ab)_2$, 50 = 50 kDa Human Fc. **B:** 100µg DN-MFG-E8 protein from 293 F transiently transfected cells was loaded on a Superdex column and eluted in 24 ml. **C:** 100µg DN-MFG-E8 protein from CHO-K1 stable transfected cells was loaded onto a Superdex column and eluted in 24 ml.

3.2.3 Determining an effective method for the induction of apoptosis.

Following isolation and purification of recombinant DN-MFG-E8 protein, it was necessary to first confirm that it was fully functional. Initially this was determined by ensuring that the protein bound preferentially to apoptotic cells.

To determine the most effective method of inducing apoptosis in lymphoma cells, a number of cell lines (A20, J558L, πBCL.1, and EG.7) and also primary cells (thymocytes), were exposed to either 25 Gy caesium γ-radiation or 50 J/m^2 UVC irradiation. The percentage of cells that had become apoptotic and had consequently translocated PS to their surface was determined by treatment with 10 μg/ml fluorescently labelled annexin V and analysed using flow cytometry. In all cell lines, apoptosis could be induced using both stimuli. Overall however, the percentage of cells from tumour cell lines that had become apoptotic 24 h post treatment was slightly increased when the cells had been treated with UVC radiation (Figure 3.8). This method of inducing apoptosis was therefore used in subsequent assays. Thymocytes were however significantly more sensitive to gamma radiation when compared to UV radiation 24 h post treatment (Figure 3.8). Gamma radiation was therefore used to render thymocytes apoptotic in any subsequent assays.

A time course following treatment with both UV and gamma radiation was also carried out to determine the time required for cells to become apoptotic. After 2, 4 and 8 hours there was no significant change in the percentage of cells that had become apoptotic. 48 h post treatment, the cells were no longer cellular, so could not be used in phagocytosis assays. 24 h was therefore considered to be the optimal time to incubate all cells post treatment with radiation.

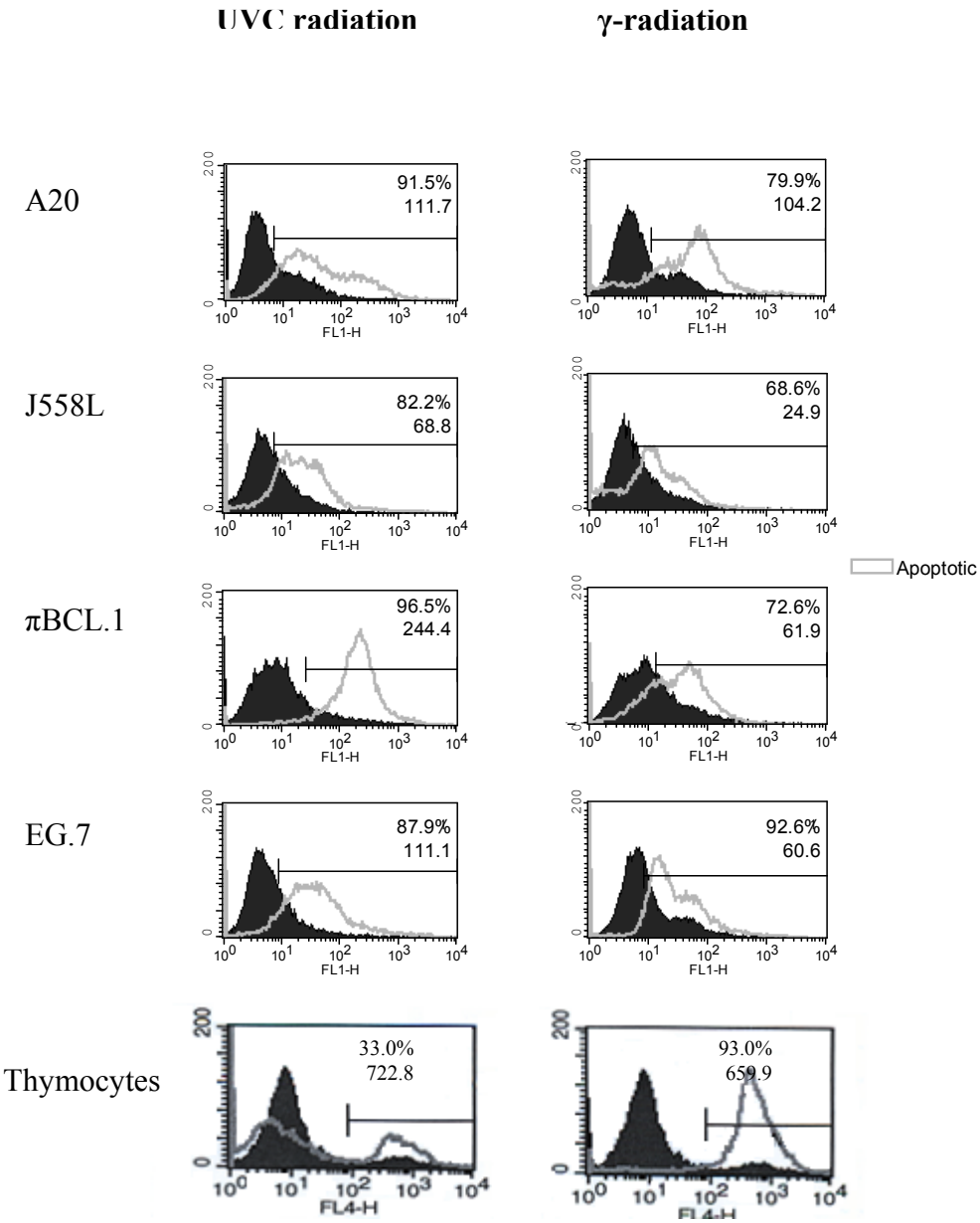


Figure 3.8 FACs analysis comparing the effect of UVC or γ-irradiation to induce apoptosis in a range of tumour cell lines and thymocytes. All cell lines were analysed 24 h post treatment with either 50 J/m^2 UVC radiation or 25 Gy caesium radiation. The percentage of cells apoptotic cells was measured by the level of Annexin V-FITC binding (FL-1). Thymocytes were prepared from 4 week old BALB/c mice and were analysed 24 h post treatment with either 50J/m^2 UVC radiation or 25 Gy caesium radiation. The percentage of apoptotic cells was measured by the level of Annexin V-Biotin binding, which was detected using APC labelled Streptavidin as the secondary antibody (FL-4). Solid histograms indicate untreated cells. Open histograms indicate irradiated cells. Numbers indicate % of Annexin V positive irradiated cells (top) and the mean fluorescence of the cells (bottom).

3.2.4 Recombinant DN-MFG-E8 binds specifically to apoptotic cells.

To ensure that DN-MFG-E8 protein was able to bind specifically to apoptotic cells, a number of tumour cell lines (πBCL.1, A20 and J558L) and primary cells were either left untreated or rendered apoptotic using 50J/m^2 UVC or 25Gy gamma irradiation, respectively. In all cases, DN-MFG-E8 protein was able to bind specifically to apoptotic cells to levels comparable to that seen with annexin V (figure 3.9). All cells bound both DN-MFG-E8 protein and annexin V to different degrees, suggesting that the level of PS exposure either varies between cell types or that different cells expose PS on their surface at different time points after exposure to apoptotic stimuli. Both transiently and stably produced proteins bound to apoptotic cells to the same degree (figure 3.10).

3.2.5 The binding of DN-MFG-E8 to PS is not dependent on extra-cellular calcium.

Previous studies have shown that the ability of Annexin V protein to bind to PS on the surface of apoptotic cells is dependent on the presence of extra cellular calcium [176]. To determine whether the same is also true for DN-MFG-E8, the binding of recombinant DN-MFG-E8 to apoptotic Jurkat and EG.7 cells with or without buffer containing 2.5mM $CaCl_2$, was determined.

Jurkat cells were either left untreated or treated with 500ng/ml CH-11 (IgM anti Fas) and incubated over night at 37^0C rendering them 70% apoptotic (as determined by Annexin V-FITC staining). 10µg/ml DN-MFG-E8 was added per sample (5 x 10^5 cells) for 1 h at room temperature either in buffer containing calcium (0.01M Hepes, pH7.4; 0.14M NaCl; 2.5mM $CaCl_2$) or without calcium (0.01M Hepes, pH7.4; 0.14M NaCl). Binding of DN-MFG-E8 was then determined by staining with 5µg/ml rat anti-CD4 FITC (YTA.3.1.2). EG.7's were treated as described in section 3.2.4; however, the binding was also determined using buffer that did not have additional calcium. The results shown in figure 3.11 illustrate that additional extra-cellular is not required for binding of DN-MFG-E8 to apoptotic cells.

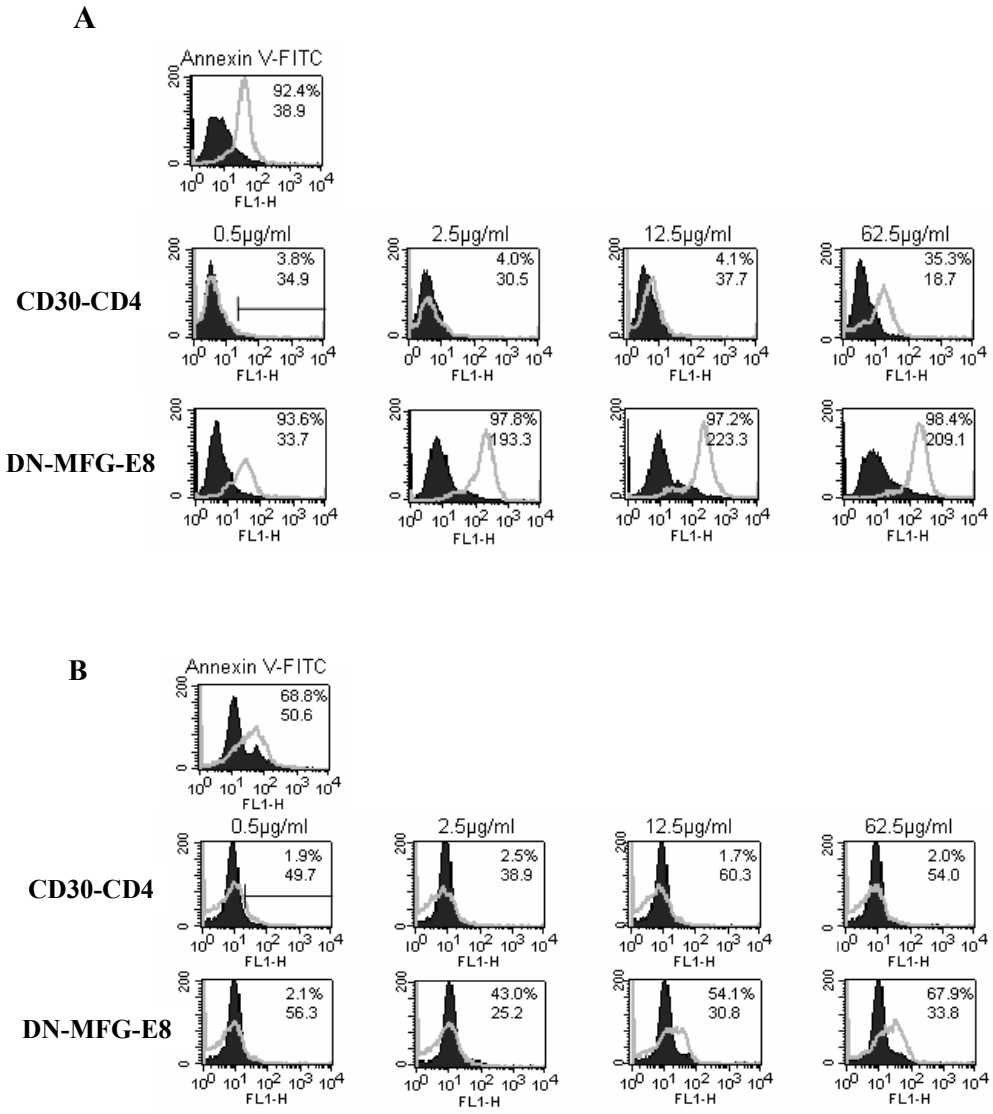
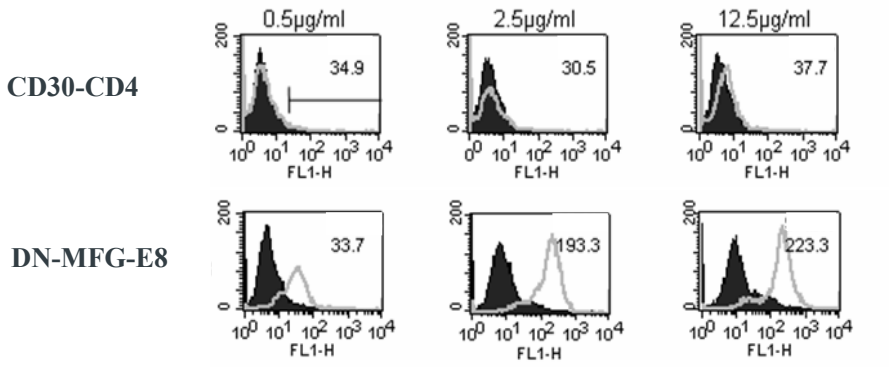


Figure 3.9 Recombinant DN-MFG-E8 protein binds to an array of apoptotic cells. A: πBCL.1 cells were either untreated (solid histogram) or exposed to 50J/m^2 UVC radiation (open histrgram) and incubated at 37^0C for 24 h. Cells were then washed with calcium buffer and 1 x 10^6 cells were used per sample. Positive control - 1µl Annexin V-FITC was added to 1 x 10^6 cells/100µl for 5 min. Negative control – CD30-CD4 protein was added to 1 x 10^6/100µl at concentrations ranging from 0.5µg/ml to 62.5µg/ml. Cells were incubated for 1 h at room temperature and were then treated with 2µg/ml anti-CD4 antibody (YTA 3.1.2) for 30 min at room temperature before being analysed by FACS. DN-MFG-E8 protein was added to 1 x 10^6 cells/100µl at concentrations ranging from 0.5µg/ml to 62.5µg/ml. Cells were incubated for 1 h at room temperature and were then treated with 2µg/ml anti-CD4 antibody (YTA 3.1.2) for 30 min at room temperature before being analysed by FACS. Top number on histogram indicates % of treated cells which bind to Annexin V, CD30-CD4 or DN-MFG-E8. Bottom number indicates the mean fluorescent intensity of binding. **B:** Assay to determine binding of DN-MFG-E8 to live versus apoptotic A20 cells. The assay was performed as described in figure 3.8A.

C



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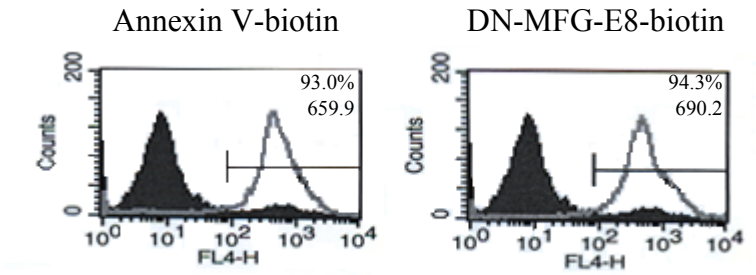


Figure 3.9 cont… C: Assay to determine binding of DN-MFG-E8 to live versus apoptotic J558L cells. The assay was performed as described in figure 3.8A. **D:** Thymocytes obtained from an 8 week old BALB/c mouse were either untreated (solid histogram) or treated with 25 Gy caesium radiation (open histogram) and incubated at 37^0C for 24 h. Cells were then washed with calcium buffer and 1 x 10^6 cells were used per sample. Cells with either treated with 10µg/ml Annexin V-biotin or 10µg/ml CD4-DN-MFG-E8-biotin for 30 min at room temperature. Cells were then washed and then treated with 10µg/ml APC labelled Streptavidin (FL-4). Top number on histogram indicates % of treated cells which bind to Annexin V or CD4-MFG-E8. Bottom number indicates the mean fluorescent intensity of binding.

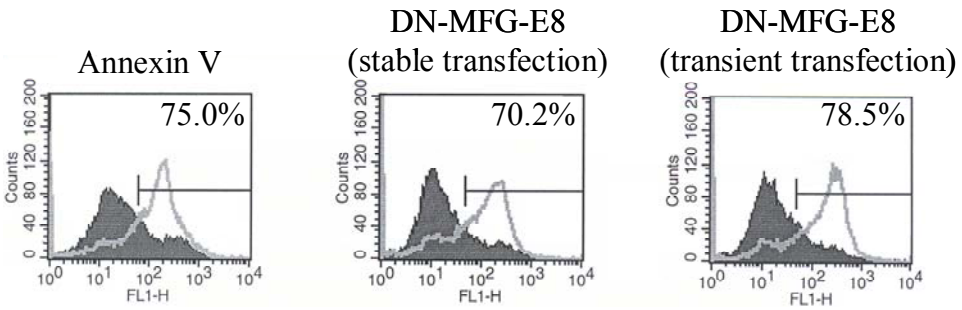


Figure 3.10 DN-MFG-E8 proteins produced either transiently or by stable cell transfection bind specifically to apoptotic cells.

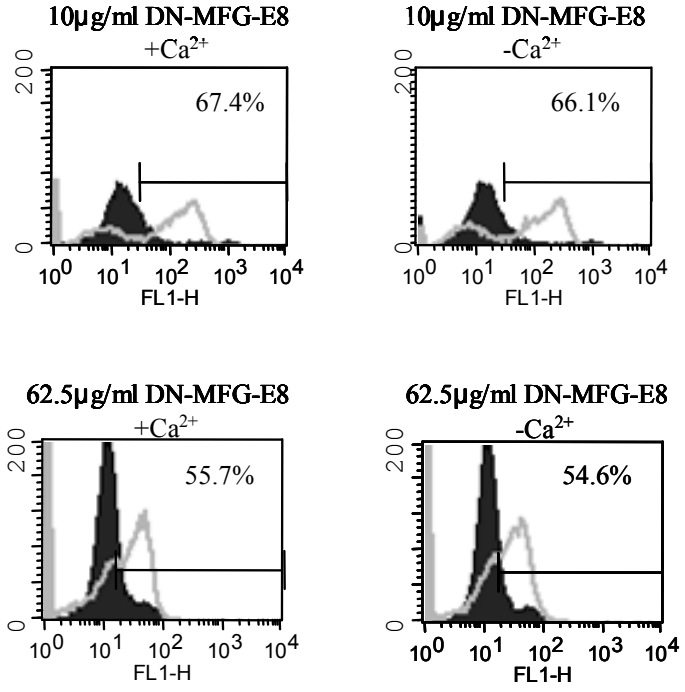


Figure 3.11 Additional extra-cellular calcium is not required for DN-MFG-E8 to bind to apoptotic cells *in vitro*. A: Comparing the ability of recombinant CD4-DN-MFG-E8 to bind to live (solid graph) versus apoptotic (open graph) Jurkat cells. **B:** EG.7's were used instead of Jurkat cells.

3.2.6 DN-MFG-E8 competes with recombinant Annexin V for binding to PS.

To examine whether the binding of DN-MFG-E8 to apoptotic cells was specific to phosphatidylserine, UVC induced apoptotic EG.7 cells were treated with various concentrations of DN-MFG-E8 protein either in the absence or presence of excess recombinant annexin V protein (50μg/ml). The ability of DN-MFG-E8 to bind to PS in the presence of Annexin V was then determined using YTA3.1.2-FITC anti-CD4 antibody. As figure 3.12 shows, the ability of DN-MFG-E8 to bind to phosphatidylserine on apoptotic cells was significantly reduced in the presence of excess Annexin V. These results demonstrate that recombinant DN-MFG-E8 protein binds specifically to PS.

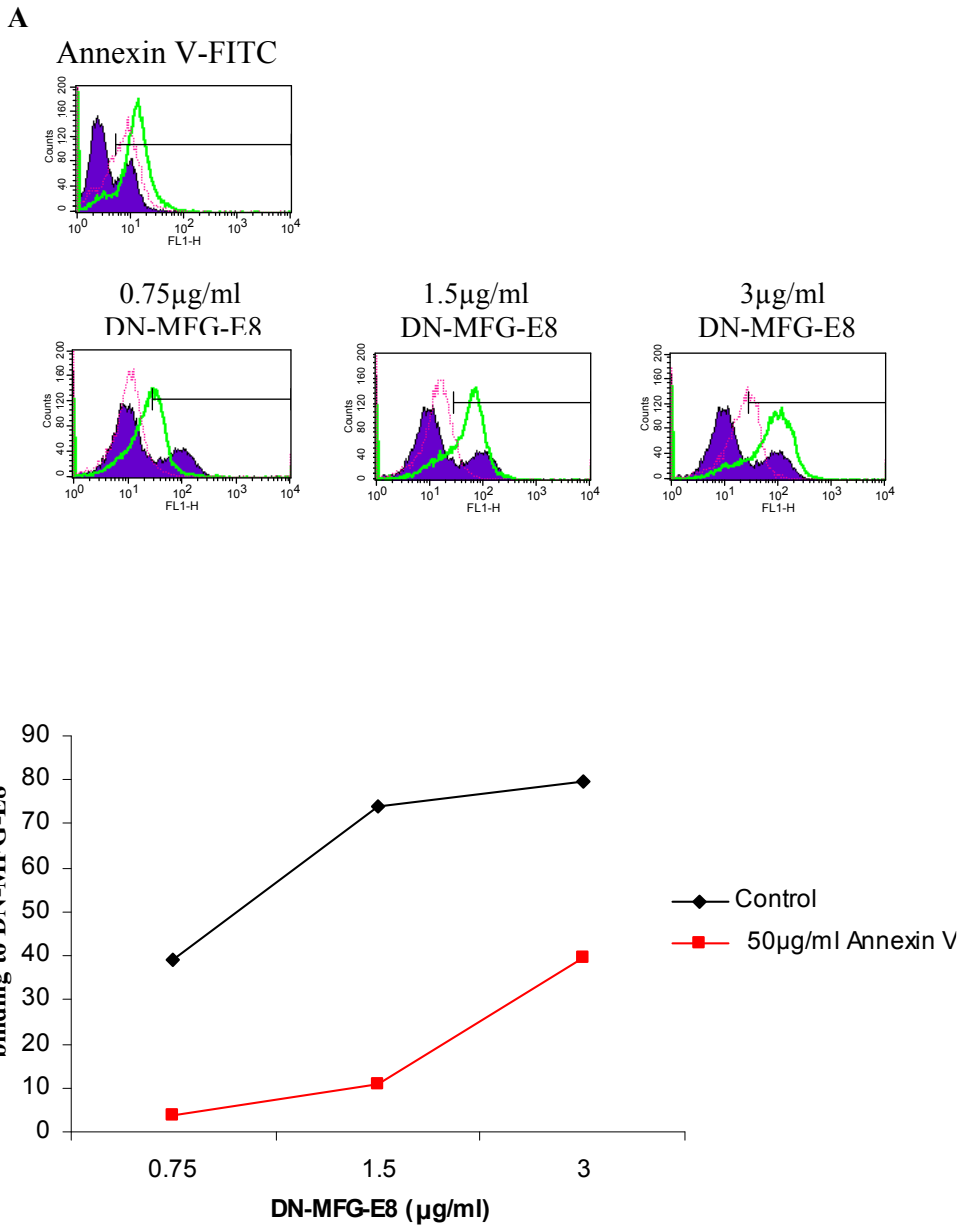


Figure 3.12 DN-MFG-E8 competes with recombinant Annexin V for binding to PS. 0.5 x 10^6 live or UVC induced apoptotic EG7's were treated with either Annexin V-FITC or a titration of recombinant CD4-DN-MFG-E8 protein (3µg/ml, 1.5µg/ml and 0.75µg/ml) with or without 50µg/ml recombinant Annexin V protein. Binding of CD4-DN-MFG-E8 to cells was determined using 2µg/ml YTA3.1.2-FITC anti-CD4 antibody. **A:** FACs analysis. Purple: live cells; Green: Apoptotic cells with a titration of CD4-DN-MFG-E8; Pink: Apoptotic cells with a titration of CD4-DN-MFG-E8 + 50µg/ml recombinant Annexin V. **B:** Graph representing data from FACs data.

3.3 DN-MFG-E8-Fc protein.

3.3.1 Cloning and expression of recombinant DN-MFG-E8-Fc protein.

In addition to inhibiting uptake of apoptotic cells by phagocytes though PS mediated mechanisms, the next aim was to determine whether this uptake could be re-targeted via Fcγ receptors, with the idea that this would promote access of cell-associated antigens into the cross-presentation pathway for effective cross priming of CD8+ T cells.

The DNA generated previously from the full length MFG-E8 L was used to PCR the PT,C1 and C2 domains and to include an *EcoR1* site at the N-terminus, a $(Gly)_4Ser$ flexible linker, and then a Bgl II site at the C-terminus. This was then fused via the $(Gly)_4Ser$ linker to a mouse IgG2a Fc region comprising of the CH2 and CH3 domains of the IgG heavy chain and the hinge region followed by its cloning into a PEE14 expression vector so that it could be used to generate stable cell lines. A schematic representation of the construct is shown in figure 3.13.

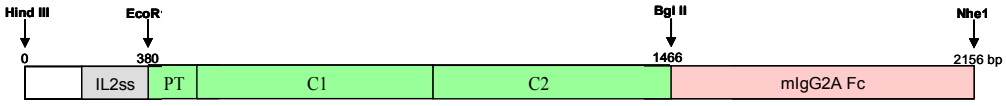


Figure 3.13 Schematic representation of the primary structure of recombinant DN-MFG-E8-Fc fusion protein. The PT, C1 and C2 domains were isolated from the TOPO Blunt II vector using *EcoR1* and Bgl II restriction enzymes and were then cloned into a *Pfu*SE-mFc2 (IL2ss) plasmid. The PT, C1, C2 and Fc region was then excised using Hind III and Nhe1 to include the IL2 signal sequence and was ligated into a pEE14 vector.

3.3.2 Generation of stable cell lines secreting DN-MFG-E8-Fc protein.

The pEE14-MFG-E8-Fc construct was used to stably transfect CHO-K1 cells following the protocol described in section 2.3.3.1. 10 days after transfection, clones which were resistant to treatment with 25μM MSX were expanded and the secretion of MFG-E8-Fc protein was determined by anti-mouse IgG sandwich ELISA (Figure 3.14). The highest secreting and

most viable clone was then expanded. The protein was purified by Protein A column chromatography as described in section 3.2.2. As figure 3.15 shows, the non-reduced protein resolved at greater than 175kDa and the reduced protein resolved at approximately 70kDa, suggesting that Fc region of the protein had formed a dimer. A schematic representation of the predicted protein structure is shown in figure 3.17.

3.3.3 Recombinant DN-MFG-E8-Fc protein binds specifically to apoptotic cells.

To determine the ability of the recombinant DN-MFG-E8-Fc protein to bind specifically to apoptotic cells, J558L plasmacytoma cells were rendered apoptotic by treatment with 50J/m^2 UVC radiation followed by incubation overnight in serum free medium. A titration of DN-MFG-E8-Fc protein was then added to either live or apoptotic J558L cells and its binding ability was determined by treating the cells with a fluorescently labelled (FITC) anti-IgG antibody. DN-MFG-E8-Fc protein was able to bind specifically to apoptotic cells, which was concentration dependent, as shown in figure 3.16A. In addition to this, the ability of the protein to bind specifically to apoptotic thymocytes when compared to annexin V and recombinant DN-MFG-E8 protein was also examined. As figure 3.16B shows, DN-MFG-E8-Fc protein was able to bind specifically to apoptotic thymocytes and the binding observed was comparable to that with annexin V and recombinant DN-MFG-E8 protein.

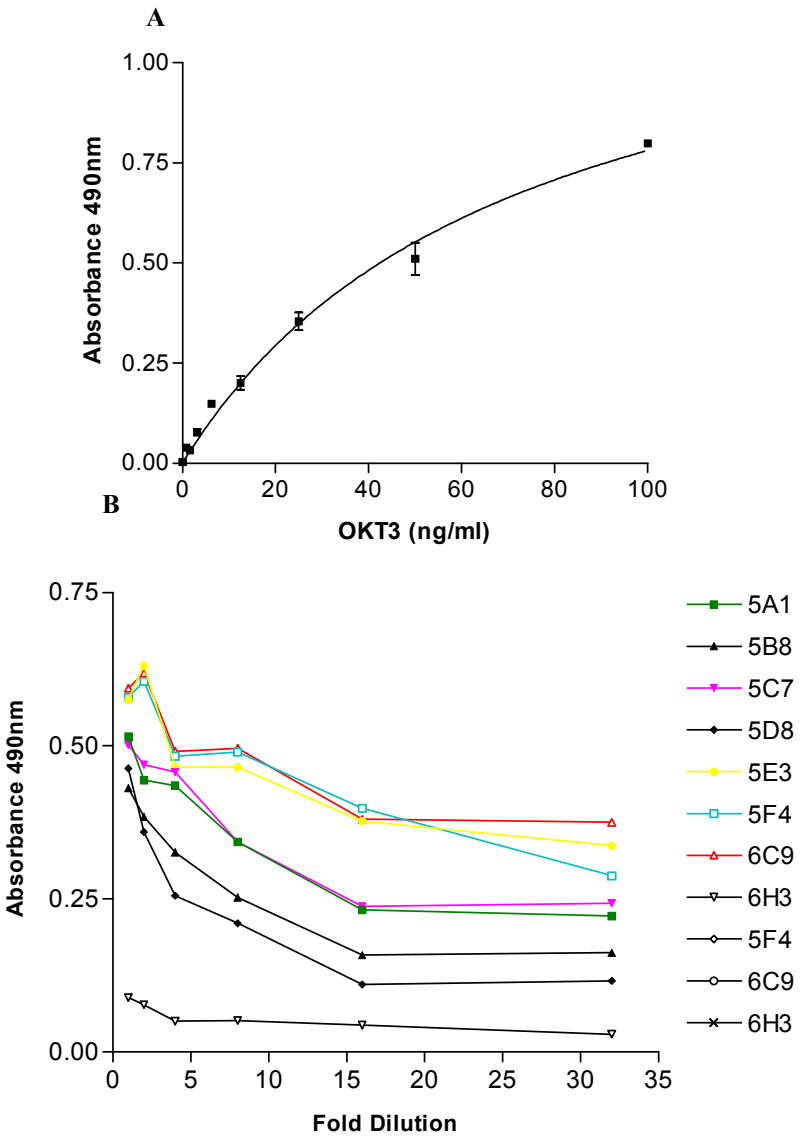


Figure 3.14 Sandwich ELISA to determine secretion levels of DN-MFG-E8-Fc protein produced by stably transfected CHO-K1 cell clones. A: Standard curve using mouse anti-human CD3 mAb; IgG2a. (OKT3) **B:** Dilution curve; Supernatant was removed from each clone that had been stably transfected with pEE14-DN-MFG-E8-Fc and then had successfully grown in selection medium (+ 25µM MSX). Graph B alone determines the cell lines which are expressing the protein, whilst graph A can be used to then calculate the approximate final concentration of protein in the supernatant. Each line represents a separate clone.

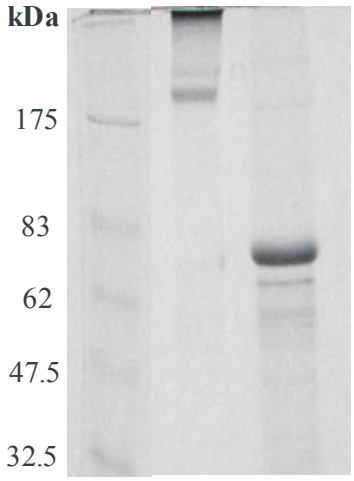


Figure 3.15 Non-reduced and reduced purified DN-MFG-E8-Fc protein. 10% SDS-PAGE gel. Lane 1, 10μg purified DN-MFG-E8-Fc protein. Lane 2, 10μg purified DN-MFG-E8-Fc protein reduced with 40μM DTT.

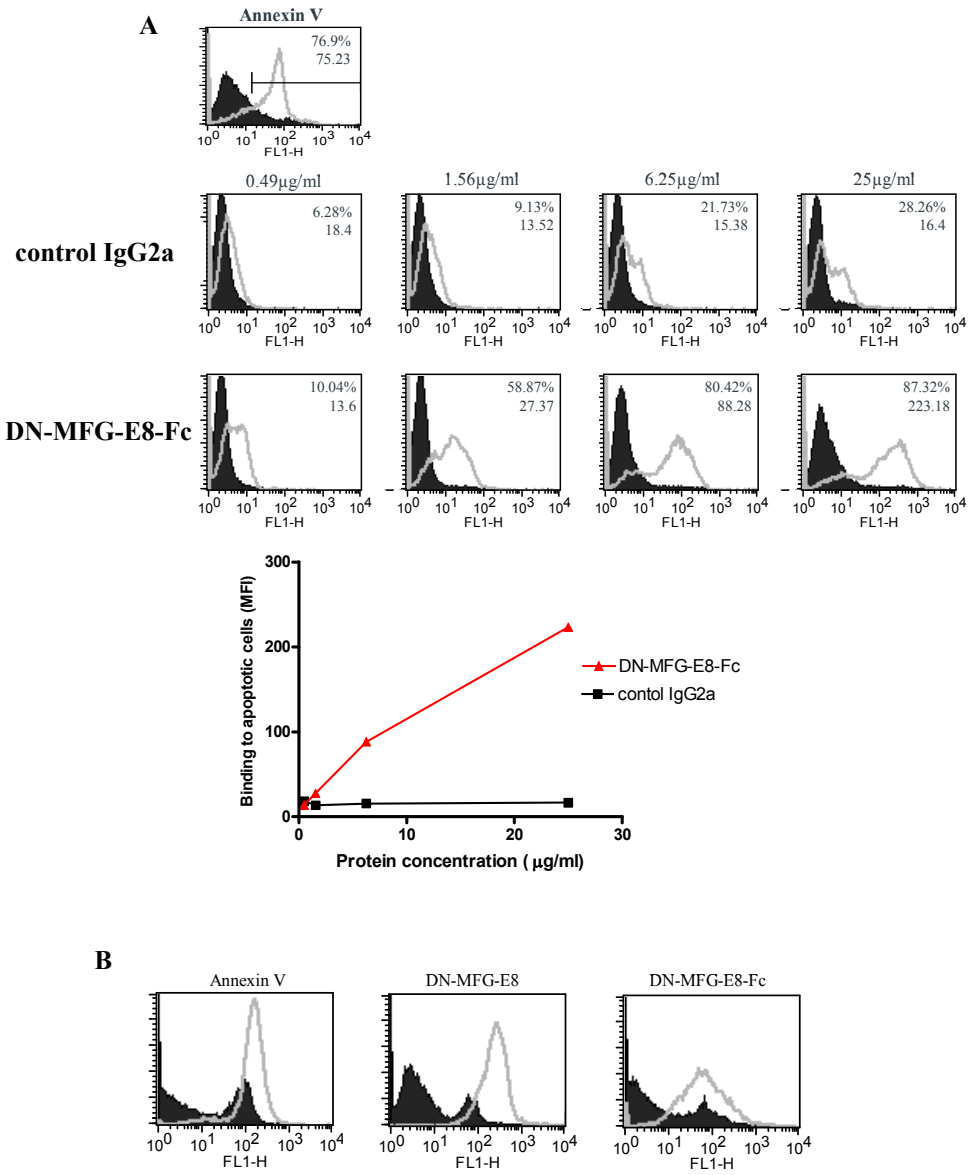


Figure 3.16 DN-MFG-E8-Fc protein binds specifically to apoptotic cells. A: J558L plasmacytoma cells were either live or rendered apoptotic with 50J/m^2 UVC radiation. 1 x 10^6 cells were then treated with a titration of either control protein (anti-CD20 IgG2a) or recombinant DN-MFG-E8-Fc protein. After washing non-bound protein from the cells, bound protein was detected by staining the cells with a fluorescently labelled (FL-1) anti-IgG antibody. Binding to live (solid histogram) or apoptotic (open histogram) was then determined by flow cytometry. Graph below represents data from histograms. **B:** Thymocytes were either untreated or rendered apoptotic by treatment with 25 Gy γ-radiation. 1 x 10^6 cells were then treated with either FITC-labelled annexin V, 60 µg/ml DN-MFG-E8 protein or 60 µg/ml DN-MFG-E8-Fc protein. Binding of the DN-MFG-E8 protein was determined by staining the cells with FITC-labelled anti-CD4 antibody. Binding of the DN-MFG-E8-Fc protein was determined by staining the cells with FITC-labelled anti-IgG antibody. Top number indicated % of cells which have bound protein. Bottom number indicates mean fluorescence intensity (MFI) of open histogram peak.

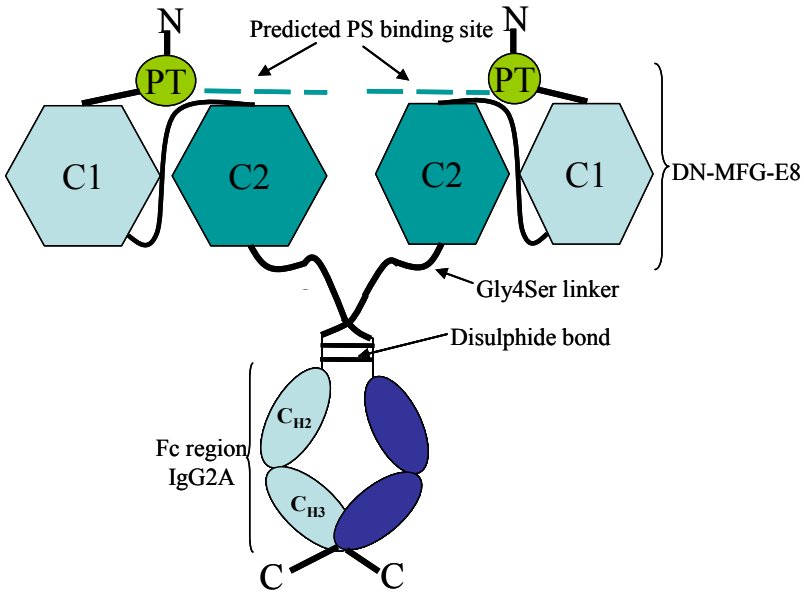


Figure 3.17 Schematic representation of the predicted protein structure of DN-MFG-E8-Fc protein. The PT,C1 and C2 domains of murine MFG-E8 are encoded upstream of the CH2 and CH3 domains of mouse IgG2a. The IgG domains form a dimer in solution (as determined in figure 3.15).

3.3.4 Endotoxin levels in DN-MFG-E8 and DN-MFG-E8-Fc preparations.

To ensure the immunological effect of both the DN-MFG-E8 and DN-MFG-E8-Fc proteins was not caused as a result of the presence of LPS, both proteins were passed through an Endotrap Red endotoxin removal system (Lonza). After which 25 µl of each sample was tested using an endosafe-PTS gram detection assay (Charles River Laboratories). Both protein endotoxin levels before purification were 60 Eu/ml, however after purification they both measured <5.00 Eu/ml, which is accepted as endotoxin low.

3.4 Discussion

A fundamental characteristic of cells which become apoptotic is their ability to expose phosphatidylserine on their cell surface for recognition by a plethora of phagocyte receptors [177]. This study aimed to exploit this feature by using it as a means to specifically target apoptotic tumour cells and re-direct their uptake via a PS-independent pathway; so as to enhance their immunogenicity in a manner that would not require a specific target for each tumour.

The clearance of apoptotic cells via PS has been studied extensively and it is widely accepted that this pathway is associated with a resulting immune tolerance against cell-associated antigens [27, 178]. In addition to examining the immunological effect of blocking this pathway, it was also of interest to determine whether an alternative phagocytic pathway would be more suitable in generating an immune response against tumour cells which have undergone apoptosis.

The binding of the Fc regions of IgG antibodies to their receptors on antigen presenting cells has long been known to augment immune responses including phagocytosis, NK cell antibody-dependent cellular cytotoxicity, activation of neutrophils, and the inhibition of B cell activation by IgG immune complexes (as reviewed in [49]). The targeting of Fcγ receptors on dendritic cells is therefore a plausible target for therapies due to their ability to regulate whether an immunogenic or tolerogenic response is initiated after antigen presentation.

In mice, there are 4 subclasses of IgG (IgG1, IgG2a, IgG2b and IgG3) which bind with differential affinities for both activating and inhibitory Fcγ receptors [179]. Although FcγRIA is the highest affinity receptor for IgG molecules in both mice and humans, it does not make a suitable target for therapy due to the fact that it is always saturated with its ligand *in vivo* (the cell does however become activated through this receptor until the antibody is cross-linked with antigen). All other FcγRs have 100-1000 fold

lower affinity for their antibody which prevents monomeric antibodies in the serum from binding. IgG2a and IgG2b are the most proinflammatory IgG molecules, showing greatest activity in the mouse than either IgG1 or IgG3 [47]. The preferential binding of IgG2a to FcγRIV [180] has been shown to be an effective target in mediating antibody-dependent cellular cytotoxicity (ADCC) in a metastatic melanoma model [47]. With this in mind, it was decided that IgG2a was the most suitable IgG subclass for binding to Fcγ receptors on dendritic cells, owing to its preferential binding to FcγRIV and its ability to generate high immunostimulatory response.

In addition to generating a dominant-negative MFG-E8 protein for blockade of apoptotic cell clearance via PS, the domains of both the DN-MFG-E8 and the Fc region of IgG2a were linked together using an amino acid linker, Gly_4Ser. This is a widely used linker for the production of bi-specific antibodies and allows the movement of both arms of the secreted protein to move and retain their separate functional integrity [181].

As described in this chapter, both DN-MFG-E8 and DN-MFG-E8-Fc DNA constructs were successfully used to transfect cell lines so that the protein was expressed and secreted at detectable levels by SDS-PAGE (figure 3.3 and 3.14). Size exclusion chromatography used to analyse both proteins showed that both DN-MFG-E8 and DN-MFG-E8-Fc had a higher molecular mass. This was more apparent in the proteins which were produced by CHO-K1 cells. This high molecular fraction may represent the formation of protein multimers, the nature of which was not fully addressed.

Other CD4-fusion proteins made in house did not form aggregates under the same conditions, which suggested that it was the structure of the MFG-E8 region which was causing this. This multimer formation did not however appear to affect the binding sites of the protein to PS on apoptotic cells (figures 3.8 and 3.15).

Additionally, the binding of the PT, C1 and C2 domains to apoptotic cells was specific to PS, as the binding could be inhibited in the presence of recombinant annexin V protein (Figure 3.11).

The next stage in this study was to then determine what effect both recombinant MFG-E8 proteins would have on apoptotic cell clearance, both *in vitro* and *in vivo*.

Chapter 4 – Results

Manipulating the phagocytosis of apoptotic cells by professional phagocytes

4.1 Introduction

One of the fundamental problems in achieving immunity against tumours is due, in part, to the fact that most tumour cells die when treated with radiotherapy or chemotherapeutic drugs by apoptosis. It is widely accepted that the clearance of apoptotic cells promotes tolerance against cell-associated antigens, including those associated with tumours, rather than immunity [44]. When cells become apoptotic, they expose a number of receptors on their surface, commonly referred to as "eat me" signals, which are then recognised by professional phagocytes. The classical route for apoptotic cell uptake is via phosphatidylserine exposed on the cell surface at early stages of apoptosis. PS is recognised by a myriad of receptors and opsonising bridging molecules to ensure that apoptotic cells are cleared rapidly and efficiently so as to prevent their break down by secondary necrosis [182].

Inhibiting apoptotic cell clearance *in vivo* can lead to the development of autoimmune diseases such as system lupus erythematosus (SLE) [126, 183]. Necrotic cells promote the maturation of dendritic cells and in turn convert the tolerogenic response associated with the presentation of self-antigens, to the cross-priming pathway which promotes expansion of naïve self-antigen specific CD8+ T cells [184, 185]. If an immune response to self-antigens can be established in this manner, it is conceivable that an immune response to tumour-associated antigens could be generated through the same means by manipulating the clearance pathway of apoptotic tumour cells.

The ability to prime tumour-specific CTLs *in vivo* by *ex vivo* matured peptide-pulsed dendritic cells has already been shown to generate immunity against tumours. This effect was however short lived and did not permit long lasting protection against subsequent tumour challenge [42]. As discussed in chapter 1 (section 1.6.3) the tumour microenvironment is highly immunosuppressive. This prevents the maturation of DCs and thus elicits a tolerogenic effect upon tumour-associated antigen uptake and presentation to tumour-specific CTLs [186-188].

Before the generation of a CD8+ T cell response against a tumour antigen can be examined, the first step towards this goal was to control the manner in which apoptotic tumour cells are cleared by phagocytes. This chapter describes the way in which this was achieved. Firstly, the effect of DN-MFG-E8 on apoptotic cell uptake by both macrophages and dendritic cells was studied. Additionally, re-directing apoptotic tumour cell clearance through Fcγ receptors using the DN-MFG-E8-Fc protein was also examined.

4.2 Recombinant MFG-E8 proteins can manipulate the clearance of apoptotic cells by macrophages.

4.2.1 DN-MFG-E8 protein inhibits phagocytosis of apoptotic cells by macrophages

4.2.1.1 DN-MFG-E8 binds to PS on apoptotic EG.7 cells and inhibits their clearance by resident peritoneal macrophages.

In 2002, Nagata and colleagues [98] showed that MFG-E8 carrying a point mutation in the RGD motif, required for binding to integrins, behaved as a dominant-negative protein and inhibited the phagocytosis of apoptotic cells by peritoneal macrophages *in vitro* and *in vivo*. The initial step therefore, was to first determine whether this was also true for the recombinant DN-MFG-E8 protein generated in this study. In the first instance, the ability of DN-MFG-E8 to block uptake of UVC-induced apoptotic EG.7 cells by resident peritoneal macrophages *in vitro* was determined. CFSE-labelled apoptotic cells which had either been treated with a titration of recombinant DN-MFG-E8 protein or left untreated, were added to resident peritoneal macrophages at a ratio of 1 macrophage to 4 apoptotic EG.7 cells. Using light microscopy it was possible to observe the uptake of apoptotic cells into the macrophage cytoplasm after 1 h 15 min at 37^0C. The level of phagocytosis was then determined using FACs analysis by staining macrophages with fluorescently labelled (APC) anti-CD11b antibody and determining the percentage of cells that were positive for both CFSE and APC fluorescence.

DN-MFG-E8 protein inhibited PS-mediated phagocytosis of apoptotic cells by resident peritoneal macrophages in a dose-dependent manner as seen in figure 4.1. This resulted in up to 40% inhibition of apoptotic cell clearance by resident peritoneal macrophages when compared to untreated apoptotic cells.

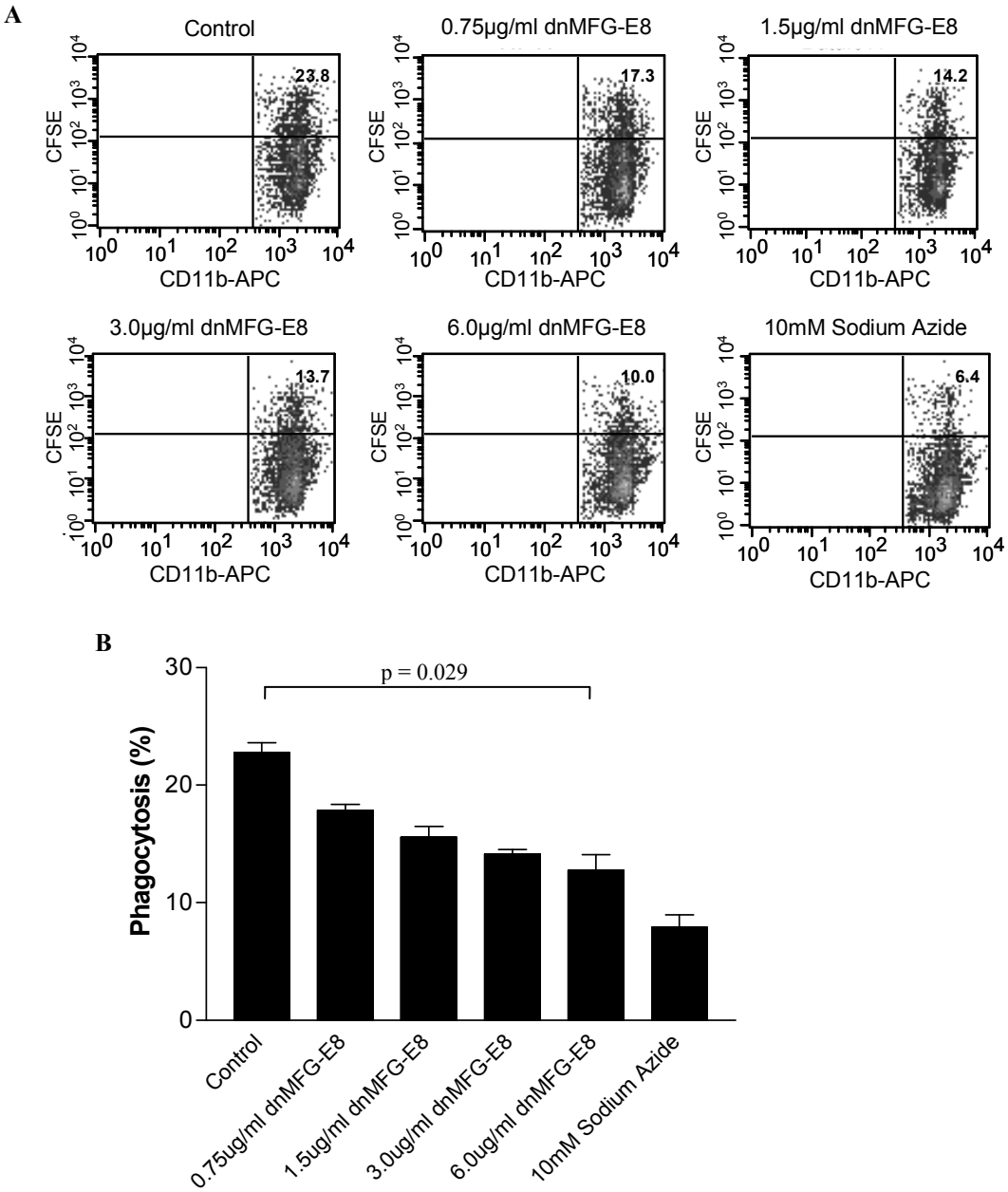


Figure 4.1 Recombinant DN-MFG-E8 inhibits phagocytosis of apoptotic cells by macrophages *in vitro* in a dose-dependent manner. A: FACs analysis. X-axis indicates CD11b +ve macrophages. Y-axis indicated CFSE labelled thymocytes. Double positive cells indicate that macrophages have phagocytosed apoptotic thymocytes. A total of 10,000 events were collected per sample. B: Graph representing data from FACs analysis, showing the percentage of phagocytosis seen either from a positive control, negative control (10mM Sodium Azide), or with a titration of DN-MFG-E8 protein (0.75 – 6µg/ml). Errors bars indicate the results from 3 separate experiments.

4.2.1.2 Inhibition of apoptotic cell clearance by macrophages in the presence of DN-MFG-E8 protein is confirmed using confocal microscopy.

In addition to determining the ability of recombinant DN-MFG-E8 protein to inhibit the percentage of PS mediated apoptotic cell phagocytosis by peritoneal macrophages, the effect of the recombinant protein on the phagocytic index (where the phagocytic index refers to the average number of apoptotic cells phagocytosed per macrophage) was also determined *in vitro*.

Although in the previous phagocytosis assay EG.7 cells were used as a source of apoptotic cell, for this assay cells of smaller size were used so that the number of cells internalised per macrophage could be calculated accurately. For this purpose, thymocytes were used as a source of apoptotic cell. CFSE-labelled apoptotic thymocytes were treated with or without a titration of recombinant DN-MFG-E8 protein and were added to resident peritoneal macrophages, prepared as described in section 2.1.1; which instead of being allowed to adhere to the bottom of a 48 well plate, were placed on Poly-L-Lysine (PLL) cover slips. After 75 min, non-phagocytosed apoptotic thymocytes were washed off and the peritoneal macrophage membrane was stained with 10 μg/ml biotinylated F4/80 antibody followed by a fluorescently labelled Streptavidin Alexa 546. The number of apoptotic thymocytes which had been phagocytosed was then determined using Confocal laser scanning microscopy (CLSM) as described in section 2.5.2.

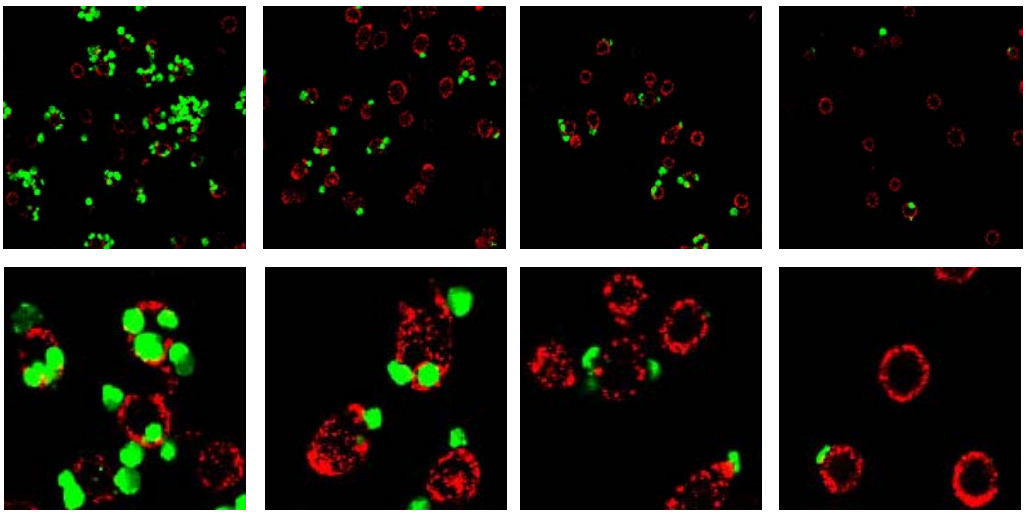
Consistent with the previous experiment, the blocking of PS on the apoptotic cell surface by DN-MFG-E8 protein was able to cause a 53% reduction in phagocytosis (figure 4.2a). The effect of DN-MFG-E8 inhibition was again proven to be dose dependent (figure 4.2b). Additionally, using confocal imaging, the number of thymocytes internalised per macrophage was also calculated. The phagocytic index was significantly reduced in the presence of the DN-MFG-E8 protein. At a

concentration of 6 μg/ml, recombinant DN-MFG-E8 was able to reduce the phagocytic index from 1.21 to 0.48 (figure 4.2c).

From these images alone it was not possible to determine whether the apoptotic cells were adhered to the macrophage cell surface or had been internalised. Successful phagocytosis was confirmed when images were taken from different focal points throughout the cells so that 1-dimensional images taken at different planes cell could be transformed into a 3-dimensional image (known as Z stacks).

Noticeably, the level of blocking in both *in vitro* assays could not be abrogated entirely by treatment with 10 mM Sodium Azide. Studies by Hanayama's group have also shown that phagocytosis cannot be completely inhibited and have shown that treatment with 2 μg/ml of D89E recombinant DN-MFG-E8 protein reduced phagocytosis of apoptotic thymocytes by NIH3T3 cells by up to 60% [98]. This suggests that either other competing PS receptors operate during PS-mediated clearance of apoptotic cells or that there is a partial redundancy in the clearance pathway. In any case, Nagata and colleagues have shown in their work that total phagocytic blockade is not necessary in order to observe an altered immune response.

A



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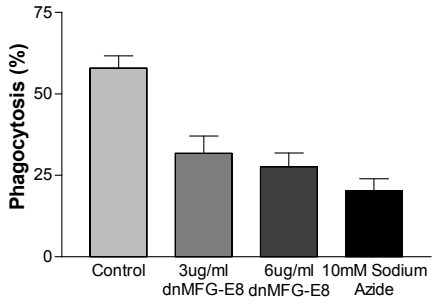

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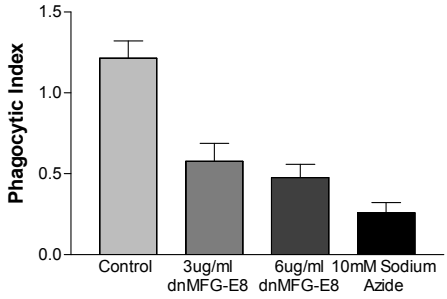

D

	Number of macrophages	Phagocytosis (%)	Number of thymocytes per macrophage					Phagocytic Index
			0	1	2	3	≥4	
Control	175	57.88	76	40	27	14	18	1.21
3µg/ml DN-MFG-E8	123	31.81	85	17	14	4	3	0.58
6µg/ml DN-MFG-E8	121	27.63	89	18	7	5	2	0.48
10mM Sodium Azide	109	20.33	86	17	5	1	0	0.26

Figure 4.2 Confocal microscopy showing that recombinant DN-MFG-E8 is able to inhibit the phagocytosis of apoptotic thymocytes *in vitro*. Resident peritoneal macrophages from 8 week old BALB/c mice were co-cultured with γ-irradiated apoptotic, CFSE labelled thymocytes treated with or without DN-MFG-E8 protein. The macrophage membranes were stained with biotinylated F4/80 antibody and then treated with fluorescent Streptavidin Alexa546. A: Pictures obtained from Confocal microscopy showing the number of apoptotic thymocytes (Green) taken up by each macrophage (Red) B: Graph showing the percentage of cells that had phagocytosed apoptotic cells after each treatment. C: Phagocytic index = the average number of engulfed apoptotic thymocytes per macrophage. D: Table showing data used to plot graphs.

4.2.1.3 Inhibition of phagocytosis with DN-MFG-E8 protein is also observed *in vivo*.

To examine the effect of recombinant DN-MFG-E8 protein on apoptotic cell clearance *in vivo*, 1 x 10^7 CFSE-labelled γ-irradiated apoptotic thymocytes that had been untreated or pre-treated with either a titration of recombinant DN-MFG-E8 protein (100 µg/ml or 25µg/ml) or 100µg/ml a control CD4-CD30 protein were injected into the peritoneum of 8 week old BALB/c mice. 30 min post injection, mice were sacrificed and the peritoneal macrophages were harvested. To determine the percentage of peritoneal macrophages that had phagocytosed the apoptotic thymocytes, macrophages were labelled with 10 µg/ml biotinylated F4/80, followed by 10 µg/ml APC-labelled Streptavidin. The cells were then examined using flow cytometry to determine the percentage of double-positive (CFSE + APC) cells and thus the level of phagocytosis.

As shown in figure 4.3, 100µg/ml of recombinant DN-MFG-E8 consistently reduced the level of apoptotic cell uptake by resident peritoneal macrophages by approximately 44% when compared to both the untreated control and the negative control. In contrast, at 25µg/ml the ability of DN-MFG-E8 to inhibit macrophage uptake *in vivo* was variable. Interestingly, after 60 min all apoptotic cells had been cleared from the peritoneal cavity regardless of their treatment with either the control or DN-MFG-E8 protein. This suggested that either the protein did not remain bound to PS or that there was direct competition for PS with other PS binding proteins within the peritoneum.

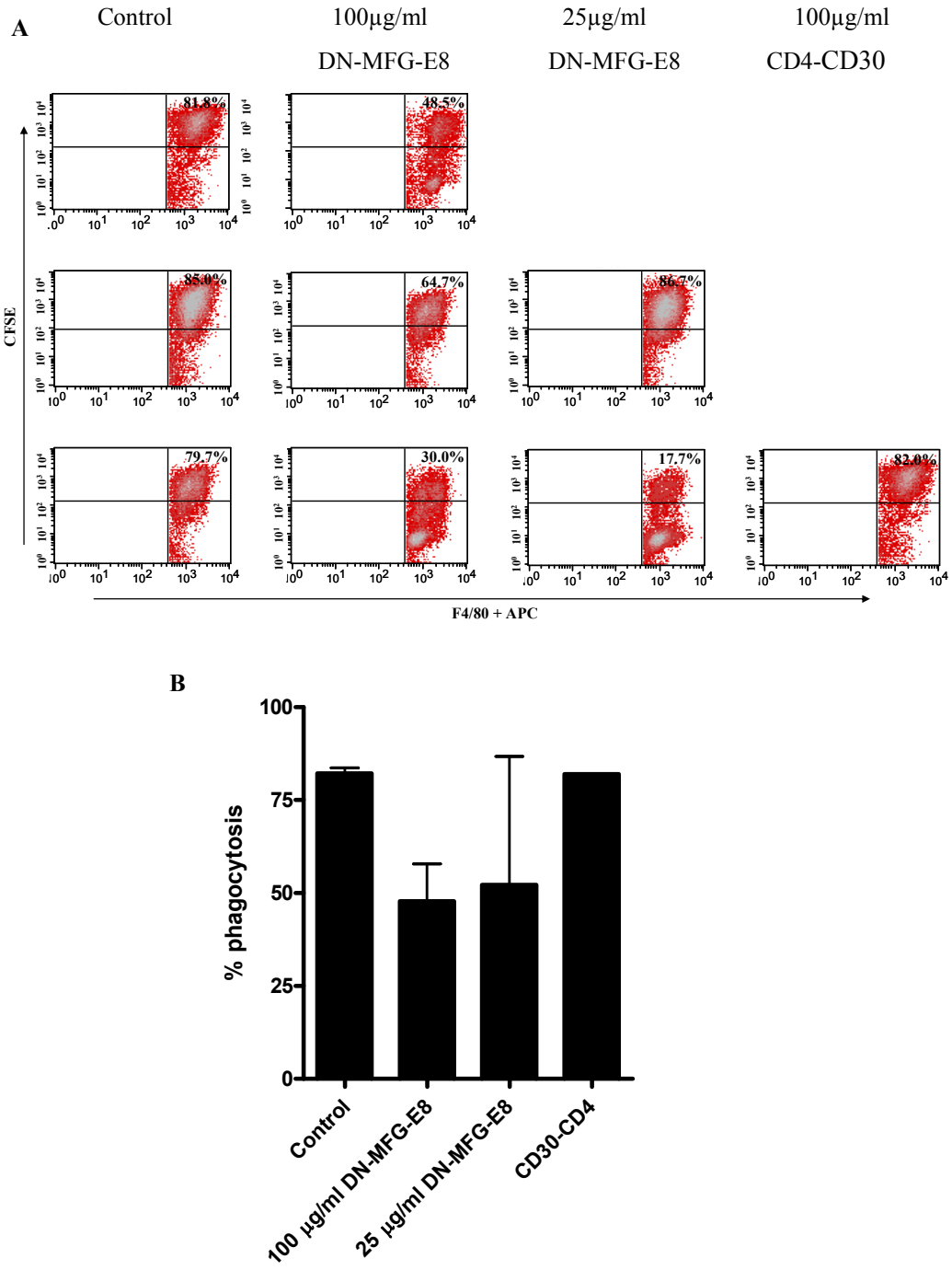

Figure 4.3 FACs analysis demonstrating the ability of DN-MFG-E8 protein to inhibit macrophage mediated apoptotic cell clearance *in vivo*. A: 3 mice were injected per group with either 1 x 10^7 CFSE (y-axis) labelled apoptotic thymocytes (control), 1 x 10^7 CFSE labelled apoptotic thymocytes treated with 100 µg/ml DN-MFG-E8 protein, 1 x 10^7 CFSE labelled apoptotic thymocytes treated with 25 µg/ml DN-MFG-E8 protein, or 1 x 10^7 CFSE labelled apoptotic thymocytes treated with 100µg/ml CD30-CD4. Macrophages were pooled together in each group and labelled with 10 µg/ml biotinylated F4/80 antibody; followed by 5 µg/ml Streptavidin labelled APC antibody (x-axis). Double positive (CFSE + APC) were considered as macrophages that had engulfed apoptotic thymocytes. Number indicates percentage of macrophages that had engulfed apoptotic thymocytes. Each graph is representative of one mouse **B:** Graph representing data from A. This experiment was performed once.

4.2.3 DN-MFG-E8-Fc protein can inhibit phagocytosis through PS receptors and facilitate clearance through Fcγ receptors.

4.2.3.1 DN-MFG-E8-Fc increases the uptake of apoptotic cells by macrophages *in vitro*.

DN-MFG-E8 protein successfully reduced PS-mediated clearance of apoptotic cells by macrophages both *in vitro* and *in vivo*. To examine what effect this protein would have once linked to an Fc domain, DN-MFG-E8-Fc protein was used to treat CFSE-labelled apoptotic thymocytes which were then cultured with resident peritoneal macrophages at a ratio of 4:1. After 75 min, the percentage of phagocytosis was determined by labelling the macrophages with fluorescently labelled (APC) anti-CD11b antibody. The percentage of phagocytosis was then determined by enumerating cells that displayed double staining.

In contrast with the effects of DN-MFG-E8, DN-MFG-E8-Fc protein enhanced the clearance of apoptotic cells by macrophages *in vitro* in a dose-dependent manner. (figure 4.4). To determine whether this enhanced phagocytosis was sustainable, uptake was determined over a time period of 3 hours. As shown in figure 4.5, the presence of DN-MFG-E8-Fc was able to enhance uptake of apoptotic cells consistently over a period of 3 hours when compared to non-treated apoptotic cells. The inhibitory effect of DN-MFG-E8 was again clearly apparent in this experiment and was comparable to the inhibition seen with sodium azide.

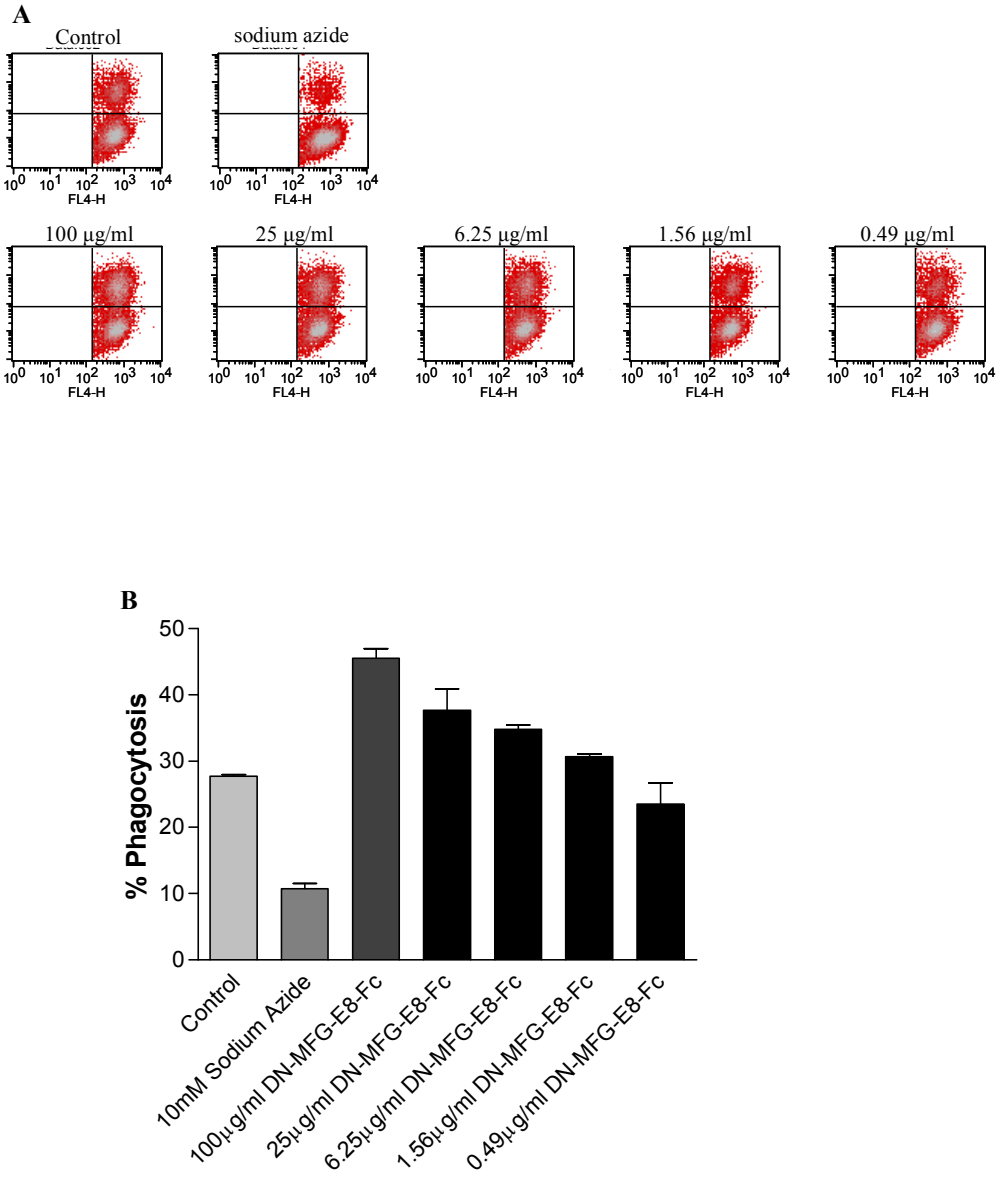

Figure 4.4 DN-MFG-E8-Fc increases phagocytosis by macrophages in a dose-dependent manner *in vitro*. **A:** FACs analysis showing the effect of DN-MFG-E8-Fc protein on resident peritoneal macrophage-mediated clearance of CFSE-labelled apoptotic thymocytes *in vitro*. Apoptotic thymocytes were either untreated or treated with a titration of recombinant DN-MFG-E8-Fc protein (100 µgml – 0.49 µg/ml) and then added to macrophages at a ratio of 4:1 for 75 min at 37^0C. Percentage of phagocytosis was determined by cells staining double positive for CFSE and APC-labelled CD11b. **B:** Graph representing data from FACs anaylsis. n = 3.

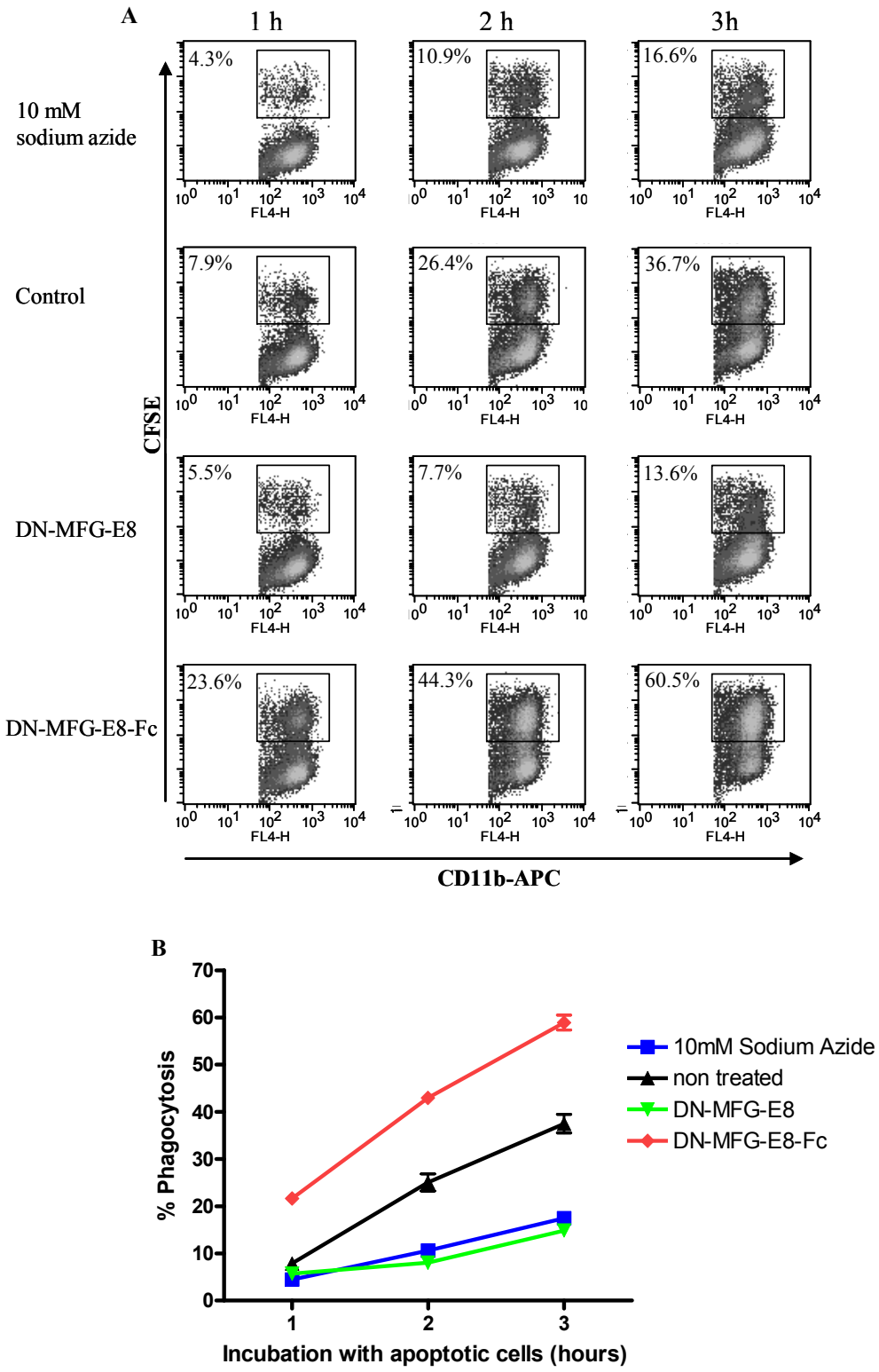

Figure 4.5 DN-MFG-E8-Fc enhances phagocytosis of apoptotic cells. **A:** CFSE-labelled apoptotic thymocytes were added to resident peritoneal macrophages at a ratio of 4:1 over a time course of 1 to 3 h. The percentage of phagocytosis was determined by labelling the macrophages with APC-conjugated CD11b antibody and then analysing the cells by flow cytometry. Number indicates percentage of double-positive cells shown in gated region of plot. **B:** Graph representing data from triplicate samples.

4.2.3.2 The effect of DN-MFG-E8-Fc protein on the uptake of apoptotic cells by peritoneal macrophages *in vivo*.

To investigate the functional role of DN-MFG-E8-Fc protein *in vivo*, CFSE-labelled apoptotic thymocytes were either left untreated or treated with 100 μg/ml DN-MFG-E8, DN-MFG-E8-Fc or annexin V protein prior to being injected into the peritoneal cavity of BALB/c mice. 20 min post injection, the resident peritoneal macrophages were harvested and the percentage of macrophages which had phagocytosed apoptotic cells was determined by staining the macrophages with a fluorescently labelled (APC) anti-CD11b antibody.

DN-MFG-8E-Fc was unable to significantly increase the percentage of apoptotic cell clearance by resident peritoneal macrophages *in vivo* (figure 4.6) when compared to the control where an increase of only 8% was observed. It is noteworthy, however, that uptake of apoptotic cells in the control group was particularly efficient reaching 70%, even in the relatively short time that the apoptotic cells were present in the peritoneal cavity (20 min). It is plausible that an upper limit of phagocytic clearance of apoptotic cells had already been reached in this model which can not be enhanced by re-directing uptake to Fcγ receptors.

Interestingly, whereas DN-MFG-E8 inhibited uptake by approximately 22.5% in this experiment, recombinant annexin V protein failed to block uptake of apoptotic cells by peritoneal macrophages. Overall, the evidence would suggest that the peritoneal cavity is a highly phagocytic environment that is capable of efficiently removing of apoptotic cells.

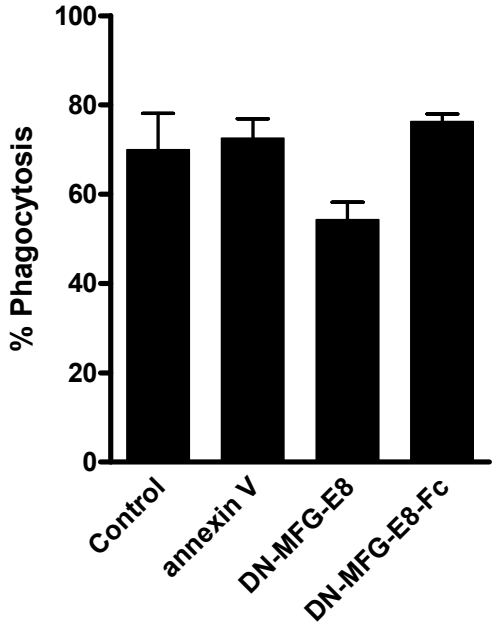

Figure 4.6 DN-MFG-E8-Fc protein is unable to increase phagocytosis of apoptotic cells *in vivo*. Graph representing data from FACs analysis where by CFSE-labelled apoptotic thymocytes were either untreated or treated with 100 μg/ml annexin V protein, DN-MFG-E8 protein, or DN-MFG-E8-Fc protein prior to being injected into the peritoneum of BALB/c mice. 20 min post injection, resident peritoneal macrophages were harvested and the percentage of phagocytosis was determined by labelling the macrophages with APC-labelled anti CD11b antibody and calculating the percentage of CFSE and APC double-positive cells. There were 3 mice per group.

4.3 Investigating the effect of recombinant DN-MFG-E8 proteins on apoptotic cell uptake by dendritic cells.

4.3.1 Blocking PS on apoptotic cells does not inhibit phagocytosis by bone marrow-derived dendritic cells.

Dendritic cells are considered the dominant cell type *in vivo* for cross-presentation of antigen owing to their unique ability to capture antigen and then migrate to secondary lymphoid organs, where they present the antigen to CD8+ T cells [29]. In the case of cell-associated antigen, the cross-presentation by dendritic cells of antigen derived from apoptotic cells typically renders antigen-specific T cells tolerant [44] (examined further in chapter 5).

Bone marrow from C57Bl/6 mice was grown in GM-CSF-supplemented culture medium for 6 days. Dendritic cells were then harvested and re-suspended into serum free X-vivo medium, supplemented with GM-CSF. This was to ensure that any manipulation of apoptotic cell clearance would not be as a consequence of other clearance mechanisms such as complement-mediated phagocytosis [87]. After the percentage of CD11c+ cells had been determined by labelling the cells with fluorescently labelled anti-CD11c antibody and analysed by flow cytometry, BMDCs were seeded at 2.5 x 10^5 CD11c+ cells / ml in a 48 well culture plate. CFSE-labelled thymocytes were then treated with or without 100 μg/ml DN-MFG-E8 or DN-MFG-E8-Fc proteins in 100 μl of serum free medium for 1 h at 37^0C prior to being cultured with the BMDCs. The final concentration of the recombinant proteins in the culture medium was 10 μg/ml. As a negative control 10 mM sodium azide was added to BMDCs 1 h prior to the addition of apoptotic thymocytes. BMDCs were harvested at different time points and the percentage of BMDCs which had phagocytosed apoptotic thymocytes was determined by flow cytometry.

In contrast to macrophages, the blockade of PS on apoptotic cells using DN-MFG-E8 protein was unable to inhibit their phagocytosis by CD11c+

BMDCs. As shown in figure 4.7, the percentage of phagocytosis observed was modestly increased when PS was blocked when compared to the control. This suggested that an alternative clearance pathway by an as yet unknown receptor on the dendritic cells is more efficient at mediating apoptotic cell clearance, or that PS-blockade forced the DC to use a different clearance pathway. Similar work using annexin V as a blockade for PS-mediated apoptotic cell clearance by splenic CD11c+ dendritic cells also showed enhanced that this enhanced phagocytosis.; the explanation of which was not confirmed [189].

4.3.2 DN-MFG-E8-Fc enhances phagocytosis of apoptotic cells by bone marrow-derived dendritic cells.

DN-MFG-E8-Fc protein efficiently binds to PS on apoptotic cells and is able to significantly increase the percentage of phagocytosis by macrophages *in vitro* in a dose-dependent manner (figure 4.5). This enhancement was also observed when apoptotic thymocytes were treated with DN-MFG-E8-Fc protein and added *in vitro* to bone marrow-derived dendritic cells from C57BL/6 mice. Phagocytosis was higher by 43% after 2 h when compared to control non-treated apoptotic thymocytes and also when apoptotic thymocytes were treated with an isotype control (IgG2a mouse anti-human CD20). After 18 h however, the percentage of double positive cells after treatment with DN-MFG-E8-Fc protein had slightly decreased, most likely as a result of apoptotic cell degradation within the phagosome.

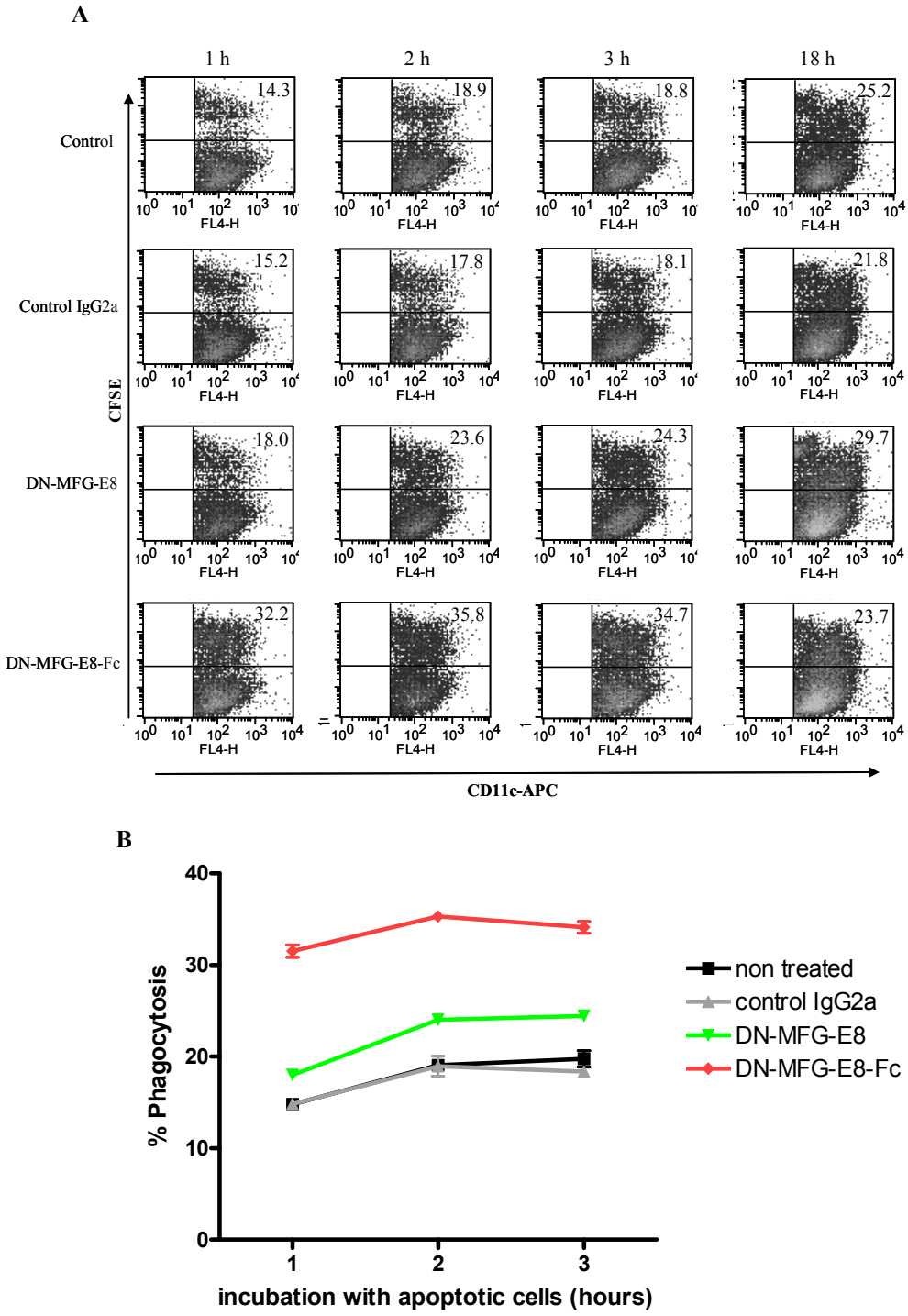

Figure 4.7 Recombinant MFG-E8 protein can be used to manipulate phagocytosis of apoptotic cells by BMDCs. **A:** CFSE-labelled apoptotic thymocytes were either untreated or treated with 100 µg/ml DN-MFG-E8 or DN-MFG-E8-Fc protein prior to being added to BMDCs over a time period of 1-3 h at 37^0C. BMDCs were harvested and the percentage of phagocytosis was determined by labelling the BMDCs with a fluorescently (APC) labelled anti-CD11c antibody. The cells were then analysed by flow cytometry. Number on density plots indicates percentage of double positive cells. **B:** Graph representing data from triplicate samples.

4.3.3 Inflammatory stimuli do not increase the capacity of phagocytosis by BMDCs

In an inflammatory environment, the phagocytic activity of macrophages is markedly increased to resolve the inflammation as quickly as possible [63]. To determine whether this also holds true for dendritic cells *in vitro*, the assay as shown in figure 4.7 was repeated, however the assay was performed in the presence or absence of 25 ng/ml LPS. As shown in figure 4.8, the presence of a toll-like receptor agonist did not increase the phagocytic capability of CD11c+ BMDCs *in vitro*. Again, DN-MFG-E8-Fc protein increased the phagocytosis of apoptotic thymocytes by BMDCs over a time period of 18 h when compared to control. Blockade of PS with DN-MFG-E8 protein again demonstrated that BMDCs are able to phagocytose through PS-independent mechanisms.

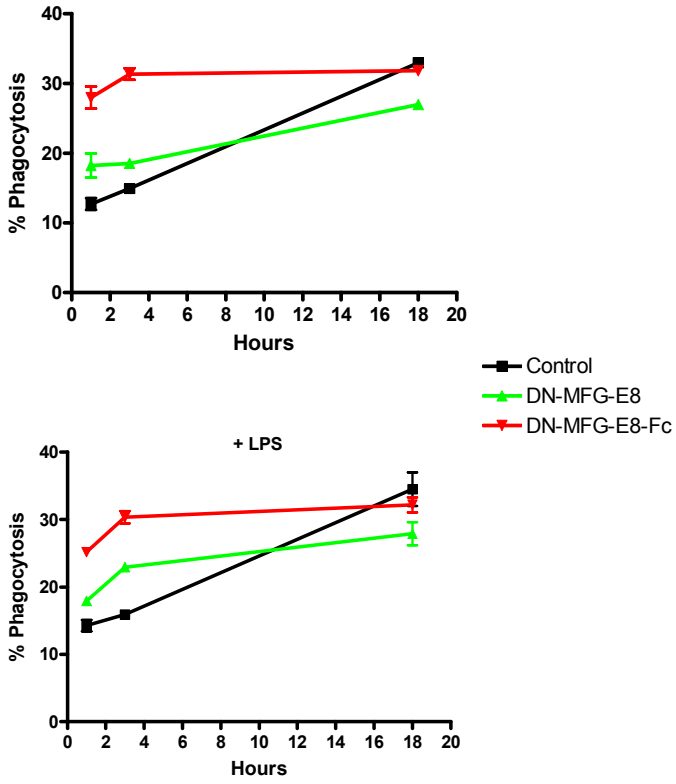

Figure 4.8 Inflammatory stimuli do not affect the phagocytic capability of BMDCs *in vitro*. Graphs representing flow cytometry analysis where CFSE-labelled apoptotic thymocytes were either untreated or treated with 100 µg/ml DN-MFG-E8 or DN-MFG-E8-Fc protein prior to being added to BMDCs (apoptotic thymocytes: BMDC ratio 4:1) over 1,2,3 and 18 h. After incubation at 37^0C, percentage phagocytosis was determined by cell which stained double positive for CFSE and CD11c. n=3

4.3.4 DN-MFG-E8-Fc protein enhances phagocytosis of apoptotic cells by FcγR$^{-/-}$ BMDCs due to the presence of the inhibitory FcγRIIb.

The treatment of apoptotic cells with DN-MFG-E8-Fc protein clearly enhances their uptake by BMDCs *in vitro*. To address the role of Fcγ receptors in this assay, BMDCs from Fcγ receptor knock out mice were cultured for 6 days as described previously. BMDCs were harvested and the assay as described from 4.3.1 was repeated with either wild type or Fcγ receptor$^{-/-}$ BMDCs. As figure 4.9 shows, DN-MFG-E8 protein failed to inhibit the uptake of apoptotic cells. More surprisingly however, apoptotic cells treated with DN-MFG-E8-Fc protein were still cleared more efficiently by BMDCs generated from FcγR-deficient mice compared to that seen with BMDCs generated from wild-type mice.

Of the three groups of Fcγ receptors in mice, (FcγRI, II and III), the activating Fcγ receptors I and III require co-expression of the common γ-chain for their assembly and signalling functions. The inhibitory receptor, FcγRII is, in contrast, a single chain receptor which does not require the γ-chain [190]. The result obtained here may therefore be as a result of apoptotic cell clearance through the inhibitory Fcγ receptor IIb, the immunological outcome of which is examined further in chapter 5.

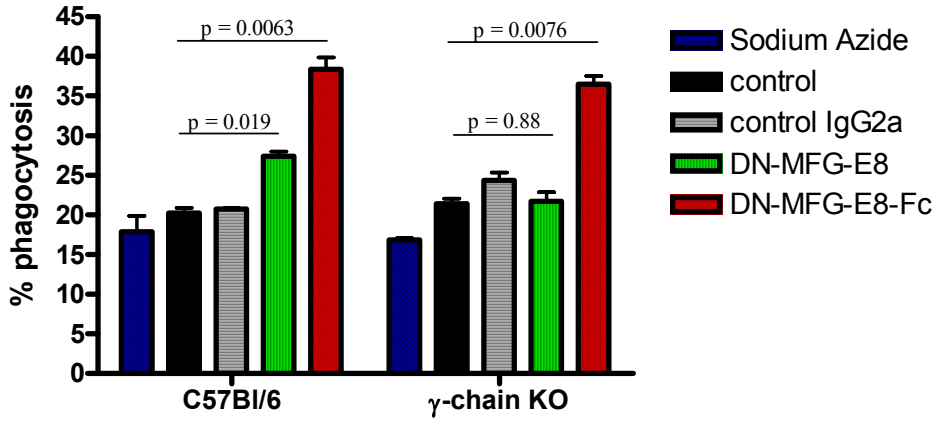

Figure 4.9 DN-MFG-E8-Fc can still increase phagocytosis of apoptotic cells in γ-chain knock out BMDCs. Graph representing flow cytometry analysis where CFSE-labelled apoptotic thymocytes were either untreated or treated with 100 µg/ml DN-MFG-E8, DN-MFG-E8-Fc protein or an isotype control (anti-human CD20 IgG2a) prior to being added to BMDCs (apoptotic thymocytes: BMDC ratio 4:1) for 2 h at 37^0C. Percentage phagocytosis was determined by cells which stained double positive for CFSE and CD11c. P values are generated from students T test where $p<0.05$ is statistically significant. Results are shown in triplicate where the error bars represent the standard deviation of the mean.

4.3.5 **FcγRIIb-deficient dendritic cells have enhanced phagocytic properties compared to wild-type dendritic cells.**

To elucidate the potential role of the inhibitory Fcγ receptor, FcγRIIb (CD32b), in mediating apoptotic cell clearance by dendritic cells in the presence of the DN-MFG-E8-Fc protein, BMDCs from FcγRIIb-deficient mice were grown in culture as described previously. CFSE-labelled apoptotic thymocytes were treated in the absence or presence of DN-MFG-E8, DN-MFG-E8-Fc, or an isotype control IgG2a protein, anti-CD20 IgG2a (at 100 μg/ml) prior to being added at a ratio of 4 apoptotic cells: 1 CD11c+ BMDC *in vitro* for up to 3 h. As shown in figure 4.10, DN-MFG-E8-Fc protein was able to enhance the uptake of apoptotic cells over the period of 1-3 h when compared to all other treatments. It was clear from this result and that observed in figure 4.9 that to obtain blockade of Fcγ receptor-mediated apoptotic cell clearance in the presence of DN-MFG-E8-Fc protein, BMDCs which were deficient in both activation and inhibitory Fcγ receptors would have to be used; an option that was not available at the time of study.

Additionally, the level of phagocytosis observed with all treatments was markedly higher than that observed in either wild type or FcγR-deficient BMDCs; suggesting a potential role of FcγRIIb in limiting the level of apoptotic cell clearance; in an attempt to limit autoreactivity to self-antigens.

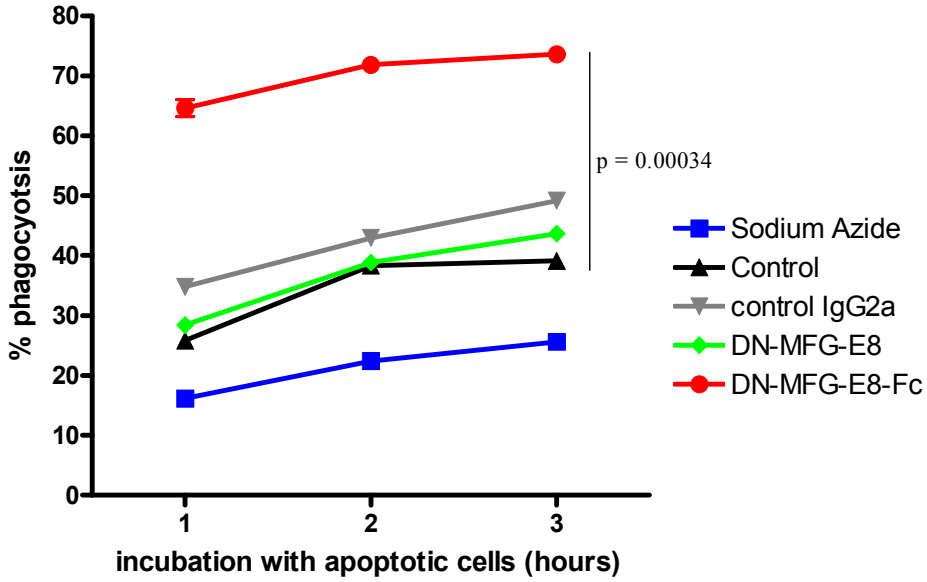

Figure 4.10 DN-MFG-E8-Fc protein enhances uptake of apoptotic cells by CD32b-deficient BMDCs. Graph representing flow cytometry analysis where CFSE-labelled apoptotic thymocytes were either untreated or treated with 100 µg/ml DN-MFG-E8, DN-MFG-E8-Fc protein or an isotype control (anti-human CD20 IgG2a) prior to being added to CD32b-deficient BMDCs (apoptotic thymocytes: BMDC ratio 4:1) for 1-3 h at 37^0C. Percentage phagocytosis was determined by cells which stained double positive for CFSE and CD11c. P values are generated from students T test where $p<0.05$ is statistically significant. Data is representative of triplicate samples.

4.4 Discussion

The engulfment of apoptotic cells has been shown to involve a two-step process. The first step involves the tethering of apoptotic cells to the macrophage by receptors such as CD14 and CD36 and the second step, referred to as the 'tickle' step, requires the exposure of PS on the apoptotic cell membrane [191]. The binding of PS via bridging molecules to integrins on phagocytes induces reorganisation of the actin-based cytoskeleton through activation of the CrkII-DOCK180-Rac1 complex allowing successful internalisation of apoptotic cells [120]. The 'tickle' step is, alone, not sufficient to mediate apoptotic cell clearance, highlighted by the accumulation of apoptotic cells in the spleen of MFG-E8-deficient mice, unable to be cleared effectively by tingible-body macrophages [126].

In support of this well documented model, this study has also shown that the blockade of PS by recombinant DN-MFG-E8 is able to inhibit the uptake of apoptotic cells by macrophages *in vitro* in a dose-dependent manner by up to 40% (figure 4.1 and 4.2). The use of Confocal laser scanning microscopy confirmed that the method used to determine phagocytosis in this assay showed that apoptotic cells were internalised and not simply bound to the macrophage cell surface. Additionally, it was also possible to determine the number of thymocytes that had been engulfed per macrophage; referred to as the phagocytic index [98]. In this study, the percentage of phagocytosis was reduced from 57.9% to 27.6% when thymocytes were treated first with recombinant DN-MFG-E8 protein. The phagocytic index was also reduced from an average of 1.21 thymocytes per macrophage down to 0.48 thymocytes per macrophage (Figure 4.2). This effect was then also observed using an *in vivo* system (figure 4.3) where by DN-MFG-E8 protein was able to inhibit phagocyte mediated apoptotic cell clearance *in vivo* by approximately 42% using a saturating concentration of DN-MFG-E8 protein (100 μg/ml). The ability for DN-MFG-E8 protein to inhibit phagocytosis at lower concentrations such as 25μg/ml was however highly variable, suggesting that only complete PS blockade is sufficient to inhibit apoptotic cell clearance mediated by all other PS receptors and bridging molecules found in the peritoneum. Recent studies have shown

that resident peritoneal macrophages preferentially secrete the PS-receptor molecule, TIM-4, for mediating apoptotic cell clearance and that MFG-E8 is only secreted when the macrophages have been stimulated with thioglycollate [96]. This would suggest that when DN-MFG-E8 protein is used at lower concentrations there is still PS exposed on the apoptotic cell membrane which can bind to TIM-4; facilitating their clearance. Additionally, the inhibition shown using 100 μg/ml DN-MFG-E8 protein could only be observed within the first 20 min of injecting treated apoptotic cells into the peritoneal cavity; after which time, all apoptotic cells were cleared regardless of their treatment. This again would suggest that there is direct competition within the peritoneal cavity for the binding to PS, the most likely competitor being TIM-4.

With that in mind, it is important to consider that only a single defect within the apoptotic cell clearance pathway is sufficient to promote the onset of autoimmunity and that complete inhibition is not an absolute requirement [134], it may therefore not be necessary to induce complete blockade of apoptotic cell clearance in order to observe an immune effect. As mentioned previously, MFG-E8$^{-/-}$ mice develop autoimmune disease in an age-dependent manner [126]. Similarly, mice deficient in the complement receptor, C1q, develop excessive inflammation and SLE-like autoimmunity [87, 192]. Conversely, defects in other apoptotic cell clearance pathways, such as those mediated through CD36 or mannose-binding lectin (MBL) show increased levels of apoptotic cell bodies in tissues, however their deficiency in mice does not lead to detectable autoimmunity [193, 194]. This indicates a possible redundancy within this system or that there is an incomplete characterisation of apoptotic cell receptors (explored further in the general discussion).

Although the blockade of PS in some tumour models with annexin V has already been shown to enhance their immunogenicity [189], additional studies have shown that targeting of cells through activating Fcγ receptors expressed on dendritic cells induces effective tumour immunity [46]. The aim of this study was to determine whether tumour cells which die by

apoptosis can also be targeted to Fcγ receptors by taking advantage of the PS which becomes exposed on the cell membrane.

Numerous studies have shown that the targeting of activating Fcγ receptors on dendritic cells promotes their maturation; as determined by their activation status, costimulatory molecule expression, and cytokine secretion [46, 52, 54, 56, 195]. This study was therefore aimed at targeting the activating Fcγ receptor clearance pathway with the intention of enhancing DC maturation and therefore immunity against cell-associated antigen upon apoptotic cell uptake.

The treatment of apoptotic cells with DN-MFG-E8-Fc protein was able to bind to PS on apoptotic cells (as demonstrated in chapter 3) and increase the percentage of phagocytosis by macrophages *in vitro* in a dose-dependent manner (figure 4.4). This enhanced uptake was also observed over a time period of 1-3 h where by apoptotic cells treated with DN-MFG-E8-Fc were cleared by more than 2.5 fold more efficiently than untreated apoptotic cells under the same conditions (figure 4.5). This would support that DN-MFG-E8-Fc protein is able to block PS on apoptotic cells and re-direct their uptake through Fc receptors on macrophages.

To then examine the effect of this protein on apoptotic cell uptake by dendritic cells, BMDCs were cultured *in vitro* with GM-CSF and their ability to phagocyte apoptotic cells in the presence or absence of either DN-MFG-E8 or DN-MFG-E8-Fc proteins was examined. DN-MFG-E8-Fc consistently enhanced the uptake of apoptotic cells by BMDCs *in vitro* (figure 4.7). However, in contrast to the data obtained using resident peritoneal macrophages, the presence of DN-MFG-E8 protein was unable to reduce apoptotic cell uptake by BMDCs *in vitro* (figure 4.7). In fact, the ability of BMDCs to phagocytose apoptotic cells was actually modestly enhanced in the presence of DN-MFG-E8, a similar finding that has been observed upon PS blockade on apoptotic cell with annexin V protein [189]. This evidence is however in stark contrast to the finding by Miyasaka *et al*

[196] in which MFG-E8-/- BMDCs showed a clear reduction in their ability to phagocyte apoptotic cells.

The presence of LPS in this assay did not affect the phagocytic ability of the BMDCs in either the presence or absence or DN-MFG-E8 or DN-MFG-E8-Fc protein when compared to non-stimulated BMDCs (figure 4.8). This would suggest that BMDCs are able to internalise material when they are at the same time exposed to inflammatory stimuli. Studies done by other groups have already shown however that if BMDCs are matured with inflammatory stimuli prior to their culture with apoptotic cells, their phagocytic capacity is significantly down-regulated [37]. However, in constrast, studies have also shown that within the first hour, the presence of LPS caused significantly enhanced endocytosis by dendritic cells, followed by its rapid decline [30]. This evidence, including that which is shown in this study, would suggested that the time of exposure, amount of stimulus, variants in culturing of the dendritic cells, all induce variable outcomes.

One of the key aims in this study was to assess the effect of re-directing apoptotic tumour cell uptake through activating Fcγ receptors on dendritic cells, with the goal of facilitating tumour-associated antigens into the cross-presentation pathway for effective priming to CD8+ T cells (and to mature or activate DCs). To determine whether DN-MFG-E8-Fc protein utilised the activating Fcγ receptor pathway, BMDCs from FcγR-deficient mice were used in an *in vitro* phagocytosis assay. $Fc\gamma R^{-/-}$ BMDCs still showed enhanced clearance of apoptotic cells when treated with DN-MFG-E8-Fc protein, comparable to that seen from wild type BMDCs (figure 4.10). Because $Fc\gamma R^{-/-}$ mice still express the inhibitory FcγRIIb, it was therefore likely DN-MFG-8-Fc protein was re-directing their uptake through this receptor. To determine whether this was the case, BMDCs from FcγRIIb-deficient mice were used in an *in vitro* phagocytosis assay. Again, DN-MFG-E8-Fc protein was able to enhance the clearance of apoptotic cells when compared to untreated apoptotic cells. To note, the total percentage of phagocytosis by FcγRIIB-deficient BMDCs was much greater that that

seen by wild type or FcγR$^{-/-}$ BMDCs suggesting a potential role of the inhibitory receptor in controlling the capacity for phagocytic cell clearance.

It was not possible from this data alone to determine the preferential receptor that would be utilised when apoptotic cells are re-targeted to Fcγ receptors using the DN-MFG-E8-Fc protein. It was however clear that the protein is able to facilitate their clearance through both activating and inhibitory Fcγ receptors; a factor that may influence the immunological outcome upon subsequent cross-presentation of antigen to antigen-specific CD8+ T cells. Previous studies have shown that the presence of the inhibitory Fcγ receptor on dendritic cells induces T cell tolerance to internalised antigen due to its ability to inhibit effective DC maturation upon its engagement [51]. It was therefore of key interest to assess the balance between activating and inhibiting Fcγ receptors on dendritic cells when apoptotic cells were treated with both DN-MFG-E8 and DN-MFG-E8-Fc proteins.

Chapter 5 - Results

Investigating the effect of re-directing apoptotic cell uptake via Fc receptors on their immunogenicity.

5.1 Introduction

Dendritic cells have the privileged ability of being able to initiate *in vivo* CD8+ T cell responses through either direct priming, or through cross-presentation; involving the presentation of peptide-MHC class I complexes derived from exogenous proteins.

The cross-presentation of exogenous antigen to CD8+ T cells has one of two outcomes; antigenic tolerance (cross-tolerance) or immunity (cross-priming). Research published in 1998 [38] provided the first evidence that antigens that are expressed by apoptotic cells can be presented by dendritic cells to CD8+ T cells via cross-presentation. In mice, cross-presentation is exclusive to a subset of CD8α+, CD11c+ dendritic cells, which have the ability to internalise dying cells and cross-present the cell-associated antigen onto MHC class I complexes, whilst retaining the ability to form peptide-MHC class II complexes for presentation to CD4+ T cells [39].

The evidence that apoptotic cells can provide a source of antigen to prime CD8+ T cells *in vivo* lead to subsequent research to determine whether apoptotic tumour cells could be used as a suitable cancer vaccine. With this is mind, Ronchetti and colleagues examined the therapeutic outcome of immunising mice with apoptotic cancer cells. They discovered however that this induced a decreased level of cytotoxic T cell response compared to the immunisation with live, growth arrested tumour cells [40]. In support of this knowledge, it is well established that apoptotic cells have immunosuppressive properties, owing to their rapid elimination by monocytes/macrophages which, in turn, show enhanced expression of anti-inflammatory cytokines, such as IL-10 and TGFβ, while decreasing their

expression of pro-inflammatory cytokines, such as TNFα, IL-1 and IL-12 [197]. It is therefore not surprising that the efficient presentation of apoptotic-derived antigens by dendritic cells is extremely limited when apoptotic cells are administered alone as a vaccine.

The blockade of phosphatidylserine on apoptotic cells has been shown to at least partially enhance their immunogenicity. The treatment of apoptotic tumour cells with Annexin V protein prior to their administration *in vivo* both reduced their phagocytosis by macrophages whilst maintaining their ability to be cleared by dendritic cells, leading to an increased level of protection against subsequent tumour challenge [189]. In further support of this, BALB/c mice which were immunised with xenogeneic apoptotic human T cells treated with Annexin A5 protein produced enhanced secondary antibody responses when compared to apoptotic cells alone. Although this study showed that PS blockade enhanced the immunogenicity of apoptotic cells upon secondary challenge, this effect was not observed in the primary response [198], highlighting the need for more investigation into this model.

One of the determining factors dictating whether tolerance or immunity ensues after antigen capture is largely owing to the maturation state of the dendritic cell during DC-T cell interactions. In the absence of acute inflammation and/or infection, most dendritic cells *in vivo* are functionally immature, giving them the ability to capture antigens in the form of whole cells or opsonised particles. In the absence of either endogenous or exogenous stimuli, T cells which are engaged by the cross-presenting DC are rendered tolerant [44, 199]. Conversely, in the presence of stimuli such as inflammatory cytokines, CD40 ligand, and viral and microbial components such as d.s RNA and LPS, dendritic cells undergo a state of active maturation where by the expression of costimulatory molecules and cytokine secretion is induced. The engagement of CD40 with its cognate ligand, expressed by CD4 T cells, leads to the enhanced expression of costimulatory molecules including CD86, CD80, CD70, 4-1BB ligand and OX40 ligand on the APC cell surface. The upregulation of CD70 on APCs

for example is required for effective CD40-mediated priming of CD8+ T cells *in vivo* [200].

One of the aims in this study was to determine if re-directed uptake of apoptotic cells via FcγRs would lead to maturation of DCs. Previous studies have shown that targeting of soluble antigen via immune complexes through activating Fcγ receptors, expressed on the DC cell surface, is able to alter the balance of DC-mediated peripheral T cell tolerance by provision of both antigen loading and DC maturation [201]. Targeting immune complexes through activating FcγRs has already been shown to induce both CD4+ and CD8+ T cell responses that can confer tumour immunity [46].

FcγR-mediated activation of DCs is also able to alter the tolerogenic outcome of cognate DC-T cell interactions by the induction of enhanced costimulatory molecules and cytokine production [202]. DCs which take up antigen through FcγRs permits rapid targeting of antigen to the endosomal/lysosomal compartment for cross-presentation [52], whilst engagement of activating Fcγ receptors induces DC maturation as assessed by increased surface expression of CD40 and CD86 as well as secretion of IL-12 [203].

This chapter describes the effect of targeting apoptotic cell-associated antigens to dendritic cells, either via PS-independent mechanisms using DN-MFG-E8 protein, or via activating Fcγ receptors on DCs using DN-MFG-E8-Fc protein with the overall objective of developing an approach to enhance the immunogenicity of apoptotic tumour cells.

5.2 Observing the effect of different clearance mechanisms on apoptotic cell-associated antigen presentation to CD8+ T cells *in vitro*.

5.2.1 Clearance of apoptotic cells in the presence of DN-MFG-E8-Fc enhances CD8+ T cell proliferation, in particular at sub-optimal levels of stimulation.

Chapter 4 described how the use of recombinant MFG-E8 proteins is able to re-direct the clearance of apoptotic cells by dendritic cells through alternative receptors other than the classical phosphatidylserine pathway. To elucidate the possible effects that these alternative clearance pathways may have on subsequent antigen presentation, the first experiment examined the ability of APCs to process cell-associated antigen from apoptotic cells treated with DN-MFG-E8-Fc protein and then present antigen to antigen-specific CD4+ and CD8+ T cells *in vitro*. The model antigen used for this assay was Ovalbumin (OVA), which when processed by APCs is degraded into a peptide of 9 amino acids (SIINFEKL) which is presented with MHC class I to transgenic OVA-specific CD8+ T cells (OT-I). Similarly, the CD4 helper epitope for OVA ($OVA_{323-339}$ – ISQAVHAAHAEINEAGR) is also processed by antigen presenting cells and is presented with MHC class II molecules to transgenic OVA-specific CD4+ T cells (OT-II).

OVA-expressing EG.7 tumour cells were rendered apoptotic by treatment with UVC radiation and either left untreated or treated with 100 μg/ml DN-MFG-E8-Fc protein. A titration of apoptotic EG.7 cells were then added *in vitro* to lymph node and spleen cells from either OT-I or OT-II mice. Two independent experiments, (Figure 5.1 and 5.2), suggested that apoptotic cells expressing the model antigen OVA induced better proliferation of OVA-specific CD8+ T cells in the presence of DN-MFG-E8-Fc.

This *in vitro* assay did not however exclude the possibility that EG.7 cells are themselves able to directly present the SIINFEKL peptide on MHC class I to the OT-I CD8+ T cells. Additionally, when the protein was tested for endotoxin contamination, there were higher than background levels of endotoxin detectable (43 Eu/ml). All subsequent antigen presentation and T cell proliferation studies were performed using apoptotic splenocytes from H2-K^{bm1}mice, which were then loaded with OVA (see section 2.5.4 for protocol). APCs from bm1 mice, unlike those from C57BL/6 mice, are unable to present OVA peptides to OT-1 T cells [204]. The endotoxin in the protein preparation was also removed using an Endotrap column, thereby eliminating the possibility of non-specific T cell stimulation.

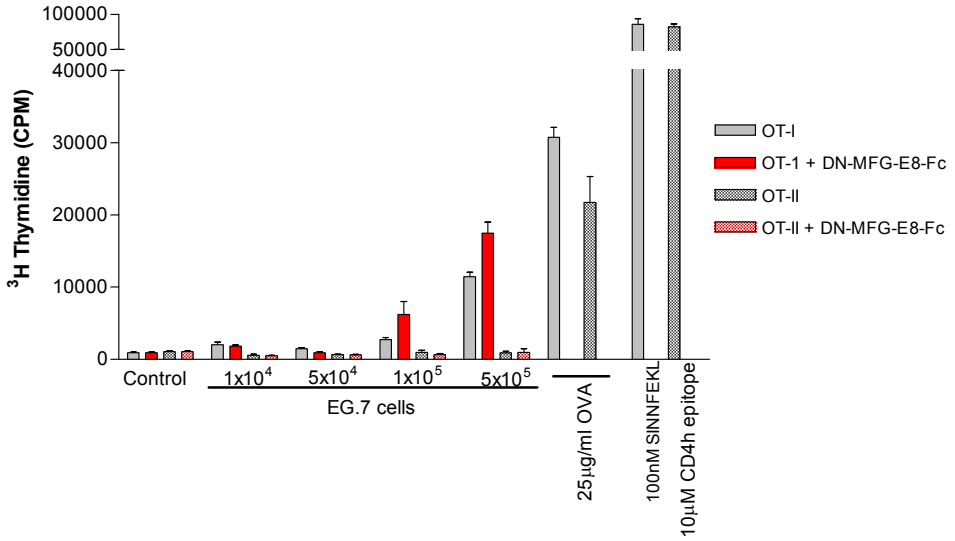

Figure 5.1 DN-MFG-E8-Fc protein enhances antigen presentation to OT-I T cells *in vitro*. Graph representing the level ^{3}H Thymidine (CPM) incorporated into proliferating OT-1 and OT-II T cells, stimulated with apoptotic OVA-expressing EG.7 cells treated with or without 100 µg/ml DN-MFG-E8-Fc protein. n=3 for each bar. Error bars represent the standard error of the mean.

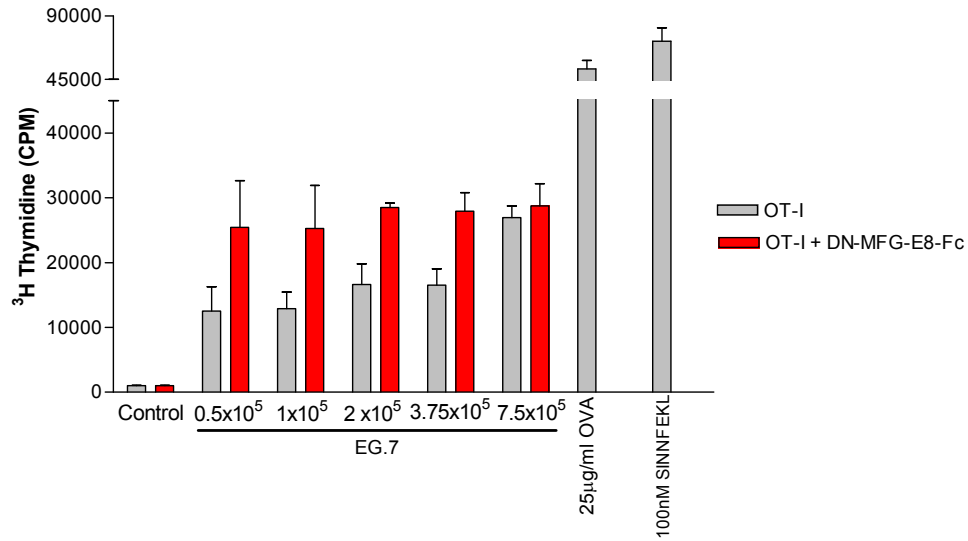

Figure 5.2 Effect of DN-MFG-E8-Fc protein on antigen presentation is limited to a range of apoptotic cell doses. Graph representing the level ^{3}H Thymidine (CPM) incorporated into proliferating OT-I T cells, stimulated with apoptotic OVA-expressing EG.7 cells treated with or without 100 µg/ml DN-MFG-E8-Fc protein. n=3 for each bar. Error bars represent the standard error of the mean.

5.2.2 Cultured BMDCs are able to cross-present cell-associated antigen from apoptotic cells to CD8+ T cells *in vitro*.

In addition to using bm1 splenocytes as a source of apoptotic cell to prevent direct MHC class I-OVA-peptide presentation, it was also important to ensure that the phagocytes which clear the apoptotic cells were professional dendritic cells, capable of cross-presentation. For this purpose, dendritic cells were cultured from murine bone marrow (BMDCs) and used in future cross-presentation assays.

To firstly assess the efficiency of BMDCs to cross-present antigen from syngeneic apoptotic cells *in vitro*, and then subsequently determine whether this can be manipulated in the presence of either DN-MFG-E8 or MFG-E8-Fc protein, BMDCs were cultured with OVA-loaded apoptotic bm1 splenocytes alone, or in the presence of 100 μg/ml DN-MFG-E8 or DN-MFG-E8-Fc protein. The ability of CD11c+ apoptotic cell-loaded BMDCs to cross-present MHC class-I-OVA peptide complexes was then assessed by then culturing them *in vitro* with purified CFSE-labelled OT-I cells. After 72 h, OT-I cell proliferation was determined by flow cytometry in which the dilution of CFSE fluorescence was measured.

The data shown in figure 5.3 indicated that CD11c+ BMDCs were able to effectively cross-present apoptotic cell-associated OVA-peptide to antigen-specific CD8+ T cells *in vitro*. This did not however appear to be dependent on a particular apoptotic cell clearance pathway, where by apoptotic cells phagocytosed via both PS-independent pathways (as determined by their treatment with either DN-MFG-E8 or DN-MFG-E8-Fc proteins) did not promote, nor inhibit the ability of BMDCs to cross-present antigen when compared to using apoptotic cells alone.

The ratio of OT-I: DC appeared to be an important factor in this assay, as the highest ratio (1 OT-I: 4 DC) did not confer successful cross-presentation, possibly due to a high cell concentration which could have reduced specific cell-cell contact interactions. This ratio was therefore excluded in the following experiment.

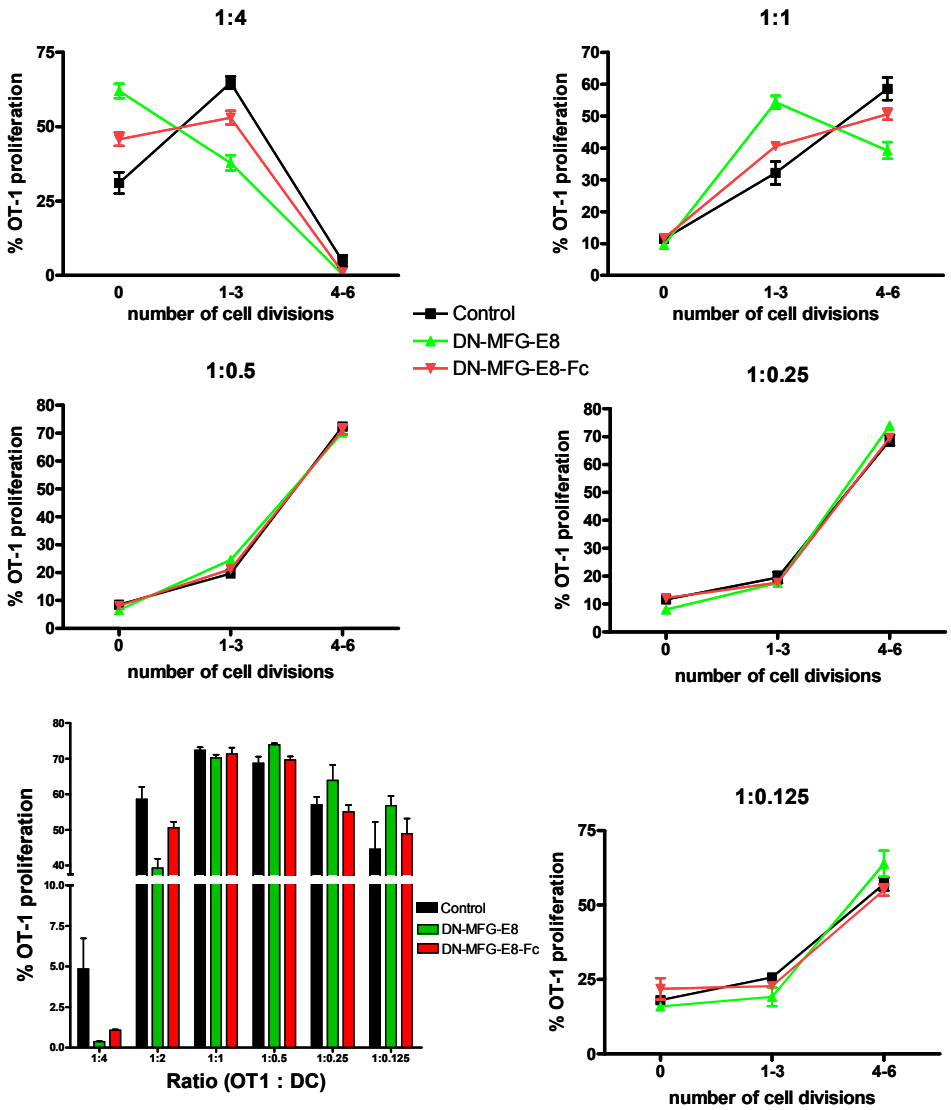

Figure 5.3 Cross-presentation of OVA-peptide to purified CD11c+ BMDCs to transgenic OT-I cells *in vitro*. BMDCs from C57BL/6 mice were loaded with OVA-loaded apoptotic bm1 cells that had been either untreated or treated with 100 µg/ml DN-MFG-E8 or DN-MFG-E8-Fc protein (at a ratio of 1 DC: 4 apoptotic cells) for 3 h at 37^0C. After BMDC purification (by CD11c MACs separation), they were added to CFSE-labelled transgenic OT-I cells at different ratios. The ratio of OT-I: DC used is shown above each graph. OT-I proliferation was determined using flow cytometry where one CFSE dilution was equivalent to one round of cell proliferation. Bottom-left graph shows the total percentage of OT-I cells which had undergone proliferation compared to non-stimulated cells. Each sample shown was performed in triplicate. Error bars represent the standard error of the mean.

To further probe the role of Fcγ receptors on facilitating apoptotic cell clearance by dendritic cells in this model, BMDCs from γ-chain knock out mice were grown in culture and their ability to cross-present antigen derived from OVA-loaded apoptotic cells compared to wild type BMDCs was examined. The use of BMDCs from γ-chain knock out mice was able to establish what the effect of targeting through the inhibitory Fcγ receptor in the absence of FcγRI and III, and IV would have on subsequent antigen cross-presentation to CD8+ T cells. Previous studies have shown that expression of inhibitory FcγRIIb on dendritic cells enforces peripheral tolerance by inhibiting effector T cell responses, where by the coengagement of activating Fcγ receptors with the inhibitory receptor can suppress both human and mouse ITAM-induced DC maturation [51].

As demonstrated in chapter 4, figure 4.9, γ-chain knock out BMDCs phagocytosed apoptotic cells more readily in the presence of the DN-MFG-E8-Fc protein compared to their wild-type control. As shown in figure 5.4, this did not however enhance nor inhibit their ability to cross-present antigen to antigen-specific CD8+ T cells *in vitro*. Similarly, the blockade of PS by DN-MFG-E8 protein had no effect on antigen cross-presentation when compared to loading γ-chain KO BMDCs with apoptotic cells alone.

An additional observation obtained from this assay, suggested that cross-presentation by wild-type BMDCs was more effective at high OT-I: DCs ratios, whereas the converse was true for γ-chain KO BMDCs. It was not however possible at this stage to ascertain a plausible reason for this finding. It is possible that this BMDC/OT-I *in vitro* system is highly efficient in processing and presenting OVA to T cells. This contrasts with using whole OT-I splenocytes which have fewer DCs and are likely to be less activated than BMDC and therefore in this setting the effects of Fc protein-targeting/activation can be observed. BMDC are well known to have a partially activated phenotype. Thus the effects of the Fc protein could be seen in the first but not second in vitro culture system.

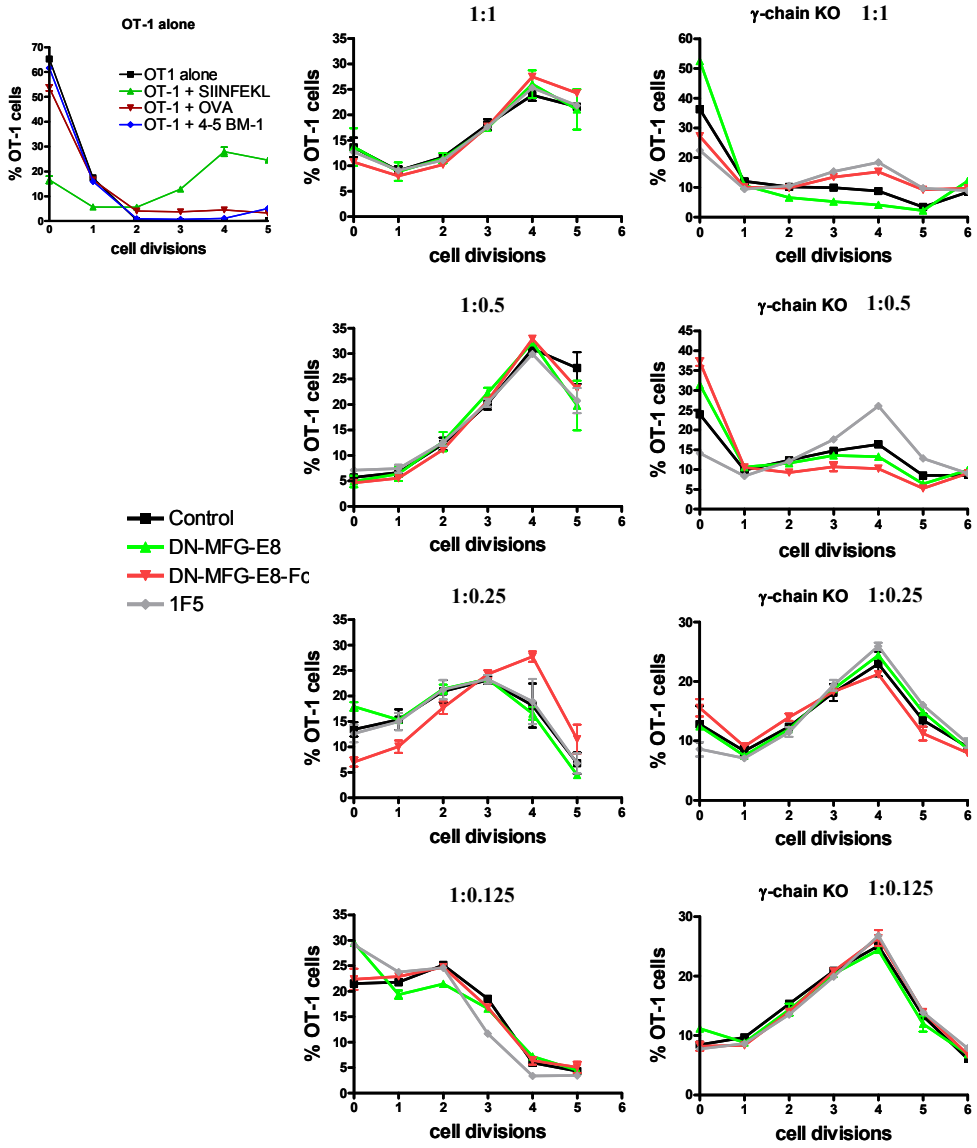

Figure 5.4 Cross-presentation of OVA peptide by CD11c+ BMDCs to transgenic OT-I cells *in vitro*. BMDCs from C57BL/6 or γ-chain knock out mice were loaded with OVA-loaded apoptotic bm.1 cells that had been either untreated or treated with 100 μg/ml DN-MFG-E8, DN-MFG-E8-Fc, or an isotype control protein, 1F5 (anti-human CD20 IgG2a) at a ratio of 1 DC: 4 apoptotic cells for 3 h at 37^0C. After BMDC purification (by CD11c MACs separation), they were added to CFSE-labelled transgenic OT-I cells at different ratios for 72 h. The ratio of OT-I: DC used is shown above each graph. OT-I proliferation was determined using flow cytometry where one CFSE dilution was equivalent to one round of cell proliferation. Top-left graph is without BMDCs. Each sample shown was performed in triplicate. Error bars represent the standard error of the mean.

5.3 Loading BMDCs with apoptotic cells in the presence of DN-MFG-E8 or DN-MFG-E8-Fc protein reduces their ability to stimulate CD8+ T cell proliferation *in vivo*.

5.3.1 Apoptotic cells administered to mice intravenously are cleared by APCs, permitting cross-presentation of cell-associated antigen to CD8+ T cells.

To establish whether apoptotic cells cleared through Fcγ receptors (using DN-MFG-E8-Fc protein) or via blockade of PS (using DN-MFG-E8 protein) would result in enhanced antigen cross-presentation to CD8+ T cells *in vivo*, OVA-loaded apoptotic bm.1 splenocytes were treated with either DN-MFG-E8 or DN-MFG-E8-Fc protein, prior to being administered intravenously into syngeneic mice. As shown in figure 5.5, cell-associated OVA was efficiently processed and presented *in vivo* to adoptively transferred CFSE-labelled OT-I cells, promoting CD8+ T cell proliferation in a dose-dependent manner. The effect of the two recombinant MFG-E8 proteins did not however appear to have any significant difference in the cross-presentation of the antigen in this model.

Apoptotic cells injected alone are not directed to a particular phagocyte and could therefore have been cleared by alternative scavenger phagocytes such as macrophages, unable to efficiently cross-present antigen and migrate to draining lymph nodes. Alternatively, the DN-MFG-E8 or DN-MFG-E8-Fc proteins may not have remained bound to the apoptotic cells once *in vivo* due to potential competition between other PS-binding proteins (as discussed in chapter 1, section 1.4.5).

To try and overcome this, the loading of apoptotic cells to dendritic cells was orchestrated in a more controlled fashion by using cultured BMDCs and loading them with apoptotic cells *in vitro*, prior to their administration.

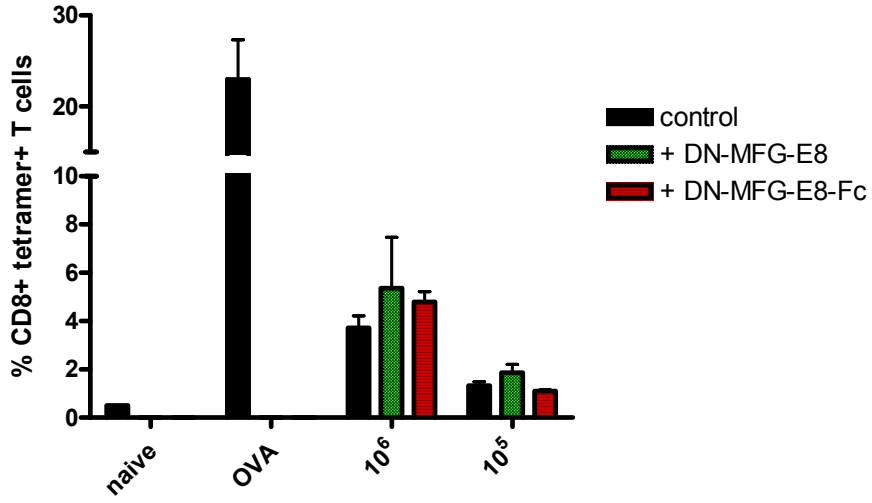

Figure 5.5 Cross-presentation of antigen to CD8+ T cells *in vivo*. 2 x 10^6 CFSE-labelled OT-I cells were administered i.v per C57Bl/6 mouse. 24 later, OVA-loaded apoptotic bm.1 cells treated with or without DN-MFG-E8 or DN-MFG-E8-Fc protein were administered at either 10^5 or 10^6 cells / mouse. After 72 h, the spleen from each mouse was removed and the percentage of OVA-specific CD8+ T cells was determined by staining the cells with 10 µg/ml APC-labelled anti-CD8 antibody and 20 µg/ml PE-labelled tetramer. Cells which stained double positive are indicated on the graph. Results represent data from triplicate values. Error bars represent the standard error of the mean.

5.3.2 CD8+ T cells show reduced accumulation in the spleen after stimulation with BMDCs loaded with apoptotic cells via a PS-independent clearance pathway.

BMDCs cultured *in vitro* have the ability to phagocytose apoptotic cells and cross-present the cell-associated antigen to antigen-specific CD8+ T cells (see section 5.2.2). To determine whether this could also be achieved in an *in vivo* setting, BMDCs were loaded with apoptotic cells *in vitro* in the presence of either DN-MFG-E8 or DN-MFG-E8-Fc proteins, prior to their administration *in vivo*. Their ability to migrate to the spleen and cross-present the antigen to CD8+ T cells *in vivo* was then determined by measuring CFSE dilution of OT-I T cells.

As shown in figure 5.6, BMDCs loaded *in vitro* with OVA-loaded apoptotic cells were efficient at migrating to the lymphoid organs and cross-presenting antigen to transgenic OT-I cells *in vivo*, as determined by their ability to promote both accumulation and proliferation of OT-I T cells in the spleen. Interestingly, the loading of BMDCs with apoptotic cells in the presence of both DN-MFG-E8 or DN-MFG-E8-Fc protein caused inhibition of antigen cross-presentation as determined by both OT-I accumulation (which showed a reduction by 60% and 71%, respectively) and proliferation (which showed a reduction by 41% and 46%, respectively) in the spleen.

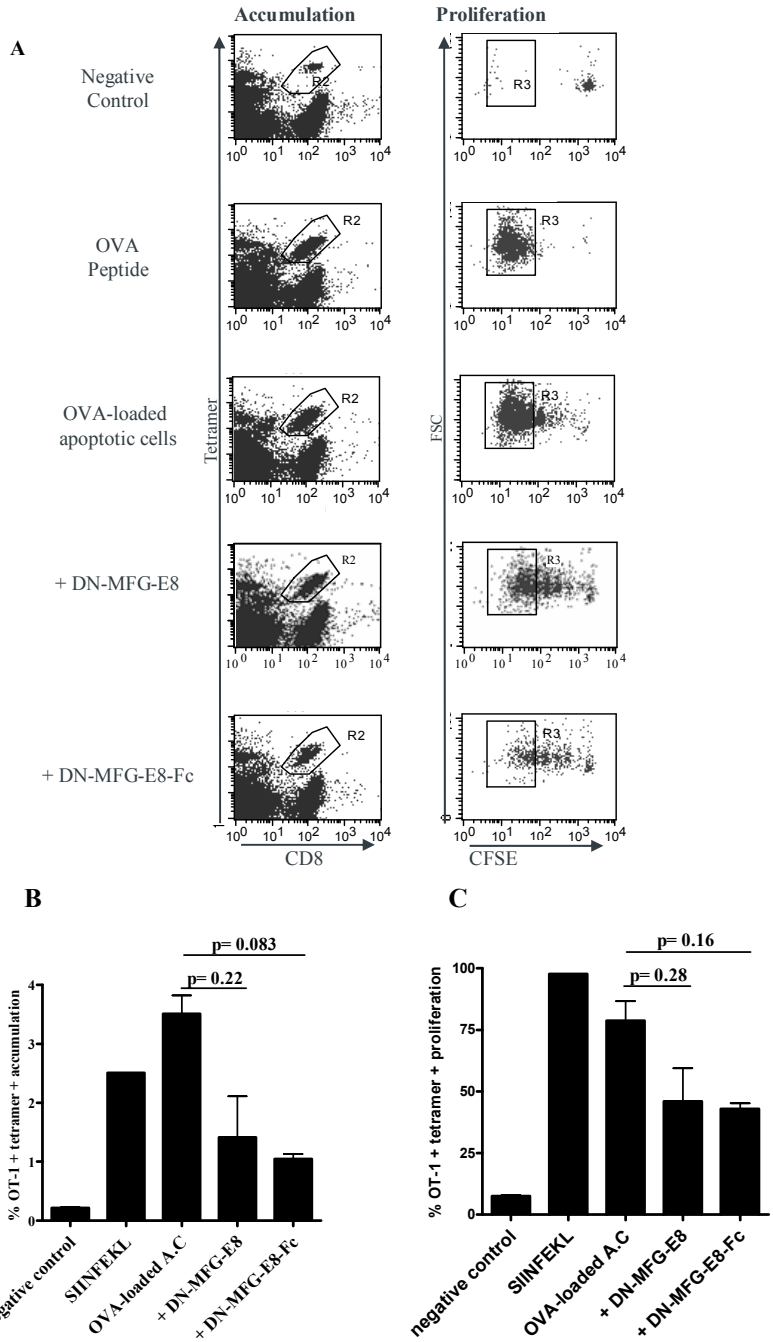

Figure 5.6 CD8+ T cell accumulation and proliferation is inhibited by recombinant MFG-E8 proteins. 2 x 10^6 CFSE-labelled OT-I cells were adoptively transferred i.v per C57Bl/6 mouse. CD11c+ BMDCs, loaded with OVA-loaded apoptotic bm.1 cells in the presence or absence of 100 µg/ml DN-MFG-E8 or DN-MFG-E8-Fc protein for 3 h, after which 5 x 10^5 CD11c+ BMDCs were administered i.v per mouse. On day 3, spleens were harvested and OVA-specific CD8+ T cell accumulation and proliferation was determined by analysis using flow cytometry. **A:** Left panel shows flow cytometry dot plot, gating double positive CD8+ and tetramer + cell population. Right panel shows CFSE-dilution of gated double positive cells from left panel. **B:** Graph representing data obtained from gated cells from left panel from A. **C:** Graph representing data obtained from gated cells from right panel from A. P value is representative of a two-tailed paired t-test. Graph is representative of 3 different mice in each group.

As only 25-30% of apoptotic cells were phagocytosed by BMDCs in this assay within 3 h (see chapter 4, figure 4.7), this resulted in the co-administration of 'free' OVA-loaded apoptotic cells that have not been internalised by the BMDCs. To confirm the previous result (figure 5.6) and to eliminate any effect observed by apoptotic cells injected alone, the assay was repeated, however after BMDCs were loaded with apoptotic cells, they were purified by CD11c+ selection prior to their administration.

As shown in figure 5.7, the accumulation of antigen-specific CD8+ T cells within the spleen 3 days post stimulation with BMDCs was markedly reduced when BMDCs were loaded with apoptotic cells in the presence of either DN-MFG-E8 or DN-MFG-E8-Fc proteins.

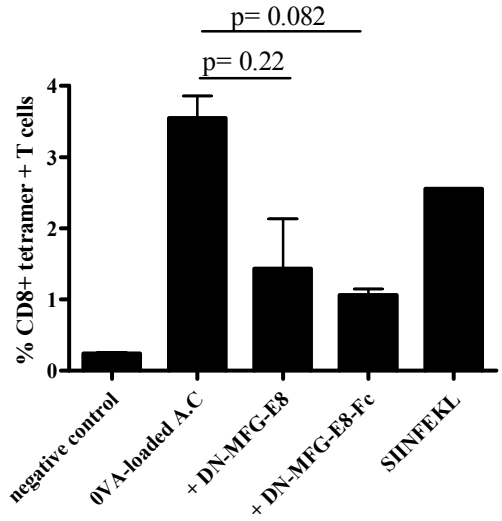

Figure 5.7 Cross-presentation of antigen to CD8+ T cells is orchestrated primarily by dendritic cells loaded *ex vivo*. 2 x 10^6 CFSE-labelled OT-I cells were adoptively transferred i.v per C57Bl/6 mouse. CD11c+ BMDCs, loaded with OVA-loaded apoptotic bm.1 cells in the presence or absence of 100 µg/ml DN-MFG-E8 or DN-MFG-E8-Fc protein for 3 h. After which BMDCs were purified to remove non-phagocytosed apoptotic cells and 5 x 10^5 CD11c+ BMDCs were administered i.v per mouse. On day 3, spleens were harvested and OVA-specific CD8+ T cell accumulation was determined by analysis using flow cytometry. Graph represents data from flow cytometry analysis showing the total percentage of CD8+ tetramer + T cells present in the spleen. P-value is representative of a two-tailed paired t-test. Graph represents 3 mice in each group.

It was clear from the results obtained that the loading of apoptotic cells either via a mechanism other than PS or targeting apoptotic cells directly to Fcγ receptors caused a reduction in the ability of DCs to cross present antigen to CD8+ T cells in the spleen. As shown in chapter 4 (figure 4.7), apoptotic cell clearance by BMDCs was enhanced in the presence of both DN-MFG-E8 and DN-MFG-E8-Fc proteins. The observation seen here was therefore not as a result of insufficient antigen loading. One possible explanation is that the route of antigenic uptake using these alternative clearance pathways inhibited access of antigen into the cross-presentation pathway and was transferred to highly acidic lysosomal compartments for rapid degradation. Alternatively, this method may have favoured loading antigen onto MHC class II molecules for presentation to CD4+ T cells; a theory that was not tested in this model.

An additional explanation for this result is that the clearance mechanism utilised did not promote a state of DC activation sufficient to induce homing of the DCs to the secondary lymphoid organs upon their administration *in vivo* [205]. This explanation is explored further in the discussion.

5.4 Apoptotic cell clearance via Fcγ receptors in the absence of the inhibitory FcγRIIb induces DC maturation *in vitro*.

5.4.1 FcγR-mediated clearance of apoptotic cells by FcγRIIb-deficient DCs induces expression of costimulatory molecules, CD86 and CD70.

Previous studies have shown that FcγR-engagement on dendritic cells by IgG-immune complexes induces DC activation as determined by their increased surface expression of MHC, CD40, CD80 and CD86 [52]. It is widely accepted that the expression of costimulatory molecules on dendritic cells is required for priming T cell responses. More recently, it has been shown that CD70, a member of the TNF family is induced upon activation of DCs by CD40 and TLR engagement. Interestingly, CD70 was not induced following activation of DCs by type I interferon, a stimulant that upregulates CD86 expression [206]. Furthermore, CD70 expression on dendritic cells was shown to be important for CD40 and TLR-mediated priming of antigen specific CD8+ cytotoxic T cells [207]. In light of those findings, I sought to investigate the effect of uptake of apoptotic cells by different receptors on the expression of the costimulatory proteins CD70 and CD86. Because FcγRIIb can inhibit DC maturation induced by Fcγ receptors, the effect of the route of uptake of apoptotic cells on DC maturation was compared in BMDC cultures obtained from FcγRIIb deficient and sufficient mice.

As a positive control, LPS was used in the following DC maturation assays. LPS is a well characterised stimulator of dendritic cells, inducing DC maturation as determined by the increased expression of costimulatory molecules, as well as inducing inflammatory cytokine secretion [208].

Wild type (WT) and FcγRIIb$^{-/-}$ BMDCs were grown and cultured as described previously. After 6 days in culture, CD11c+ BMDCs were

purified and loaded *in vitro* with apoptotic thymocytes either alone, or treated with DN-MFG-E8 or DN-MFG-E8-Fc protein. Additionally, it was also important to ensure that there was no presence of endotoxin which would favour DC maturation, hence syngeneic thymocytes were used as a source of apoptotic cell which were therefore were free from exogenously loaded antigen.

The surface expression of CD86 and CD70 on BMDCs from WT or FcγRIIb$^{-/-}$ BMDCs was determined after 18 h *in vitro* in the presence of apoptotic cells treated with or without DN-MFG-E8 or DN-MFG-E8-Fc protein. As shown in figure 5.8, the incubation of cells with the DN-MFG-E8-Fc protein caused further upregulation of CD86 in wt DCs and to a greater extent in CD32KO DCs. The most striking data, however, was that DN-MFG-E8-Fc coated apoptotic cells induced a clear increase in CD70 expression, but only in CD32KO DCs. This assay provides strong evidence in which the presence of the inhibitory Fcγ receptor is able to control dendritic cell maturation, and suggests for the first time that upregulation of CD70 expression on the cell surface can be controlled by the engagement of Fcγ receptors; another mechanism which could mediate effective CD8+ T cell priming.

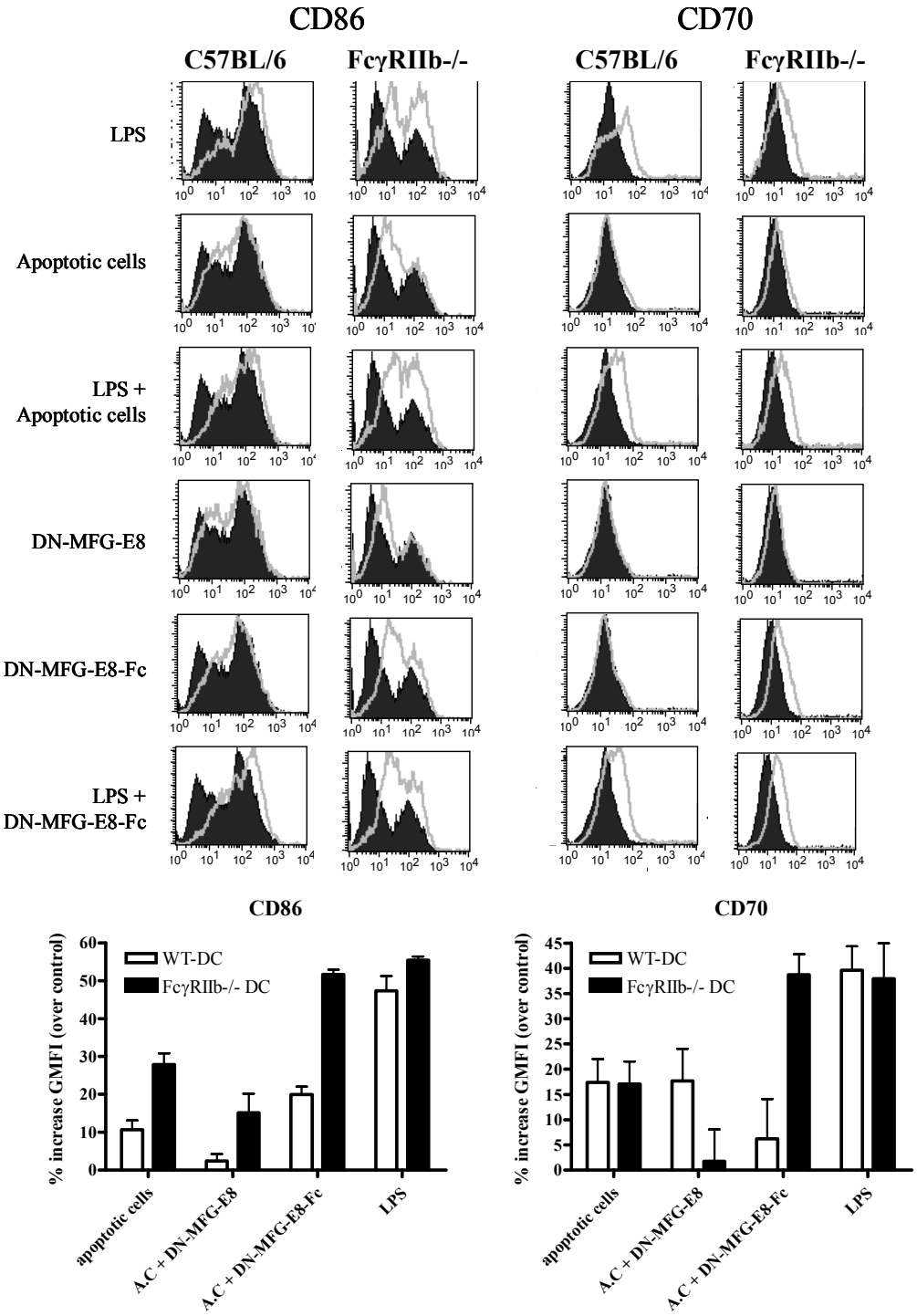

Figure 5.8 FcγR-mediated clearance of apoptotic cells induces full maturation of FcγRIIb$^{-/-}$ DCs but not WT-DCs. A: FACs histograms indicating expression of CD86 and CD70 as detected using 10 μg/ml fluorescently-labelled anti-CD86 (FL-1) or anti-CD70 (FL-2) antibodies. Closed histogram represents control, non-treated BMDCs. Open histogram represents BMDCs treated with either LPS or apoptotic cells +/- DN-MFG-E8 or DN-MFG-E8-Fc proteins. **B:** Bar graphs show the increase of the geometric mean fluorescence intensity (GMFI) from duplicate results in 2 independent experiments.

As a matter of caution, it was important to determine that the effect of dendritic cell maturation upon removal of the inhibitory Fcγ receptor was as a result of apoptotic cells being cleared through activating Fcγ receptors in its absence and not due to the cross-linking of the Fcγ receptors by the presence of the DN-MFG-E8-Fc protein on the cell surface. Previous studies have shown that targeting antigens to Fcγ receptors on mouse bone marrow-derived dendritic cells leads to the significant enhancement of cross-presentation [209]. To confirm this, and eliminate the possibility that the result shown in this study was not due to the effect of cross-linking the DN-MFG-E8-Fc on BMDC, BMDCs were treated in the presence of DN-MFG-E8-Fc which had been immobilised onto a tissue culture plate.

As shown in figure 5.9, DN-MFG-E8-Fc protein efficiently bound to the tissue culture plate surface, the saturated concentration (30 μg/ml) was then used in the subsequent maturation assay.

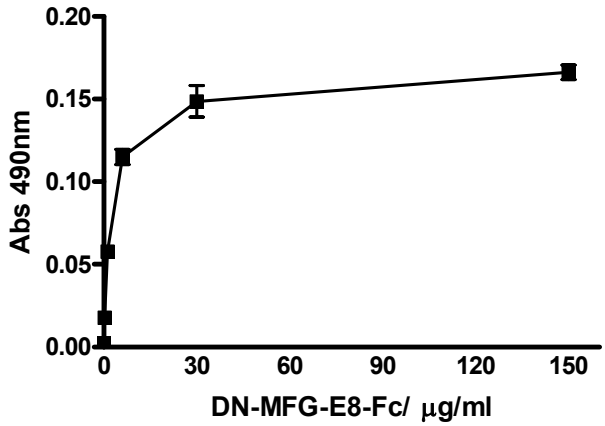

Figure 5.9 Saturation binding curve of DN-MFG-E8-Fc to a tissue culture plate. Graph showing the results of a sandwich ELISA performed to determine the concentration of DN-MFG-E8-Fc protein required to saturate a 24 well tissue culture plate.

Both WT and FcγRIIb$^{-/-}$ BMDCs were placed onto immobilised DN-MFG-E8-Fc protein for 18 h, after which they were then analysed for the surface expression of both CD86 and CD70 using flow cytometry. Cross-linking the Fcγ receptors on either WT or FcγRIIb$^{-/-}$ BMDCs using immobilised DN-MFG-E8-Fc protein was unable to induce a significant change in either CD86 or CD70 surface expression (figure 5.10).

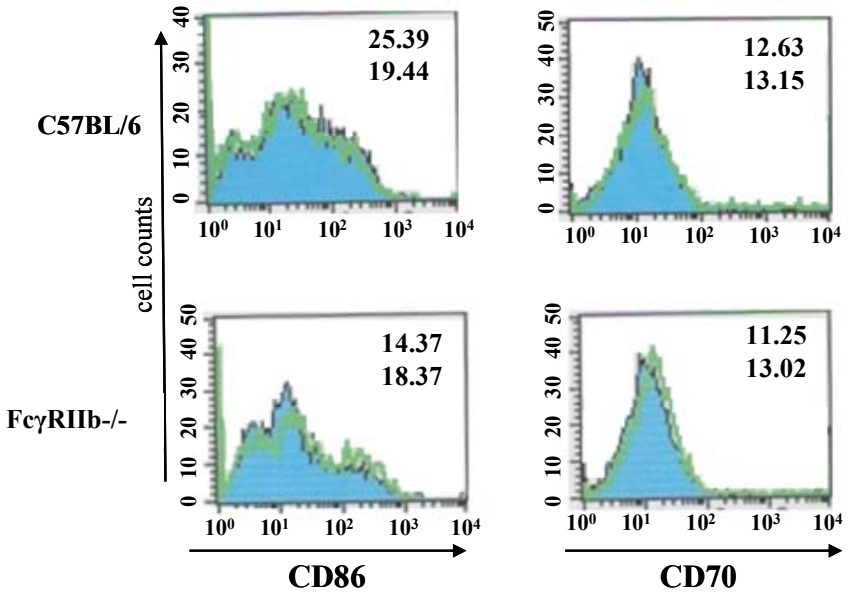

Figure 5.10 Effect of cross-linking Fcγ receptors on BMDCs with DN-MFG-E8-Fc protein *in vitro*. Histograms representing the effect of cross-linked DN-MFG-E8-Fc protein on surface expression of CD86 and CD86 on both CF57BL/6 and CD32b$^{-/-}$ CD11c+ BMDCs *in vitro*. 10 μg/ml fluorescently labelled anti CD11c (FL-4), anti-CD86 (FL-1) and anti-CD70 (FL-2) was used in the assay. Top number indicates geometric mean fluorescence (GMFI) of control BMDC histogram. Bottom number indicates GMFI of treated BMDC histogram.

5.5 Immunising mice with apoptotic cells suppresses antibody production in mice.

The exposure of PS on apoptotic cells is a key factor in inhibiting inflammation, a response which is required for the survival of antigen-specific T cells and the subsequent activation of the adaptive immune response [210]. Additionally, the clearance of apoptotic cells via PS has also been shown to have an effect on the humoral immune response, where by the presence of apoptotic cells dampens the production of serum IgG antibodies; a response which is eliminated upon blockade of PS on apoptotic cells with annexin V [167]. The clearance of apoptotic cells via PS induces anti-inflammatory responses including the release of TGFβ. Studies by Hoffman *et al* [178] have shown that mice immunised with PS liposomes prior to antigenic challenge showed a reduction in the amount of antibody produced against the antigen by approximately 1.5 fold. Similarly, work by Gray *et al* [211] demonstrated that the administration of apoptotic thymocytes up to 4 weeks prior to challenge with collagen-induced arthritis, showed enhanced protection against disease severity through the generation of regulatory B cells and subsequent IL-10 production. These studies would suggest that the presence of PS is, alone, able to suppress an inflammatory response against an antigen and as consequence promotes the release of anti-inflammatory cytokines, leading to a reduced humoral response.

To observe the effect of blocking PS on apoptotic cells using either DN-MFG-E8 or DN-MFG-E8-Fc protein on the levels of serum IgG generated in response to antigen, BALB/c mice were immunised with a strong immunogen, KLH (Keyhole limpet Haemocyanin) which was suspended in complete Freund's adjuvant (CFA). Syngeneic apoptotic thymocytes were then either left untreated or were treated with 100 μg/ml DN-MFG-E8 or DN-MFG-E8-Fc protein. 2×10^7 thymocytes were then administered intravenously per BALB/c mouse, whilst at the same time being challenged with KLH in CFA. Days 1 and 2 post challenge, mice were again treated

with apoptotic thymocytes as described for day 0. On days 10 and 20, the serum was then analysed for anti-KLH antibodies.

As shown in figure 5.11, by day 10 the blockade of PS on apoptotic cells with either DN-MFG-E8 or DN-MFG-E8-Fc proteins promoted enhanced anti-KLH antibody production compared to the control or apoptotic cells alone. This was further enhanced by day 20, where by the treatment of mice with apoptotic cells alone lead to reduced antibody production compared to the treatment of KLH alone, or upon PS blockade.

There is a non-significant trend observed in this experiment which supports that PS exposure on apoptotic cells is able to dampen the humoral response against a model antigen. Additionally, the blockade of PS on apoptotic cells is able to alleviate this response, and in fact suggests that a modestly enhanced antibody response against a non-specific antigen can be achieved by simple blockade of PS alone.

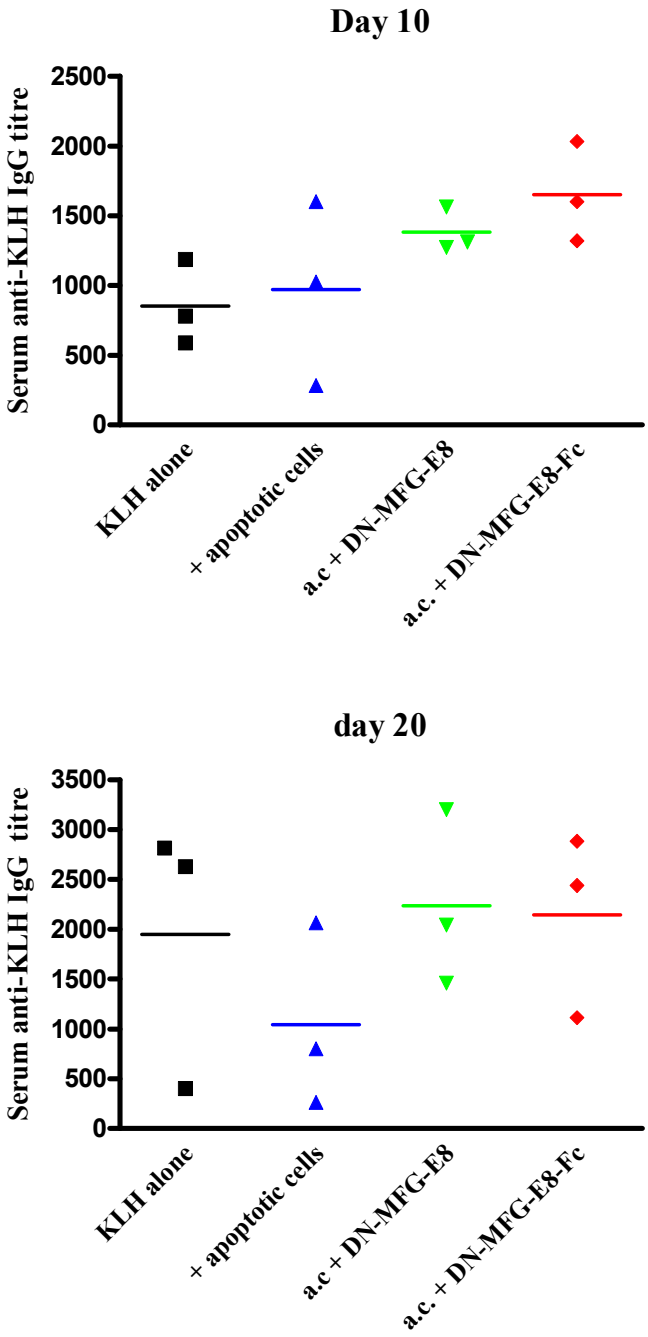

Figure 5.11 Influence of PS blockade on apoptotic cells on the primary humoral immune response. Graphs representing the relative titre of anti-KLH antibodies in the serum detected by anti-KLH sandwich ELISA day 10 and day 20 post KLH challenge where BALB/c had been injected on day 0, 1 and 2 with apoptotic cells either untreated or treated with DN-MFG-E8 or DN-MFG-E8-Fc protein in 200 µl PBS per BALB/c mouse. Graphs show comparable levels of anti-KLH antibodies at a standard absorbance (0.15 on day 10 and 0.210 on day 20 at 490nm). Each point represents one mouse.

5.6 Discussion

A potentially important role of dendritic cells for use as a vaccine in cancer immunotherapy is attributed to their ability to process and present cell-associated antigens, including those associated with tumours [212]. Depending on the milieu of costimulatory molecules and cytokine profile, dendritic cells have the capacity to either promote or inhibit tumour immunity [29, 199]. One of the caveats when designing a dendritic cell-based vaccine is the requirement for an adjuvant (either endogenous or exogenous) to be present at the time of antigen capture and presentation, to ensure that the balance between tolerance and immunity is tipped towards the latter. Matzinger, among others, initially proposed the danger hypothesis, where by immunity only ensues when there is a risk to the host [213]. This includes the presence of factors such as inflammatory cytokines, CD40L, or viral and microbial products [44]. Although this is not optimal when generating cancer vaccines, it is of key importance to the host in maintaining peripheral tolerance to self antigens.

This chapter investigated whether an immune response against a cell-associated antigen could be generated in the absence of an adjuvant by the manipulation of the classical immune tolerance pathway, used primarily to remove apoptotic cells, with the aim of applying this technique to apoptotic tumour cell removal; thereby enhancing their immunogenicity.

To examine the effect of re-directing apoptotic cell clearance via a pathway other than phosphatidylserine, initial experiments were performed to establish a system in which the ability of cell-associated antigen to be cross-presented to antigen-specific CD8+ T cells could be determined. In the first instance, apoptotic OVA-expressing tumour cells (EG.7) which were treated with DN-MFG-E8-Fc were presented more efficiently via splenic antigen presenting cells to OVA-specific CD8+ T cells *in vitro* when compared to apoptotic tumour cells alone (figure 5.1 and 5.2). As explained previously, this assay did not eliminate the possibility of direct antigen presentation, nor could the number or type of cells which were

processing and presenting the antigen be accurately determined. There may have in fact been no dendritic cells present in this assay, suggesting that this not an appropriate model for this study. To improve this assay further the rest of this study focused on the use of targeting apoptotic cells specifically to dendritic cells for use as a source of antigen presenting cell. Only a small number of dendritic cells can be found within lymphoid organs, skin and mucosa. It is therefore difficult to isolate a sufficient quantity of dendritic cells to be used as effective vaccines. For this reason, murine bone marrow was cultured in the presence GM-CSF *in vitro*. This generated a large number of dendritic cells derived from haematopoietic stem cell precursors which are capable of cross-presenting antigen to cytotoxic CD8+ T cells [214].

As shown in section 5.2.2, BMDCs loaded with apoptotic cells were able to effectively cross-present apoptotic cell-associated antigen to CD8+ T cells *in vitro* when loaded with apoptotic cells via either PS or PS-independent pathways; however in contrast to the first experiment, the level of CD8+ T cell proliferation was not enhanced in the presence of either DN-MFG-E8 or DN-MFG-E8-Fc protein. One possible explanation for this is that the splenic APCs used in section 5.2.1 had a more immature phenotype compared to cultured BMDCs used in this assay, allowing more antigen loading and subsequent presentation to the T cells. Further investigation showed that the loading of BMDCs in the absence of the Fcγ receptors (γ-chain knockout mice) still conferred effective antigen cross-presentation of apoptotic cell associated antigen when apoptotic cells were treated with DN-MFG-E8-Fc protein (figure 5.4). This suggested that the clearance pathway being utilised could in fact be via the interaction with the only remaining Fcγ receptor, inhibitory FcγRIIb.

To examine what effect both proteins would have on MHC class I-restricted antigen presentation in an *in vivo* setting, apoptotic cells were treated with either DN-MFG-E8 or DN-MFG-E8-Fc protein prior to their administration *in vivo*. Their ability to then be processed and presented by endogenous APCs was however unaffected when compared to the injection

of apoptotic cells alone, as determined upon comparison between the proliferation of adoptively transferred OT-I CD8+ T cells (figure 5.5). It is possible within this setting that there may have been competition with PS on the apoptotic cells with other PS binding proteins upon administration *in vivo*. As explained in chapter 4, apoptotic cell clearance in the peritoneal cavity after treatment with DN-MFG-E8 protein was unable to inhibit phagocytosis for a sustained period of time, suggesting the displacement of the protein with other high affinity proteins such as TIM-4, which is expressed by resident peritoneal macrophages [96]. To overcome this problem, BMDCs were loaded with apoptotic cells *in vitro* prior to their administration *in vivo*, thereby ensuring that re-direction of the apoptotic cells in the presence of both recombinant proteins was unaffected by other PS-binding proteins.

Upon transfer of apoptotic cell-loaded BMDCs *in vivo* it was apparent that the loading of apoptotic cells via a PS-independent pathway (using DN-MFG-E8) or via Fcγ receptors (using DN-MFG-E8-Fc) caused inhibition of effective T cell stimulation (figure 5.6 and 5.7). This suggested that either the route of antigen processing and presentation utilised via different clearance receptors altered the ability for the dendritic cells to stimulate the CD8+ T cells, or that by changing the receptors used for apoptotic cell clearance, caused a phenotypical change in the DC leading to their loss of ability to migrate to lymphoid organs.

DCs capture antigens through multiple endocytic pathways such as phagocytosis and macropinocytosis; both of these pathways of which are reliant on the rearrangement of the actin cytoskeleton [215]. In addition to endocytosis, the actin cytoskeleton is also required for DC migration [216] where by actin polymerisation and depolymerisation generates extensions, called podosomes, which induce DC movement towards the advancing edge of the cell [217, 218]. Upon DC maturation, podosome formation is lost [30], because they have assumingly reached their destination for antigen presentation.

DC migration is imperative for the activation of lymphocytes within secondary lymphoid organs with antigenic material that has been captured and processed from the periphery (as reviewed in [205]). The access of CD8α+ DCs into the spleen occurs upon their maturation by their increased expression of the chemokine receptor, CCR7 [219], which promotes their migration towards CCL19 and CCL21 gradients, produced by stromal cells within the T cell zone [220]. Although it is not possible to determine from the experimental data obtained thus far, a potential hypothesis for the results observed here could suggest that apoptotic cell clearance in the presence of either DN-MFG-E8 or DN-MFG-E8-Fc protein inhibited an essential cell-cell interaction which is vital for the upregulation of chemokine receptors, such as CCR7.

An *in vitro* experiment investigating the upregulation of costimulatory molecules on dendritic cells (figure 5.8) confirmed that apoptotic cells cleared via Fcγ receptors in the presence FcγRIIb exhibited a lower maturation phenotype, as determined by a reduction in the level of both CD86 and CD70 surface expression; which could be rescued by the removal of FcγRIIb. T cells which are primed by mature dendritic cells, as determined by their increased surface expression of MHC and costimulatory molecules, are able to proliferate and survive more efficiently than those stimulated by immature dendritic cells [26]. The signalling through TLRs or the ligation of CD40 on dendritic cells with CD40L, provided by CD4+ T cell help, has shown to promote the upregulation of costimulatory molecules including CD80, CD86, CD70, 4-1BBL, and OX40L on the surface of APCs. Studies have also shown that the expression of CD70 on dendritic cells through either CD40 or TLR stimulation is also able to promote CD8+ T cell responses without the requirement for CD4+ T cell help [221]. This study now suggests that the upregulation of both CD86 and CD70 can be controlled by the balance between the surface expression of activating and inhibitory Fcγ receptors when apoptotic cells are targeted through these receptors.

CD86 binds to CD28 on T cells and promotes cell-cycle progression and survival of primed T cells whilst CD70 interacts with the CD27 receptor on T cells, promoting their survival during successive divisions. This is supported by evidence where CD70 blockade leads of the inhibition of both primary and memory CD40-mediated CD8+ T cell responses [200]. CD28 and CD27 are constitutively present on naïve CD8+ T cells, highlighting their importance in T cell priming. Together these signalling pathways are complementary in determining the magnitude of both effector and memory CD8+ T cell responses [222].

Finally, this study focused on the role of apoptotic cell clearance on the humoral immune response. Previous studies have shown that the masking of PS on apoptotic cells with a dominant-negative MFG-E8 protein promoted a reduction in apoptotic cell clearance and lead to the production of autoantibodies against phospholipids and DNA [134]. It is clear from this data and work by others, that the exposure of PS on apoptotic cells is, alone, able to prevent inflammation and suppress the development of antibodies against self or a model antigen. The results in this study also suggest a similar phenomenon, where by the blockade of PS on apoptotic cells with either DN-MFG-E8 or DN-MFG-E8-Fc proteins reduced the immunosuppressive effect of using apoptotic cells alone to suppress the development of antibodies against a model antigen (KLH). (figure 5.11).

It is clear from the results obtained in this chapter that the type of apoptotic cell clearance pathway utilised by dendritic cells is able to have numerous and unpredictable outcomes. The concept that simple blockade of PS alone is sufficient to enhance the immunogenicity of apoptotic cells does not account for a number of factors which have been encountered here when this system is tested *in vivo*. The manipulation of antigen processing, presentation, subsequent DC maturation and migration to secondary lymphoid organs weaves a complex web of pathways, the control of which requires that each pathway behaves in a predictable fashion. The use of dendritic cells as potential cancer vaccines must therefore account for all of these processes, as each one is critical in obtaining a therapeutic result.

General Discussion

The clearance of apoptotic cells is a highly controlled process of removing unwanted, unnecessary, or potentially dangerous cells without invoking an inflammatory response. This essential function of the immune system only becomes obvious when this understated, yet complex process is disrupted. Defective apoptotic cell clearance is strongly associated with the pathogenesis of a number of diseases such as systemic lupus erythematosus (SLE) [70], type I diabetes, cystic fibrosis [223], and obstructive pulmonary disease (COPD) [224].

Ensuring that cells which undergo apoptosis are cleared by phagocytes both efficiently and rapidly is the shared responsibility between a number of ligands and opsonising bridging proteins [225]. MFG-E8 has been characterised in detail as a protein secreted by a sub population of macrophages and immature dendritic cells [196] that acts to provide a bridge between phosphatidylserine on apoptotic cells and integrins on phagocytes to facilitate their uptake [98]. An early event in apoptosis is the exposure of phosphatidylserine on the outer plasma membrane [177]. Removal of cells via this pathway promotes tolerance, whereby cell-associated antigens which are processed and presented by APCs to CD4+ and CD8+ T cells in the absence of costimulation, whilst also in the presence of anti-inflammatory cytokines such as IL-10 and TGF-β, promotes T celldeletion or anergy. Whilst this favours protection from autoimmunity, this immunosuppressive environment highly favours the success and advancement of tumours by preventing effective anti-tumour immunity [199].

Mice deficient in receptors which engage with PS on apoptotic cells and mediate their clearance have highly variable and contrasting outcomes. Whilst MFG-E8-/- mice develop auto-antibodies and splenomegaly in an age-dependent manner [126], CD14-/- mice which are also deficient in apoptotic cell clearance do not develop an obvious autoimmune response,

owing to the compensatory anti-inflammatory cytokines which are released by macrophages as a result [106]. Similarly CD36-/- mice have no signs of defective phagocytosis or antigen cross-presentation [193], clearly suggesting a redundancy in this clearance pathway. However, in stark contrast to these models is the role of the complement protein, C1q. C1q binds to calreticulin on apoptotic cells and facilitates their phagocytosis through the interaction with CD91 [87]. Additionally, C1q also binds complement where IgM antibodies bind to apoptotic cells which then recruit C1q. C1q-deficiency, both in mice and humans, leads to excessive inflammation and the onset of SLE-like autoimmunity [226]. Whilst the mannose-binding lectin, MBL, also interacts with CD91 to mediate apoptotic cell clearance, its deficiency, unlike C1q deficient mice, does not lead to autoimmunity. It appears therefore in this model that the binding of complement could be important in the role of preventing autoimmunity as opposed to the successful clearance of apoptotic cell bodies [87, 194].

The therapeutic potential of using proteins which abrogate the normal clearance pathway of apoptotic cells has been discussed in the scientific community for over a decade, the ultimate aim being to provide improvement of cancer immunotherapy techniques and to allow a clearer understanding of autoimmune disease models. Interfering with the recognition of PS on apoptotic cells promotes the reduction of phagocytosis by macrophages, a relative increase in their clearance by dendritic cells, and modulates inflammatory responses, all of which suggest a potential therapeutic benefit in the development of effective cancer vaccines. Previous studies have shown that interference of this pathway with annexin V abrogates effective clearance of apoptotic tumour cells and as a consequence, enhances their immunogenicity [167].

The advantage of using apoptotic tumour cells in the development of cancer immunotherapy is owing to the fact that whole cells contain a milieu of cancer epitopes specific for their unique mutated sequences. They also have the added advantage of being able to generate both CD4+ and CD8+ T cell responses without the need to know which epitope is

responsible. The aim of this study was to elucidate the potential role of MFG-E8 protein in the clearance of apoptotic cells, and if proven to be important, attempt to manipulate this system in a manner which could be used to render apoptotic tumour cells immunogenic.

The use of a dominant-negative MFG-E8 protein to study the effect of PS-mediated apoptotic cell clearance was first accomplished by Asana *et al* [134]. In this study they generated a dominant-negative MFG-E8 protein which had a mutation in the RGD motif which is required to bind to integrins on phagocytes. Here the masking of PS on apoptotic cells lead to defective apoptotic cell clearance and the development of antibodies against DNA and phospholipids. Inspiration from their study initiated the hypothesis for this study, in which the blockade of PS on apoptotic cells could lead to the development of an immune response against a cell-associated antigen.

It is widely accepted that the immunisation with an antigen alone is not sufficient to stimulate an immune response against the antigen, and can instead induce a state of tolerance. To elicit an effective immune response the presence of an adjuvant, whether it be exogenous or endogenous, is typically required at the time of antigen exposure [227]. It has been postulated that cell necrosis may explain how activation of the adaptive immune system occurs in an apparently pathogen-free environment, such as allograft transplantation or in therapy-induced tumour rejection [228]. Apoptotic cells which are not cleared rapidly lose their cellular integrity and release their contents into the local environment; a process referred to as secondary necrosis. Necrotic cells release danger signals into the local environment that provide a source of endogenous adjuvant which are absent from apoptotic cells. HMGB1, uric acid, gelectins, S100 proteins, thioredoxin, and heat shock proteins (HSPs) are all released from necrotic cells and activate the innate immune system [62, 227, 229, 230]. Additionally, HMGB1 and HSPs have also been shown to stimulate the adaptive immune system via signaling through TLR2 and TLR4 [231, 232].

More recent data have now suggested that late apoptotic/secondary necrotic cells express a ligand which binds to dendritic cells and promotes effective antigen cross-presentation. This ligand, identified as a cytoplasmic ubiquitous preformed acid-labile protein-associated ligand, becomes accessible upon cell necrosis for binding to a C-type lectin on CD8α+ murine dendritic cells called DNGR1 (also called CLEC9A). The signaling through DNGR1, like the mannose receptor [21], was shown to divert antigenic cargo away from lysosomal compartments to allow their retrieval for cross-presentation [233]. This data would support the hypothesis that apoptotic cell blockade, which induces secondary necrosis, would promote enhanced cross-presentation of antigen to generate a cell-associated antigen immune response.

In the first part of this study, recombinant dominant-negative MFG-E8 protein was generated so that it could bind to PS on apoptotic cells but no longer encoded the domains required for binding to integrins on phagocytes. As a result, DN-MFG-E8 protein inhibited PS-mediated apoptotic cell uptake by macrophages by up to 44% in a dose-dependent manner (Figure 4.2 and 4.3), whilst showing a slight enhancement of apoptotic cell clearance by BMDCs (figure 4.7). This blockade was however only transient *in vivo* and could not abolish this clearance pathway completely. Similar observations have also been observed in studies which examined the blockade of apoptotic cell clearance by TIM-4-deficient mice (as discussed by Gordon Freeman, British Society of Immunology, 2008). This suggested that *in vivo*, there is likely to be competition between other PS binding proteins. Inducing enhanced immunity to apoptotic cells may therefore only be achievable if one were to develop a DN-MFG-E8 construct that has a much higher avidity for PS than other PS binding proteins, thereby removing this limiting step.

In parallel, the uptake of apoptotic cells through an alternative clearance pathway was also investigated. The targeting of Fcγ receptors on dendritic cells using antigen complexed to IgG molecules (IgG immune complexes)

has already been shown to be an effective method at eliciting tumour immunity. This finding arose from initial studies which demonstrated that the coating of myeloma cells with tumour-specific antibodies promoted cross-presentation of cellular antigens by DCs that would then elicit a strong CD8+ T cell response, the T cells of which were specific for the cancer-testis antigens expressed in the tumour. This method of generating tumour-specific immunity proved more effective than the use of DCs loaded with peptides or apoptotic tumour cells alone [56]. Further investigation has shown that it is the selective engagement of activating Fcγ receptors on dendritic cells which promotes enhanced antigen cross-presentation [234]. This was supported by data which showed that DCs which were deficient for the inhibitory FcγR, FcγRIIb, which were pulsed *ex-vivo* with immune complexes, were able to generate antigen-specific CD8 T cells *in vivo* specific against a murine melanoma model [46].

With this in mind, a second recombinant DN-MFG-E8 protein was also generated which had the additional capability of binding to Fcγ receptors on APCs, referred to as DN-MFG-E8-Fc. Treatment of apoptotic cells with DN-MFG-E8-Fc protein conferred significantly enhanced uptake by both macrophages and BMDCs *in vitro* (figure 4.5 and 4.7, respectively).

The subsequent treatment of apoptotic cells with either DN-MFG-E8 or DN-MFG-E8-Fc proteins was unable to augment antigen cross-presentation by BMDCs *in vitro* compared to the use of apoptotic cells alone (figure 5.3). The treatment of apoptotic cells with either protein which were then administered *in vivo* was also unable to mask or promote antigen cross-presentation to transgenic OT-I cells when this system was reliant on endogenous APCs. A possible explanation for this is that the transient ability of either DN-MFG-E8 or DN-MFG-E8-Fc protein to block PS recognition on apoptotic cells *in vivo* was not sufficient to augment antigen uptake and cross-presentation to CD8+ T cells due to competition with other PS binding proteins. Interestingly however was that BMDCs which were loaded firstly *in vitro* with apoptotic cells via either a PS-independent pathway or through Fcγ receptors, caused a dramatic reduction

in their ability to cross-present antigen to CD8+ T cell responses *in vivo* (figure 5.6 and 5.7) as determined by the ability of transgenic OT-I cells to both accumulate and proliferate within the spleen. As was postulated in chapter 5, this apparent inhibition of antigen cross-presentation could have been the result of alternative antigen processing within the DC which meant that less antigen was available for cross-presentation. This however would not account for the fact that BMDCs were able to promote OT-I proliferation *in vitro* in the presence of absence of either DN-MFG-E8 proteins. This suggested therefore that the loading of apoptotic cells via these alternative pathways may have had an effect on the activation status of the dendritic cells and their subsequent ability to migrate to the spleen.

After much speculation, a possible explanation for the inhibition of CD8+ T cell responses observed in this study came to light by the comparison of dendritic cell maturation upon apoptotic cell loading in either the presence or absence of the inhibitory Fcγ receptor (FcγRIIb). Whilst activating Fcγ receptors on dendritic cells have been shown to be effective targets for enhancement of antigen loading and presentation, research has also shown that the presence of the inhibitory Fcγ receptor is able to determine whether this response ensues or is rendered tolerant [202].

It was clear from phagocytosis studies that γ-chain deficient BMDCs which only express the inhibitory Fcγ receptor were still able to phagocytose apoptotic cells more readily in the presence of DN-MFG-E8-Fc protein (figure 4.9) and additionally were still able to cross-present antigen to OT-I cells *in vitro* (figure 5.4). To evaluate whether the balance between activation and inhibitory signals through FcγRs on dendritic cells is important in determining dendritic cell maturation, DCs were generated from bone marrow-derived from either C57BL/6 WT or FcγRIIb-deficient mice.

In comparison to LPS-induced maturation, WT BMDCs were inefficiently matured in response to apoptotic cells cleared in the presence of DN-MFG-E8-Fc protein (figure 5.8) as determined by their surface expression of

CD86 and CD70. This is consistent with previous reports which target IgG immune complexes to WT BMDCs *in vitro* and examine their maturation [46]. However, FcγRIIb-/- BMDCs displayed a maturation phenotype that was comparable to that seen with LPS when apoptotic cells were phagocytosed in the presence of DN-MFG-E8-Fc protein, showing upregulation of both CD86 and CD70 surface expression (figure 5.8). The possibility that FcγRIIb-/- BMDCs were more sensitive to maturation in general could be excluded by the fact that FcγRIIb-/- BMDCs showed equivalent maturation in response to LPS compared to WT controls.

Although the main focus of this study was to determine whether a CD8+ T cell response could be generated against apoptotic cell-associated antigens without the requirement of additional exogenous adjuvants, it was also of interest to examine what effect these proteins could have on the humoral immune response which can also be manipulated by the presence of apoptotic cells. The exposure of PS on apoptotic cells has been shown to alleviate both inflammation and the generation of antibodies against self or model antigens [178, 211]. In support of this, the blockade of PS with either DN-MFG-E8 or DN-MFG-E8-Fc protein alleviated the ability of apoptotic cells to suppress the generation of antibodies against the model antigen, KLH (figure 5.11).

This study, with the corroboration of others, signifies the ever apparent stark contrast between an immune response observed *in vitro* to that observed *in vivo*. The loading of dendritic cells ex vivo with apoptotic tumour cells for use as tumour vaccines will eradicate the potential interference of macrophages and other phagocytes, whilst also ensuring that dendritic cells are functionally mature before being administered, so as to limit the risk of inducing further tumour tolerance. It is clear that the route of antigen uptake, the amount of antigen load and the subsequent dendritic cell maturation, are all vital components for generating immunity. In particular, this study, again supported by other groups [46] has shown that Fcγ receptor-mediated DC maturation *in vitro* is critically dependent on the balance between opposing activating and inhibitory receptors. The

pivotal role of FcγRIIb in this process is clearly paramount where by studies which involve loading of DCs ex vivo with tumour antigens prior to administration *in vivo* have only been successful using either DCs from FcγRIIb-deficient mice [46], or when an additional adjuvant such as TLR ligand or anti-CD40 has been used to ensure activate DC maturation [235]. Research has already started to focus; the aim of a particular study was to identify IgG1 Fc domains which showed improved binding to the low-affinity activating Fc receptor FcγRIIIa and reduced binding to the low-affinity FcγRIIb [236].

In order to safely and effectively use apoptotic tumour cells as a source of antigen for cross-priming by dendritic cells in tumour therapy, further investigation into mechanisms which control receptor-mediated clearance must be further investigated. The milieu of factors which can influence the immune outcome *in vivo* must also raise caution when trying to make predictions from *in vitro* data.

Future work

This work, supported strongly by a recently published study [237] would suggest that the application of either DN-MFG-E8 or DN-MFG-E8-Fc proteins is, alone, likely to prove difficult in generating an effective cancer immunotherapy. This does not however rule out their potential use in combination with other cancer therapeutic regimens such as chemotherapy. A recent study has shown that an anti-MFG-E8 antibody used in combination with the chemotherapy drug, gemcitabine, worked effectively in a murine colon carcinoma model, showing long term protection in a synergistic manner. Interestingly, its effectiveness was most dramatic when anti-MFG-E8 antibody was administered either at the same time, 3 or 7 days post treatment with gemcitabine [237]. Their study, as well as the results obtained within this study suggests that a cohort of different variables *in vivo* dictate the immune outcome.

In order to take this research forward, it would be of key interest to determine whether the treatment of either DN-MFG-E8 or DN-MFG-E8-Fc proteins used in parallel with a chemotherapy drug in a murine tumour model is able to synergistically induce tumour regression and potentially long term survival. A question which immediately arises from the study using an anti-MFG-E8 antibody with gemcitabine, is why the combination therapy is only successful when the antibody is used at particular time points throughout the experiment.

The amount or type of tumour cell death caused in response to the chemotherapy drug will affect the level of binding of the MFG-E8 protein to bind to phosphatidylserine on apoptotic cells. Similarly, the plethora of cytokines and chemokines which are present during the treatment course are also likely to vary, again, influencing the possible effectiveness of either PS blockade or the clearance of tumour cells through Fcγ receptors on phagocytes.

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