Modulation of Dendritic Cells and Autoimmunity by Apoptotic and Necrotic Cells

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List of Abbreviations

AC	Apoptotic cell
Ag	Antigen
ANOVA	Analysis of variance
AnV	Annexin V
APC	Antigen-presenting cell
B6	C57Bl/6 strain of mouse
BMDC	Bone marrow-derived dendritic cell
CD(e.g. CD40)	Cluster of differentiation
CM	Conditioned media
ConA	Concanavalin A
CpG	Oligodeoxynucleotide (ODN) of unmethylated CpG
CytD	Cytochalasin D
D e.g. D6	Day
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
F/T	Freeze-thaw cycle
FACS	Fluorescence-associated cell sorting
FBS	Fetal bovine serum
FDCP	Factor-dependent cell Paterson 1
GMCSF	Granulocyte macrophage colony stimulating factor
GP	Glycoprotein
Gp33	Peptide 33-41 (immunodominant epitope) of the LCMV GP
GVHD	Graft versus host disease
HK	Heat kill (specifically, 30mins at 60°C)
HSP	Heat shock protein
ICOS	Inducible co-stimulatory
IFNγ	Interferon γ
IL (e.g. IL6)	Interleukin
IL12p40	Subunit p40 of IL12
ILN	Inguinal lymph node

IP	Intraperitoneal
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
MACS	Magnetic bead-associated cell sorting
NC	Necrotic cell
PAMP	Pathogen-associated molecular pattern (inc. LPS and CpG)
PDLN	Pancreatic draining lymph node
PI	Propidium Iodide
PRR	Pattern recognition receptor
PS	Phosphatidylserine
RIP	Rat insulin promoter
ROS	Reactive oxygen species
SC	Subcutaneous
SFasL	Super Fas ligand
SLE	Systemic lupus erythematosus
TGFβ	Transforming growth factor β
Thym	Thymocyte
TLR	Toll-like receptor
TNFα	Tumour necrosis factor α
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
Treg	Regulatory T lymphocyte
UVC	Ultraviolet light, shortwave (280 nm–200 nm)

NB. 'Current study/investigation/etc' refers to that detailed in this thesis.

Abstract

Modulation of Dendritic Cells and Autoimmunity by Apoptotic and Necrotic Cells

As the principal antigen-presenting cells to T cells, dendritic cells (DCs) have a key role in the balance of immunity and autoimmunity. They are essential in two major, converse roles – eliciting T cell immune responses to pathogenic material, and maintaining peripheral tolerance to self-tissue by inhibiting self-reactive T cells. These functions involve the processing of pathogenic or self antigens and subsequent presentation of antigenic peptides on MHC to antigen-specific T cells. DC recognition of conserved pathogenic markers induces a mature phenotype that governs immunogenic presentation to T cells and, consequently, the adaptive immune response. In contrast, DC recognition of self tissue suppresses maturation, instead inducing a tolerogenic phenotype that induces self antigen-specific T cell to die, become anergised, or converted to T regulatory cells. Apoptotic cells are the major source of self-antigen for the maintenance of peripheral tolerance, and their defective clearance by DCs is implicated in autoimmunity. Apoptotic cells are thought to actively suppress maturation of DCs and inhibit the possible immune responses promoted by proinflammatory mediators released from necrotic cells. However, the immune function of apoptotic cells and their relative influence over necrotic cells are highly contested, partially due to the complex nature of immunogenicity arising from the sourcing and generation of apoptotic cells.

In this investigation, various methods of inducing apoptosis and necrosis are evaluated. Definitive methods of inducing well-characterised cell death are then employed to compare the effects of apoptotic and necrotic cells on dendritic cells and *in vitro* and *in vivo* immune responses. Reported here are *in vitro* findings that support previous reports of the anti-inflammatory response of DCs to apoptotic cells, and the inflammatory response of DCs to necrotic cells. The previously-reported inhibitory effect of apoptotic cells on LPS-induced secretion of Th1 cytokines is supported here, but the inhibitory effect of apoptotic cells on LPS-induced upregulation of co-stimulatory molecules is contested. Novel findings describe the upregulation of DC expression of co-inhibitory molecules induced by both apoptotic cells and necrotic cells. Apoptotic cells, but not necrotic cells, had a suppressive effect on CpG-induced upregulation of co-stimulatory molecules and pro-inflammatory cytokines. Apoptotic cells suppressed the capacity of untreated and CpG-treated, but not LPS-treated, DCs to elicit IFNγ production by T cells. Apoptotic cells, but not necrotic cells, induced regulatory T cells and partially restored their CpG-suppressed induction. Finally, apoptotic cell-modulation of DCs inhibited the induction of autoimmunity in a novel modification of an *in vivo* model of diabetes. Interestingly, novel evidence for the possibility of necrotic cell-induced tolerance by means of direct T cell killing is addressed.

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"He is brave who knows what is sweet and what is bitter in life,

and goes out undeterred to meet both."

Pericles.

CHAPTER 1 – Introduction

1.1 Dendritic cells

1.1.1 Overview of the roles of dendritic cells

Dendritic cells (DCs) are antigen-presenting cells (APCs) which, upon detection and engulfment of pathogenic material, undergo a process of maturation whereby they up-regulate surface expression of co-stimulatory molecules and production of proinflammatory cytokines. DCs present antigen to antigen-specific, naïve T cells leading to T cell activation and proliferation – the initiation of the adaptive cell-mediated immune response. DCs are the principal initiators of the adaptive T cell response due to their unequalled proficiency in presentation of antigenic peptides on MHC to T cells. This same ability also ensures DCs' crucial function in maintaining peripheral tolerance. In this role, immature DCs take up antigen by clearance of apoptotic cells, then process and cross-present antigenic peptides to cytotoxic T cells (1). Because dendritic cells that have ingested apoptotic cells do not mature, and therefore do not express the 'second signal' of co-stimulatory molecules, autoantigen is presented in a tolerogenic manner which causes the anergy or deletion of autoantigen-specific (autoreactive) T cells. In this way, peripheral tolerance is maintained. Presentation by mature DCs and immature DCs is therefore synonymous with immunogenic and tolerogenic presentation, respectively, and thus perturbations in the maturation status of DCs can profoundly alter the immunological outcome (2).

A number of endogenous substances have been identified as being immune regulators in that they are capable of initiating or inhibiting DC maturation. Alarmingly, self-reactive T cells that have escaped the regulatory mechanism of thymic deletion have only to be presented with antigen in the right stimulatory circumstances for autoimmunity to ensue (3). The pivotal role of DCs in the prevention of autoimmunity by maintaining peripheral tolerance has been demonstrated clearly (1, 4). In this role, DCs phagocytose dead cells, both apoptotic and necrotic. The role of dead cells in peripheral tolerance and immunity is highlighted in numerous associations between various autoimmune diseases and defective clearance of apoptotic cells (5-8), and the interaction between dead cells and dendritic cells has been shown to induce diverse immunological outcomes (1, 9-16).

Due to ever increasing evidence, a widely accepted dogma exists that states that apoptosis is an anti-inflammatory, tolerising stimulus (13, 17), and necrosis is a proinflammatory, immunity-activating stimulus (18). The increasingly approved view is that apoptotic cells actively inhibit DC maturation, which assists in minimising the possibility of immunogenic presentation of autoantigens, subsequent inflammation during apoptotic cell clearance, and autoimmunity. However, the field is rife with contradictions and these are discussed later. The dead cell-associated factors that influence DCs in directing the course of the immune response are becoming increasingly identified, and this is also discussed later. However, the effect that these factors have on DCs and how they govern the selection between immunity and tolerance is not clear, and is the subject of this investigation. First, the two responses are discussed in more detail, and then the factors themselves in the context of apoptosis and necrosis are considered, before the controversy over their effects is examined.

1.1.2 Immunogenic dendritic cells

Dendritic cells recognise pathogens via pattern-recognition receptors (PRRs) by binding pathogen-associated molecular patterns (PAMPs), which are highly conserved, recognisable markers on pathogens (2). Toll-like receptors (TLRs) are the predominant family of PRRs, between them recognising a wide variety of both exogenous (e.g. microbes) and endogenous (e.g. heat shock proteins (HSPs)) inflammatory mediators (19).

TLRs play a key role in many PAMP-recognition pathways. TLR2 synergises with CD14 to respond to peptidoglycan and lipoteichoic acid, surface components of gram-positive bacteria, leading to NF-$\varkappa$B activation in macrophages (20, 21). TLR4 recognises LPS from gram-negative bacteria once it is bound to LPS-binding protein (LBP) and leads to inflammation via the NF-$\varkappa$B pathway (22-24). TLR4 also has an important role in IL12 secretion by DCs that have taken up necrotic tumour cells (25). The receptor has been implicated in inflammation by ligation of Hsp60, (26) as has CD14 (27), which is required to associate with TLR4 in the receptor complex that mediates LPS-induced inflammation (28). Incidentally, CD14 can also mediate ingestion of apoptotic cells (29). TLR3 has been shown to promote DC cross-priming of T cells against viruses. TLR3 recognises double-stranded RNA from viruses and directs it into the cross-presentation pathway, so allowing DCs to prime T cells that are specific to viruses that do not directly infect DCs (30). TLR5 is stimulated by bacterial flagellin proteins, which act via TLR5 on DCs to induce surface co-stimulatory marker expression and inflammatory cytokine production (31). TLR7 enables DCs to recognize single stranded RNA and elicit immunity against viruses (32). Furthermore, some guanine nucleoside analogues have been revealed to be ligands for TLR7 (33), so TLR7 may well also be capable of recognising oxidised lipid moieties that are specific to apoptotic cells. TLR9 facilitates the response to non-mammalian DNA as identified by its unmethylated CpG motifs (34).

Ligation of TLRs and other PRRs stimulates DC maturation, which is characterised by up-regulation of co-stimulatory molecules at the cell surface, up-regulation of antigenic peptide-bearing MHC molecules, production of inflammatory cytokines such as IL6, IL12 and TNFα, changes in antigen trafficking and presentation and decreased proficiency in phagocytosis (2). DCs are highly efficient at presenting antigen to both $CD4^+$ and $CD8^+$ T cells. DCs are especially proficient at cross-presentation, where exogenous antigen (i.e. from outside the cell) can enter the endogenous pathway and be presented on MHC class I molecules to $CD8^+$ cytotoxic T cells (35). In this way, DCs mature to become exceptionally adept at priming $CD8^+$ T cells (the cross-presentation pathway is also a mechanism for tolerising CD8+ T cells to self-Ag, which is a constant process in the steady state (36)). The NF-κB signaling pathway is the foremost pathway involved in mature DC immune function, as it is a principal transcriptional regulator of proinflammatory cytokines and other important immunological molecules (37), (38, 39). In this pathway, the intracellular domains of activated TLR receptors recruit MyD88 (or TRIF, in the MyD88-independent pathway, which reconvenes with the MyD88-dependent pathway at NF-κB activation), forming an activation complex involving IRAK and TRAF6. This complex activates IκB kinase β, which phosphorylates the NF-κB inhibitor IκB, leading to the degradation of IκB and allowing NF-κB to enter the nucleus and direct proinflammatory transcription (40).

According to the two-signal model of T cell activation first proposed over four decades ago, T cell activation by APCs requires antigen-specific recognition between the APC MHC-peptide complex and the T cell receptor (TCR), *plus* interaction between co-stimulatory molecules on the APC and their appropriate receptors on the T cell (41). Numerous co-stimulatory molecules have since been identified on DCs, with key molecules including CD40(42), CD83(43), and CD80 and CD86 (also known as B7.1 and B7.2, respectively) (44, 45). All of these molecules engage in complex positive feedback signalling that promotes further activation of both the DC and T cell. CD40 ligation on DCs by the CD40 ligand molecules on T cells, for example, induces increased expression of C80 and CD86 which are prominent in cytotoxic T cell priming and anti-tumour immunity (46, 47).

CD80 and CD86 themselves interact with CD28 on the T cell surface for promoting production of the cytokine IL2, which is crucial for expansion of both T cells and regulatory T cells (Tregs) (48) (though Treg suppressive activity is inhibited (49)). However, CD80 and CD86 also interact with the T cell molecule CTLA-4, which is upregulated once T cells are activated. Signalling via CTLA-4 inhibits T cell proliferation (50) and is essential for Treg function (51). The relatively new molecule CD83 is associated with induction of T cell proliferation and production of IFNγ by active T cells (52). These molecules can serve functions away from the DC-T cell synapse too. For example, by inducing the up-regulation of chemokine receptors on DCs, CD40 signalling aids DC migration to secondary lymphoid tissues where the DC-T cell interactions occur (53).

Many other molecules are up-regulated on the surface of DCs as a result of proinflammatory signalling. CD25 is the receptor for IL2, and the usual source of IL2 for DCs is the antigen-specific T cells with which DCs have begun to interact. Ligation of CD25 leads to increased proinflammatory cytokine production (54) by DCs and is thus a further positive feedback signal mechanism that perpetuates T cell expansion. CD25 is more celebrated in $CD4^+CD25^+$ T regulatory cells, one of the functions of which is to suppress IL2 production (55).

As well as IL2, other DC-produced cytokines are essential inflammatory mediators of the immune response, including IL12, IL6, IL1 and TNFα. IL12 is a well established mediator of inflammation by activating natural killer (NK) cells, generating lymphokine-activated killer cells (LAKs), and inducing T cell proliferation and IFNγ production. It is primarily produced by DCs and other phagocytes upon microbial stimulation (56). Production is then perpetuated and guides Th1 differentiation during DC interaction with T cells (57).

IL6 aids Ig production by B cells (58), enhances proliferation and survival of Th1 and Th2 T cells (59) but preferentially promotes Th2 differentiation (60), and recruits leukocytes to sites of inflammation by modifying the chemoattractant properties of local cells (61), as well as having

effects on many other body systems. Accordingly, IL6-deificient mice have been shown to be unable to control viral and bacterial infections (62).

1.1.3 Tolerogenic dendritic cells

Tolerogenic dendritic cells are currently the subject of intense research and debate. Tolerogenic DCs express co-inhibitory molecules that are thought to counter co-stimulatory molecules in the immunity-tolerance balance. Investigations have shown that these molecules can be contradictory, complex, and certainly controversial, and each is worthy of its own review. For conciseness, only key and representative findings are discussed in detail here.

B7DC (also known as PD-L2) and B7H1(also known as PD-L1) are both expressed on human and mouse myeloid DCs, and both are ligands for the PD-1 (programmed death-1) receptor that is present on T cells and is up-regulated on activated T cells (63, 64). Ligation of PD-1 by either ligand may negatively regulate the activation events induced by IL2 or by ligation of T cell CD28 by the DC co-stimulatory molecules CD80 and CD86 (63) . B7H1 has been shown to dampen immune responses by inhibiting IL12 secretion, increasing IL10 promotion, and promoting generation of Tregs (65). Ligation of PD-1 may also, as the name suggests, lead to direct cell death (66). Tumour cells can take advantage of this mechanism by expressing B7H1, for example, and inducing apoptosis in tumour-specific T cells (67).

However, both molecules have been implicated in immunity. Transgenic overexpression of B7DC on tumour cells in mice significantly enhanced anti-tumour T cell-mediated immunity, though curiously this was via a PD-1-independent mechanism (68).

B7H2 is also known as ICOS-ligand (ICOSL) as it interacts with ICOS on T cells, and its position as either stimulatory or inhibitory has been highly contentious. Extensive study has revealed a

complex and diverse role. Its absence is associated with impaired $CD4^+$ and $CD8^+$ T cell function (69, 70) and T cell-dependent B cell responses (71) . However, investigations suggest that the stimulatory role of B7H2 is restricted to acting on previously-activated T cells, as B7H2-ICOS signalling is not required in early activation events (72) and naïve T cells are low in ICOS (73). Therefore, B7H2 appears to be limited to promoting survival of differentiated T effector cells, rather than activation. In contrast, both Treg activation and expansion is induced by B7H2 expression on myeloma cells, leading to tumour immune evasion (74). Survival of splenic transplant mice was promoted by inhibiting B7H2 from Day 3 after surgery, but this effect was ablated if inhibition was commenced at Day 0 (75). However, Tregs were not involved in this discrepancy between early and late intervention, indicating that Treg induction may not be the only inhibitory effect of B7H2. The role of B7H2-ICOS signaling in both T cells and Tregs is excellently illustrated in work by Prevot et al (2010), who demonstrated protection against diabetes due to defects in diabetogenic effector T cells in $ICOS^{(-/-)}$ NOD, but accelerating diabetes in BDC2.5 $ICOS^{(-/-)}$ NOD mice due to a dominant defect in Treg (76). However, protection against diabetes was accompanied by onset of autoimmunity towards the neuromuscular system, indicating that B7H2 may be more crucial to inhibiting rather than promoting immunity.

Witsch et al (2002) demonstrated on immature human Langerhans cells a low expression of B7H2 that was not up-regulated upon maturation (77). In the interaction of mature DCs with T cells, B7H2-ICOS signaling made no contribution to the up-regulation of IL2 secretion but instead regulated CD28 signalling and helped to promote IL10 secretion. B7H2 has also been associated with tolerogenic DCs in Chlamydia infection (78) and with myeloma immune evasion (79). Despite the historical role of B7H2 in T cell stimulation, more recent investigations are providing increasing evidence of a role of DC B7H2 in Treg induction and tolerance. The opposing roles appear to be separated both temporally and in terms of effector cells.

B7H3 expression is associated with the high resistance of malignant mesothelioma (80), indicative of an immunosuppressive nature. However, transfection of B7H3 into tumour cells can elicit highly effective anti-tumour immunity (81), and its presence on DCs has been reported to stimulate T cell activation and elicit IFNγ production (82). Precisely the opposite results have been reported elsewhere, namely inhibition of T cell activation and cytokine production (83). Interference of B7H3 function has been observed to exacerbate EAE in various models (83, 84). The conflicting evidence may have arisen due to multiple receptors for B7H3 with contrasting functions. Although B7H3 is as yet an orphan ligand, TLT-2 has been identified as a potential receptor for B7H3 in an immunostimulatory role (85), whilst another may yet be discovered to perform an immunoinhibitory function, much like the contrasting functions of CD28 and CTLA-4.

The tolerogenic capacity of B7H2 and B7H3 is highly contested. However, because of increasing support towards prominent regulatory roles, B7H2 and B7H3 are considered in the Results chapters alongside co-inhibitory molecules. Nonetheless, in ascertaining and discussing its role in this investigation, it is considered in the context of all available evidence.

B7H4 is relatively established as a co-inhibitory molecule. Expression of B7H4 is strongly associated with numerous tumours (86-88). Its immunosuppressive effects have been utilised in improving allograft survival in mice (89), but are yet to be seen in suppression of autoimmunity in association with DCs.

Anti-inflammatory cytokines are crucial to tolerance, namely IL10 and TGFβ. IL10 treatment of DCs is sufficient to induce tolerogenesis and prolong survival after intestine allograft (90), and both IL10 and TGFβ is essential for the development of regulatory T cells (91, 92). Another mechanism by which dendritic cells regulate immunity is by expression of the tryptophan metabolising enzyme indoleamine 2,3-dioxygenase. Indeed, ACs have been shown to suppress DCs by IFN-induced IDO (93). IDO is an enzyme that degrades the essential amino acid

tryptophan, and is thought to suppress the proliferation of facultative intracellular pathogens, tumour cells and activated T cells alike by depriving them of the nutrient (94). Amongst the first evidence of an immunoregulatory function for IDO was the finding of its role in inhibiting maternal immunity to foetal tissues (95).

1.2 Apoptotic cells and necrotic cells in the periphery

Apoptotic cells are recognised and selectively phagocytosed by DCs (96) and then cross-presented to T cells (97, 98) in a tolerogenic manner, inducing anergy (99) or deletion (apoptosis) (100, 101) of autoreactive T cells. Animal model studies demonstrate that this is a constitutive and continual process (102, 103).

Just as DCs are subject to endogenous inhibitory modulation, so they may be influenced by endogenous activation signals. Endogenous 'danger signals' represent a possible group of additional stimuli that may play a pivotal role in dichotomising DC responses by receptors that have both pro- and anti-inflammatory functions [Figure 1.1]. The majority of endogenous danger signals are simply intracellular components that elicit an inflammatory response when released from necrotic cells, whereas others are up-regulated during cellular stress and may be secreted to alert the innate immune system (18), rendering otherwise-inhibitory apoptotic cells stimulatory. This may represent a major regulatory system for differentiating between situations that warrant immune responses and those that do not. For that reason, associations between dead cells and danger signals will be examined here.

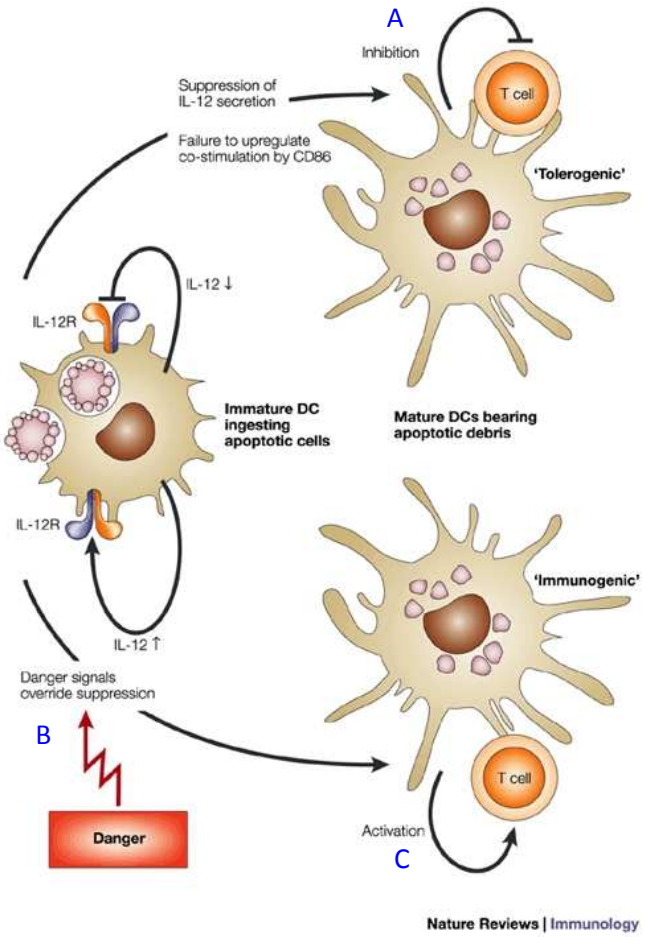

Figure 1.1 Danger signals, immunity and tolerance. Dendritic cells initiate immunity or tolerance to antigens by presenting antigenic peptides to antigen-specific T cells. Usually, peptides from pathogens are presented in conjunction with co-stimulatory molecules such as IL12 and CD86 on 'mature' DCs, leading to T cell activation into armed effector T cells. A) In healthy individuals, uptake of self-antigen from apoptotic cells actively suppresses DC maturation and hence production of co-stimulatory molecules, so self-antigen is presented without co-stimulation, leading to T cell deletion or anergy. B) Danger signals are thought to be capable of inhibiting the suppressive signalling of apoptotic cells. C) Inhibition of DC suppression causes the switching of tolerogenic presentation to immunogenic presentation. Image from (1)

Subsequent to tissue damage or injury *in vivo*, DCs and other cells of the innate immune system are exposed to a broad range of stimuli, most of which is probably endogenous, including autocrine cytokines and cytokines from other innate immune cells. Consequently, DCs are subject to a host of opposing activating and inhibitory signals that compete with each other at either the level of receptor ligation or intracellular signalling. Loss of this balance between these endogenous signals in favour of DC activation may lead to chronic inflammatory disorders and autoimmunity. In contrast, if this balance is biased in tumours towards tolerance, as is often the case, then the immune system fails to elicit an effective immune response against dangerous cells. Ascertaining how these stimuli interact to determine the immunological outcome would help to substantiate the relative significance of each of the large number of recently recognised endogenous stimuli of

DCs. This would consequently help to establish effective methods of DC modulation behaviour *in vivo* for therapy of autoimmune diseases and cancer.

It is important to acknowledge the similar role of macrophages in apoptotic cell clearance. In a model of experimental autoimmune encephalitis (EAE), injection of apoptotic cells pulsed with myelin oligodendrocyte glycoprotein (MOG) antigen induced MOG-specific tolerance and suppressed development of the disease. In this example, tolerance required apoptotic cell uptake by two particular subsets of macrophages in the spleen, as well as by DCs (16).

DCs, with superior antigen-presenting capability, may bear the greater responsibility for induction of regulatory T cells and the persistence of tolerogenesis. The greater importance of macrophages may lie in the initial anti-inflammatory response: DNA from apoptotic cells has been shown to bind MHCII on macrophages and inhibit antigen presentation (104).

1.2.1 Necrosis and endogenous danger signals

Current concepts of immunology revolve around a central principle known as the 'danger model.' The introduction of this model saw a paradigm shift from the notion that the immune system distinguishes between infectious non-self and non-infectious self to the notion that it distinguishes between harmful and non-harmful (105). This model was adopted thanks largely to the fact that it encompassed plausible accounts of transplant rejection, tumour immunity and autoimmunity. This theory also allowed for regulation of adaptive immunity by endogenous factors. It is well documented that certain infections are able to break host tolerance to self-tissues, but endogenous danger signals may also have sufficient influence on DCs to regulate tolerance and immunity (18). Endogenous danger signals are molecules that are derived from the host and are capable of

activating DCs. They are released or produced by cells undergoing stress, damage or abnormal cell death, and can be distinct from the antigen(s) that are subsequently presented to T cells (105).

Necrotic cells are those which have undergone acute, rapid death caused by mechanical damage, osmotic lysis or viral lysis, and are characterised by loss of membrane integrity and subsequent release of intracellular contents. Constitutive danger signals are normal, exclusively-intracellular components released by necrotic cells, hence their extracellular presence conveys information of tissue damage, and therefore infection risk, to DCs, causing their maturation (17, 18). For example, ATP and UTP, components of energy metabolism, can be released from the cell due to inflammation, hypoxia and mechanical stress (106). This causes proinflammatory cytokine production by murine DCs (107) via the DC purinergic receptor P2X7 (108).

Some endogenous danger signals are inducible – during stress, danger signals are newly synthesised or innocuous molecules are modified to become immunostimulatory (109, 110). Covering both the inducible and constitutive classes of danger signals are the heat shock proteins (HSPs). HSPs are the most abundant and conserved proteins in mammals, and are involved in protein folding, protection and chaperoning. HSPs are soluble and restricted to intracellular compartments. During viral infection, the immunogenicity of HSPs from an infected cell *in vivo* is partially explained by the association of HSPs with antigenic peptides specific to the virus. This is supported by reports of the influenza protection elicited by complexes of the HSP gp96 isolated from influenza-infected cells (111), and similarly-induced protection against intracellular bacteria (112). However, HSPs also have innate adjuvanticity. Necrotic cell death releases the HSPs Hsp70, Hsp90, gp96 and calreticulin, which cause murine DC maturation and Nf-κB activation *in vitro* at concentrations comparable to physiological concentrations available after necrotic cell death *in vivo* (113). Gp96 induces DC maturation in both murine and human DCs, as shown by their ability to cause DC secretion of TNFα and IL12, up-regulation of co-stimulatory molecules and improved T cell activating capabilities (114) via TLR2/4 (115). *In vivo*, transgenic overexpression of Hsp70 can

promote autoimmunity (116). Release of HSPs has been implicated in the adjuvanticity of necrotic tumour cells (117). This helps to explain the low adjuvanticity of apoptotic tumour cells, though tumour cells are often found to express remarkably high levels of HSP. It has been shown that increase of Hsp70 content of a tumour cell undergoing heat shock (to increase HSP expression) increases its adjuvanticity substantially (118, 119). It is postulated that surface expression of heat shock proteins by stressed and cancer cells (120, 121) may serve as a mechanism of direct immune activation without the requirement for preceding cell death (122).

Uric acid is recognised as another endogenous danger signal. Uric acid, a constitutive end-product of purine degradation found in the cytosol, can induce inflammation when released from necrotic cells (123). It induces the inflammasome (a signalling pathway, in which caspases are instrumental, leading to IL-1β activation and secretion (124)) via NLRP3 (cias1) (125). The P2X7 receptor is also involved in the inflammasome process (126). Also, cell injury due to DNA and RNA degradation increases uric acid generation and correlates with increased adjuvanticity of stressed cells (127).

DNA acts as a potent endogenous inflammatory mediator, being an obvious sign of cell rupture if located extracellularly, and one that is implicated as a major target of autoantobodies in SLE (128). Chromatin has been identified as an immunogenic constituent of DNA (129). DNAse 1 and the complement component C1q act together to degrade chromatin that has been released from necrotic cells (130). Efficient degradation of DNA reduces the immunogenicity of dead cells, as signified by the systemic autoimmunity that arises in DNAse I-deficient mice, which develop an SLE-like autoimmune illness (131). HMGB1 (high mobility group box 1) is another component of the nucleus whose extracellular presence following necrosis triggers stimulation of DCs [Rovere 2004].

Oxidated LDLs (oxLDLs) are found on apoptotic cell surfaces and are immunogenic and proinflammatory (110). Oxidative damage can sufficiently alter nucleic acids and proteins to cause autoimmunity. In SLE patients some anti-DNA antibodies are found to preferentially bind 8-

hydroxydeoxyguanosine (8OHDG), a product of oxidative damage to guanine (132), rather than normal DNA.

Proteases contribute to the adjuvanticity of necrotic cells. When lysed cells are treated with selected serine protease inhibitors their immunogenicity is abrogated and they are even modulated to act in an anti-inflammatory manner similar to apoptotic cells (133).

Virtually any incidence of infection or tissue damage will inevitably lead to the release of potentially immunostimulatory cell constituents within a proinflammatory environment. However, the prevalence of autoimmunity in humans is relatively infinitesimal given the frequency of such exposure. This demonstrates the importance of peripheral tolerance and the effectiveness with which dendritic cells limit immunogenic presentation to T cells of self-Ag.

1.2.2 Apoptosis and apoptotic cell clearance

Apoptosis is a strictly regulated and complex form of cell death, which is appropriately also known as 'programmed cell death.' Cells may be induced or may self-induce to undergo apoptosis for many reasons, including redundancy, improper development, DNA damage or viral infection. Apoptosis is characterized by autodigestion of the cell due to controlled degradation of intracellular components by endogenous proteases and DNA degradation by endogenous endonucleases. This leads to DNA fragmentation, chromatin condensation, cytoskeletal disruption, cell shrinkage, and cytoplasmic membrane blebbing, but one of the most apparent characteristics is also the most immunologically significant – the preservation of membrane integrity throughout the process (134, 135). Maintaining membrane integrity prevents leaking of potentially inflammatory intracellular contents, and ensures that only apoptotic cell signals are available to local immune cells. One such signal is the outer-leaflet membrane expression of the

phospholipid phosphatidylserine (PS), which in healthy cells is confined to the inner-leaflet and is therefore not accessible (136).

DCs have a number of receptors capable of binding PS. Although external PS is a key marker of apoptotic cells, it is also accessible in necrotic cells. Necrotic cells do not flip PS to the outer-leaflet as it is an active process performed by a lipid scramblase (137) that first requires transcription of apoptosis genes (138), and hence is not possible in necrotic cells. However, necrotic cells lose membrane integrity and thus expose inner-leaflet PS. Therefore, any anti-inflammatory properties that PS has may be countermanded by endogenous danger signals released in necrotic cell lysates, thus PS-mediated anti-inflammatory signalling appears to remain exclusive to apoptotic cells.

Links have been well established now between defective clearance of apoptotic cells and autoimmunity, thanks largely to investigations into the pathology of the autoimmune disease systemic lupus erythematosus (SLE). Defects in apoptotic cell clearance strongly correlate with SLE (5-7, 10). These links are discussed in more detail with the apoptotic cell clearance receptors below. The link between defective apoptotic cell clearance and autoimmune and inflammatory conditions may largely be due to apoptotic cells undergoing secondary necrosis. Secondary necrosis occurs when caspases and intracellular ATP decrease in availability over time, resulting in energy-dependent apoptotic death switching to energy-independent necrotic death (139, 140) – the cells and blebs lose membrane integrity and release intracellular contents into the extracellular milieu. Thus, defective clearance results in an abundance of autoantigens during potentially inflammatory conditions.

1.2.3 DC receptors for dead cells and bridging molecules

Interestingly, some DC surface receptors are receptors for both apoptotic cell clearance and exogenous antigen. To establish how DCs are modulated by the plethora of endogenous factors, it must be verified whether receptors involved in apoptotic cell clearance are ever inflammatory, or indeed if known PRRs are ever involved in apoptotic cell clearance. To address these issues, it is necessary to recognize the receptors involved in apoptotic cell clearance, PAMP/endogenous danger signalling, and to especially note those that are involved in both.

1.2.3.1 Scavenger receptors

Scavenger receptors bind modified lipoproteins, which enables them to clear altered-self (141). On macrophages, CD36 mediates phagocytosis of apoptotic cells, requiring thrombospondin 1 (TSP-1) which acts as a bridging molecule between CD36 and PS on apoptotic cells (142, 143). Albert et al (2008) found CD36 and αvβ5 integrin to be responsible for DC uptake of apoptotic cells and subsequent trafficking for MHCI cross-presentation (144), though the role of CD36, αvβ3 and αvβ5 in antigen trafficking and cross-presentation has more recently been challenged (145). Other studies suggest that CD36 is not required for apoptotic cell uptake by DCs at all, which is instead mediated preferentially by the vitronectin receptor, mentioned below (11).

Strikingly, CD36 is shown to be directly ligated by the clonally variant protein PfEMP-1 expressed by Plasmodium falciparum-infected erythrocytes, leading to the inhibition of DC maturation (146). DCs bound in this way release TNF-α but also secrete IL10 and fail to prime T cells. The same group later showed that apoptotic cell uptake, like PfEMP-1-ligation, induces an anti-inflammatory response via CD36 and TSP-1 (146). Regardless of its role in actual clearance of apoptotic cells, CD36 represents an influential suppressor of DC maturation and one that is exploited by pathogens for that very reason.

Despite an anti-inflammatory role, CD36 is also involved in some inflammation. One group demonstrated that, in human vascular endothelial cells, H. pylori- and P. gingivalis-derived LPS is able to antagonise TLR4, and induce formation of heterodimer receptor complexes of TLR1, TLR2 and CD36, leading to NF-κB activation and TNFα secretion (147). It is interesting that CD36, a typically anti-inflammatory receptor, should associate with typically inflammatory receptors. However, with measurement of TNF-α secretion alone and no possibility of performing T cell-activating assays on the cell types used it is not compelling that DCs activated in this way could not still be tolerising in function. However, there is more evidence that suggests that CD36 may be involved in proinflammatory signalling. CD36 also has an important role in clearance of oxidised low-density lipoprotein (oxLDL) (148), and oxidation of lipoproteins on apoptotic cell surfaces is key to their recognition and subsequent ingestion by macrophages (109). PS, the seminal marker to aid recognition of apoptotic cells, must itself be oxidised before macrophages can phagocytose apoptotic cells efficiently, and it is done so by the reactive oxygen species that are generated during Fas-induced apoptosis (149). Apoptotic cells display oxidised moieties of lipids and lipid-proteins. The oxidation-specific epitopes of oxidised phospholipids (oxPLs), which are increased on the apoptotic cell surface, are found to induce $CD4^+$ T cell cytokine production and activate endothelial cell adhesion of monocytes. Oxididation therefore has the potential to make apoptotic cells proinflammatory and immunogenic (110). Moreover, oxidative modification of poly(G) (polyguanylic acid) causes autoantibody production against both the native poly(G) and the modified poly(G) in scleroderma, and autoantibodies in SLE that preferentially bind the modified poly(G) (150). In keeping with the recurrent theme of multiple ligands, CD36 also binds the heat shock protein gp96 (151).

The class-A scavenger receptor (SRA) has been shown to contribute to apoptotic T cell clearance by macrophages in the thymus (152). SRA also plays a role in pathogen recognition, as it binds LPS (153) and enables phagocytosis of E. coli by macrophages (154). Another scavenger receptor with inflammatory properties has been implicated in HSP-mediated immunity. Scavenger receptor

LOX-1 is a promising target for cancer immunotherapy, as it binds Hsp70 and promotes cross-presentation, and has been loaded with tumour antigen to induce antitumour immunity *in vivo* (155).

Also critical in macrophage phagocytosis of apoptotic cells is the vitronectin receptor (αvβ3), which was the first receptor to be identified as mediating phagocytosis of apoptotic cells (156) and was later found to cooperate with CD36 and TSP-1 (143). MFG-E8 (milk fat globule-epidermal growth factor 8) acts as bridging molecule between macrophage αvβ3 and PS (157), and autoimmune disease due to impaired apoptotic cell clearance in MFG-E8 knockout mice suggests an active involvement in suppression of inflammation (158). It has been shown that immature DCs secrete MFG-E8 in exosomes, a property which is lost in the mature state, and this correlates with phagocytic ability, and that a MFG-E8 knockout transgenic mouse has greatly diminished phagocytic capabilities in apoptotic cell clearance (157). This data suggests a similar role in DCs for MFG-E8 as in macrophages, and it may yet be found that αvβ3 receptor on DCs mediates anti-inflammatory signalling.

CD31 also is involved in apoptotic cell clearance (159). Mice with CD31 deficiency develop autoimmunity and exhibit hyper-responsive B cells when challenged with LPS or IgM, indicating a possible active inhibitory function (160). Further, CD31 from Jurkat cells, a human T cell line, has been shown to contain an (ITIM), which, when activated, inhibits protein tyrosine kinase (PTK)-dependent signal transduction mediated by the T cell receptor (TCR) complex, which contains an immunoreceptor tyrosine-based activation motif (ITAM) (161). DCs have long been known to express CD31 (162, 163), but any anti-inflammatory effects in DCs are yet to be reported.

TAM (Mer), Tyro3 and Axl are related members of a family of receptor tyrosine kinases. All have roles in regulation of cytokine production in macrophages, and have also been shown to have roles in the recognition and phagocytosis of apoptotic cells (164, 165). Mer has been shown to be present on bone marrow-derived DCs but Behrens group found that it is not essential for DC

engulfment of apoptotic cells (165), whereas macrophages derived from $Mer^{-/-}$ mice were 90% less efficient at clearing apoptotic cells, leading to anti-nuclear antibodies but no autoimmunity-caused tissue damage (166). Mice deficient in all of the receptors Mer, Tyro3 and Axl develop autoimmunity (167), suggesting that these receptors act synergistically but are independently functional.

1.2.3.2 Complement

C1q and C4 are components of the classical complement pathway and their separate or joint deficiencies due to inherited null mutation are associated with strong susceptibility to systemic lupus erythamatosous (SLE) in humans (168). Increasing accumulation of C1q on maturing blebs from apoptotic keratinocytes (169) directed the way for the link between C1q and autoimmunity to be attributed to the requirement of C1q for apoptotic cell clearance by macrophages (6, 170). Poor clearance could subsequently result in an abundance of necrotic factors capable of stimulating inflammatory signalling in DCs. C1q, as well as mannose-binding lectin (MBL), stimulate macrophage ingestion of apoptotic cells by binding both the apoptotic cell surface and macrophage surface calreticulin, which in turn is bound to CD91 (171). CD91 also mediates antigen trafficking by binding the heat shock proteins gp96, Hsp90, Hsp70 and calreticulin (172).

IC3b opsonises PS on apoptotic membranes, and binds to CR3 and CR4 on macrophages, leading to phagocytosis (8). Morelli et al (2003) found that not only do the scavenger receptors CR3 (CD11b/CD18), and to a lesser extent, CR4 (CD11c/CD18), mediate phagocytosis of apoptotic cells in DCs via the opsonising aid of iC3b among other opsonants, but iC3b-CR3 interaction resulted in decreased levels of mRNA and suppressed secretion of proinflammatory cytokines IL1α, IL1β, IL6, IL12p70 and TNFα, with no change in the secretion of TGFβ1 (9). This is in keeping with the findings that CR3 ligation suppresses IL12 and IFNγ secretion in macrophages

(173). This anti-inflammatory response is no doubt aided by the binding of C-reactive protein (CRP) to apoptotic cells, which is shown to induce anti-inflammatory responses in macrophages (174). Here, CRP enhanced complement activation but protected the integrity of the cell membrane from later complement components by preventing formation of the membrane-attack complex. Furthermore, CRP augmented macrophage phagocytosis of apoptotic cells, which was associated with secretion of TGFα. Additionally, these anti-inflammatory effects were not effective once the cells became necrotic, indicating that CRP protects against necrotic factor-induced inflammation but will not counter it if it nonetheless occurs. This is some of the most promising evidence yet that signalling through mediators of apoptotic cell uptake actively suppresses inflammation. However, Gaipl et al (2001) demonstrate that complement binding does not occur until late apoptosis or early secondary necrosis (175). Complement may therefore be acting as an important but late suppressor of immunity when phagocytes are overburdened by apoptotic cells and many of those apoptotic cells have progressed to later stages of apoptosis and secondary necrosis. Certainly, most other receptors involved in the regulation of apoptotic cells, such as the PS receptor, appear to recognise apoptotic cells much earlier on, and apoptotic cell clearance is usually extremely efficient and rapid as to make the progression to late-stage apoptosis and secondary necrosis an infrequent occurrence (176, 177). Being a 'last resort' method of apoptotic cell clearance, or possibly one that is specific to only those cell types that progress rapidly to secondary necrosis, complement-mediated clearance may have obscured the true importance of other clearance receptors in the earlier stages of apoptosis. This may go some way to explaining why reports may differ in their accounts of the importance of the same receptors when using different apoptotic cell types for *in vivo* clearance assays. Regardless, it is interesting to think that the inhibitory effect of complement may even offset the proinflammatory signalling induced by necrotic factors, and warrants further investigation as to how the signalling pathways of both interrelate.

1.2.3.3 Fcγ receptors

Autoimmune conditions sometimes have autoantibodies that bind the surfaces of apoptotic cells, for example anti-phospholipid autoantibodies (aPLs) bind exposed PS (178). These can then facilitate apoptotic cell recognition by macrophages and DCs via Fc receptors and in this way apoptotic cell ingestion may instigate inflammation by the release of TNF-α (179). Fcγ receptors bind the Fc portion of immunoglobulin G, are involved in clearance of immune complexes, phagocytosis of antibody-opsonised pathogens, enhancement of antigen presentation, and antibody-dependent cellular cytotoxicity, and are found on most immune cells (180). FcγRI (CD64) and FcγRIII (CD16) belong to the activation class of Fc receptors, which transmit activation signals via immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytosolic domain. The FcγRII (CD32) subset receptor FcγRIIa also belongs to the activating class of Fc receptors, but FcγRIIb belongs to the inhibitory class of Fc receptors, which transmit inhibitory signals via immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (181, 182). Generation of balanced immune responses is dependent on the synchronized expression of these sets of receptors on the same cell (183). FcγRIIb is particularly important for tolerance, and may have an important role in preventing spontaneous DC maturation, by raising the threshold for activation. (184). FcγRI, FcγRII and FcγRIII are all expressed on DCs (185).

FcyRIIa is required for apoptotic cell-IgG complex-mediated IFNa production by DCs (apoptotic cell-IgG complex serves as a substitute for lupus sera immune complexes), where DNA-containing immune complexes signal through both FcγRIIa and TLR9 (186). DC FcγR ligation by IgG-associated apoptotic cells triggers maturation and induces efficient MHC class I and II-restricted antigen presentation (187). These findings show that a proinflammatory receptor is involved in clearance, though IgG is no doubt the (predominant) DC activator. However, given that most immune receptors discussed so far appear to be modulated by endogenous danger and/or apoptotic factors, the FcγR family merit further investigation. Indeed, intravenous Ig (IVIg)

therapy has been used successfully for many years to treat several autoimmune and systemic inflammatory diseases that are characterised by excessive tissue necrosis (185). From unpublished findings [Miller JI and Millar DG] that FcγR blocking abrogates necrotic cell-induced DC maturation, it is tempting to speculate that endogenous danger signaling may act via FcγR, and that further elucidation of endogenous danger signaling may extend the application of IVIg therapy to more autoimmune diseases.

1.2.3.4. Others

The TIM (T cell/transmembrane, immunoglobulin and mucin) family of receptors specifically recognise PS on apoptotic cells, but differ in their expression and function, appearing to have both anti-inflammatory and highly co-stimulatory roles (188). Expression is mainly by T cells and APCs, where cross-linking has been demonstrated to lead to various effects including potent co-stimulation (Th2 cells), cross-tolerance (APCs) and apoptosis (Th1 cells), amongst many other findings.

CD205 (also known as DEC-205) has been identified as an important receptor in the phagocytosis of dead cells, recognising ligands on both apoptotic cells and necrotic cells (189).

DC phagocytosis of ACs has been associated with a semi-mature DC state that is in conflict with the established bimodal model, and these semi-mature DCs have been implicated in tolerance. DCs exposed to a respiratory antigen expressed high levels of co-stimulatory molecules but also produced high IL10 and induced antigen-specific CD4+ T cell tolerance in a murine model of respiratory hypersensitivity and asthma (190). DCs matured by TNFα increased co-stimulatory molecule expression but were poor producers of proinflammatory cytokines. Upon repeated injections into mice, these DCs induced antigen-specific protection against EAE (191). These findings indicate that the cytokine profile, rather than the surface phenotype consisting of a repertoire of co-stimulatory and co-inhibitory molecules, is more instrumental in determining the

subsequent immune response. This is an interesting addendum to the long-established two-signal model – it is now arguable that the eponymous two signals (the antigen-receptor signal induced by antigen-specific recognition, and the non-specific signal from co-stimulatory molecules) are necessary for a T cell response but are not sufficient to commit the T cell response in the appropriate direction of either immunity or tolerance. It is the cytokine profile that directs and is crucial in ensuring the appropriate immune response.

1.3 Immunogenicity of apoptotic cells

The immunogenicity of apoptotic cells is contested due to a wealth of conflicting reports. Here, the literature is examined for patterns that can reveal distinct circumstances for both the anti- and the proinflammatory behaviour of dead cells, whether or not this distinction correlates with apoptosis and necrosis, and whether or not it can satisfyingly reconcile the observed immunogenic and tolerogenic effects on DCs

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1.3.1 Poor characterization of dead cells and/or progression to secondary necrosis

Apoptotic cells and necrotic cells are two very distinct forms of cell death with, in theory, two very distinct sets of immunological properties. Unfortunately, studying one form without contamination of the other is extremely difficult. Furthermore, some reports disappoint with poor initial characterisation of the type of dead cell being used in the investigation. For example, Takahashi et al (2003) report that apoptotic cells, which did not induce up-regulation of co-stimulatory molecules, induced up-regulated secretion of IL10 (as did live cells, but not necrotic cells) and increased production of IL12p40 and, greatly so, IL6 (unlike live cells and necrotic cells) (15). However, some of the apoptotic cells used were described by the authors as 92% positive for Annexin V and 61% positive for PI. The cells being used were, in fact, mostly necrotic, which accounts well for the induction of proinflammatory cytokines. Indeed, the IL6 production is maximum at the highest proportion of 'apoptotic' cells. IL12p40 production is at a maximum at the second highest proportion of 'apoptotic' cells but inexplicably falls dramatically at the highest proportion. So in this study, poor characterisation of the dead cells has given rise to false allusions as to immunogenicity of apoptotic cells. It is still, however, difficult to reconcile the necrotic cell-induced cytokine profile with the lack of up-regulation of co-stimulatory molecules. In other

studies, apoptotic cells are incubated with DCs for up to 48 hours or more, but no evidence is provided to demonstrate that apoptotic cells that are not phagocytosed in this time (and many will not be) do not progress to secondary necrosis whilst in culture with DCs. Physiological secondary necrosis occurs usually only in cases of defective apoptotic cell clearance, which allows apoptotic bodies the time to begin to disintegrate, releasing potential endogenous antigen and danger signals much like necrosis does. Unfortunately, the semantics of apoptosis and necrosis are not always sufficient in the immunological context.

1.3.2 Temporal specificity

Work by Lucas' group highlights some of the complexities of the effect of apoptotic cells on APCs (192). Apoptotic cells stimulated TGFβ1, although only late in phagocytosis, whereas various proinflammatory ligands of TLRs 2, 4 and 9 induced early and sustained secretion of TNFα, macrophage-inflammatory protein (MIP) 1α and MIP-2, and later secretion of IL10, IL12, and TGFβ1. Interestingly, combined apoptotic cells and TLR ligands augmented early secretion of TNFα, MIP-1α, and MIP-2 and increased late TGFβ1 secretion, while inhibiting late TNFα, IL10, and IL12 secretion. The authors propose that apoptotic cells thus aid recruitment of immune cells for inflammation as well as ensure subsequent resolution. This would certainly be beneficial in order to avoid advantageous conditions for pathogens. Indeed, it has been shown that trypanosomes may take the opportunity to flourish during apoptotic cell-mediated immune suppression (193). This report reveals that the concomitant pro-and anti-inflammatory responses of APCs may not be completely simultaneous, and emphasises the importance of monitoring responses.

1.3.3 Tumour cells

Innate recognition of tumour antigens as danger signals may be the driving force behind the immunogenic properties of some apoptotic cells. Many immunotherapy studies in humans report the successful treatment of tumours by administration of DCs pulsed with apoptotic tumour cells. Accordingly, the immunogenic properties of apoptotic cells has been shown in the *ex vivo* apoptotic cells of many cancers (e.g. from patients with ovarian cancer (194), leukemia (195) or mesothelioma (196), among many others). However, this is testament more to the immunogenicity of tumour cells than of apoptotic cells. For example, UV appears to generate innocuous apoptotic cells (197, 198) unless those apoptotic cells are tumour-derived *ex vivo*, whereby they are immunogenic in many studies (for example (199), (200), (201). In fact, investigations have demonstrated *in vitro* that the apoptotic and necrotic state is irrelevant in the use of tumour cells for DC loading and priming (with a view to use in DC-based vaccines), as tumour cells are immunogenic regardless of the mode of death (201, 202) and are phagocytosed at equivalent rates (203). These results suggest that the provenance of the cells is of more immunological consequence than the death state of the cell. That is, apoptotic cells may be able to suppress DCs, but this is irrelevant in apoptotic tumour cells because the tumourous nature of the cell offsets any inherent suppressive capability arising from its apoptotic state. However, controversy still exists as to the relative merits of pulsing DCs with apoptotic or necrotic cells. Some studies maintain that tumour lysates induce DCs to generate a more potent antitumour response than DCs treated with apoptotic tumour cells (204, 205). Still others have observed apoptotic tumour cells displaying superior immunogenicity than necrotic tumour cells(206), and producing more efficient vaccines (207).

The immunogenicity of apoptotic tumours can in many studies be attributed to tumour-specific overexpression of particular molecules, which DCs may perceive as danger. For example, the immunogenicity of apoptotic cells of an allogeneic lung cancer cell line used to pulse DCs in the

treatment of lung cancer (208) may reflect the tumour specific overexpression in that cell line of several molecules, including Her2, WT1 and survivin. The implication is that danger signals can overpower apoptotic signals. However, this hierarchy of signaling cannot be assumed. Of course, it is well known that tumours influence the local microenvironment so as to inhibit inflammation and immunity. This is not fully understood but many different mechanisms have been implicated. Danger signals and altered-self can be masked from immune detection, or cell death can be promoted in immune cells. Alternatively, apoptotic signals can overpower danger signals. In fact, it appears to be the mimicry of apoptotic cells that allows escape from immune detection by some tumourous cells. As such, many tumour cells are not immunostimulatory at all. For example, apoptotic cells from squamous cell carcinoma (SCC) may up-regulate some DC co-stimulatory molecules but cannot induce the IL12 secretion necessary for a complete immune response (209).

As such, when apoptotic cells have been used to pulse DCs in DC-based immunotherapy in the treatment of cancers, often it is necessary to prime these DCs with, or administer them with, adjuvants. In the SCC example above, necrosis of the tumour cells was sufficient to induce full DC maturation *in vitro* (209) . SSC has successfully been treated in mice by pulsing DCs with apoptotic cells from a syngeneic cell line and co-administering them with IL2 (210). Without IL2, DCs treated with the UV-induced apoptotic cells were poorly immunogenic. In humans, clinical responses have been seen in indolent B cell lymphoma patients in a pilot study of vaccinations with DCs pulsed with apoptotic autologous tumour cells (211). However, before apoptosis is induced by UVC-irradiation, tumour cells required heat shock treatment for one hour in order to increase expression of HSPs. *In vitro*, cellular stress has been necessary to improve the immunogenicity of apoptotic leukaemia cells, where the up-regulation of heat shock-induced Hsp60 and 72 was implicated as a required stimulus to promote DC maturation and induction of T cell activation (212).

Finally, production of novel autoantigens (213), often present in tumourous cells, or which can caused by unusual post-translational protein modifications during apoptosis (214), may induce immunity if released from the cell before efficient and full degradation. The process of ordered degradation of intracellular proteins during apoptosis provides much opportunity for the generation of novel autoantigens. T cells will not have been tolerised to these autoantigens and antigens from immune privileged sites. Therefore, if the first exposure of these antigens to the immune system is under inflammatory conditions they can be registered as being dangerous and invoke lasting immunity.

1.3.4 Cell stress and the method of apoptosis induction

The method and intensity of apoptosis induction can be reflected in the up-regulation of danger signals prior to cell death, and, accordingly, may affect the immunogenicity of the apoptotic cells (215). For example, apoptosis of 67NR mouse carcinoma cells via the Fas (CD95) pathway or induced by the anticancer drug bortezomib has been associated with DC maturation whereas the same cell type induced to undergo apoptosis by UV has not (197). Furthermore, UV-induced apoptotic cells have been seen to promote tolerance *in vivo* (198). However, UV irradiation can induce dose-dependent *de novo* synthesis of HSPs to protect against UV-induced damage (216), which can lead to enhanced immunity (217, 218). The dose-dependency may reconcile the contradictions in the literature as to the immunogenicity of UV treated apoptotic cells. Similarly, some proteins are unusually phosphorylated during apoptosis and can be immunogenic, as seen in the autoantibody production in SLE (215).

In another example, uptake of irradiation-induced apoptotic cells by macrophages causes inflammation as shown by the infiltration of neutrophils to sites of uptake (219). Lorimore et al (2001) showed that this effect was not caused directly by the ionizing radiation to which tissue was

exposed, but by the uptake of radiation-induced apoptotic cells. Ionising radiation generates reactive oxygen species (ROS) in cells by the oxidation of water and other molecules (220). Oxidative stress occurs to a cell when ROS production exceeds the cells anti-oxidant defense capability and results in tissue damage, potentially causing cancer, amongst other diseases (220) . Although ROS (particularly oxidation of PS) are required during apoptosis, including for creating lipid moieties at the cell surface to allow for efficient recognition and ingestion by phagocytes (109, 149), oxidation-modified lipids can induce inflammation (110). Given that most apoptotic cell clearance appears to be immunologically silent, and that significant necrosis was not detected in radiated tissues by Lorimore et al (2001), it is possible that oxidation-modified lipids and proteins themselves may cause inflammation and DC maturation in the unusual circumstance that their quantity exceeds a particular threshold, above which apoptotic signalling is surmounted and negated.

Most stresses induce up-regulation of stress proteins until the stress subsides or continues to a fatal level and apoptosis begins. Indeed, the proinflammatory properties of those proteins that are up-regulated to limit damage by e.g. oxidative stress, heat stress, damage, or any other stress that may be indicative of possible DNA damage or viral infection, may ensure that a stress-induced fatality is immediately succeeded by an inflammatory, rather than innocuous or anti-inflammatory, response to that cell. It makes immunological sense that apoptotic cells are inhibitory by default, but are conferred immunostimulatory properties by stress-induced proteins in order to stimulate immunity against cells that harbour danger such as viruses or properties characteristic of tumour cells. Stress and intracellular factors might even be more of a determinant than the mode of cell death, as discussed in 1.3.3.

1.3.5 Variety in DC subsets and responses

An additional complication is that different combinations of stimuli could result in different types of immune response, as illustrated by the number of permutations of inhibitions or up-regulations of the various cytokines and co-stimulatory molecules seen incurred by different treatments. Although intriguing, this variation in responses makes comparisons between stimuli difficult, and requires that techniques are optimised to monitor the production and effect of cytokines and other DC-mediated immune molecules *in vivo*. Also, certain DC subsets are more specialised at apoptotic cell capture (4) (221) or at T cell stimulation (222), therefore some studies may not be qualitatively comparable with others. DC subtypes may also account for discrepancies and dissimilarities in the literature due to differences in anatomical location and related functions. For example, plasmacytoid DCs, which are $CD11c^{-}CD123^{hi}$, reside primarily in blood and lymphoid organs and are important for innate antiviral immunity. Myeloid DCs, which are $CD11c^{+}CD123^{lo}$, include Langerhans Cells, which reside in the skin and probably deal with oxidation-specific epitopes more than any other DC subtype, and interstitial, dermal and submucosal DCs, which bear their name according to their anatomical location and face their own unique repertoire of antigens. Furthermore, DCs subtypes may have to behave differently as they may have to rely on different regulatory mechanisms *in vivo* to avoid eliciting autoimmunity (223).

On a more practical level, accurate conclusions may be elusive due to the sensitivity of DCs. Normal experimental routine, for example fresh plating, can cause mechanically-induced maturation and cytokine production that may mature further DCs within the same experimental sample (18).

1.4 Concluding remarks

The uptake of apoptotic cells has a clear involvement in tolerance and autoimmunity. *In vitro* and *in vivo* studies have shown that apoptotic cell uptake actively suppresses DC maturation and hence promotes tolerance. In contrast, cell debris from necrotic cells contains danger signals which cause inflammation and cross-priming of autoreactive T cells by autoantigen-presenting, activated DCs. These danger signals may counteract inhibitory apoptotic cell signals, and so apoptotic cells may potentially contribute autoantigen when in necrotic, inflammatory conditions. Danger signals may also accumulate within apoptotic cells and have sufficient immunogenicity to negate inhibition without cell lysis or preceding inflammation. The importance of the difference in immunogenicity between apoptotic and necrotic cells is highlighted by the demonstrations that necrotic cell lysates or derivatives can induce anti-tumour immunity, and the implication of necrosis in SLE-like autoimmune illnesses. Clarification of apoptotic and endogenous danger signalling could therefore generate monumental progressions in tumour vaccination and autoimmunity.

There are many danger signals released in injury and probably much immunogenic presentation of autoantigens which is, nonetheless, regulated due to the prior and constant maintenance of peripheral tolerance, as well as negative feedback mechanisms that limit inflammation and return sites of infection or injury to the steady state (224). It appears unlikely that danger signals have sufficient power or longevity to initiate or maintain autoimmune responses without supplementary abrogation of one or more of several regulatory mechanisms, including T regulatory cells, or without a multitude of activating signals (225). However, endogenous danger signals are nonetheless powerful as evidenced by their ability to induce anti-tumour immunity, hence their experimental use in clinical anti-tumour vaccines. Further, danger signals might predispose to autoimmunity by lowering the threshold at which DCs may be activated, making DCs more susceptible to activation by otherwise innocuous material. Alternatively, endogenous danger signals may act by negating the inhibitory signalling induced by apoptotic cells. Understanding how to

manipulate DCs to induce tolerance via the pathway of apoptotic cell-induced suppression will hopefully redress this immune imbalance and provide practical applications in the treatment of autoimmune disorders.

As mentioned, apoptotic cells are currently in active clinical use for immunotherapy. Given the mounting evidence of the tolerogenic properties of apoptotic cells, it is extremely perilous that they are being used for immunostimulation in clinical settings. In successful trials, their immunostimulatory effect is probably down to other factors like the presence of HSPs, but these are by no means universal in apoptosis. In fact, apoptotic tumour cells do not constitute vaccines as potent as those that use tumour RNA (226), which is perhaps on account of the inhibitory effects of apoptotic cells. In many DC-based tumour vaccines, DCs must be treated with apoptotic cells alongside immunostimulants such as LPS, or apoptotic cell-treated DCs are co-administered with immunostimulants, in order to maximise the possibility of initiation of immunity. However, the present study demonstrates that the suppressive capacity of apoptotic cells should be a major consideration in the preparation of these vaccines.

At the core of the unpredictability of DC immunotherapy is the unique and complex immunogenicity of any one tumour. As such, DC priming with tumour cells is largely haphazard. At best, it is providentially effective, but at worst it is potentially dangerous and counterproductive. Progress in understanding and controlling the mechanisms by which DCs are primed for immunity, or suppressed for tolerance, represents new opportunities to direct the immune response against cancers effectively and assuredly.

This investigation was designed to evaluate various methods of induction of cell death, in order to find consistent methods of generating exclusive populations of well-characterised apoptotic cells and exclusive populations of well-characterised necrotic cells. These cells were used to illustrate the effect of each dead cell type on DCs, by establishing the effect on surface expression of co-stimulatory molecules and secretion of major cytokines, and the previously unreported effect of

apoptotic and necrotic cells on DC expression of co-inhibitory molecules. The effect of apoptotic and necrotic cells on LPS-induced maturation of DCs was determined in order to support or oppose previous reports of apoptotic and necrotic cell-mediated modulation of dendritic cell maturation. Also, the previously unreported effect of apoptotic and necrotic cells on CpG-induced maturation was determined, and the consequences of this interaction on CpG-induced immune responses *in vitro* and *in vivo*.

CHAPTER 2 – Methods and Materials

2.1 Cell culture

All cell culture incubations referred to are at 37˚C, 5% CO_2, in a humidified incubator. All recoveries of cells by centrifugation refer to centrifugations at 400g for 5 minutes unless otherwise stated. 'GMCSF' and sGMCSF refer to GMCSF purified from the supernatant of cultures of the GMCSF-secreting cell line Ag8653 (227). 'Recombinant GMCSF' and 'rGMCSF' refer to recombinant GMCSF (Sigma-Aldrich). Bone marrow dendritic cells (BMDC) were generated according to the Erlangen Method (228), with minor modifications as described. Bone marrow from the femurs and tibias of C57Bl/6J mice was treated with ACK (0.15M NH_4Cl, 10mM KHCO3 and 0.1mM EDTA) for 2 minutes to lyse erythrocytes, then the cells were washed and recovered by centrifugation and resuspended in complete media (cRPMI — RPMI-1640 (Gibco-Invitrogen), 2mM L-glutamine, penicillin/streptomycin, 10% heat-inactivated FBS (unless otherwise stated) and 0.5μM BME) containing 100-200ng/ml GMCSF. Cultures were incubated for 6-8 days. On day 3 cells were supplemented with fresh cRPMI and GMCSF, and on d6/7 non-adherent BMDC were recovered by centrifugation and resuspended in fresh cRPMI, with no GMCSF unless otherwise stated. If intended for use on d8, non-adherent BMDC were replated with fresh cRPMI and GMCSF on d6 for further incubation.

FDCP-1 (factor-dependent cell Paterson-1, FDCP) cell line was cultured in cRPMI and 100-200ng/ml GMCSF in culture flasks and incubated. FDCP were split and replenished with fresh cRPMI and GMCSF twice a week. Splenocytes were obtained by removing the spleen of C57Bl/6J mice. The spleens were crushed using frosted glass slides into HBSS (Gibco-Invitrogen), filtered through a disposable nylon mesh strainer then washed and recovered by centrifugation. The cells were treated with ACK for two minutes to lyse erythrocytes, then washed and recovered by

centrifugation, and resuspended in cRPMI ready for incubation with or without dead cells. Thymocytes were obtained by removing the thymi from C57Bl/6J mice. Thymi were then crushed using frosted glass slides into HBSS, washed and recovered by centrifugation, treated with ACK to lyse erythrocytes, washed and recovered by centrifugation again, and resuspended in cRPMI ready for induction of apoptosis.

2.2 Induction of apoptosis and necrosis

FDCP were induced to undergo apoptosis/necrosis during exponential growth phase to minimise baseline levels of apoptosis and necrosis. For heat kill (HK) treatment, FDCP were resuspended in cRPMI at ~$1x10^7$ cells/ml in 1.5ml Eppendorf tubes and placed in a heat block (AccuBlock, Labnet International Inc) at 60˚C for 30 minutes, then allowed to cool prior to dilution with cRPMI and coculture with BMDC. For freeze-thaw (F/T), FDCP were recovered by centrifugation and the dry pellets were placed in a -80˚C freezer for 20 minutes until pellets were solid, then allowed to defrost at room temperature before repeated twice to total three cycles of freeze-thaw. For growth factor withdrawal, FDCP were washed twice in HBSS, resuspended in cRPMI and incubated for various lengths of time as stated. For camptothecin treatment, the adopted method after preliminary experiments was the incubation of FDCP in fresh cRPMI and GMCSF with 14.4μM camptothecin (Sigma-Aldrich) for 24 hours. For UV exposure, Petri dishes of FDCP culture in fresh cRPMI and GMCSF were positioned under UVC-emitting light bulbs in a Stratolinker UV machine for 1-1000 seconds in order to receive UV doses of 0.2-200J/m^2, before being returned to incubation for 2-72 hours. Thymocytes were induced to undergo apoptosis by incubation with 5μg/ml superFas ligand (sFasL, Sigma-Aldrich) for four hours. All spins of cells after death induction treatment were 800g for 10 minutes in order to include small apoptotic bodies in the pellet (NB. Cells *and* supernatants of HK treatments were used, in order to not lose intracellular components escaped from cell into media).

2.3 Surface antibody staining and fluorescence-activated cell sorting (FACS)

Samples of variously-treated BMDC were washed and recovered by centrifugation, resuspended in 100µl FACS buffer (PBS, 2% FBS, 0.1% NaN3) plus anti-IgG (Fc block) and incubated at 4˚C for 30 minutes. Cells were washed again in FACS buffer and recovered by centrifugation before being resuspended in FACS buffer plus antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), biotin, and/or allophycocyanin (APC). Variously-conjugated antibodies were specific for CD40, CD80, CD83, CD86, CD25, MHCII, B7DC, B7H1, B7H2, B7H3, B7H4, B220, CD54, CD69, CCR5, and TRAIL (all eBiosciences). Samples were incubated at 4°C or on ice in the dark for 20-30 minutes, cells washed with FACS buffer, cells resuspended in 100µl FACS buffer + secondary antibodies conjugated with streptavidin-cychrome and incubated at 4°C or on ice in the dark for 20-30 minutes, washed with FACS buffer then acquired within one hour on a BD Biosciences FACSCalibur, using Cell Quest software. Alternatively cells were resuspended in 200µl neutral buffered formalin, stored at 4°C and acquired within two weeks. The data was analysed using FlowJo analysis software. The detected amount of antibody at and above which a cell was considered 'high' for the specific cell surface marker was determined in each experiment by the use of untreated negative control BMDCs and TLR ligand-treated positive maturation control BMDCs. For co-cultures of BMDC and FDCP, BMDCs were gated on CFSE if they had been stained with CFSE, or were gated on CD11c and appropriate forward-scatter and side-scatter in order to exclude dead cells and debris.

Occurrence and type of cell death was determined by incubating cells with Annexin V (AnV, eBiosciences) and propidium iodide (PI, BD Pharmingen) at the manufacturers recommended dilutions in binding buffer (10mM HEPES pH7.4, 140mM NaCl, 2.5mM $CaCl_2$, 0.1% NaN_3) at room temperature (RT) for approximately 20 minutes before being washed and analysed according to the protocol described above.

2.4 Gel electrophoresis and fluorescence microscopy

Death-induced FDCP were adjusted to $2x10^6$ cells per sample and spun before the supernatant was removed. Each sample was resuspended in 1ml ice cold 70% EtOH and incubated at -20°C overnight. Samples were then thawed at RT and the EtOH removed, then the samples were resuspended in 40µl phosphate-citrate buffer (24 parts 0.2M Na_2HPO_4, 1 part 0.1M citric acid (pH 7.8)) and incubated RT 30 minutes. Samples were spun at 1000G for 5 minutes and the supernatant was transferred to new tubes and supplemented with 3µl of 0.25% Nonidet NP-40 and 3µl of 1mg/ml RNase then incubated for 30 minutes at 37°C. 3µl of 1mg/ml proteinase K was added to samples which were then incubated for a further 30 minutes at 37˚C. 12µl of 6x loading buffer (0.25% bromophenol blue, 30% glycerol) was added to samples which were then loaded on to agarose gel alongside DNA standard size samples (100bp, 1000bp) and run at 80V for 1 hour. Gel was visualised using a UVP Transilluminator.

DNA was visualised by fluorescent microscopy by first washing cells and recovering them by centrifugation on to glass slides. 300µl of 300nM DAPI staining solution was added to each cell spot, before incubation at RT in the dark for 5 minutes. Slides were rinsed three times with PBS before visualisation by microscope at a 460nm wavelength.

2.5 Enzyme-linked immunosorbent assay (ELISA)

Experimental culture samples in 12-, 24-, or 96-well plates were spun in a refrigerated (4˚C) centrifuge for 5 minutes at 800g and the supernatants transferred to new plates and incubated at -20˚C for at least 6 hours to ensure freezing and lysis of unwanted cells. Plates were stored for up to 8 weeks before ELISA. 96-well ELISA plates (Nunc) were coated with capture antibody (anti-IL10, anti-IL12, anti-TGFβ, anti-IL6, all eBiosciences), and incubated 4˚C overnight, then washed

five times with wash buffer (PBS, 0.1% Tween20) using a plate washer and incubated for 2 hours at RT with blocking buffer (PBS, 3% bovine serum albumin), then washed five times again. After thawing, supernatants were added to plates as neat, 2-, 5- or 10-fold dilutions with binding buffer (PBS, 1.5% BSA), alongside serial dilutions of soluble cytokine controls (all eBiosciences). Plates incubated for 2 hours at RT or overnight at 4˚C, washed five times, then incubated for 2 hours at RT with binding buffer plus avidin-horseradish peroxidise (AV-HRP, BD Pharmingen) and biotinylated detection antibody (anti-IL10-bio, anti-IL12-bio, anti-TGFβ-bio, anti-IL6-bio, all eBiosciences). Plates were washed five times then incubated at RT for 2-20 minutes with TMB substrate (Uptima), treated with stop solution (1M H_2SO_4), and the developed colour was measured on a spectrophotometer (MRXII, Dynex Technologies, using Revelation 4.21 software) at OD490.

2.6 Phagocytosis assay

BMDC were incubated with 10ng/ml carboxyfluorescein diacetate succinimidyl ester (CFSE, Fluka) for 10 minutes at 37˚C, washed twice in HBSS, and resuspended in RPMI at $1x10^6$ cells/ml. Death-induced FDCP were incubated with PKH26 (Sigma-Aldrich) for 5 minutes according to manufacturer's instructions, washed twice in cRPMI and resuspended in cRPMI at various cell densities as indicated, before being added to BMDC in equal volumes (100µl each) in a 96-well plate. Duplicate samples were supplemented with the phagocytosis inhibitor cytochalasin D at 50µg/ml. Plates were then incubated for 24hrs before antibody staining and FACS analysis.

2.7 T cell assays

Splenocytes were plated in 96-well plates at $4x10^6$ cells/ml, with or without doses of concanavalin A (Sigma-Aldrich) at 0.625 – 10μg/ml. The conditioned media (CM) from DC+(AC/NC)±(LPS/CpG) incubations was added to T cells at various concentrations, then the plates were incubated for three days, before the supernatants were isolated and frozen, and analysed for cytokines by ELISA. If Tregs were to be measured, 5ng/ml IL2 (Peprotech) was added to each sample on the third day of incubation and incubation was continued for a further 2 days. Cells were then stained according the protocol described in 2.3 above, with CD4, CD25 and Foxp3 antibodies (eBiosciences).

2.8 *In vivo* model of diabetes

DCs (6 days old) were co-incubated with ACs/NCs (or neither) at a AC/NC:DC ratio of 2:1 for 8-12 hours (overnight) as described above, then treated with 100ng/ml CpG/LPS/neither for 24 hours. DCs were then washed and separated by magnetic associated cell sorting (MACS) using CD11c-specific, high-gradient immunomagnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. Isolated DCs were pulsed with gp33 peptide by incubating with 10^{-7}M gp33 for one hour. DCs were then washed and resuspended in HBSS and injected subcutaneously into the flanks (unless otherwise stated) of RIP-GP transgenic mice (C57Bl/6J mice expressing the LCMV glycoprotein under control of the rat insulin promoter, first described by Ohashi et al (1991) (3)) on Day 0 at a volume of 300μl and a cell count of $2\text{-}5x10^6$ DCs per injection [Fig. 2.1A]. This process was repeated in a staggered manner, such that the second injection on Day 2 used 8 day-old DCs (9 day-old by time of injection) and a third injection on Day 4 used 6 day-old DCs (7 day-old by time of injection) from a second harvest of bone marrow [Fig. 2.1B]. Experiments were undertaken with age- and gender-matching as much as possible.

Blood glucose levels of mice were measured using Accu-Check strips (Roche Diagnostic Systems) one day prior to the first injection then 2-3 times per week until the end of the experiment or death, for indication of onset of diabetes. Diabetes was defined, as according to veterinary recommendations, as three consecutive measurements of blood glucose concentrations of 15mM or above. At three such measurements, or any one such measurement accompanied by clinically significant signs of illness (whichever was first), mice were euthanized by CO^2 asphyxiation or cervical dislocation. Clinically significant signs of illness included notable weight loss, substantial reduction of motor activity, and dishevelled coat. All mice were kept under specific pathogen–free conditions.

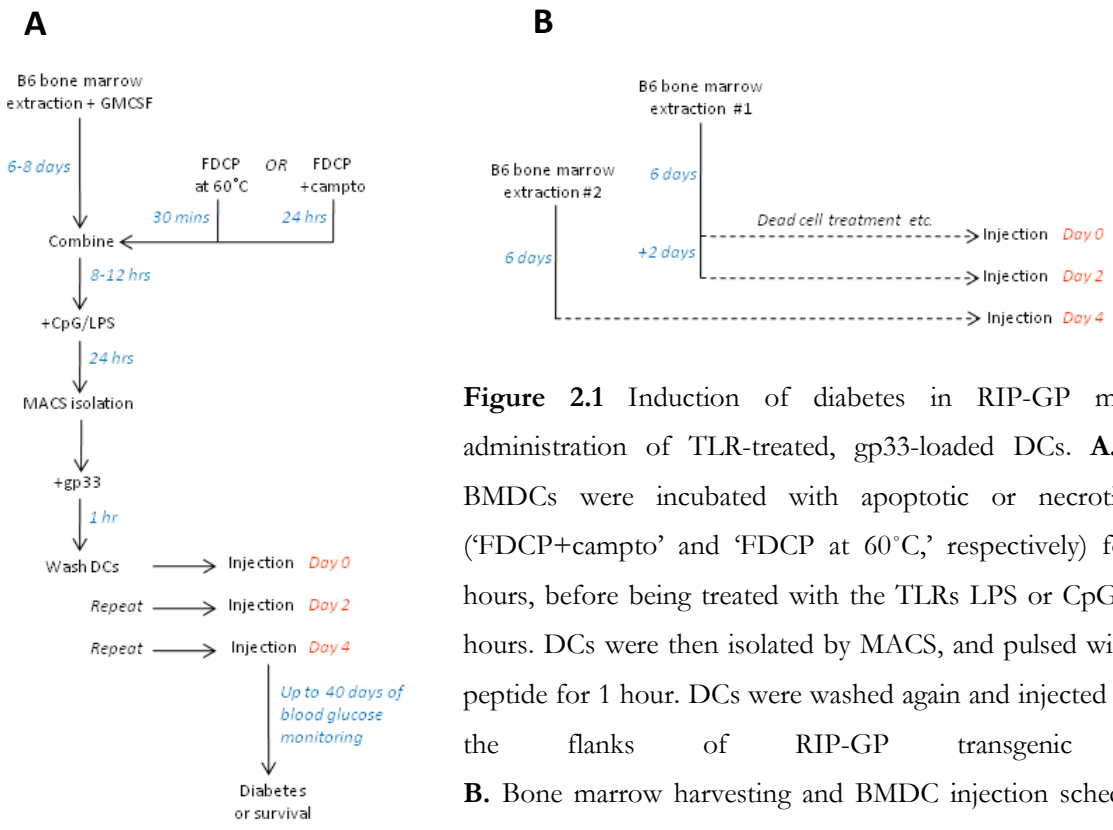


Figure 2.1 Induction of diabetes in RIP-GP mice by administration of TLR-treated, gp33-loaded DCs. **A.** D6/8 BMDCs were incubated with apoptotic or necrotic cells ('FDCP+campto' and 'FDCP at 60°C,' respectively) for 8-12 hours, before being treated with the TLRs LPS or CpG for 24 hours. DCs were then isolated by MACS, and pulsed with gp33 peptide for 1 hour. DCs were washed again and injected SC into the flanks of RIP-GP transgenic mice. **B.** Bone marrow harvesting and BMDC injection schedule: In order for injections to be made on Days 0, 2 and 4 using appropriately aged (differentiated) BMDCs, bone marrow was harvested from two separate mice 4 days apart, such that BMDCs injected on Day 0 were 8 days old (6 days culture, two days of treatments), those on Day 2 were 10 days old (8 days culture, 2 days of treatments), and those on Day 4 were 8 days old (second BM harvest, 6 days of culture, 2 days of treatment).

2.9 Statistics

In Chapters 3 and 4, all comparisons of *No treatment* (None) vs. *Apoptotic cell-treatment* (AC) vs. *Necrotic cell-treatment* (NC) were performed using one-way ANOVA with Tukey's multiple comparison test. All comparisons of LPS/CpG+None vs. LPS/CpG+AC vs. LPS/CpG+NC were performed using one-way ANOVA with Tukey's multiple comparison test. All comparisons of None vs. LPS/CpG+None, None vs. LPS/CpG+AC, and None vs. LPS/CpG+NC were performed using ANOVA with Dunnett's multiple comparison test where None was the control. In Results figures, "separate experiments" is defined as experiments using BMDCs derived from different mice.

CHAPTER 3 – Dead Cells and Their Effect on Dendritic Cell Surface Co-Stimulatory and Co-Inhibitory Molecules

3.1 Apoptosis and necrosis of FDCP

3.1.1 Introduction

In order to ascertain the effects of dead cells on DC behaviour, apoptotic cells (ACs) and necrotic cells (NCs) had to be generated consistently and separately. Several methods of death induction were investigated in order to find the techniques that could most reliably produce cell populations consisting of chiefly ACs or chiefly NCs. Producing a combination of AC and NC would not have been satisfactory due to the fact that it is difficult to sort AC from NC (the main marker of apoptosis, phosphatidylserine, is also accessible by antibodies/dyes in necrotic cells) without either: obstructing markers necessary for being recognised for phagocytosis; or, increasing the proportion of AC progressing to secondary necrosis due to mechanical stress or time. It was therefore necessary to establish cell-killing methods that induce high proportions of either type of cell death with little presence of the other.

The FDCP-1 (factor-dependent cell Paterson-1) cell was identified as an ideal candidate for the generation of dead cells. FDCP-1 (FDCP) is an immortalised murine cell line and non-tumorigenic haemopoietic progenitor. It is fast growing, easy to culture and its requirements for culture are conveniently similar to DC. Its growth and survival are dependent on GMCSF, which immediately provides a straightforward method of potential cell death in the form of growth factor withdrawal. Another method of cell death investigated was UV exposure - a common method of inducing

apoptosis in various cell types and one that is prominent in the literature of DC modification. This warranted particular attention given the apparent conflictions in the literature of the effect of UV-induced apoptotic cells on DC behaviour. Other killing methods investigated, although not all shown, included high temperature (heat kill - HK), repeated freeze-thaw cycles, the apoptosis-inducing drug camptothecin (cam), the ER-stress-related apoptosis-inducing drug azetidine, and the apoptosis-inducing protein CD95 ligand (CD95L), also known as Fas ligand.

The effect of each killing method was characterised in terms of the proportion of apoptotic and necrotic cells after a given time. Those methods of particular interest were then subject to further examination, particularly to determine the time-frame in which AC were in the majority. Levels of apoptosis and necrosis were determined by incubating cells with Annexin V-FITC and propidium iodide (PI) stain, then analysing cells by flow cytometry. Annexin V-FITC is a FITC-conjugated ligand that specifically binds to phosphatidylserine (PS). In live cells PS is restricted to the inner leaflet of the plasma membrane and therefore inaccessible to antibody. Apoptotic cells express PS on the outer leaflet of the plasma membrane, whereas necrotic cells have lost membrane integrity (making inner leaflet PS accessible to Annexin V) and so both forms of cell death stain positive for Annexin V. PI intercalates into DNA. It cannot permeate intact plasma membrane and is therefore used to distinguish between apoptotic and necrotic/secondary necrotic death [See Fig. 3.1].

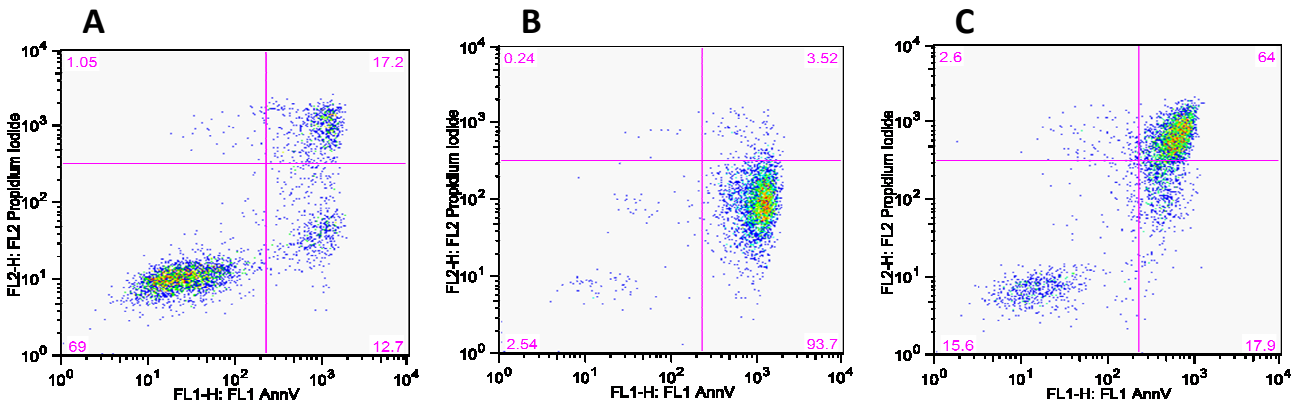


Figure 3.1 Example Annexin V-PI FACS plots. For each graph, the cells in the lower-left quadrants have stained low for both AnV and PI and hence were alive upon staining. Cells in the lower-right quadrant have stained high for AnV (therefore express PS) and stain low for PI (therefore have membrane integrity), therefore these cells were apoptotic. The upper right quadrant cells stained high for AnV and PI, therefore these cells were dead. **A.** These cells were FDCP cells taken straight from culture, and it can be seen that 69% were alive. **B.** FDCP were treated with camptothecin for 48 hrs, resulting in apoptosis in 93.7% of cells. **C.** FDCP were exposed to high heat for 30 mins, resulting in necrosis in 64% of cells.

3.1.2 Results

3.1.2.1 High levels of necrosis are induced in FDCP by heat kill and repeated freeze-thaw cycles

Incubating FDCP in cRPMI for 30 minutes at 60˚C caused necrosis in 94% of cells [Figure 3.2: 'Heat kill']. Exposing FDCP to three cycles of alternating incubations of -80˚C and room temperature caused necrosis in approximately 85% of cells (Figure 3.2: 'Freeze-thaw'). 10% of cells survived freeze-thaw, appearing as neither apoptotic nor necrotic according to AnV-PI staining. Freeze-thaw caused cells to aggregate heavily and become difficult to manipulate and pipette.

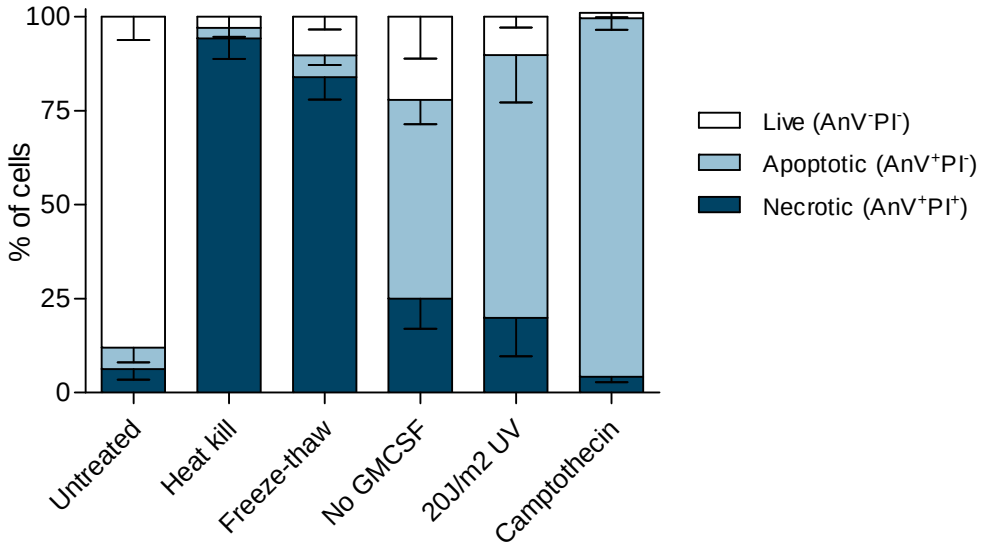


Figure 3.2 Apoptosis and necrosis of FDCP after various death-inducing treatments. FDCP were killed by heat treatment (60˚C, 30mins), three freeze-thaw cycles, 24hr GMCSF withdrawal ('No GMCSF'), 20 J/m² UVC plus 24hr incubation, or 48hr exposure to 14.4μM camptothecin. The percentage and type of cell death in untreated and killed FDCP was determined by Annexin V and PI staining. Data is from at least three experiments for each treatment, bars indicate SD.

3.1.2.2 Exposure to camptothecin induces high levels of apoptosis in FDCP

Incubation with 14μM of the topoisomerase I-inhibitor (229), camptothecin, induces a very high percentage (95%) of FDCP to undergo apoptosis, as indicated by $AnV^{+}PI^{-}$ staining [Figure 3.2]. It required a minimum of approximately 24 hours constant camptothecin exposure to commit to apoptosis the proportion of cells that were detectable as apoptotic at 48 hours [Figure 3.3]. Furthermore, there is no increase in the presence of necrotic cells throughout the incubation period, unlike in GMCSF withdrawal. Full execution of the apoptotic pathway was confirmed by demonstration of the late-stage process of DNA fragmentation, as illustrated by the laddering of ethidium bromide-stained DNA on agarose gel [Figure 3.6]. Thus camptothecin treatment provides a broad interval of time wherein ACs constitute a very high percentage of the cell sample.

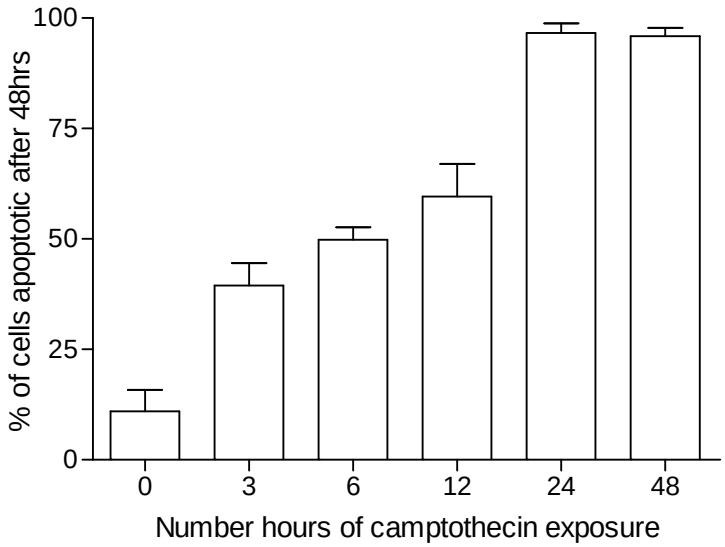


Figure 3.3 All FDCP that are apoptotic after 48 hrs of camptothecin-treatment are committed to apoptosis within 24 hrs of the start of treatment. FDCP were incubated with 14.4μM camptothecin for the indicated number of hours before being washed and incubated in fresh media for the remainder of a total 48hr incubation. 24hrs of camptothecin exposure was necessary and sufficient for virtually all cells to be detected as apoptotic 48hrs after beginning of treatment. Percentages of cells undergoing apoptosis were signified by Annexin V-positive, PI-negative, staining. Data is from at least three experiments for each treatment, bars indicate SD.

3.1.2.3 GMCSF withdrawal induces apoptosis and secondary necrosis in FDCP

FDCP underwent apoptosis after withdrawal of the growth factor GMCSF. Within 48 hours, approximately 46% of cells displayed an apoptotic phenotype according to AnV-PI staining [Figure 3.2]. However, many FDCP progressed to secondary necrosis while others were yet to begin apoptosis. The proportion of apoptotic cells was at a maximum at 24 hours post-withdrawal (data from other times not shown). This finding required confirmation using non- AnV-PI methods because AnV-PI data alone was misleading: In monitoring progression of apoptosis/necrosis at numerous intervals for all cell death methods (data not shown), it was originally shown that virtually all cells were apoptotic after approximately 68 hours of GMCSF withdrawal [Figure 3.4A]; however, the data also showed an initial increase in the proportion of NC (a maximum, at 24 hours, of 38%) which then declined (to 9% − similar to the proportion of NC prior to any GMCSF withdrawal).

Fluorescence microscopy was used to compare the staining of DNA of live FDCP, apoptotic (48hr camptothecin-treated) FDCP, and the suspect 68-hour GMCSF-withdrawn FDCP. Live cells demonstrated very clear staining of the DNA by DAPI in compact and distinct nuclei [Figure 3.4B]. Camptothecin-treated cells showed clear staining of DNA that had disintegrated and was spread throughout the cell [Figure 3.4C], and close inspection shows DNA being packaged into apoptotic bodies forming at the cell surfaces (inset, yellow arrows). By contrast, very few GMCSF-withdrawn FDCP showed evidence of DNA staining at all [Figure 3.4D]. This revealed that negative PI staining was due to a loss of DNA, which could most probably be attributed to secondary necrosis. Accordingly, GMCSF-withdrawal was dismissed as a method of apoptosis induction.

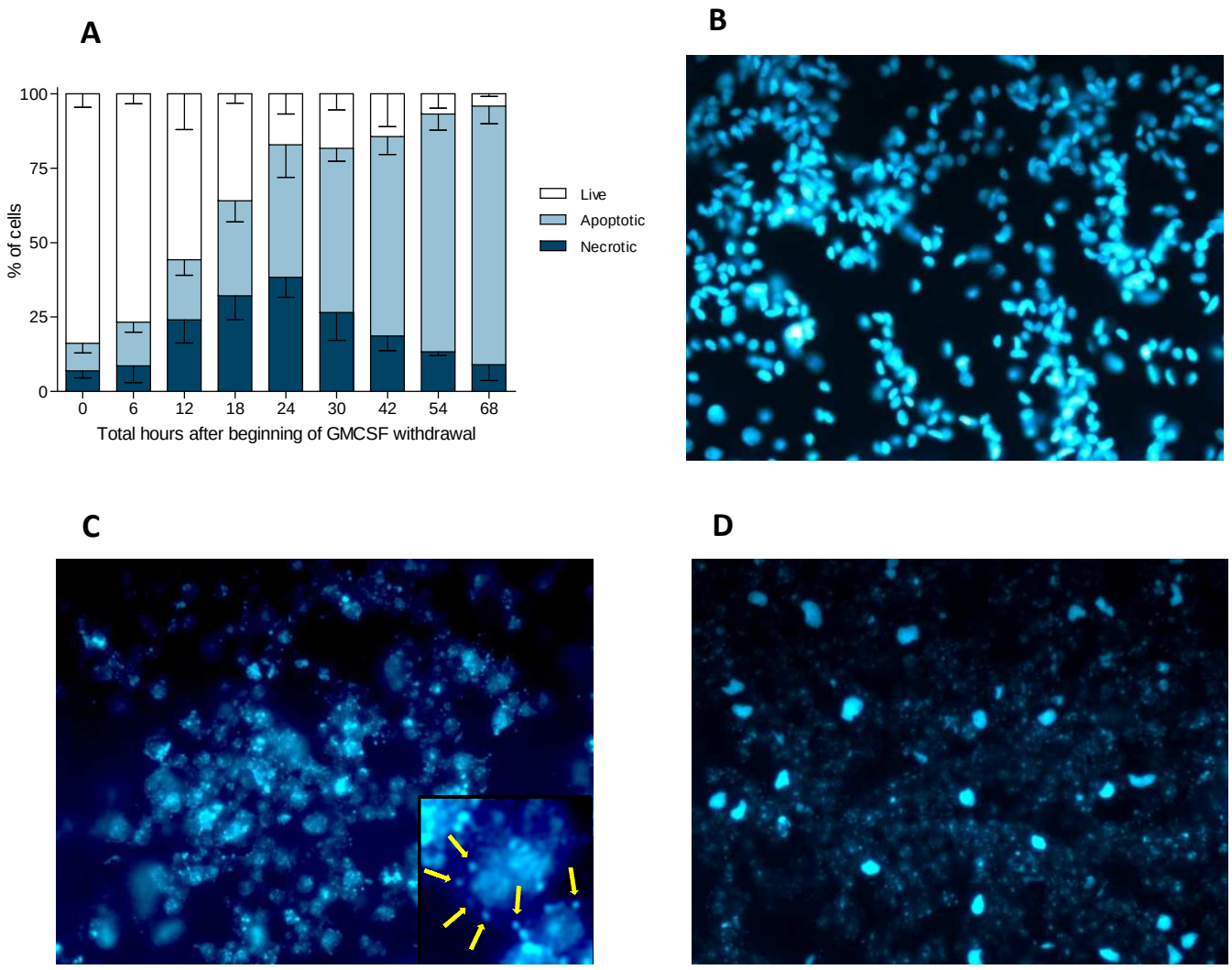


Figure 3.4 GMCSF-withdrawal causes necrosis in FDCP but AnV-PI staining falsely indicates apoptosis. **A.** FDCP were cultured without GMCSF for up to 68hr and the percentage and type of cell death determined by AnV-PI staining, which indicated that the percentage of apoptotic FDCPs increased steadily over time (and nears 100% of the total cell population), whereas the percentage of necrotic FDCPs inexplicably decreased after 24hr. Fluorescence microscopy and DAPI staining of DNA was then used to compare DNA disintegration of live (untreated) FDCP, apoptotic (48-hour camptothecin-treated) FDCP, and GMCSF-withdrawn FDCP. **B.** Live cells had visibly intact nuclei. **C.** Nuclei of 48hr camptothecin-treated FDCP disintegrated into distinct fragments. DNA was seen to be packaged into apoptotic vesicles (yellow arrows, inset). **D.** The nuclei of 72hr GMCSF-withdrawn FDCP were undetectable by DAPI staining, indicating either complete disintegration of DNA and/or loss of DNA from the cell body. Data and images are representative of at least two experiments for each treatment, bars indicate SD.

3.1.2.4 UV exposure dose-dependently induces apoptosis and necrosis in FDCP

UV exposure was investigated at various doses (0.5-200J/m^2) and subsequent lengths of time of incubation (2-72 hours). The maximum percentages of UV-induced ACs observed were apparent at 24 hours post-UV (data not shown), which was the post-UV incubation time used for the UV dose experiments summarised here. The incidence of cell death increased with increasing dosage of UV, which raised the percentage of dead cells from 25% (untreated FDCP,) to 57% at 1 J/m^2, 78% at 10 J/m^2, and plateauing at ~90% at 20 J/m^2 and upwards [Fig. 3.5]. The increase in dead cells comprised increases in both ACs and NCs. The maximum proportion of ACs (73%) was observed at a UV dose of 20 J/m^2. Apoptosis at 2 J/m^2 and 20 J/m^2 was confirmed by banding of ethidium bromide-stained DNA on agarose gel [Figure 3.6]. Proportions of necrotic cells increased slowly from 7% of untreated cells and reached a maximum of 43% of cells at the highest tested UV dose, 200 J/m^2 [Fig. 3.5]. UV exposure successfully induced apoptosis but also caused damage to cells such that necrosis and/or secondary necrosis incidence increased concurrently with apoptosis. This method of cell death was therefore insufficient for the purposes of this study.

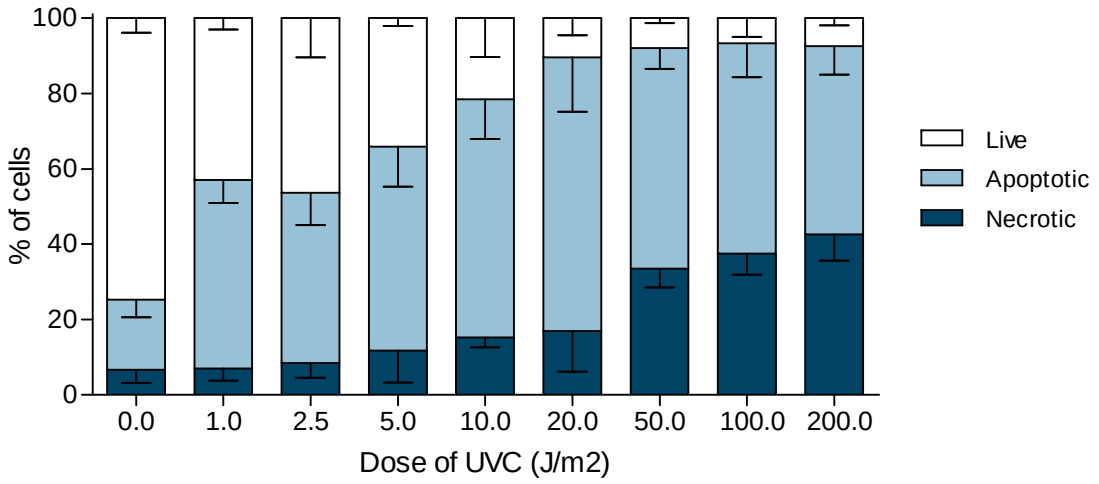


Figure 3.5 UV exposure dose-dependently induces apoptosis and necrosis in FDCP. FDCP were exposed to UV at various doses, incubated for 24hrs, and then analysed for cell death by AnV-PI staining. The maximum percentage of apoptotic cells (73%) is achieved at 20 J/m^2, whereas higher doses of UV induce increasing proportions of necrosis. Data is from three separate experiments, bars indicate SD.

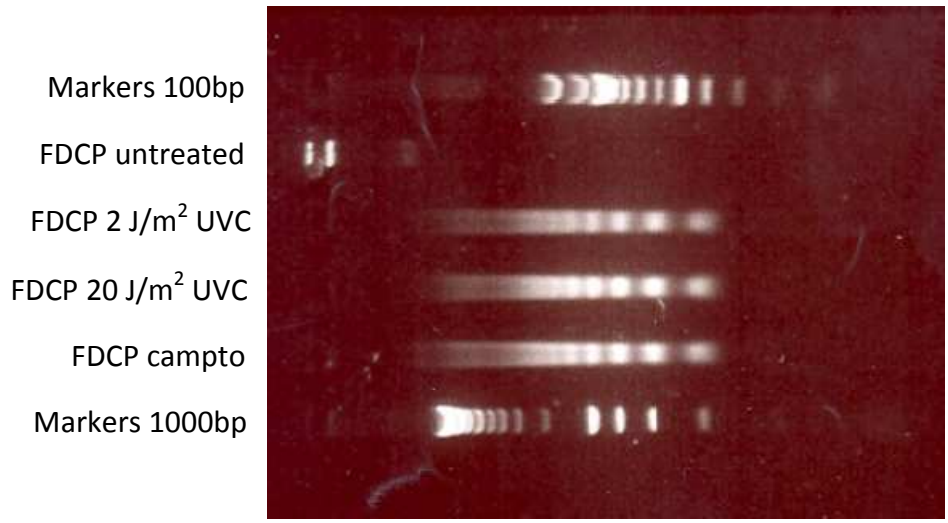


Figure 3.6 UV and camptothecin treatment caused DNA fragmentation in FDCP. FDCP were exposed to 2 J/m^2 UVC or 20 J/m^2 then incubated for 24hrs, or exposed to 14.4μM camptothecin then incubated for 48hrs. Untreated FDCP had intact, unfragmented DNA that, after ethidium bromide staining, did not separate into different bands on agarose gel during electrophoresis. The DNA of the UV-treated and the camptothecin-treated FDCP separated into multiple, distinct bands on the gel, indicating DNA fragmentation. This image is representative of two separate experiments.

3.2 Alteration of dendritic cell phenotype by apoptotic and necrotic cells

3.2.1 Introduction

DCs express a range of cell surface proteins that literature demonstrates will be up- or down-regulated in response to pro- or anti-inflammatory stimuli, and in doing so act as markers of DC maturation or inhibition associated with immunogenicity or tolerance. Together, these markers include receptors for phagocytosis, receptors and ligands for T cell interactions, and receptors for chemokines and cytokines. We used murine bone marrow-derived dendritic cells (BMDC) as a model DC type to examine how apoptotic and necrotic cells alter the expression of these markers.

Across the literature, cell surface expression of CD40 and CD86 is highly sensitive to maturation stimuli and is (as are numerous other co-stimulatory molecules) consistently low on immature DCs, and high on mature DCs. As such, CD40 and CD86 are well established as standard markers of DC maturation, and are used to characterise the activation state of DC in initial experiments of this study. These early experiments were to ascertain the ideal conditions for BMDC culture and treatment. We then studied the direct effects of DC interaction with dead cells by incubating DC with apoptotic or necrotic cells at a ratio of 2:1 dead cells:DCs for 24-48 hours and examining phagocytosis and DC survival. Next, to determine the immunological effect of apoptotic cells and necrotic cells (24hr camptothecin-treated FDCP and heat-killed (60˚C for 30mins) FDCP, respectively, in all experiments hereafter, unless otherwise stated) on DCs, DCs were treated with dead cells for 24 or 36 hours and then examined for expression of a range of maturation and inhibition markers. To determine if dead cells affected DC ability to respond to exogenous maturation stimuli (in this case, TLR ligands) some DCs were treated with LPS/CpG for the last 16 or 20 hours of incubation. Dead cells were added before TLR ligand because DC maturation

inhibits phagocytosis (2) and cross-presentation of antigens(230). DCs were then examined by antibody staining and flow cytometry for levels of expression of cell surface markers.

DCs were analysed using flow cytometry to determine levels of expression of several well-established co-stimulatory proteins that act as markers of maturation. The levels of expression of these markers on resting DCs were compared alongside the levels on DCs treated with LPS or CpG, which are well known to induce up-regulation of CD40, CD80, CD83, CD86 and CD25, amongst other markers (231). For each marker, the percentage of resting DCs expressing a high level of that marker was set at an amount of approximately 10%, representing non-specifically-matured DCs (i.e. DCs that have matured in response to a mechanical, temperature or age-related stress rather than a specific exogenous stimulus) in order to normalise between experiments. This value ensured that responses to maturation stimuli could be confirmed by observing an increase in the percentage of cells expressing high levels of these markers, while still allowing for observation of any inhibition of non-specific maturation. For example, comparing CD40 expression on resting DCs with expression on LPS-treated DCs [Fig. 3.11] reveals that LPS at the given concentration induces 31% of the DCs to express high levels of CD40, compared to the 10% that are induced non-specifically in the untreated sample. In these measurements, where the response to LPS/CpG can be confidently predicted, it is the relative, rather than absolute, percentage of mature DCs that is of interest. In contrast to these well-studied markers of maturation, the role of the co-inhibitory members of the B7 family B7DC, B7H1, B7H2, B7H3 and B7H4 is not so well established, and even disputed. Although all linked to tolerance, most are the subject of debate, with conflicting evidence pointing towards dual roles. Therefore, up-regulation or down-regulation in response to maturation stimuli could not be taken for granted either way and so the resting expression level was established by comparing with antibody isotype controls. In all BMDC samples, cells were also gated on CD11c-high cells and by size, in order to gate out dead cells and cell debris.

3.2.2 Results

3.2.2.1 DCs phagocytose both apoptotic and necrotic FDCP

Apoptotic (camptothecin-treated) FDCP and necrotic (heat kill-treated FDCP) FDCP (ACs and NCs, respectively, hereafter) were stained with the red membrane dye PKH26 and incubated with CFSE-labelled DCs. Phagocytosis of dead cells by DCs was measured by detecting DCs that were PKH26-positive. ACs were phagocytosed with an incidence approximately equal to NCs (by 22% and 19% of DCs, respectively, $p>0.05$) [Figure 3.7]. Co-incubations were repeated with the addition of cytochalasin D (cytD), which blocks actin polymerisation and thus phagocytosis (232). The addition of cytD significantly decreased the association of DCs with dead cells (to 3% and 5% for ACs and NCs, respectively), confirming that the association is actin-dependent and therefore due to phagocytosis rather than surface adhesion between cells.

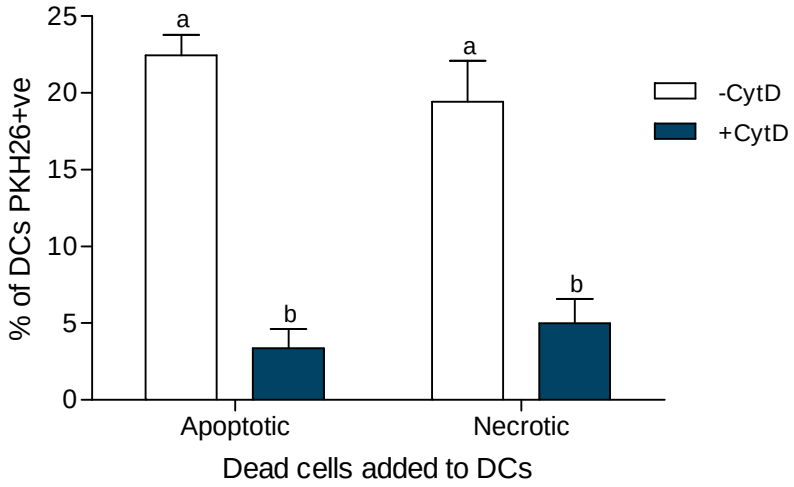


Figure 3.7 DCs phagocytose both apoptotic and necrotic FDCP. Apoptotic cells (camptothecin-treated FDCP) and necrotic cells (heat-killed FDCP) were stained with PKH26 and incubated with CFSE-labelled DCs for 24hrs. CD11c+CFSE+ DCs that were positive for PKH26, in a manner negated by the presence of the actin-inhibitor cytochalasin D (cytD), were indicative of DC phagocytosis of the dead cells. Apoptotic cells were phagocytosed by 22% of DCs, but in the presence of cytD were phagocytosed by only 3% of DCs. Necrotic cells were phagocytosed by 19% of DCs ($p>0.05$ compared to apoptotic cells), but in the presence of cytD were phagocytosed by only 5%. 'a' and 'b' signify significantly different means, two-way ANOVA. Data is from three separate experiments, bars indicate SD.

3.2.2.2 DCs remain alive after phagocytosing apoptotic cells or necrotic cells

To determine if phagocytosis of dead cells induces cell death in DCs, or if DC viability was inadvertently affected by the method of FDCP killing (for example by residual camptothecin), DC cell death was examined following 48-hour incubation with dead cells. AnV-PI staining was used to determine that the percentage of live DCs was unaffected by incubation with either dead cell type, remaining as it did at approximately 70% [Fig. 3.8]. DCs co-incubated with ACs or NCs incurred similar levels of apoptosis and necrosis (approximately 10% and 20%, respectively) as untreated DCs. Therefore, neither ACs nor NCs induce DC death. Continued population growth was observed after the 48-hour incubation period, suggesting that DCs remained viable for longer than 48 hours after incubation with dead cells.

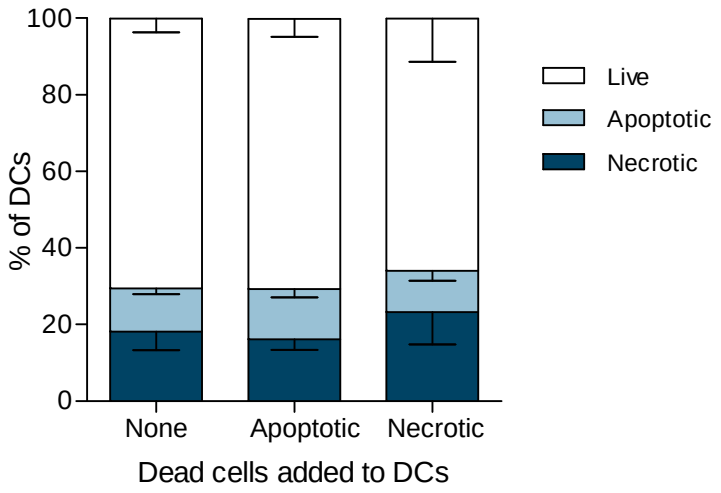


Figure 3.8 DCs remain alive after 48hr co-culture with apoptotic cells (ACs) or necrotic cells (NCs). DCs were cultured with dead FDCP for 48hrs, then the incidence of death in the DCs themselves was examined using AnV-PI staining. DCs co-incubated with either ACs or NCs incurred similar levels of apoptosis and necrosis (approximately 10% and 20%, respectively) as untreated DCs. Therefore, ACs and NCs do not induce death of DCs. Data is from three separate experiments, bars indicate SD.

3.2.2.3 Ag8-secreted GMCSF generated immature BMDCs, except in high concentrations which increases resting levels of co-stimulatory proteins

GMCSF for use in DC generation from bone marrow was produced using the GMCSF-secreting murine cell line Ag8. To validate the use of Ag8-secreted GMCSF (that is, cultured in-house) rather than use of purchased, certified endotoxin-free, recombinant GMCSF, the two were examined alongside each other for proinflammatory properties. At 40ng/ml, both recombinant and secreted GMCSF generated DCs with low levels of the cell surface maturation markers CD40 and CD86 (compared to LPS exposure in the final 24 hours) at Day 6 of bone marrow culture [Figure 3.9]. This indicated that GMCSF produced in-house from the Ag8 cell line was suitable for generating immature DCs for this investigation. 500ng/ml GMCSF induced higher amounts of CD40- and CD86-high DCs by Day 6 than low GMCSF, but also a higher yield of DCs (data not shown). ~100ng/ml was used in all subsequent DC culture for maximum production of CD11c-high, CD40/CD86-low DCs. None of the GMCSF amounts examined induced secretion of IL12p40 (data not shown).

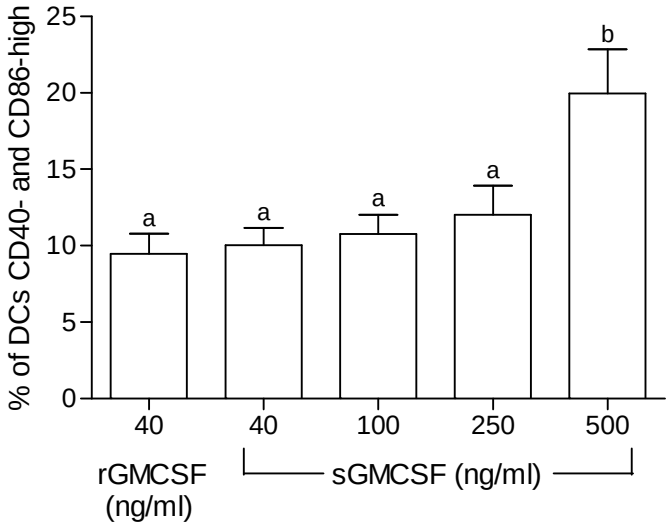


Figure 3.9 Ag8-secreted GMCSF generated immature BMDCs, except in high concentrations which increases resting levels of co-stimulatory proteins. BMDCs (DCs) were generated by culturing bone marrow for 6 days in cRPMI supplemented with either certified endotoxin-free recombinant GMCSF (rGMCSF) or various doses of GMCSF purified from the supernatant of the GMCSF-secreting cell line Ag8 (sGMCSF). Low doses (40-250ng/ml) of sGMCSF induced the same percentage of CD40/CD86-high DCs that 40ng/ml rGMCSF did (10% and 9%, respectively), demonstrating that the Ag8-produced GMCSF was adequately non-inflammatory for generation of DCs for this study. However, 500ng/ml sGMCSF generated DCs with a higher percentage of CD40/CD86-high cells (20% compared to 10-12% in low-GMCSF DCs). 'a' and 'b' signify significantly different means, one-way ANOVA. Data is from at least three experiments for each treatment, bars indicate SD.

3.2.2.4 Necrotic cells, but not apoptotic cells, induce DC up-regulation of co-stimulatory proteins

DCs incubated for 24 hours with ACs or NCs were compared with each other and with untreated DCs for changes in expression of co-stimulatory proteins.

CD40, CD80 and CD86 expression was not up-regulated on DCs that had been incubated with ACs [

]. ACs induced slight up-regulation of CD25 from 9% to 14%, though this was not statistically significant. ACs did induce significant up-regulation of CD83, increasing the proportion of DCs expressing high levels of CD83 from 9% to 15% ($p<0.05$).

NCs induced significant up-regulation of CD40 from 10% to 19%, CD80 from 11% to 20%, CD83 from 9% to 25%, and CD86 from 11% to 18% ($p<0.001$ for each). Expression of CD25 was significantly up-regulated after NC treatment from 9% to 24% ($p<0.01$). ACs and NCs had significantly different effects on the levels of DC expression of all of the selected co-stimulatory molecules, with the exception of CD25. In general, NCs induced up-regulation of co-stimulatory molecules to a highly significant degree whereas ACs did not. Statistically significant changes in expression of co-stimulatory molecules (compared to untreated DCs) are summarised in Table 1.

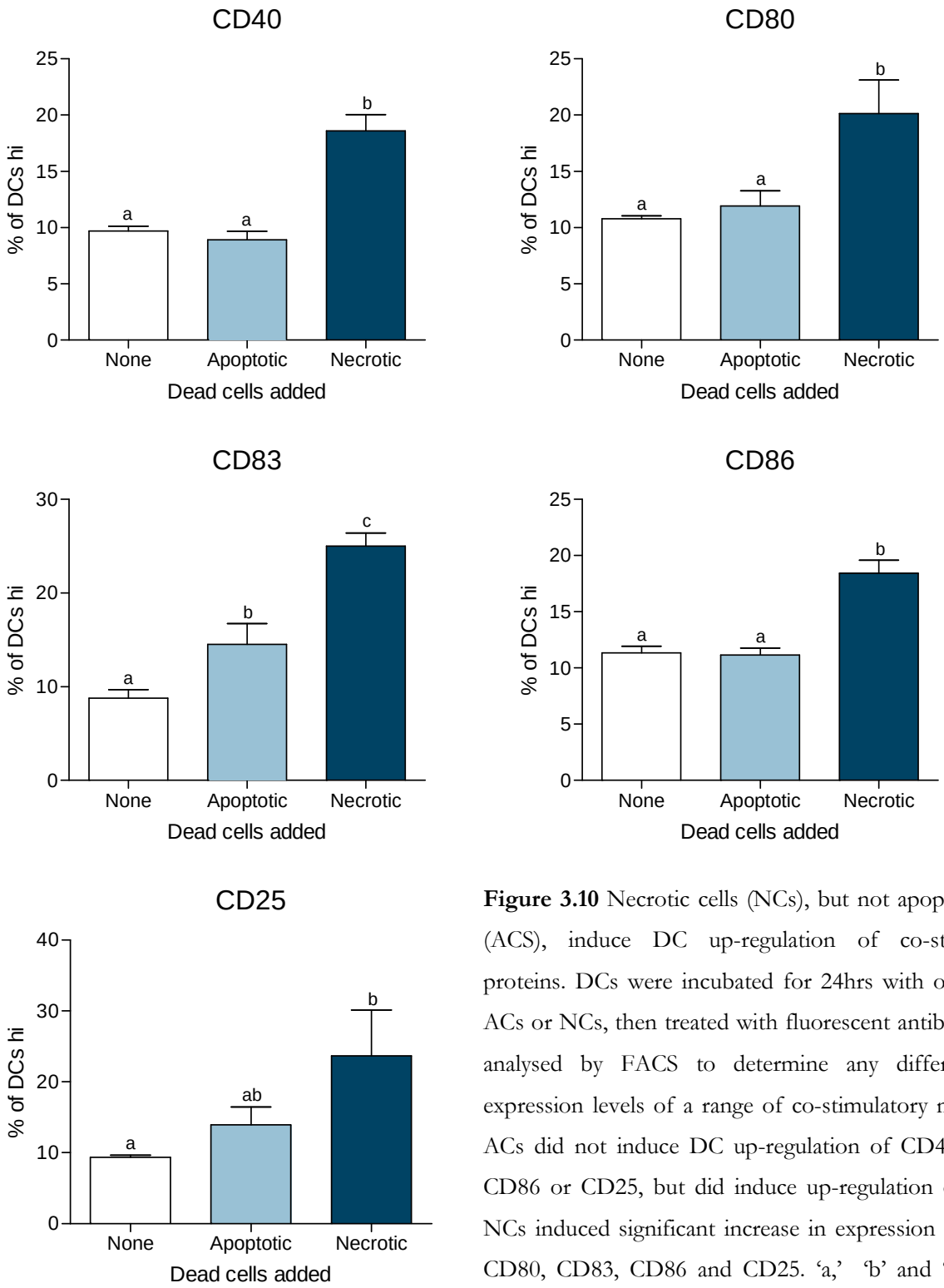


Figure 3.10 Necrotic cells (NCs), but not apoptotic cells (ACS), induce DC up-regulation of co-stimulatory proteins. DCs were incubated for 24hrs with or without ACs or NCs, then treated with fluorescent antibodies and analysed by FACS to determine any differences in expression levels of a range of co-stimulatory molecules. ACs did not induce DC up-regulation of CD40, CD80, CD86 or CD25, but did induce up-regulation of CD83. NCs induced significant increase in expression of CD40, CD80, CD83, CD86 and CD25. 'a,' 'b' and 'c' signify significantly different means, one-way ANOVA. Data for each molecule is from three separate experiments or more, bars indicate SD.

Figure 3.11 (Below) DC co-stimulatory molecule FACS plots. FACS plots representative of the results graphed in Figure 3.10 are shown below. Histograms are shown for each co-stimulatory molecule (CD40, CD80, CD83, CD86 and CD25), depicting the expression level of the relevant molecules on untreated DCs ('None'), apoptotic cell-treated DCs ('+Apoptotic'), necrotic cell-treated DCs ('+Necrotic') and LPS-treated DCs ('+LPS'), as indicated. Below these are shown the corresponding graphs of the co-stimulatory molecule expression levels (i.e. fluorescence intensity) plotted against CD11c (on which the molecules were gated during analysis, such that only CD11c+ cells were analysed).

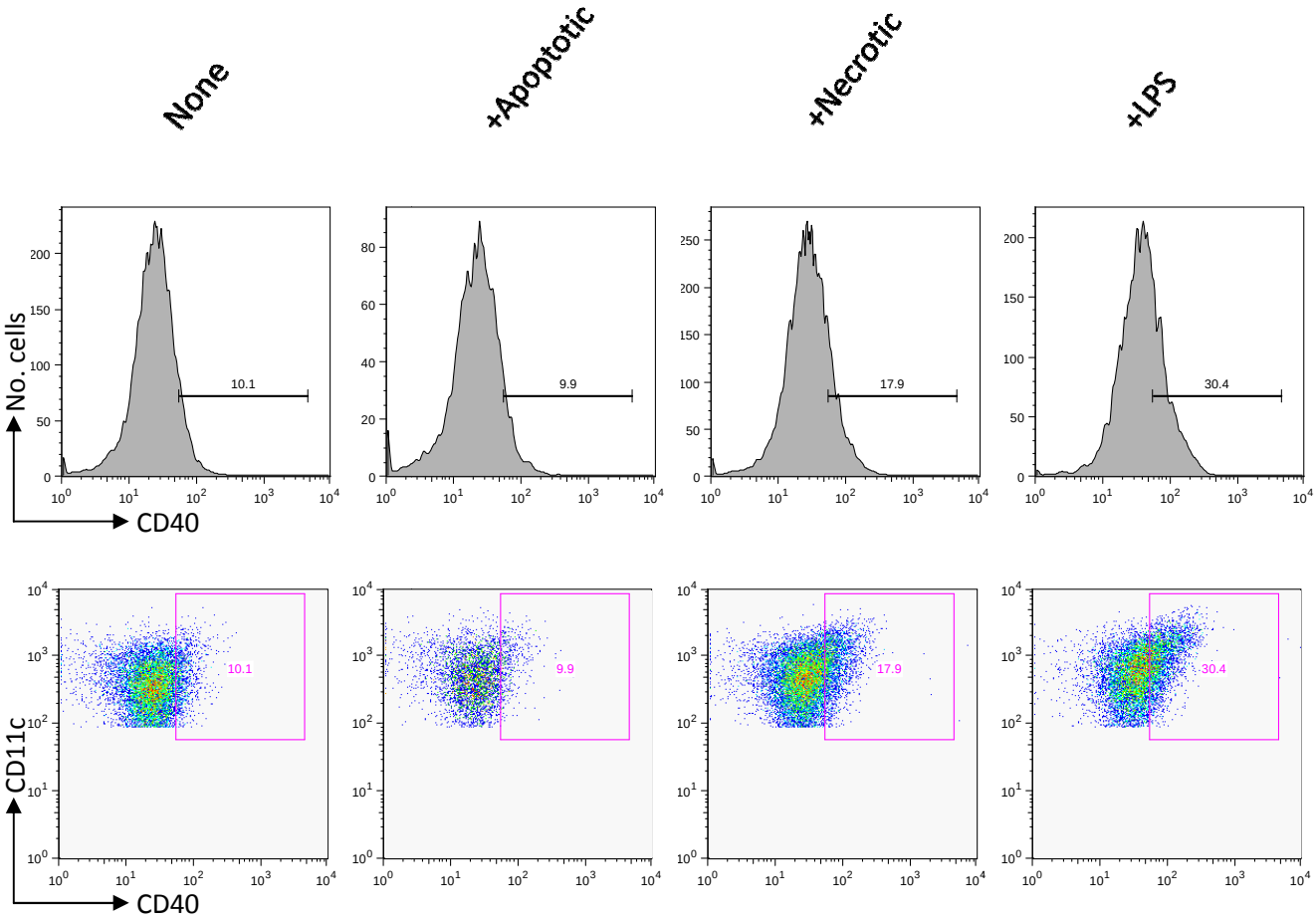


Figure 3.11 (1/3) See above.

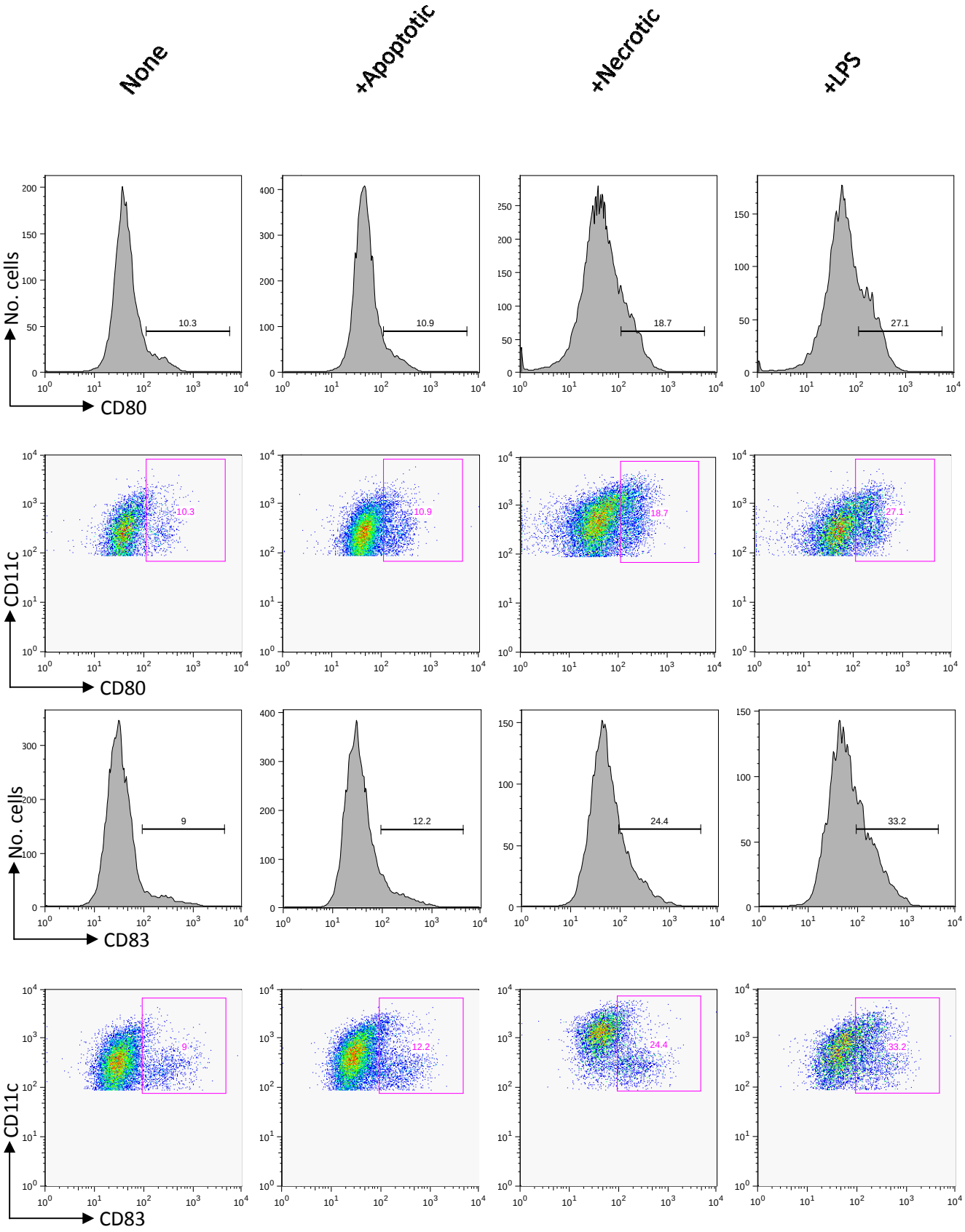


Figure 3.11 (2/3) See p68.

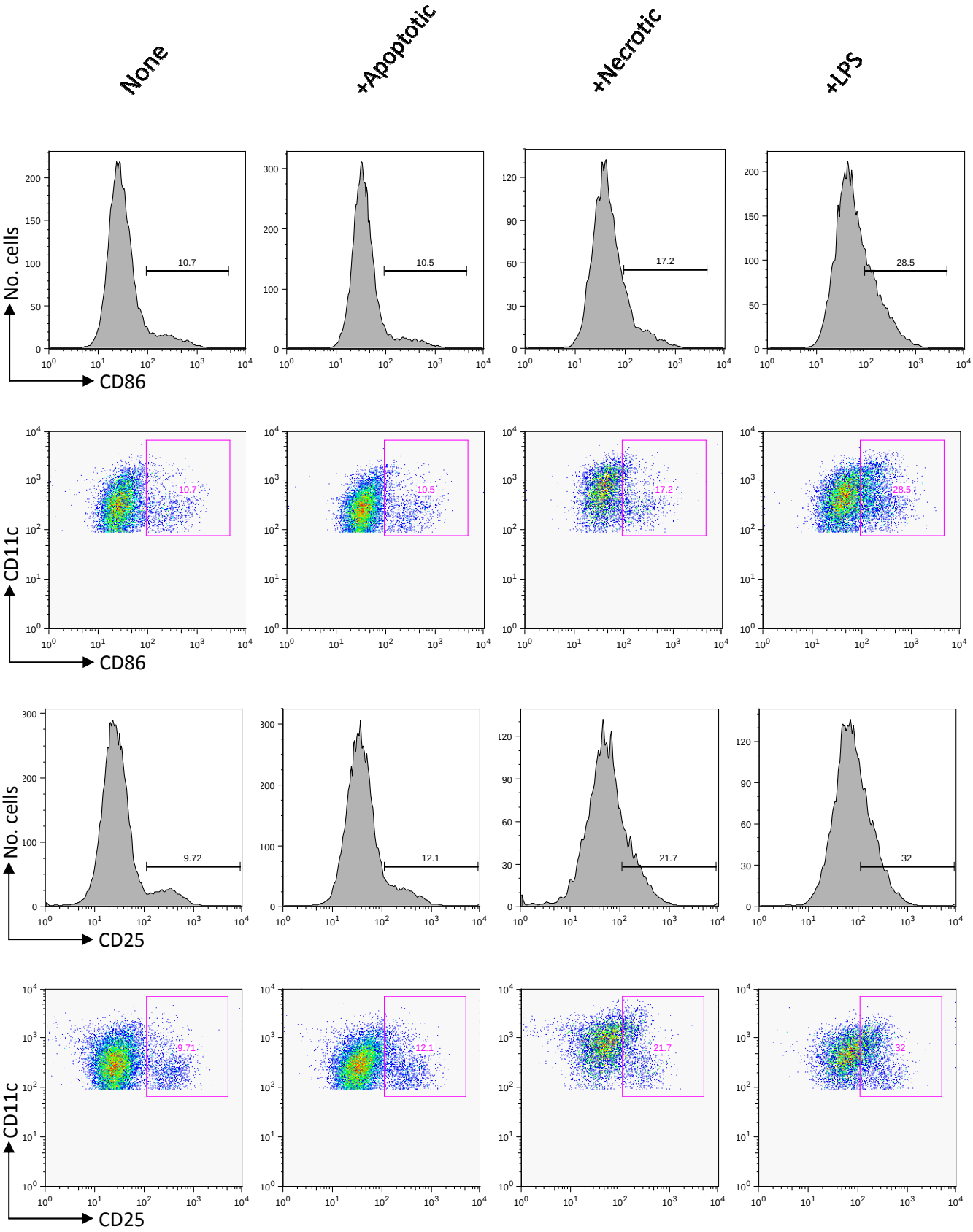


Figure 3.11 (3/3) See p68.

3.2.2.5.Apoptotic cells and necrotic cells both induce DC up-regulation of co-inhibitory proteins (but induce dissimilar combinations)

DCs incubated for 24 hours with ACs or NCs were compared with each other and with untreated DCs for changes in expression of co-inhibitory proteins.

ACs did not induce significant up-regulation of B7H1 from a resting level of 21% or B7DC from a resting level of 30%, but significantly increased B7H2 from 20% to 38% ($p<0.001$), B7H3 from 5% to 12% ($p<0.001$), and B7H4 from 5% to 10% ($p<0.001$) [**Error! Reference source not found.**].

NCs induced significant up-regulation of B7DC from 30% to 38% ($p<0.001$), B7H1 from 21% to 29% ($p<0.001$), B7H2 from 20% to 34% ($p<0.01$), and B7H4 from 5% to 8% ($p<0.05$). NCs induced up-regulation of B7H3 but not to a significant degree.

AC-induced levels of expression of B7H2, B7H3 and B7H4 were not significantly different from NC-induced levels, though AC-induced levels were consistently higher. NC-induced expression was significantly higher than AC-induced expression for B7DC ($p<0.001$) and B7H1 ($p<0.05$). In general, ACs induced high increases in co-inhibitory molecule expression, and NCs induced relatively moderate increases. Statistically significant changes in expression of co-inhibitory molecules (compared to untreated DCs) are summarised in Table 1.

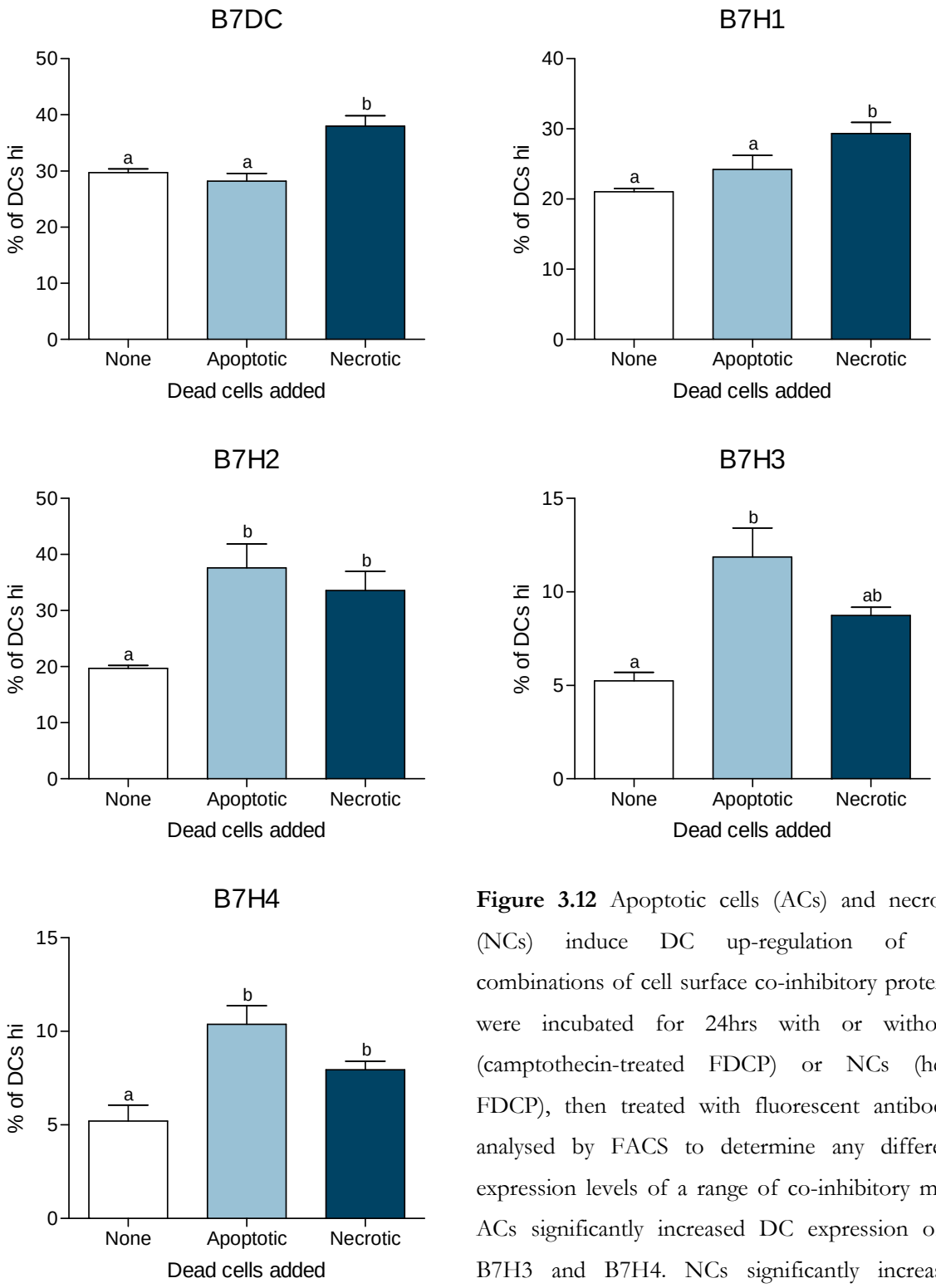


Figure 3.12 Apoptotic cells (ACs) and necrotic cells (NCs) induce DC up-regulation of different combinations of cell surface co-inhibitory proteins. DCs were incubated for 24hrs with or without ACs (camptothecin-treated FDCP) or NCs (heat-killed FDCP), then treated with fluorescent antibodies and analysed by FACS to determine any differences in expression levels of a range of co-inhibitory molecules. ACs significantly increased DC expression of B7H2, B7H3 and B7H4. NCs significantly increased DC expression of B7DC, B7H1, B7H2 and B7H4. 'a' and 'b' signify significantly different means, one-way ANOVA. Data for each molecule is from three separate experiments or more, bars indicate SD.

Figure 3.13 (Below) DC co-inhibitory molecule FACS plots. FACS plots representative of the results graphed in Figure 3.12 are shown below. Histograms are shown for each co-inhibitory molecule (B7DC, B7H1, B7H2, B7H3, B7H4), depicting the expression level of the relevant molecules on untreated DCs ('None'), apoptotic cell-treated DCs ('+Apoptotic'), necrotic cell-treated DCs ('+Necrotic') and LPS-treated DCs ('+LPS'), as indicated. Below these are shown the corresponding graphs of the co-inhibitory molecules plotted against CD11c, on which the molecules were gated during analysis, such that only CD11c+ cells were analysed.

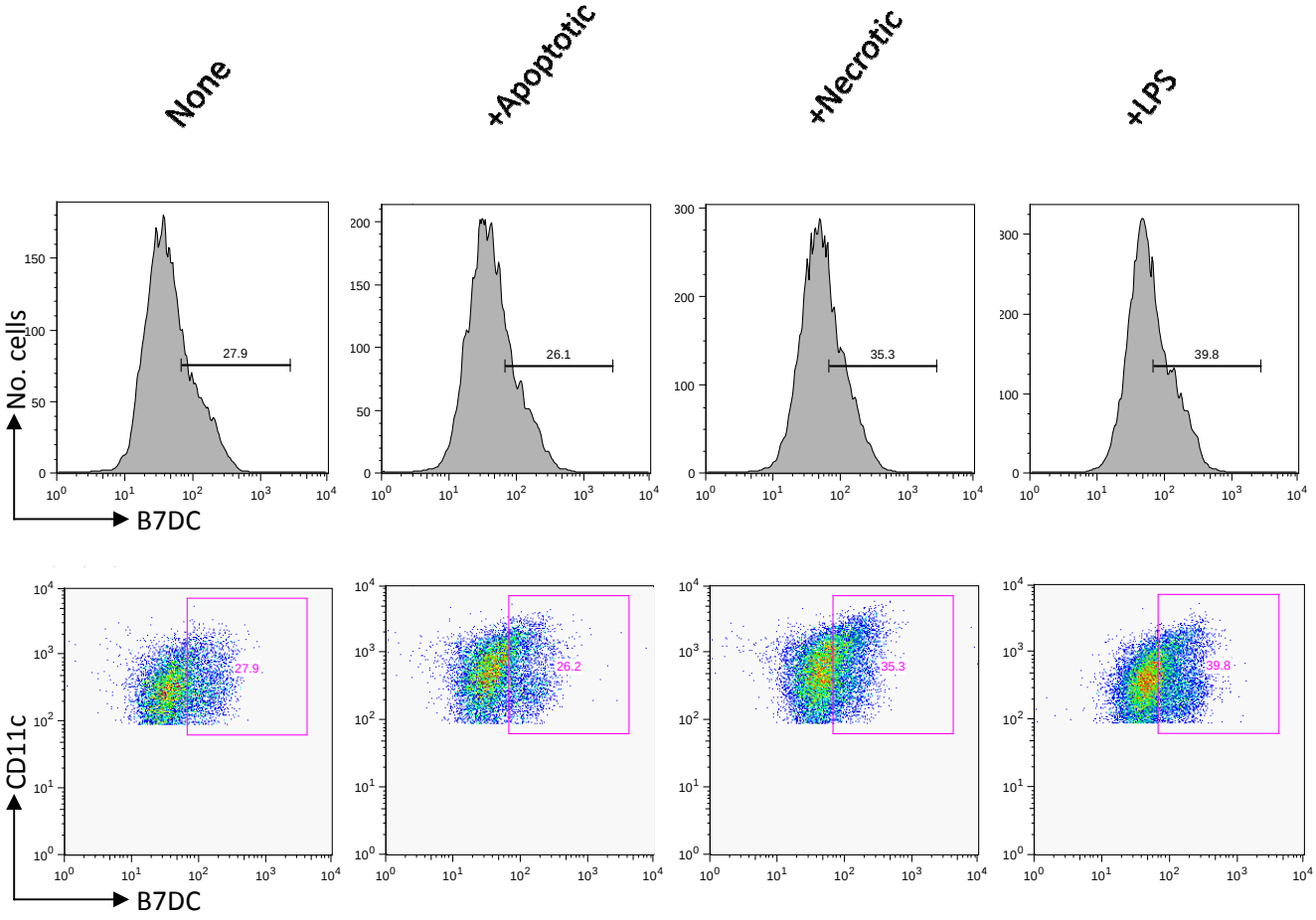


Figure 3.13 (1/3) See above.

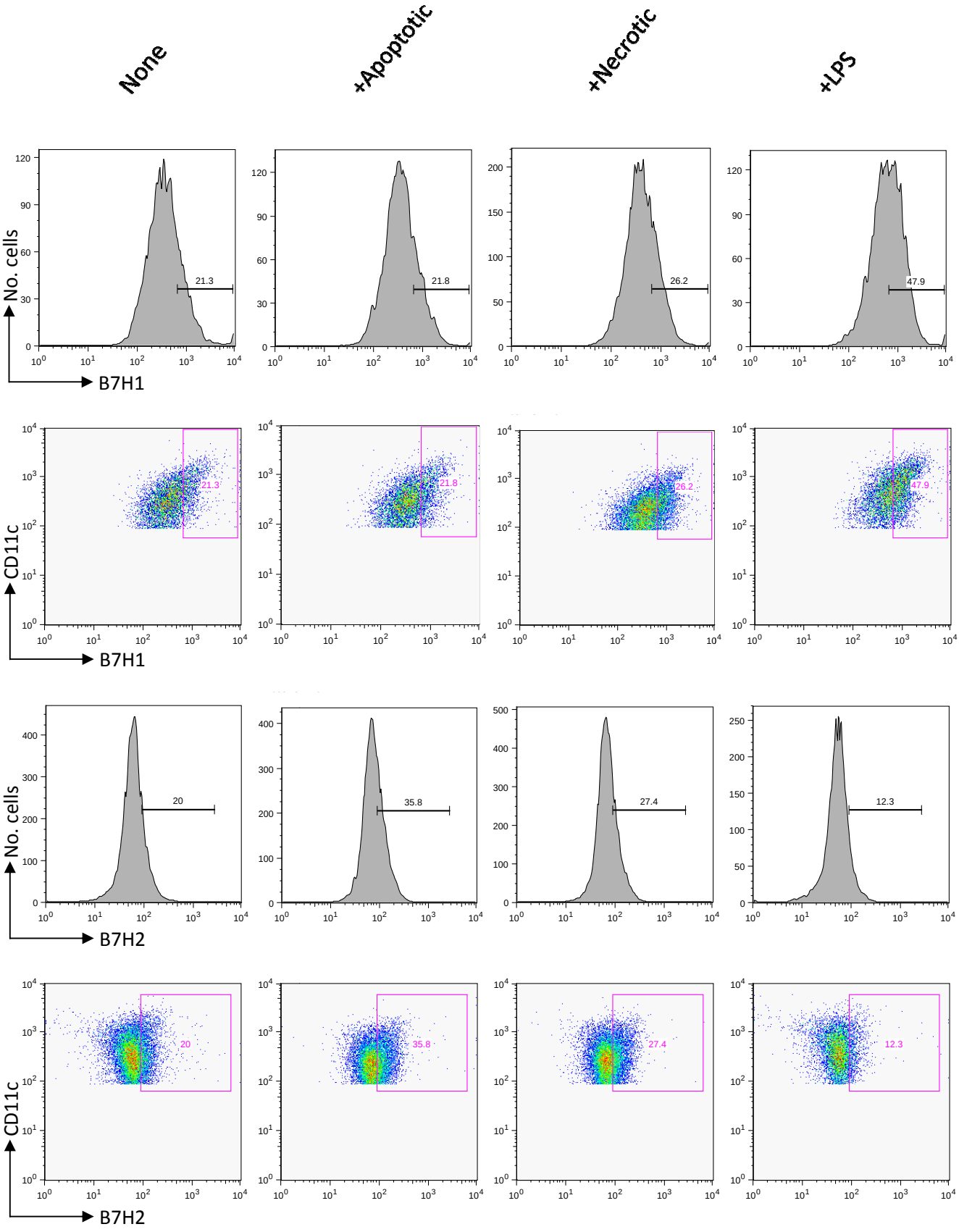


Figure 3.13 (2/3) See p73.

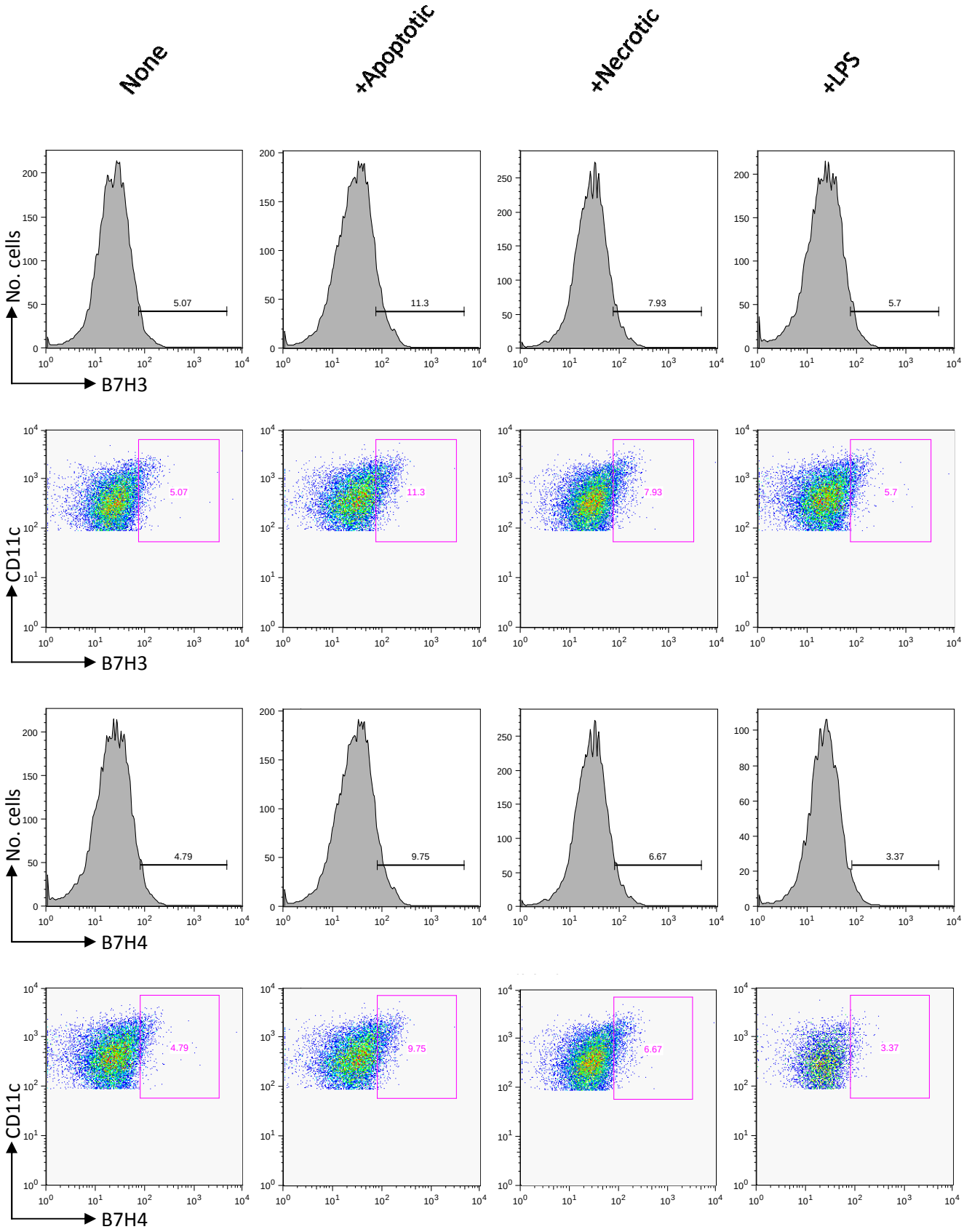


Figure 3.13 (3/3) See p73.

Table 1 Significant changes in DC surface expression of co-stimulatory and co-inhibitory molecules after 24 hour incubation with apoptotic or necrotic cells.

	CD40	CD80	CD83	CD86	CD25	B7DC	B7H1	B7H2	B7H3	B7H4
Apoptotic cells	↔	↔	↑	↔	↔	↔	↔	↑↑↑	↑↑↑	↑↑↑
Necrotic cells	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑	↑↑↑	↑↑↑	↑↑	↔	↑

↑, ↑↑, ↑↑↑ = Increased expression where $p<0.05$, 0.01, 0.001, respectively

↔ = No significant change

Red and pink indicate changes associated with inflammation in the literature

Greens indicate changes associated with tolerance/suppression/resolution of inflammation in the literature

Red and deeper green indicate increases of ≥x1.75

3.2.2.6 Neither apoptotic cells nor necrotic cells affect LPS-induced up-regulation of co-stimulatory molecules

The ability of dead cells to inhibit or enhance PAMP-induced DC maturation, as indicated by alteration of expression of co-stimulatory and co-inhibitory molecules, was investigated. First, the response to LPS alone was determined. Then DCs were incubated with ACs or NCs, and supplemented with LPS for a further incubation period. AC- and NC-treated DCs that were subsequently treated with LPS were analysed by FACS alongside LPS-treated DCs (not pre-treated with dead cells) and statistically compared by one-way ANOVA (for details of multiple comparison tests please see 2.9).

The proportion of DCs with high expression of CD40 was increased three-fold from 10% to 31% ($p<0.001$) by treatment with LPS [Fig. 3.14]. DCs incubated with ACs prior to the addition of LPS responded similarly, with a proportion of 28% CD40-high (also $p<0.001$ compared to untreated DCs), and DCs pre-incubated with NCs also responded similarly, with 32% (also $p<0.001$ compared to untreated DCs). DCs pre-treated with ACs, those pre-treated with NCs, and those not pre-treated with dead cells, increased CD40 to levels that were not significantly different from one another. That is, up-regulation of CD40 in response to LPS was not significantly different between DCs, AC-treated DCs or NC-treated DCs ($p>0.05$).

LPS treatment increased CD80 expression from 11% to 36% ($p<0.001$) [Fig. 3.14]. LPS increased CD80 in AC- and NC-treated DCs to 39% and 40%, respectively (both also $p<0.001$ compared with untreated DCs). Up-regulation of CD80 in response to LPS was not significantly different between DCs, AC-treated DCs or NC-treated DCs ($p>0.05$).

CD83 increased from 9% to 33% after LPS treatment ($p<0.001$) and to 34% and 45% in DCs pre-treated with ACs or NCs, respectively (both also $p<0.001$ compared with untreated DCs) [Fig. 3.14]. The LPS response of NC-treated DCs was notably higher than the response to LPS of

non-pre-treated DCs, though up-regulation of CD83 in response to LPS was not significantly different between DCs, AC-treated DCs or NC-treated DCs ($p>0.05$).

LPS treatment induced an increase in CD86 from 11% to 30% ($p<0.001$), and to 33% and 45% in DCs pre-treated with ACs or NCs, respectively (both also $p<0.001$ compared with untreated DCs) [Fig. 3.14]. The LPS response of NC-treated DCs was markedly higher than the response to LPS of non-pre-treated DCs (a 50% relative increase in the number of CD86-high DCs), though up-regulation of CD86 in response to LPS was not significantly different between DCs, AC-treated DCs or NC-treated DCs ($p>0.05$).

LPS treatment considerably increased CD25 expression from 9% to 54% ($p<0.001$). LPS also increased CD25 in AC- and NC-treated DCs to 54% and 54%, respectively (both also $p<0.001$ compared with untreated DCs) [Fig. 3.14]. Up-regulation of CD25 in response to LPS was not significantly different between DCs, AC-treated DCs or NC-treated DCs ($p>0.05$).

LPS-induced up-regulation of CD40, CD80, CD83 and CD86 was not significantly different between DCs, AC-treated DCs or NC-treated DCs (one-way ANOVA for each molecule, $p>0.05$). In summary, neither ACs nor NCs affected LPS-induced DC up-regulation of CD40, CD80, CD83, CD86, or CD25. However, there were non-significant synergistic effects of LPS and NCs in the up-regulation of CD83 and CD86, and, notwithstanding the one-way ANOVA results, only in CD40 was the pattern of NC-treated DCs having the highest expression levels significantly ablated by the LPS response (two-way ANOVA: CD40, $p<0.01$; all others, $p>0.05$).

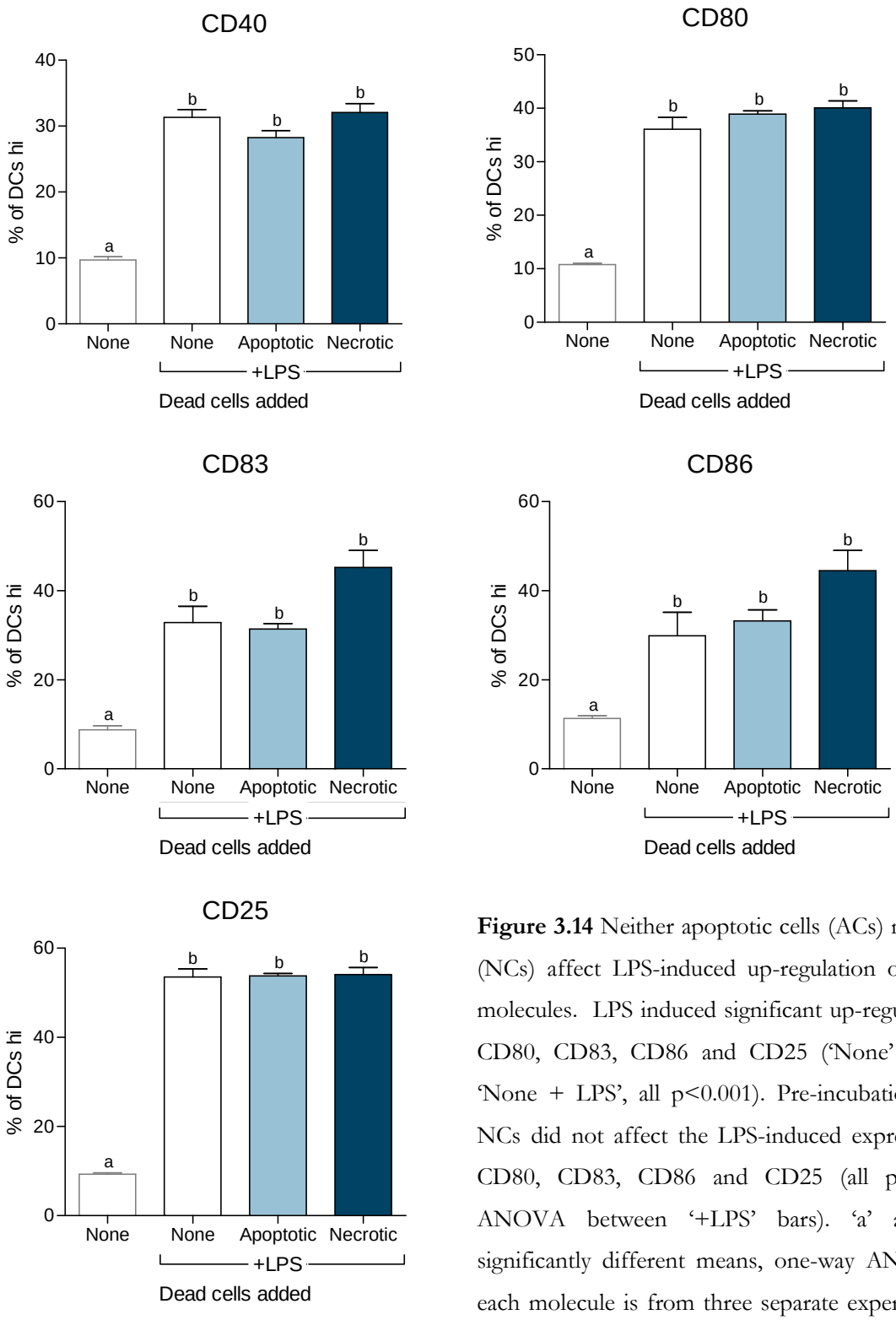


Figure 3.14 Neither apoptotic cells (ACs) nor necrotic cells (NCs) affect LPS-induced up-regulation of co-stimulatory molecules. LPS induced significant up-regulation of CD40, CD80, CD83, CD86 and CD25 ('None' compared with 'None + LPS', all $p<0.001$). Pre-incubation with ACs or NCs did not affect the LPS-induced expression of CD40, CD80, CD83, CD86 and CD25 (all $p>0.05$, one-way ANOVA between '+LPS' bars). 'a' and 'b' signify significantly different means, one-way ANOVA. Data for each molecule is from three separate experiments or more, bars indicate SD.

3.2.2.7 Apoptotic cells, but not necrotic cells, partially suppress CpG-induced DC up-regulation of co-stimulatory molecules

Similarly, to the above studies with LPS-induced maturation, the ability of dead cells to inhibit or enhance DC stimulation by PAMPs was investigated using the TLR9 ligand CpG.

The proportion of DCs with high expression of CD40 was increased more than three-fold from 10% to 34% ($p<0.001$) by treatment with CpG [Fig. 3.15]. DCs incubated with ACs prior to the addition of CpG responded with a proportion of 28% CD40-high (also $p<0.001$ compared to untreated DCs), whilst the response of DCs pre-incubated with NCs was considerably less pronounced at 23% ($p<0.01$ compared to untreated DCs). Up-regulation of CD40 in response to CpG was not significantly different between DCs, AC-treated DCs or NC-treated DCs (one-way ANOVA, $p>0.05$).

CpG treatment increased CD80 expression from 11% to 37% ($p<0.001$) [Fig. 3.15]. CpG increased CD80 in AC- and NC-treated DCs to 30% and 39%, respectively (both also $p<0.001$ compared with untreated DCs). Up-regulation of CD80 in response to CpG was not significantly different between DCs, AC-treated DCs or NC-treated DCs (one-way ANOVA, $p>0.05$).

CD83 increased from 9% to 17% after CpG treatment, but was not significant [Fig. 3.15]. CD83 increased to 15% in DCs that were pre-treated with ACs, also not significant compared to untreated DCs. CD83 increased to 38% in DCs that were pre-treated with NCs ($p<0.001$ compared with untreated DCs). CD80 expression was not affected by CpG treatment. Regardless of CpG-treatment, up-regulation of CD80 in NC-treated DCs was significantly higher than in AC-treated DCs or DCs not treated with dead cells (one-way ANOVA, $p<0.001$).

CpG treatment induced an increase in CD86 from 11% to 40% in DCs, and to 43% in DCs pre-treated with NCs (both $p<0.001$ compared with untreated DCs) [Fig. 3.15]. CpG induced a significant up-regulation of CD86 in AC-treated DCs to 25% ($p<0.01$ compared to untreated

DCs), though this was a significantly smaller increase in expression than that by both DCs and NC-treated DCs ($p<0.05$).

CpG treatment considerably increased CD25 expression from 9% to 30% ($p<0.001$) [Fig. 3.15]. CpG also increased CD25 in AC- and NC-treated DCs to 25% and 27%, respectively (both also $p<0.001$ compared with untreated DCs). Up-regulation of CD25 in response to CpG was not significantly different between DCs, AC-treated DCs or NC-treated DCs (one-way ANOVA, $p>0.05$).

CpG induced significant DC up-regulation of CD40, CD80, CD86 and CD25, but not CD83. Pre-treatment with NCs did not affect this up-regulation, other than an insignificant reduction in the up-regulation of CD40. NC-treated DC expression of CD83 after CpG treatment was significantly higher than after treatment with CpG alone ($p<0.05$) or NCs alone ($p<0.01$), suggesting a possible synergistic effect. ACs, on the other hand, caused significant reductions in CpG-induced up-regulation of CD86.

The effect of dead cells on CD40 expression is significantly different between DCs and DCs that are subsequently treated with CpG ($p<0.001$, two-way ANOVA): there is a shift from there being significant differences between dead cell treatments to there being no significant differences after CpG treatment (where the CD40 expression levels appear to have reached a maximum, regardless of prior dead cell treatment).

For CD80, CD83 and CD25 expression, the effect of dead cells on DCs is not significantly different from their effect on CpG-treated DCs ($p>0.05$, two-way ANOVA), despite the fact that ACs and NCs have significantly different effects on DCs but not after CpG treatment. Consequently, not much can be inferred from this statistic.

The effect of dead cells on CD86 expression is significantly different between DCs and CpG-treated DCs ($p<0.001$, two-way ANOVA). This is due to two shifts. Firstly, similarly to CD40

expression, there is a shift from: NC-induced expression that is significantly higher than untreated DCs; to, after CpG, DCs and NC-treated DCs having the *same, high* level of expression. Secondly, the AC-suppressed expression of CD86 is limited to CpG-treated DCs. That is, ACs appear to have no effect on resting levels of CD86, but inhibit its CpG-induced up-regulation.

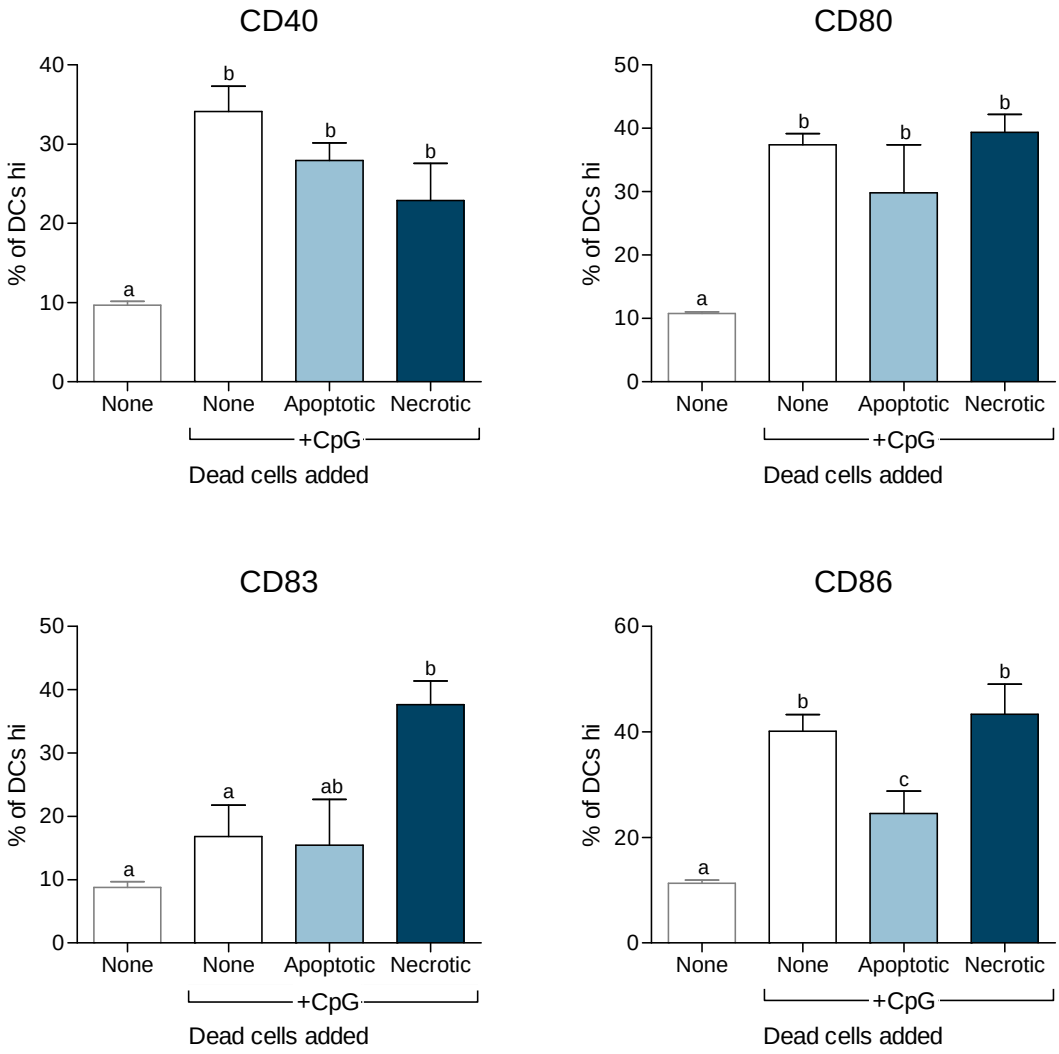


Figure 3.15 3.2.2.7 Apoptotic cells, but not necrotic cells, partially suppress CpG-induced DC up-regulation of co-stimulatory molecules. CpG induced significant up-regulation of CD40, CD80, CD86 and CD25 ('None' compared with 'None + CpG', all $p<0.001$), but did not induce significant up-regulation of CD83. Pre-incubation with ACs significantly reduced the CpG-induced up-regulation of CD86 ($p<0.05$, one-way ANOVA between '+LPS' bars), but did not significantly affect the up-regulation of CD40, CD80, CD83, and CD25 (all $p>0.05$, one-way ANOVA between '+LPS' bars). Pre-incubation with NCs significantly increased expression of CD83 ($p<0.05$) but did not significantly affect CpG-induced expression of CD40, CD80, CD86 or CD25. 'a,' 'b' and 'c' signify significantly different means, one-way ANOVA. Data for each molecule is from three separate experiments or more, bars indicate SD.

3.2.2.8 Apoptotic cell-induced up-regulation, and some necrotic cell-induced up-regulation, of co-inhibitory proteins persists through LPS treatment

The ability of dead cells to affect PAMP-induced DC alteration of expression of co-inhibitory molecules was investigated. First, the response to LPS alone was determined.

LPS treatment increased B7DC expression from 30% to 39% ($p<0.001$). LPS increased B7DC in AC- and NC-treated DCs to 38% and 46%, respectively ($p<0.01$ and $p<0.001$, respectively, compared with untreated DCs) [Fig. 3.16]. Up-regulation of B7DC in response to LPS was not significantly different between DCs, AC-treated DCs or NC-treated DCs ($p>0.05$).

Treatment with LPS induced up-regulation of the co-inhibitory molecule B7H1 from 21% to 47% ($p<0.001$) [Fig. 3.16]. LPS increased B7H1 in AC- and NC-treated DCs to 54% and 51%, respectively (both also $p<0.001$ compared with untreated DCs). Up-regulation of B7H1 in response to LPS was not significantly different between DCs, AC-treated DCs or NC-treated DCs (one-way ANOVA, $p>0.05$).

LPS treatment significantly decreased expression of B7H2 from 20% to 12% ($p<0.01$) [Fig. 3.16]. This decrease was not apparent in LPS-treated DCs that were pre-treated with ACs or NCs, where B7H2 remained at 23% and 19%, respectively ($p>0.05$ compared to untreated DCs). As such, the LPS-induced response of AC-treated DCs was significantly higher than that of DCs ($p<0.05$), however, the LPS response of NC-treated DCs was not significantly different from that of DCs or AC-treated DCs.

B7H3 after LPS treatment was 6%, compared to 5% before ($p>0.05$) [Fig. 3.16]. LPS treatment did not inhibit AC-induced up-regulation of B7H3, rising at it did to 9% ($p<0.001$) compared to untreated DCs). This was not significantly higher than B7H3 expression of LPS- (alone) treated DCs or DCs that had been pre-treated with NCs (both 6%), which were also not significantly different to untreated DCs.

B7H4 was slightly reduced from 5% to 4% after LPS treatment though not significantly [Fig. 3.16]. AC-induced up-regulation of B7H4 persisted despite LPS challenge, with expression rising to 8% (slightly, but not significantly, lower than the 10% induced in the absence of LPS). In the presence of LPS, AC-induced up-regulation of B7H4 is no longer significant compared to untreated DCs, but is significant compared to LPS- (alone) treated DCs ($p<0.01$). NC-induced up-regulation of B7H4 persisted, being significant compared to both untreated DCs ($p<0.05$) and LPS- (alone) treated DCs ($p<0.001$).

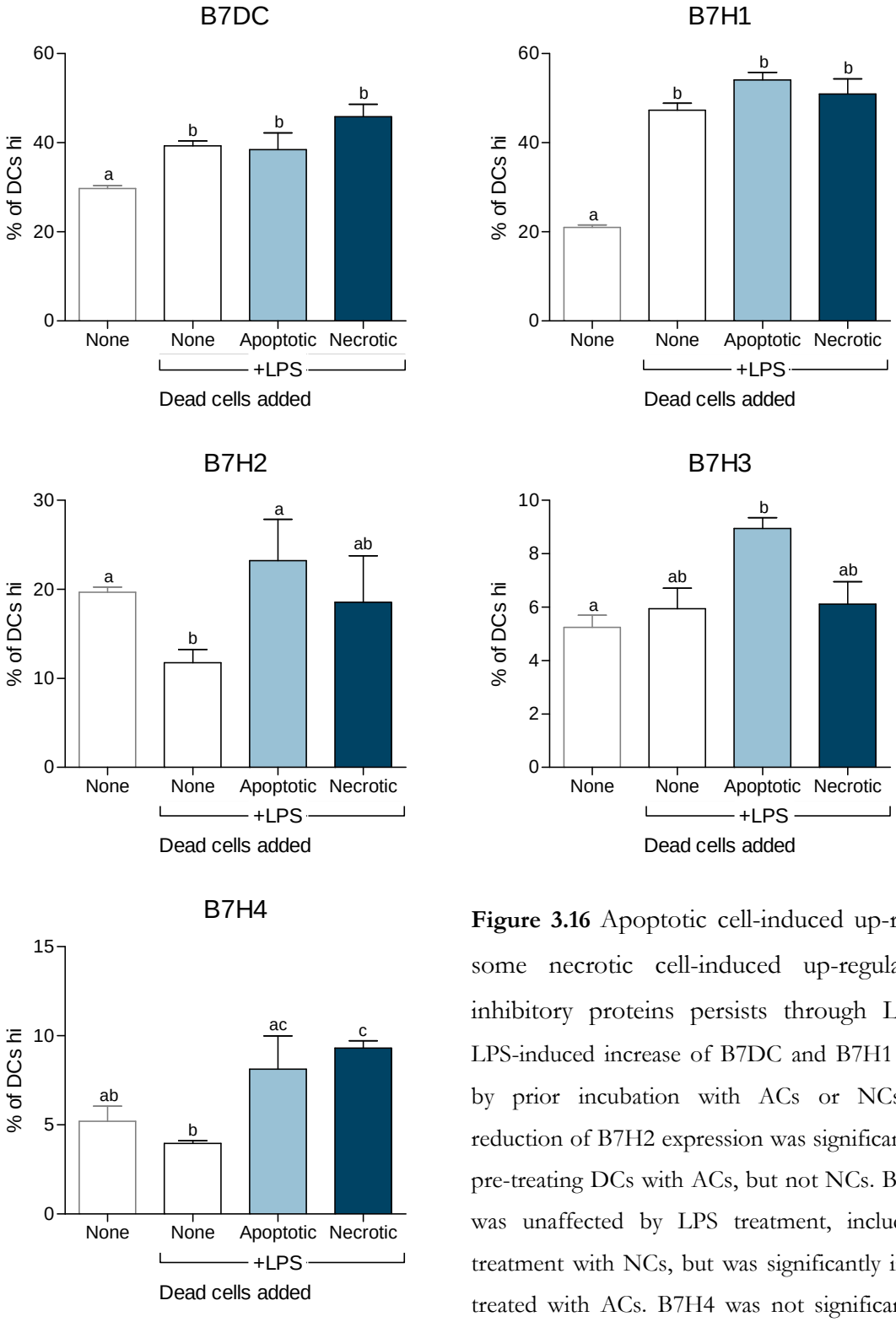


Figure 3.16 Apoptotic cell-induced up-regulation, and some necrotic cell-induced up-regulation, of co-inhibitory proteins persists through LPS treatment. LPS-induced increase of B7DC and B7H1 was unaffected by prior incubation with ACs or NCs. LPS-induced reduction of B7H2 expression was significantly inhibited by pre-treating DCs with ACs, but not NCs. B7H3 expression was unaffected by LPS treatment, including after pre-treatment with NCs, but was significantly increased if pre-treated with ACs. B7H4 was not significantly affected by LPS treatment. Pre-treatment with either ACs or NCs significantly increased LPS-induced B7H4 expression levels. 'a,' 'b' and 'c' signify significantly different means, one-way ANOVA. Data for each molecule is from three separate experiments or more, bars indicate SD.

3.2.2.9 Apoptotic cell-induced up-regulation, and some and necrotic cell-induced up-regulation, of co-inhibitory proteins persists through CpG treatment/challenge

The ability of dead cells to affect CpG-induced DC alteration of expression of co-inhibitory molecules was investigated.

CpG treatment increased B7DC expression from 30% to 36% ($p<0.001$) [Fig. 3.17]. CpG induced DC up-regulation of B7DC was significantly inhibited by pre-treating DCs with ACs (, $p>0.05$). NCs induced a slight abrogation of CpG-induced up-regulation of B7DC to 33%, which, although not itself significant, was a reduction of CpG-induced up-regulation from a significant increase to a non-significant increase – there was not a significantly lower expression of B7DC in CpG-/NC-treated DCs than in CpG-treated DCs.

Treatment with CpG induced up-regulation of the co-inhibitory molecule B7H1 from 21% to 67% ($p<0.001$) [Fig. 3.17]. CpG increased B7H1 in AC- and NC-treated DCs to 80% and 69%, respectively (both also $p<0.001$ compared with untreated DCs). Up-regulation of B7H1 in response to CpG was not significantly different between DCs, AC-treated DCs or NC-treated DCs ($p>0.05$).

CpG treatment significantly decreased expression of B7H2 from 20% to 14%. AC- and NC-induced up-regulation of B7H2 increased after CpG in AC- and NC-treated DCs to 49% and 46%, respectively (both $p<0.001$ compared to untreated DCs, and compared to CpG- (plus no dead cells) treated DCs).

B7H3 did not change after CpG treatment, remaining as it did at 5% [Fig. 3.17]. CpG treatment did not inhibit AC-induced up-regulation of B7H3, which increased to 18% ($p<0.001$ compared to untreated DCs). This was also significantly higher than B7H3 expression of CpG-treated DCs that

had been pre-treated with NCs, which remained at levels not significantly different to untreated and CpG- (alone) treated DCs (7%).

B7H4 was slightly reduced from 5% to 4% after CpG treatment though not significantly [Fig. 3.17]. AC-induced up-regulation of B7H4 persisted despite CpG challenge, with expression rising to 9% (slightly, but not significantly, lower than the 10% induced in the absence of CpG). In the presence of CpG, AC-induced up-regulation of B7H4 is no longer significant compared to untreated DCs, but is significant compared to CpG- (alone) treated DCs ($p<0.01$). NC-induced up-regulation of B7H4 was ablated after CpG-treatment, with B7H4 remaining at 5% ($p<0.05$ compared to both untreated and CpG- (alone) treated DCs).

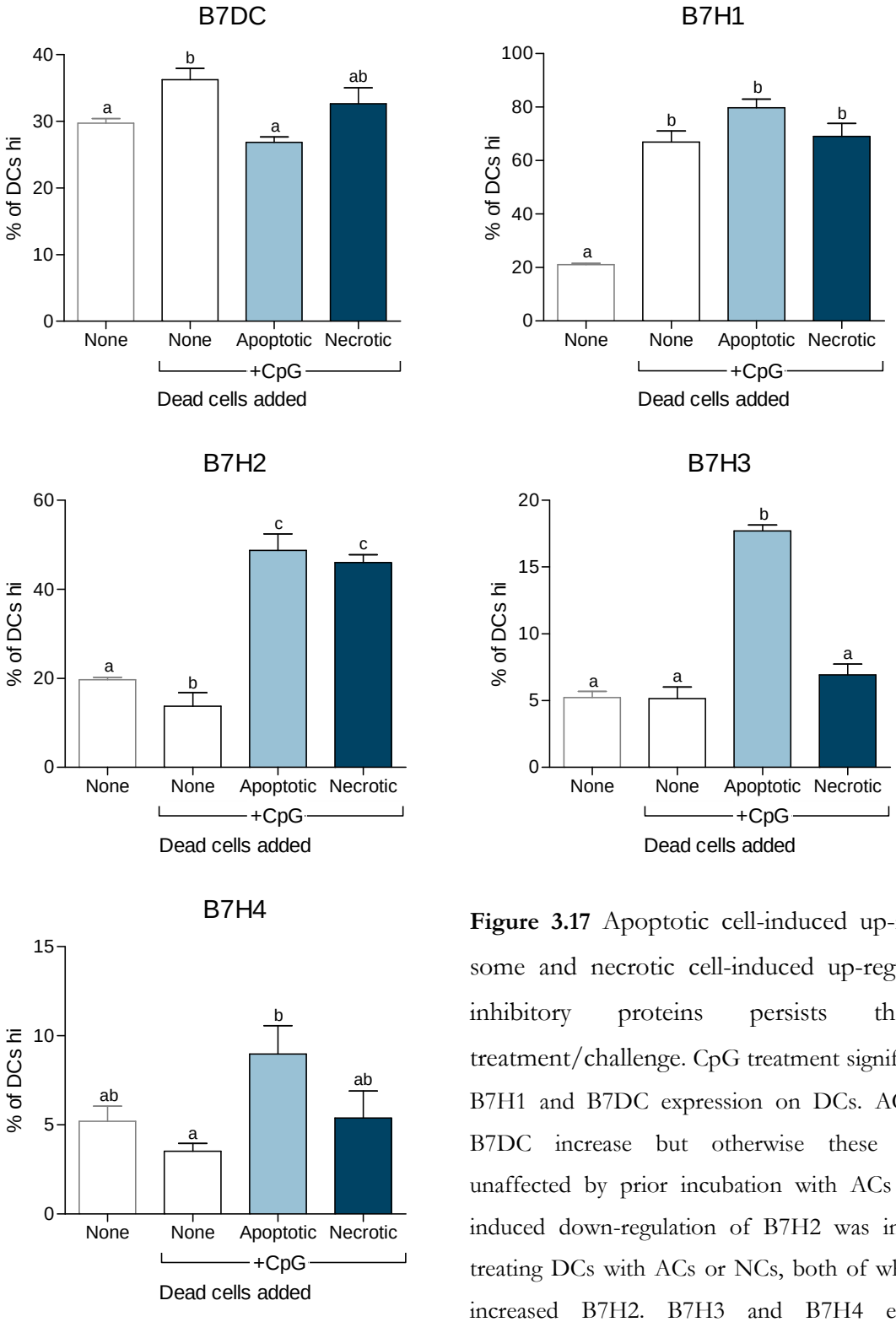


Figure 3.17 Apoptotic cell-induced up-regulation, and some and necrotic cell-induced up-regulation, of co-inhibitory proteins persists through CpG treatment/challenge. CpG treatment significantly increased B7H1 and B7DC expression on DCs. ACs inhibited the B7DC increase but otherwise these increases were unaffected by prior incubation with ACs or NCs. CpG-induced down-regulation of B7H2 was inhibited by pre-treating DCs with ACs or NCs, both of which significantly increased B7H2. B7H3 and B7H4 expression were unaffected by CpG treatment, including with NCs, but were significantly increased if pre-treated with ACs. 'a,' 'b' and 'c' signify significantly different means, one-way ANOVA. Data for each molecule is from three separate experiments or more ore, bars indicate SD.

3.3 Effects on dendritic cells of alternative apoptotic cell sources/treatments

3.3.1 Introduction

As shown above, ACs increased the expression of co-inhibitory markers in our model of AC-induced DC modification. However, maturation markers were generally unaffected in this study, in contrast to the findings of some other groups, who report the AC-induced suppression of LPS-induced up-regulation of maturation markers. The induction of co-inhibitory molecules thus far in this study suggested that camptothecin-treated FDCP would be an adequate model of AC for the further investigation of AC-induced modification of DCs (see Chapter 4). However, further improvements of the method were investigated in order to corroborate or refute published findings, and to potentially maximise the possibility of AC-induced suppression of DCs in this study. To this end, alternative incubation media supplements and sources of apoptotic cells were studied.

3.3.2 Results

3.3.2.1 Various media supplements do not aid apoptotic cell-mediated suppression of DC maturation

DC suppression by ACs has been achieved by using IMDM (Iscove's Modified Dulbecco's Medium) as the media for culture and co-incubation (233). We explored the possibility that components of IMDM endow DC-AC interactions with greater suppressive potential by using complete IMDM (cIMDM – IMDM with supplements identical to those in cRPMI), rather than cRPMI, as the media in which DCs were grown and co-incubated with ACs. The shift in DC expression of CD40 after co-incubation with ACs was insubstantially different between cRPMI and cIMDM cultures, with increases in expression of 2% and 4%, respectively [Fig. 3.18A]. This difference is even less substantial given that an expected proportion of resting cRPMI DCs (10%) were CD40-high, whereas, using the same FACS gates, 27% of resting cIMDM DCs were CD40-high. In cRPMI, LPS-induced up-regulation of CD40 to 28% was abrogated to 23% by pre-incubating DCs with ACs [Fig. 3.18B]. In contrast, in cIMDM cultures the LPS-induced up-regulation of CD40 was itself enhanced to 35%, and the pre-treatment of DCs with ACs further augmented this increase to 43%. Thus, IMDM failed to enhance AC-induced suppression of CD40 up-regulation, and, furthermore, actually itself induced high expression of CD40. However, although consistent with further observations in this study, data was not available for statistical analysis.

DCs were incubated with ACs in cRPMI supplemented with serum variations in order to investigate the role of serum factors in AC-mediated suppression of DC. This exploration was prompted by the findings of some groups indicating that serum factors such as complement may be required for DC suppression by ACs, and by the possibility that the serum used thus far may be inadequate. Heat-inactivated (HI) FBS was replaced with either: FBS that had not been heat-

inactivated (non-HI FBS); fresh mouse serum, to a final concentration of 1%; fresh mouse serum, 5%. For each media supplement, there was no significant difference in CD40 expression between DCs and DCs pre-treated with ACs [Fig. 3.18C]. This is also true of LPS-treated DCs [Fig. 3.18D]. However, non-HI FBS significantly increased the percentage of resting DC expressing high levels of CD40 from 5% to 9%, regardless of ACs being present ($p<0.001$, two-way ANOVA). Non-HI FBS made no significant difference to LPS-induced up-regulation of CD40. These findings suggest that neither additional serum nor IMDM enable or enhance AC-mediated suppression of LPS-induced up-regulation of CD40 by DCs. Moreover, IMDM and non-HI FBS appear to induce maturation of DCs.

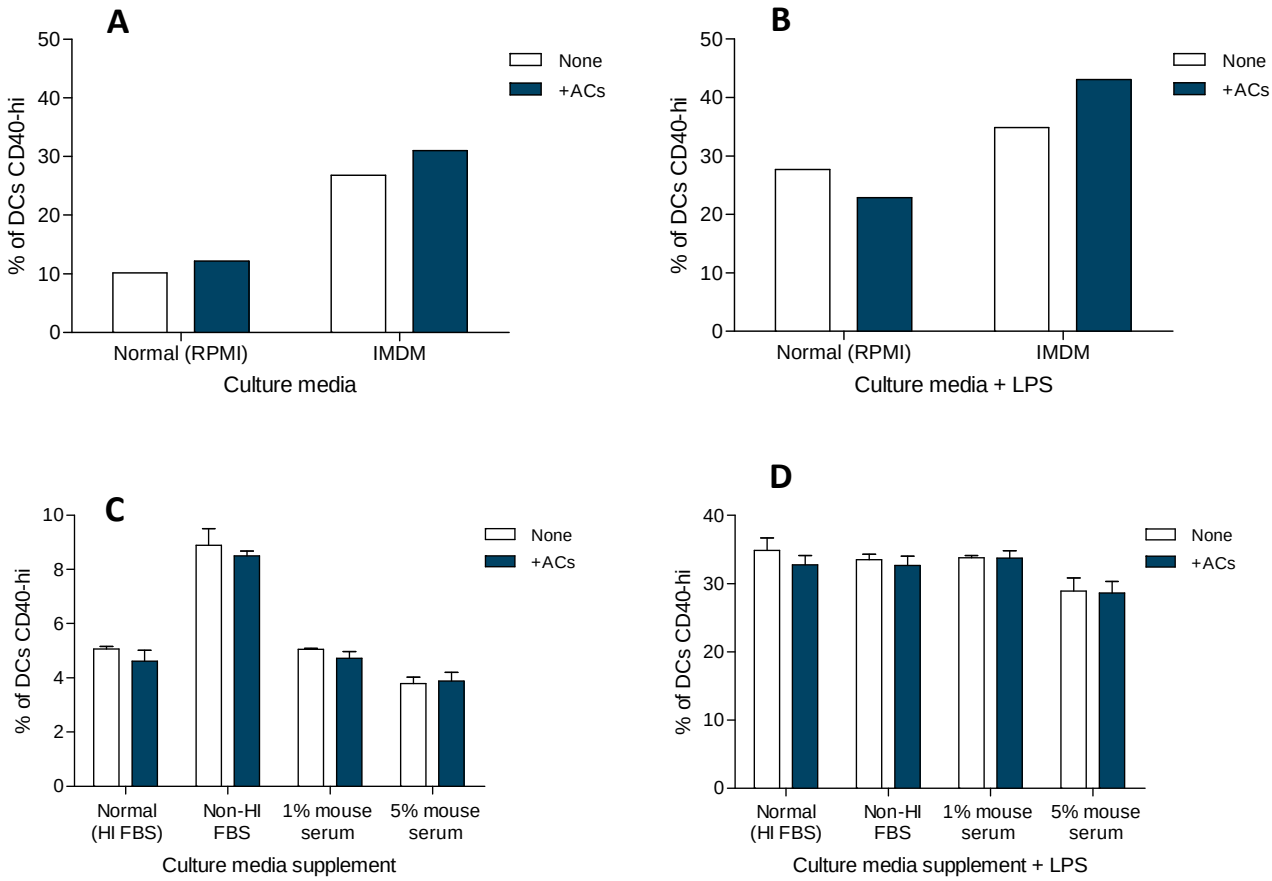


Figure 3.18 Various media supplements do not aid apoptotic cell-mediated suppression of DC maturation. DCs were cultured with apoptotic FDCP (ACs) for 24 hours in normal conditions or with various alterations of culture media and analysed by FACS antibody staining for reductions in CD40 expression. **A.** ACs did not decrease DC CD40 expression any more when co-incubated in IMDM than when co-incubated in RPMI. Moreover, IMDM appeared to increase CD40 expression of 'resting' DCs. **B.** ACs did not suppress LPS-induced DC up-regulation of CD40 expression any more when co-incubated in IMDM than when co-incubated in RPMI. **C.** DCs were co-incubated with ACs in media that had heat-inactivated FBS (HI FBS) exchanged for either: non-heat-inactivated FBS (non-HI FBS); 1% mouse serum; 5% mouse serum. Neither non-HI FBS nor either concentration of mouse serum enabled ACs to decrease CD40 expression of 'resting' DCs. Moreover, non-HI FBS increased the CD40 expression of 'resting' DCs. **D**. Neither non-HI FBS or either concentration of mouse serum enabled ACs to suppress LPS-induced DC up-regulation of CD40. Data is from two (A and B) or three (C and D) experiments for each treatment, bars indicate SD.

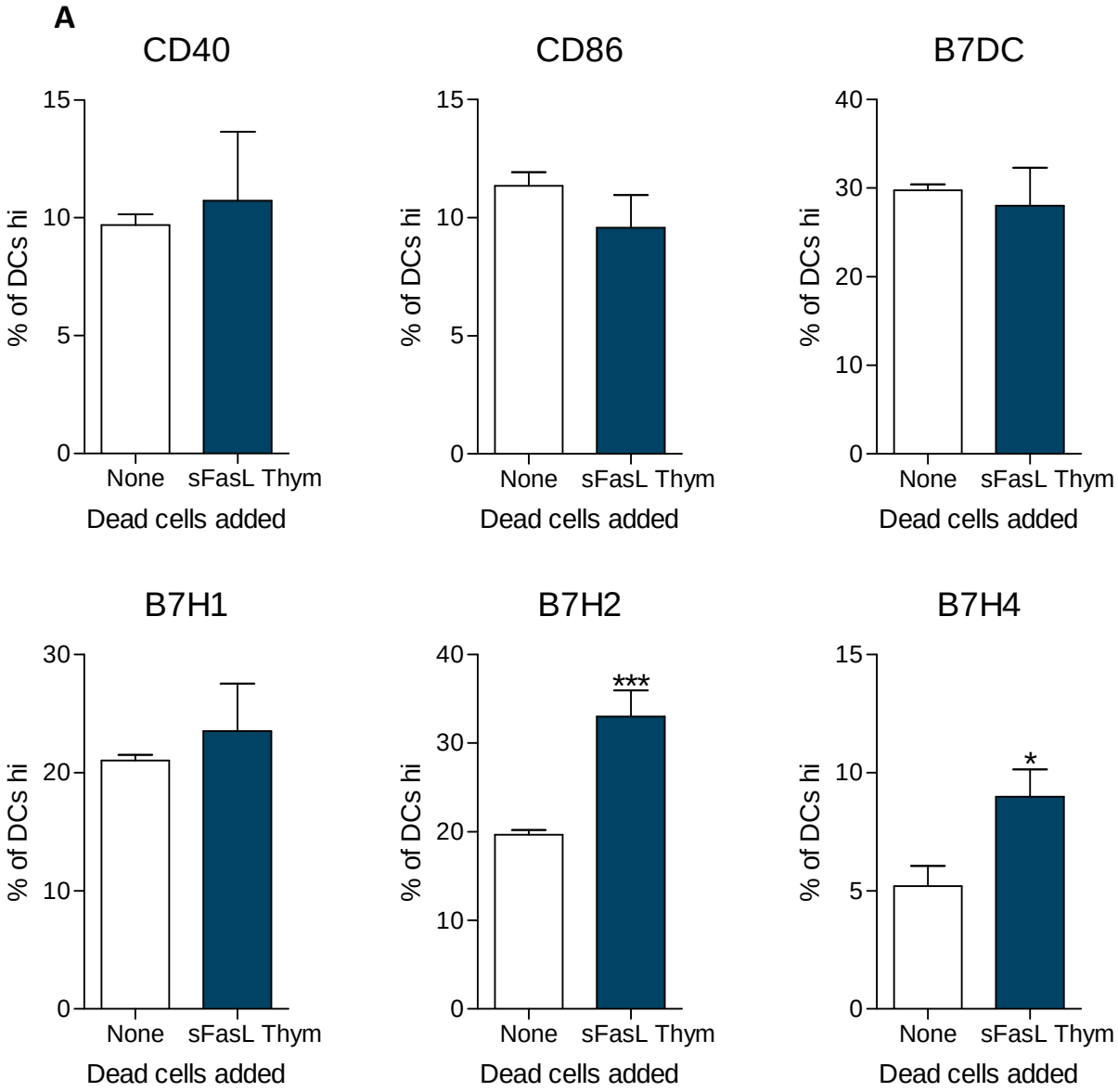
3.3.2.2 Apoptotic primary thymocytes are no more efficient than camptothecin-treated FDCP at suppressing up-regulation of DC co-stimulatory receptors or inducing up-regulation of co-inhibitory receptors

To investigate the prospect that AC-mediated immune suppression could be more successful if employing primary cells, rather than cell lines, ACs were generated from *ex vivo* thymocytes. Thymocytes were treated with super-Fas ligand (sFasL), which induced apoptosis in a high percentage of cells (84% apoptotic, 12% necrotic, 4% alive) within 6 hours – Apoptotic thymocytes were then incubated with DCs for 12 hours, with and without a subsequent 24hr LPS-treatment. DCs were then analysed by FACS for changes in expression of some of the key co-stimulatory and co-inhibitory markers.

Co-incubation with sFasL-treated thymocytes (sFasL-thymocytes) did not alter the percentage of DCs that expressed high levels of CD40, CD86 or B7DC ($p>0.05$ compared with untreated DCs), which remained at approximately 10%, 10%, and 30%, respectively [Fig. 3.19]. This was similar to DCs after co-incubation with camptothecin-FDCP, as seen in Fig. 3.10. SFasL-thymocytes did not induce up-regulation of B7H1, but did induce up-regulation of B7H2 from 20% (in untreated DCs) to 33% ($p<0.001$ and B7H4 from 5% to 9% ($p<0.05$) [Fig. 3.19A]. The proportions of DCs expressing high B7H1, B7H2 and B7H4 after co-incubation with sFasL-thymocytes were all statistically similar to the corresponding proportions of DCs after co-incubation with camptothecin-FDCP [Fig. 3.12].

Co-incubation with sFasL-thymocytes did not significantly alter LPS-induced up-regulation of CD40, CD86 or B7DC ($p>0.05$ compared with LPS-treated DCs) [Fig. 3.19B], similarly to co-incubation with camptothecin-FDCPs [Fig. 3.14], though up-regulation of CD86 was enhanced from 30% to 45% and up-regulation of B7DC was enhanced from 39% to 62%. Similarly to camptothecin-FDCP [Fig. 3.16], LPS-induced up-regulation of B7H1 was not affected by pre-treatment with sFasL-thymocytes as it increased to 49% ($p>0.05$ compared to 47% in DCs treated

with LPS alone) [Fig. 3.19B], and sFasL-thymocytes maintained up-regulation of B7H4 to 10% ($p<0.001$, compared with 4% in DCs treated with LPS alone). However, unlike treatment with camptothecin-FDCP, the up-regulation of B7H2 induced by sFasL-thymocytes was negated by LPS challenge (15%, $p>0.05$, compared with 12% in DCs treated with LPs alone). Nonetheless, expression of B7H1, B7H2 and B7H4 in LPS-treated DCs that were pre-treated with sFasL-thymocytes was statistically similar to the corresponding expression of LPS-treated DCs that were pre-treated with camptothecin-FDCP. This suggests that apoptotic primary thymocytes have no more suppressive potential than apoptotic cell line FDCP.



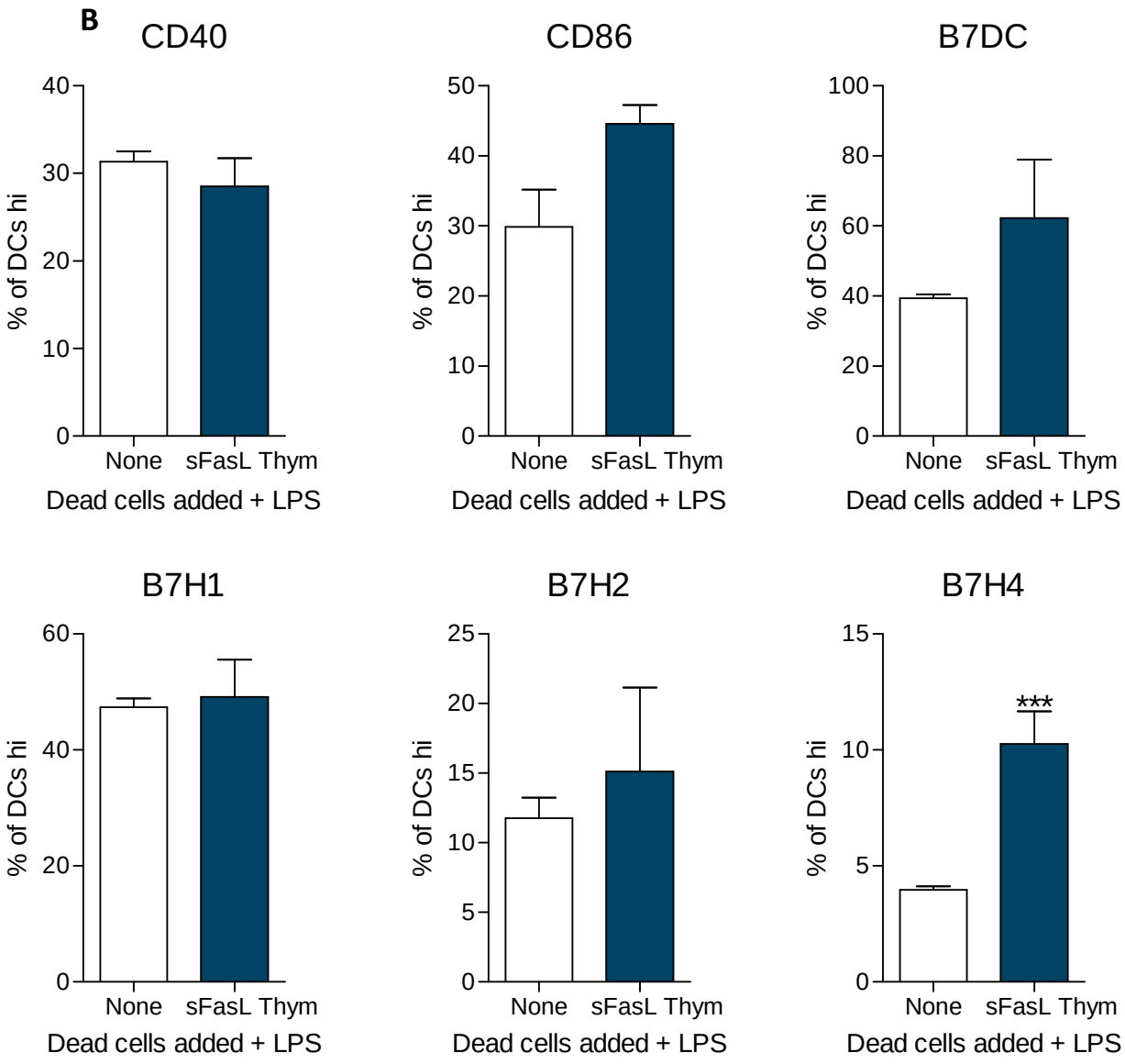


Figure 3.18 Apoptotic primary thymocytes are no more efficient than camptothecin-treated FDCP at suppressing up-regulation of DC co-stimulatory receptors or inducing up-regulation of co-inhibitory receptors. *Ex vivo* thymocytes were induced to undergo apoptosis by treating with super Fas ligand for 4hrs. DCs were incubated with apoptotic thymocytes (sFasL Thym) for 24hrs, with or without LPS for the last 12hrs. **A.** Apoptotic thymocytes made no significant difference to expression levels of CD40, CD86, B7DC or B7H1 on resting DCs. Apoptotic thymocytes induced up-regulation of B7H2 and B7H4. **B.** Apoptotic thymocytes made no significant difference to expression levels of CD40, CD86, B7DC, B7H1 or B7H2 on LPS-treated DCs. LPS-challenge negated apoptotic thymocytes-induced up-regulation of B7H2 but not of B7H4, which remained significantly higher. * = $p<0.05$, *** = $p<0.001$, referring to significant difference between the indicated and the untreated DCs (None), one-way ANOVA. Data for each molecule is from three separate experiments, bars indicate SD.

3.4 Discussion

3.4.1 Apoptosis and necrosis of FDCP

The first task in this investigation was to establish methods of producing apoptotic and necrotic cells. Due to the reportedly opposed influences of both dead cell types, it was necessary to produce cultures of as pure a dead cell type as possible. It was therefore necessary to employ methods that were both uniform and consistent in their effects. However, the challenge in producing cultures of high proportions of apoptotic cells or of necrotic cells was multifarious.

Firstly, the transient nature of apoptosis imposes certain time-frame requirements on the method of apoptosis induction. Apoptosis is transitory in that apoptotic cells can progress to secondary necrosis whereby they disintegrate and lose membrane integrity, potentially releasing proinflammatory contents. Indeed, this process has been implicated as the key trigger of pathology in SLE (234). In this study, therefore, all cells must be treated with and respond to the inducer of apoptosis with enough time left for DCs to interact with these cells before some or all of the population progress to secondary necrosis and counteract the effect of apoptotic cells. For some treatments though, many FDCP progress to secondary necrosis before others begin the apoptotic process. More specifically, many FDCP progress to secondary necrosis before others express PS. Because surface PS expression is one of the earliest stages of apoptosis, and PS is necessary for identifying AC both by the experimental observer and by dendritic cells (which do also recognise other, but later, markers), it can be supposed that surface PS expression is synonymous with the beginning of apoptosis.

The challenge in producing cultures with high proportions of apoptotic cells for a sufficient length of time was compounded by the fact that cells of the same type may respond differently towards the same stress depending on their proximity to the stress, stage of cell cycle, and/or general

health. For example, cells in culture being exposed to UV can shield other cells, further away from the UV source, from exposure. Increasing UV dose increased overall cell death, but death plateaued at 90%, possibly due to cell density preventing UV from penetrating through the entire cell culture. Furthermore, higher or longer doses of UV increased the risk of superfluous exposure to other cells that could result in secondary necrosis - necrosis increased in a dose-dependent manner and reached 40% of the cell sample (at 200J/m^2), and we may well presume that this figure would have increased with higher doses of UV [Fig. 3.5]. Lack of uniform exposure may therefore have been one reason why it was not possible to generate apoptotic cells by UV exposure without also increasing the incidence of necrosis [Fig. 3.2] – the maximum proportion of cells undergoing apoptosis at any one time was 73% (at 20J/m^2), with a continually increasing proportion of necrotic cells.

Another reason why it was not possible to generate apoptotic cells by UV exposure without also increasing the incidence of necrosis could have been the varying stages of cell cycle between different cells within the culture. This is because UV exposure triggers apoptosis through detection of DNA damage at the cell division stage of cell growth – some cells may have been already progressing to secondary necrosis when others were only just experiencing an aborted cell division and the beginning of apoptosis. However, this is unlikely considering that camptothecin-exposure, which also initiates apoptosis upon a faulty cell division attempt, induces a very high percentage of apoptosis (virtually all cells – up to 98%) in the same cell type before any secondary necrosis occurs [Fig. 3.2]. As camptothecin appeared to represent a more convenient and reliable source of apoptotic cells, it was deemed unnecessary to improve UV-induced apoptosis induction by synchronising cell cycle phases within a culture (by withdrawing growth factor for a period sufficient to halt cell growth, before reintroducing it).

The health of a cell may also have had an influence on the effectiveness and consistency of death inducing treatments. We may speculate that GMCSF-withdrawal affected cells at different rates

according to their health. For example, young cells may have been able to tolerate the lack of growth factor for longer than older cells. What is known is that many cells did not remain apoptotic for long before progressing to secondary necrosis – the percentage of necrotic cells grew from 7% to 38% within 24 hours of withdrawal [Fig. 3.4A], a surprisingly fast progression and one that rendered GMCSF-withdrawal inadequate for the purposes of this investigation.

A further complication with GMCSF-withdrawal exposed a flaw in the method of apoptosis detection itself. AnV-PI staining indicated that the proportion of necrotic cells began to decline, from a percentage of 38% at 24 hours post-withdrawal, to 9% at 68 hours [Fig. 3.4A]. Most living animal cells can repair small or even moderately large tears in the plasma membrane, and at a reasonably rapid pace (235). However, it appeared unlikely that this could have happened on such a scale as this, not least due to the absence of growth factor (which some evidence suggests is required for membrane repair (235)). Also, the 'return' of the staining pattern to not that of healthy cells but instead to that of apoptotic cells was reasonably deemed as suspect. Therefore, it was suspected that the assay demonstrated AnV-positive, PI-negative staining not due to repaired membrane integrity, but due to failed PI staining of DNA. Consequently, DAPI staining and fluorescence microscopy was used to visualise the DNA of untreated FDCP, 48-hour camptothecin-treated FDCP, and the suspect 68-hour GMCSF-withdrawn FDCP [Fig. 3.4B-D]. This showed clear and compact nuclei in the untreated FDCP, and revealed disintegration of the nuclei of camptothecin-treated FDCP into distinct fragments, indicating DNA fragmentation – a key stage late in the apoptotic process. DNA can even be seen to be packaged into small bodies budding from the periphery of the main cell bodies – these are apoptotic vesicles. In contrast, the nuclei of 68hr GMCSF-withdrawn FDCP were undetectable by DAPI staining (save in a few cells). This revealed that negative PI staining was due to the insufficient presence of DNA, which had either disintegrated into components too small to be clearly detected or had escaped out of the necrotic membranes and been lost from the degenerating cell bodies altogether. Without further investigation, advanced apoptosis could not be assumed, whilst secondary necrosis seemed most

probable. Accordingly, GMCSF-withdrawal was dismissed as a method of apoptosis induction. Furthermore, this revealed that AnV-PI staining, as a method of determining the type of cell death, is innacurrate once a cell type may start to succumb to secondary necrosis.

DNA staining with DAPI helped to reveal that there was no longer any DNA present in 68-hour GMCSF-withdrawn FDCP. Staining of DNA with ethidium bromide, and subsequent electrophoresis, would no doubt have also have shown a lack of any DNA at all. This electrophoresis method confirmed DNA fragmentation in UV- and camptothecin-treated FDCP by demonstrating DNA laddering across the gel – evidence that DNA had disintegrated and thus was able to move through the gel in separate units [Fig. 3.6].

Camptothecin, as mentioned, induced very high rates of apoptosis. Furthermore, there was no increase in the induction of necrosis throughout the 48 hour incubation period [Fig. 3.3], unlike in GMCSF-withdrawal. Thus camptothecin treatment created a broad interval of time wherein ACs represented a very high percentage of the FDCP sample. In addition, treatment was simple and possible on a large scale. However, there remained doubts that what effectively constituted chemical insult could generate a non-danger signal-inducing apoptotic cell. However, photochemotherapy has been used to generate apoptotic cells that successfully suppress graft-versus-host disease (GVHD) by inhibiting DC maturation (236). Camptothecin treatment was therefore the preferred method of apoptosis induction and was used, bar a few peripheral experiments where indicated, in all subsequent generation of apoptotic cells in this investigation.

Induction of necrosis was expectedly simple in relation to apoptosis. This is primarily because necrotic cells are by definition damaged, decaying and potentially inflammatory (this much was their purpose in this study) and cannot revert to an apoptotic cell phenotype. Also, some necrotic cell-released danger signals are base materials and chemicals that do not easily degrade extracellularly, for example uric acid. Accordingly, generation of necrotic cells was straightforward and entailed exposing cells to a stress sufficient to cause damage to all cells. In this effort,

mechanical damage was preferable to chemically-induced damage, as the washing of necrotic cell lysates would have been a time-consuming addition to the method. Also, chemicals may have altered the immunological properties of the dead cells. Heat kill (maintaining the culture at 60°C for 30mins) was not only non-chemical but ensured reasonably even exposure to the stress, and so was an ideal candidate. Heat kill produced successful results where FDCP were killed virtually totally (average 94%), uniformly and consistently [Fig. 3.2].

Necrosis induction was not without some complications. Repeated freeze-thaw cycles were employed to kill cells and were successful in doing so (inducing necrosis in 85% of FDCP) [Fig. 3.2]. However, freeze-thaw necrotic cells were adhesive and they aggregated heavily. Separation of cells into a uniform culture was impossible despite physical manipulation. This was inadequate for a method of necrosis induction, of which was required consistency and simplicity, and accordingly heat kill was used as the method of necrosis induction for the duration of the investigation.

3.4.2 Phagocytosis of apoptotic and necrotic cells by DCs

Phagocytosis of apoptotic cells and necrotic cells by DCs was examined in order to confirm that our generated DCs would indeed phagocytose camptothecin-treated (apoptotic) and heat kill-treated (necrotic) FDCP, and determine if they did so with any difference in frequency. DCs were stained with CFSE, which covalently binds cytoplasmic amines and is cleaved by intracellular esterases into a yellow-green fluorescent dye (237). Apoptotic and necrotic FDCP were stained with PKH26, a red fluorescent dye that stably integrates into the plasma membrane (238). Phagocytosis of dead cells was measured by detecting DCs (CFSE-positive cells) that were also PKH26 positive. The percentage of DCs that associated with apoptotic cells was statistically similar to the percentage associated with necrotic cells (both approximately 20%) [Fig. 3.7]. This

association was confirmed as phagocytosis by its reduction in the presence of cytochalasin D, to approximately 5% for each dead cell type. This matches another report that demonstrates that apoptotic and necrotic forms of the same cell type are phagocytosed by rat BMDCs at equal rates (203), though in that study the tumour cell line PROb was phagocytosed at a much higher rate of approximately 70-80% (239). The rates of phagocytosis in the current study are, however, comparable to the observed DC uptake of non-opsonised apoptotic neutrophils in a previous study (233). In that study, opsonisation of apoptotic cells with IgG almost doubled the number of DCs that phagocytosed apoptotic neutrophils but there was no difference in DC phenotypical changes between opsonised AC- and non-opsonised AC-treated DCs. This suggests that the number of DCs phagocytosing ACs in the current study is sufficient for the observation of DC modulation by ACs.

To determine if incubation with either apoptotic or necrotic cells induces cell death in DCs, d6 DCs were incubated for 48 hours alone or with dead cells, then the percentage and type of cell death of the DCs themselves was determined by AnV-PI staining. This experiment examined whether DC viability was inadvertently affected by the method of FDCP killing, for example residual camptothecin or cell-damaging reactive oxygen species generated by heat damage. Such effects have been caused by residual apoptosis-inducing agents in another study (240), and could possibly result in false negatives for molecule up-regulation or cytokine production in this investigation. However, the percentage of live DC was unaffected by incubation with either dead cell type, remaining as it did at approximately 70%, hence neither apoptotic nor necrotic cells induce DC death (within 48 hours).

3.4.3 Generation of immature DCs

DCs were generated by incubating murine bone marrow with GMCSF for 6-8 days. BMDCs generated thus are an artificial, in vivo model of DCs, with similarities to both myeloid and lymphoid murine DCs. GMCSF was produced in-house using the GMCSF-secreting murine cell line Ag8653 (murine myeloma cell line transfected with cDNA encoding for GMCSF (227). Using cell line-produced GMCSF from culture flasks increased the possibility of potentially proinflammatory contaminants, for example mycoplasma, being introduced into DC cultures. Resultant activation of DCs into maturity would potentially render them unresponsive to further stimuli, whether pro- or anti-inflammatory. Furthermore, high doses of GMCSF have been shown to induce DC maturation in vitro (228) and confer DC co-stimulatory activity in vivo (241). Our own GMCSF was therefore examined alongside endotoxin-free recombinant GMCSF from Peprotech for proinflammatory activity, in order to evaluate its use in this study for the generation of immature DCs. The secreted GMCSF was effective at stimulating the generation of immature DCs, and was used at ~100ng/ml in all subsequent experiments for optimal DC production.

For further validation of DCs generated from bone marrow, cells were examined for their responsiveness to TLR-ligand PAMPs. DCs exhibited responsiveness to LPS [see Fig. 3.14], CpG [see Fig. 3.15], and pam3CSK4 (as indicated by up-regulation of CD40, CD86, and secretion of IL12 [data not shown]) that was typical of immature BMDCs (231). DCs were unresponsive to the poly(I:C) used in this study (data not shown), though this was not investigated further.

3.4.4 The effect of apoptotic cells on the expression of co-stimulatory and co-inhibitory molecules of resting DCs

The apparent effect of apoptotic cells on DC expression of co-stimulatory molecules was minimal: Expression levels of CD40, CD80, CD86, and CD25 after AC treatment were, in general, extremely similar to expression levels in untreated DCs, with no significant changes [Fig. 3.10].

These findings were consistent with many published reports, including the findings of Williams et al (2008), where neither apoptotic (by incubation with anti-Fas antibody) Jurkat cells nor autologous T cells (at an AC:DC ratio of 5:1) significantly increased CD80, CD83 or CD86 (93). The present results were also consistent with those of Clayton et al (2003) where low numbers of human *ex vivo* apoptotic (by survival signal-deprivation) neutrophils (AC:DC ratio of 1:1) made no significant difference to CD40, CD80 and CD86 expression in DCs generated from peripheral blood mononuclear cells (PBMCs) (233). It is important to note, however, that high numbers of apoptotic cells (20:1) in the same study induced significant down-regulation of CD40, CD80 and CD86. Sauter et al's (2000) panel of apoptotic (by UV) cell lines also had no effect on DCs cultured from human *ex vivo* PBMCs. The exception to the present trend of no-difference observed in this study was CD83, which was up-regulated after AC treatment. This was unexpected but it does have precedent in the findings of Clayton et al (2003) (233). However, there is no evidence to suggest that CD83 may perform a regulatory role or why it would perform alone in an immune role. The lack of up-regulation of CD25 induced by ACs is consistent with a non-inflammatory role (222, 242).

The active influence of apoptotic cells can be seen much more clearly in the up-regulation of co-inhibitory molecules. Although B7DC and B7H1 were not significantly affected, the expression of B7H2, B7H3 and B7H4 were all significantly increased approximately two-fold [Fig. 3.12].

3.4.5 The effect of necrotic cells on the expression of co-stimulatory and co-inhibitory molecules of resting DCs

The response of DCs to necrotic cells was clear and invariable in the co-stimulatory molecules examined. Necrotic cells induced significant up-regulation of CD40, CD80, CD83, CD86 and CD25 [Fig. 3.10]. CD83 incurred the largest increase, having increased almost threefold from 9%

to 25%. The induced levels of all molecules were significantly higher than corresponding levels of apoptotic cell-treated DCs, except that of CD25, which, although higher, was not significantly different. These increases indicate a certain proinflammatory phenotype, and are consistent with the findings of many other groups and the generally accepted paradigm that necrotic cell lysates activate DCs (18). For example, Williams et al (2008) reported the up-regulation of CD80, CD83 and CD86 in response to F/T (four cycles) necrotic T cells (NC:DC at 5:1), regardless of whether the T cells were Jurkat cells, or allogeneic or autologous T cells (93). This is in contrast to the findings of Clayton et al (2003) where F/T (four cycles) necrotic neutrophils (NC:DC at 5:1) induced the same down-regulation of CD80 and CD86 as that induced by apoptotic cells, and a significantly larger decrease in CD40 (this was also the case for cells allowed to undergo secondary necrosis by culturing for 40 hours *ex vivo*) (233). The disparity between the Williams and Clayton studies is hard to explain with no more details about their methods being available. The present study is similar to the Williams study, but there appears to be no connection with the source of necrotic cell. We may speculate that loss of lysate may explain the Clayton findings, but it would be pure conjecture. Kushwah et al (2009) found that necrotic BMDCs did not induce maturation in live BMDCs, however, only CD86 was examined, and the method of necrosis was one cycle of F/T which, from anecdotal evidence and first-hand experience, does not reliably induce sufficient rates of necrosis (198). Sauter et al (2000) observed up-regulation of CD40, CD83 and CD86 in response to various F/T (4-5 cycles) necrotic cells lines, but interestingly this response was not echoed in the use of autologous primary cells as the source of necrotic cells (17). The up-regulation of CD25 by NCs is consistent with a proinflammatory role (222, 242).

Necrotic cells, like apoptotic cells, also induced significant up-regulation of co-inhibitory molecules, though a different combination of molecules compared to those up-regulated by apoptotic cells [Fig. 3.12]. Unlike apoptotic cells, necrotic cells induced significant, though small, up-regulations of B7DC from 30% to 38% and of B7H1 from 21% to 38%. Necrotic cells also increased B7H3 from 5% to 8%, however, again unlike ACs, this was not significant. Similarly to

apoptotic cells, necrotic cells induced significant up-regulation of B7H2 and B7H4. These increases were smaller than those induced by ACs, but statistically similar.

Although perhaps counter-intuitive, necrotic cell-induced up-regulation of co-inhibitory molecules is consistent with a proinflammatory response, which requires a slower-acting anti-inflammatory response in order to resolve the inflammation. Evidence is increasing that suggests that resolution of inflammation is an active process, rather than a passive diminishing of inflammation. The early expression of co-inhibitory molecules may play a crucial role in resolving, as well as preventing, inflammation. This is discussed further in 3.4.7.

Table 1 summarises the changes in co-stimulatory and co-inhibitory molecules induced by ACs and NCs. It can be seen that apoptotic cells appear to have a net anti-inflammatory effect, whereas necrotic cells appear to have a net inflammatory effect. However, as the relative influence of each molecule is unknown, this can only be confirmed by exploring the functions of these DCs, which is discussed in Chapters 4 and 5.

3.4.6 The effect of apoptotic cells on the expression of co-stimulatory and co-inhibitory molecules of activated DCs

After LPS challenge, DCs significantly and substantially up-regulated CD40, CD80, CD83, CD86 and CD25, with the largest increase being an increase of ~x5 (CD25) [Fig. 3.14].This was a typical mouse or human DC response to LPS (231, 242). Pre-treating DCs with apoptotic cells had no effect at all on the LPS-induced up-regulation of any of the co-stimulatory molecules examined. This was consistent with the passive role described in the findings of Sauter et al (2000) (17) and Takahashi et al (2003) (15). However, the current results were in contrast with the findings of Williams et al (2008) where ACs actively lowered the CD80, CD83 and CD86 response to LPS or

NCs (93), and Clayton et al (2003), where ACS inhibited CD40, CD80 and CD86 in response to LPS(233), and Kushwah et al (2009), where ACs suppressed LPS-induced up-regulation of CD86 (198).

CpG treatment induced significant up-regulation of CD40, CD80, CD86, and CD25 (i.e. all examined co-stimulatory molecules except CD83), all increasing by approximately threefold [Fig. 3.15]. This was generally consistent with reported responses to CpG in mouse or human DCs (54, 231). Already we can see that CpG appeared to provide a slightly less powerful stimulation of DCs than did LPS. This is substantiated by the successful suppression of some CpG-induced increases by pre-treating DCs with apoptotic cells, in contrast to LPS-induced increases. The CpG-induced up-regulation of CD86 was significantly inhibited (but not totally ablated) by apoptotic cells. Apoptotic cells also notably inhibited (though not significantly) up-regulation of CD80. To wit, no such examination has been undertaken before.

The apoptotic cell-mediated up-regulation of B7H2, B7H3 and B7H4 [Fig. 3.12] persisted through LPS and CpG challenge (though B7H3 was no longer significant after LPS) [Fig. 3.16]. There exists little evidence for the effect of CpG on co-inhibitory molecules, though it has been reported to decrease B7DC expression on *ex vivo* murine plasmacytoid DCs (243). As tests were not undertaken to determine how long it takes for expression of these molecules to begin, it is not know if LPS/CpG challenge inhibited their expression or if they caused a subsequent down-regulation of their expression.

The failure of apoptotic cells in this study to suppress LPS-induced up-regulation of co-stimulatory molecules contrasts with previous studies that specifically demonstrate otherwise. The generated DCs were certainly suppressible (in terms of co-stimulatory molecules), as observed in CpG treatment. Therefore, the LPS used in the present study, though at a lower dose than some of the previous studies, may have been from a source (E. Coli) too immunogenic for co-stimulatory molecules to be actively suppressed. The difference between LPS and CpG in the

effect on co-inhibitory molecules is less conspicuous but nonetheless present. In both treatments, up-regulation of co-inhibitory molecules is largely maintained, and PAMP-induced down-regulation of B7H2 countered. However, up-regulation of B7H2, 3 and 4 are all markedly higher in CpG-treated cells than LPS-treated cells (B7H2 significantly so, $p<0.05$).

3.4.7 The effect of necrotic cells on the expression of co-stimulatory and co-inhibitory molecules of activated DCs

Pre-treating DCs with necrotic cells had no significant effect on LPS-induced maturation [Fig. 3.14], though it did induce a non-significant but notable enhancement of the LPS-induced up-regulation of CD83 and CD86. Necrotic cells made no significant difference to the CpG-induced up-regulation of CD40, CD80, CD86 or CD25 [Fig. 3.15]. Expectedly, necrotic cells induced up-regulation of CD83. Less expectedly, given that CpG alone had very little effect on CD83, was the synergistic effect of necrotic cells and CpG, which in combination induced expression of CD83 that was significantly higher than after necrotic cells alone ($p<0.001$). Curiously, necrotic cells in combination with CpG induced less (but not significantly so) CD40 than necrotic cells in combination with apoptotic cells, or CpG alone. This is largely in contrast to the effect of necrotic cells on other co-stimulatory molecules, either alone or in combination with PAMPs.

The necrotic cell-induced up-regulation of B7DC, B7H1 and B7H4 [Fig. 3.12] persisted after LPS challenge [Fig. 3.16] (though the up-regulation of B7DC was no longer significant), whereas B7H2 up-regulation was ablated. The induced up-regulation of B7H1 and B7H2 persisted after CpG challenge [Fig. 3.17], whereas B7H4 up-regulation was ablated. The significance of this variation in co-inhibitory molecules is not yet understood, and merits detailed investigation as it may potentially indicate a distinction between two separate pathways of inhibition – one effected by

ACs, another by NCs. Also, the NC-induced up-regulation of B7H1 and B7H2 is markedly higher in CpG-treated cells than LPS-treated cells (B7H2 significantly so, $p<0.001$), whereas B7H4 is lower. It appears that necrotic cells may perhaps more easily increase the inhibitory phenotype of CpG-challenged cells than LPS-challenged cells.

The data suggests that necrotic cells do enhance PAMP-induced maturation, though in these experiments the expression level of many of the markers may already have been at or near a maximum, particularly LPS-induced maturation. The LPS stimulus may have been too strong to be inhibited, whereas CpG stimulation could to an extent be suppressed. CpG was nonetheless a substantial stimulus: The relative effects of necrotic cells on untreated DCs are significantly different to their relative effects on DCs that are subsequently treated with CpG ($p<0.001$, two-way ANOVA): For CD40 and CD86, significantly more necrotic-treated DCs are high in expression than untreated DCs; however, after CpG treatment, this significant difference no longer exists. This demonstrates that CpG treatment is powerful enough to maximise CD40 and CD86, surpassing the stimulus from endogenous inflammatory signals and rendering necrotic cell-induced up-regulation insignificant. This in itself is testament to the influence of ACs, given that AC-treatment is sufficient to significantly reduce even this effective stimulation.

3.4.8 Effects on DCs of alternative apoptotic cell sources/treatments

Previous studies have highlighted the significant role of serum proteins in apoptotic cell−DC interactions, such as opsins and other linker molecules (e.g. IgM (244)). It was contemplated that apoptotic cell-mediated suppression of DC co-stimulatory molecules (considered, at this stage in the investigation, to be crucial to tolerogenic suppression of T cell-mediated immunity) may be more successful with various media supplements. These supplements, including mouse serum, may have proved to contain crucial accessories to DC suppression. It transpired, however, that neither IMDM, nor mouse serum, nor non-heat-inactivated FBS contained anything that improved DC

suppression [Fig. 3.18]. If any linker molecules were crucial to the (later confirmed) generation of tolerogenic DCs, they were present in sufficient quantities in complete RPMI media.

This series of experiments was limited to studying CD40 alone, which was taken to represent co-stimulatory molecules in general. Although this was deemed sufficient, given the time restraints, for this branch of investigation, attention must be drawn to the fact that we have shown that co-stimulatory molecules may have dissimilar responses to the same stimuli. As such, a full study of the effects of media supplements, representing various reconstructions of the *in vivo* milieu, should include examination of a wider range of co-stimulatory and co-inhibitory molecules, as well as cytokines. Indeed, such a study constitutes a large undertaking that warrants its own dedicated investigation.

One of the key functions of DCs *in vivo* is detecting elements of potential danger, and thus DCs have an innate ability to react to non-self agents. The FDCP cells used thus far in this investigation are derived from a B6 background and are therefore, in essence, clone cells of the B6 mice from which DC were generated in this project. FDCP are therefore likely to be recognised as 'self' by our B6 DC. Also, the nature of the *in vitro* system employed here dismisses the possibility of proinflammatory responses against foreign bodies by memory cells. However, given the sensitivity of DC to exogenous material, and the possibility that the FDCP cell line has mutated sufficiently to be no longer recognised as self-tissue by B6 immune cells, there existed the possibility that cell line apoptotic cells would be less efficient at suppression of DC maturation in this model than would primary B6 apoptotic cells. Indeed, many studies of apoptotic cell-mediated suppression of DCs have employed apoptotic cells generated from primary cells.

To investigate the prospect that apoptotic cell-mediated immune suppression *in vivo* could be more successful if employing primary cells, rather than cell lines, apoptotic cells were generated from *ex vivo* thymocytes. Thymocytes were isolated from the C57Bl6/J mouse spleen and treated with super-Fas ligand (sFasL), which induced apoptosis in a high percentage of cells within 4 hours (the initiation of apoptosis after sFasL exposure does not depend on the stage of the cell cycle, unlike

camptothecin). Moreover, this method of induction of apoptosis is a reconstruction of a common and naturally-occurring mechanism – therefore, it can be argued, it has a higher physiological relevance than camptothecin-induced apoptosis. SFasL-treated thymocytes had similar properties to camptothecin-induced FDCP in that they demonstrated no significant effect on the expression of select co-stimulatory molecules on resting or LPS-treated DCs [Fig. 3.19A]. This is similar to the parity between apoptotic Jurkat cells and autologous T cells in their suppressive effects on DCs, as observed by Williams et al (2008) (93). SFasL-treated thymocytes induced significant up-regulation of B7H2 and B7H4 in resting DCs, and maintained significant B7H4 up-regulation in LPS-challenged DCs [Fig. 3.19B], similarly to camptothecin-treated FDCP. Unlike camptothecin-FDCP, sFasL-treated thymocytes failed to maintain up-regulation of B7H4 in LPS-challenged DCs. Overall, sFasL-treated thymocytes were similar to camptothecin-treated FDCP. Accordingly, it was felt that camptothecin, with similar suppressive potential but a simpler methodology, represented a far better prospect for the purposes of this investigation.

On account of their regular use as a source of apoptotic cells in previous investigations, the effect of UV-induced apoptotic cells on DCs was briefly investigated. At no tested exposure of UV did FDCP have the capacity to reduce the LPS-induced up-regulation of CD40 and CD86. The UV method used in this investigation appeared to be too destructive to maintain membrane integrity. However, photochemotherapy has been demonstrated to produce apoptotic cells that induce a tolerogenic phenotype in human DCs (245).

3.4.9 Summary

In this stage of the investigation homogenous populations of apoptotic cells and of necrotic cells were generated successfully, by means of camptothecin treatment or heat kill, respectively, of FDCP. The type of cell death was verified by several methods and was conclusive in both

treatments. Crucially, both the apoptotic state and the necrotic state could be maintained for sufficient lengths of time to allow for co-incubation with DCs and subsequent PAMP treatment. Several other methods of cell death induction were discounted as inefficient. DCs were generated successfully from bone marrow, and were responsive to both PAMPs and dead cells.

Apoptotic cells had no effect on expression of co-stimulatory molecules of resting DCs, or on the up-regulated expression on LPS-treated DCs. However, apoptotic cells did suppress some CpG-induced up-regulation of co-stimulatory molecules. In all treatments, apoptotic cells induced up-regulation of co-inhibitory molecules, but less so in LPS-treated DCs than in resting or CpG-treated DCs.

Necrotic cells induced up-regulation of co-stimulatory molecules on resting DCs. They had little effect on LPS- or CpG-induced up-regulation, though there was evidence of enhancement by necrotic cells. Necrotic cells induced up-regulation of co-inhibitory molecules on resting and LPS- and CpG-treated DCs, though, in general, not as considerably or uniformly as did apoptotic cells.

In summary, apoptotic cells appeared to induce an inhibitory phenotype in resting DCs, suppress the inflammatory phenotype of CpG-treated DCs, and have no effect on the inflammatory phenotype of LPS-treated DCs. Necrotic cells appeared to generally induce or enhance a proinflammatory phenotype, whilst simultaneously inducing anti-inflammatory features, in resting and LPS- and CpG-treated DCs. The net effect of non-up-regulated co-stimulatory molecules, and up-regulated co-inhibitory molecules, demonstrates that apoptotic cells are not merely immunologically innocuous in their failure to induce maturation of DCs, but are active inducers of a potentially tolerogenic DC phenotype.

The chief results of the current investigation, consisting of the non-inflammatory effects of apoptotic cells and the proinflammatory effects of necrotic cells, are supported by both recent and landmark studies where similar examinations of apoptotic and necrotic cells have been made. The

studies used in comparisons above were selected to cover a range of murine and human *in vitro* models using different sources of DC and a variety of methods of apoptosis and necrosis induction, but not including *ex vivo* tumour cells. The current findings are distinctive in their lack of conspicuous suppression of PAMP-induced proinflammatory phenotypes. To wit, no studies have examined the expression of co-inhibitory molecules in response to apoptotic or necrotic cells. With no precedents for comparison, these results are discussed in the context of the downstream immune events (T cell responses and *in vivo* effects) in Chapters 4 and 5.

CHAPTER 4 – The Effect of Apoptotic Cells and Necrotic Cells on Dendritic Cell-Produced Cytokines and Their Influence on T Cell Function

4.1 Introduction

Cytokine production and secretion by DCs is crucial to their function in initiating an immune response. To fully characterise dead cell-induced modulation of DCs, the cytokine profile – consisting of pro- and anti-inflammatory cytokines – was ascertained. The downstream functional effects of AC- and NC-treated DCs were then investigated. This was originally done by co-incubating DCs with ACs or NCs, then separating DCs from the dead cells by MACS, culturing the DCs with splenocytes for 3 days, and analysing the cultures for T cell-produced cytokines. These results showed no differences between DC treatments (data not shown). It was speculated that cytokine secretion by dead cell-treated DCs had ceased within the DC-dead cell co-incubation period, and that consequently DCs from different treatments were no longer functionally dissimilar (the possible implications of this are discussed in 4.3.2). Instead, T cell functional assays were performed using the culture media from DC-dead cell co-incubations. The post-incubation culture media, or conditioned media (CM), was combined with low numbers of fresh DCs and co-incubated with splenocytes treated with the lymphocyte mitogen concanavalin A (conA), which induces T cell proliferation. Significant differences were found in T cell behaviour between those incubated with AC-treated DC CM and those incubated with NC-treated DC CM.

4.2 Results

4.2.1 Necrotic cells, but not apoptotic cells, induce DC secretion of proinflammatory cytokines

Low levels of IL12p40 (IL12) and IL6 were detected by ELISA in the culture supernatant of untreated DCs (624 pg/ml and 347 pg/ml, respectively) [Fig. 4.1]. Co-incubation with NCs significantly increased the concentration of IL12 in the culture supernatant to 2660 pg/ml. Co-incubation with NCs increased the concentration of IL6 to 1600 pg/ml. Treatment of DC with ACs did not significantly alter the amounts of IL12 or IL6 detected in the culture media. The proinflammatory cytokines IL1β and TNFα were not detected in the culture supernatants of DCs treated with dead cells or with LPS (data not shown). These results demonstrate that NCs significantly increase secretion of a proinflammatory cytokine profile, whereas ACs do not.

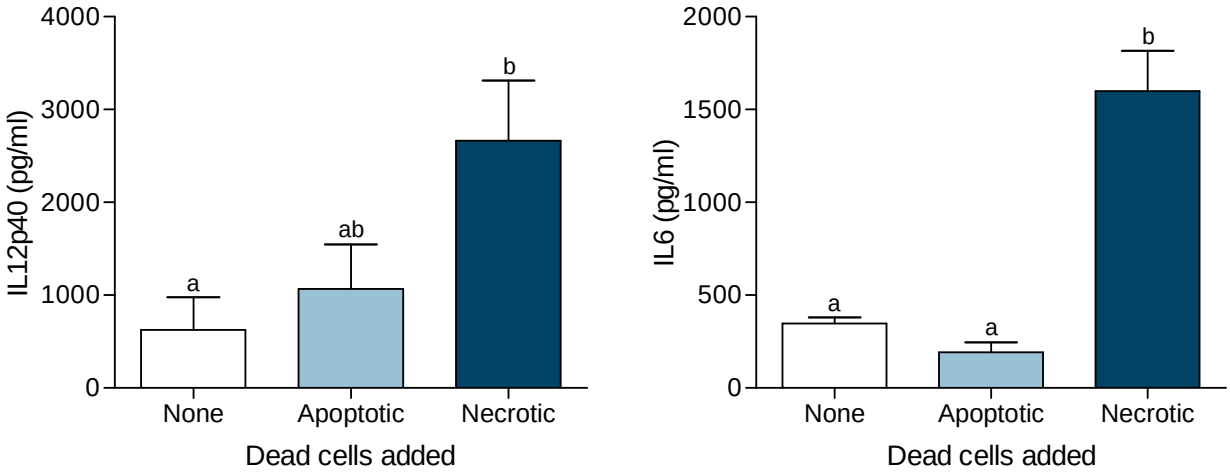


Figure 4.1 Necrotic cells, but not apoptotic cells, induce DC secretion of proinflammatory cytokines. The culture supernatant of DCs that had been incubated with or without apoptotic cells (camFDCP) or necrotic cells (HKFDCP) for 24hrs was analysed by ELISA for various cytokines. Necrotic cells induced a significant increase in secretion of both IL12p40 and IL6. Apoptotic cells did not induce any significant changes in IL12 or IL6 secretion. 'a' and 'b' signify significantly different means, one-way ANOVA. Data is from 3 separate experiments or more, bars indicate SD.

4.2.2 Apoptotic cells, but not necrotic cells, induce DC secretion of anti-inflammatory cytokines

IL10 was present in the culture supernatant of untreated DCs at a concentration of 10pg/ml [Fig. 4.2]. Treatment with ACs significantly increased this concentration to 78pg/ml ($p<0.001$). NCs increased IL10 to 15pg/ml, which was not significant.

TGFβ was present in the supernatant of untreated DCs at 780pg/ml, and was significantly increased to 2340pg/ml by pre-treating DCs with ACs ($p<0.001$) [Fig. 4.2]. NCs had the opposite effect and significantly decreased the concentration of TGFβ to 155pg/ml ($p<0.05$).

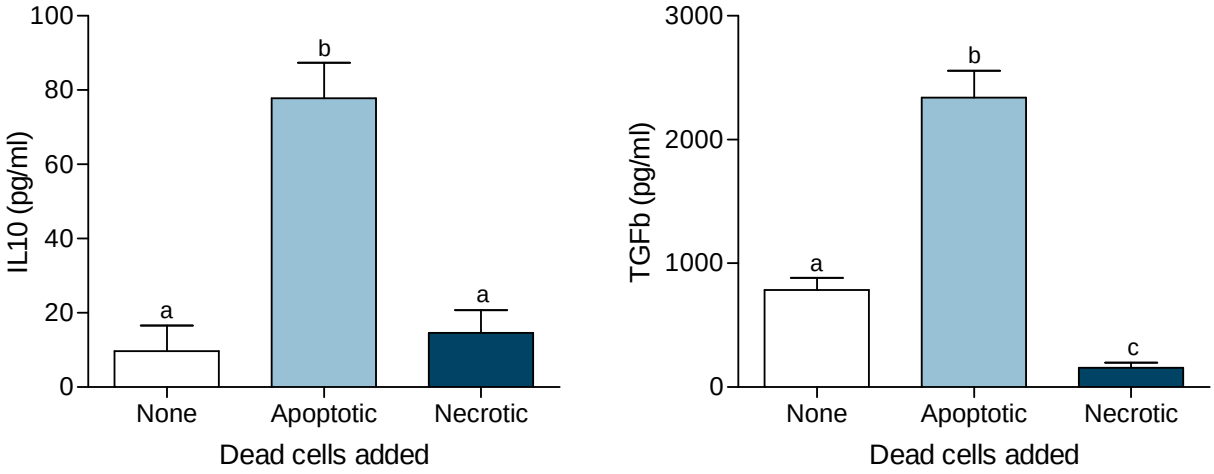


Figure 4.2 Apoptotic cells, but not necrotic cells, induce DC secretion of anti-inflammatory cytokines. The culture supernatant of DCs that had been incubated with or without apoptotic cells or necrotic cells for 24hrs was analysed by ELISA for various cytokines. Apoptotic cells induced significantly increased secretion of both IL10 and TGFβ. Necrotic cells did not induce any significant change in IL10 secretion, but significantly decreased TGFβ secretion. 'a,' 'b' and 'c' signify significantly different means, one-way ANOVA. Data is from 3 separate experiments or more, bars indicate SD.

4.2.3 DC secretion of IL12 in response to CpG is inhibited by apoptotic cells, but not necrotic cells

CpG induces significantly increased secretion of IL12 from 620pg/ml in untreated DC to 6630pg/ml [Fig. 4.3], $p<0.001$, one-way ANOVA). This up-regulated secretion was significantly abrogated to 3370pg/ml ($p<0.05$) by pre-treating DCs with ACs (but not completely inhibited, as IL12p40 secretion remained significantly higher than that by untreated DCs). In contrast, the CpG-induced secretion of IL12 was significantly enhanced to 9920pg/ml by pre-treating DCs with NCs ($p<0.05$) [Fig. 4.3].

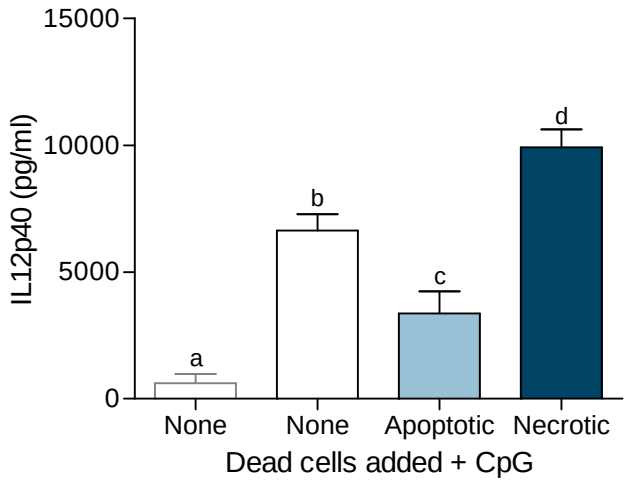


Figure 4.3 DC secretion of IL12 in response to CpG is inhibited by apoptotic cells, but not necrotic cells. CpG treatment induced a significant increase in secretion of IL12p40 by DCs. This increase was significantly inhibited by pre-treating DCs with apoptotic cells, and significantly enhanced by pre-treating DCs with necrotic cells. 'a,' 'b,' 'c' and 'd' signify significantly different means, one-way ANOVA. Data is from 3 separate experiments or more, bars indicate SD.

4.2.4 DC secretion of IL6 in response to CpG is inhibited by apoptotic cells, but not necrotic cells

CpG induces significantly increased secretion of IL6 (from ~750pg/ml in untreated DCs to ~3000pg/ml [Fig. 4.4], $p<0.001$, one-way ANOVA). This up-regulated secretion was significantly abrogated to ~1600pg/ml by pre-treating DCs with apoptotic cells (but not completely inhibited, as IL6 secretion remained significantly higher than that by untreated DCs). Surprisingly, the up-regulation of IL6 in response to CpG was slightly abrogated by pre-treating DCs with necrotic cells, though not significantly [Fig. 4.4]

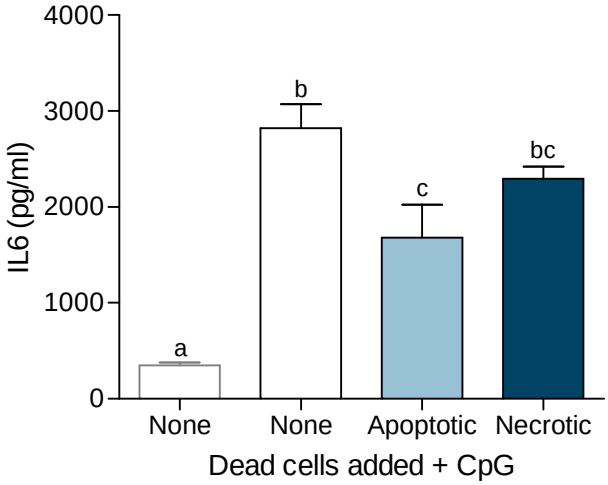


Figure 4.4 DC secretion of IL6 in response to CpG is inhibited by apoptotic cells, but not necrotic cells. CpG treatment induced a significant increase in secretion of IL6 by DCs. This increase was significantly inhibited by pre-treating DCs with apoptotic cells. The increase was slightly, but not significantly, inhibited by pre-treating DCs with necrotic cells. 'a,' 'b' and 'c' signify significantly different means, one-way ANOVA. Data is from 3 separate experiments or more, bars indicate SD.

4.2.5 Apoptotic cell suppression of IL12 production by CpG-stimulated DCs is not IL10-dependent

After induction of proinflammatory cytokine secretion in an immune response, anti-inflammatory cytokine production and secretion commences as a means to resolve inflammation. We investigated whether one such anti-inflammatory cytokine, IL10, is accountable for the AC-induced suppression of CpG-induced IL12 secretion. DCs were cultured from the bone marrow of wildtype mice and of $IL10^{-/-}$ mice from the same background (246) ($IL10^{-/-}$ DCs produced no IL10 in response to LPS, CpG, or either dead cell type, data not shown) then treated with or without dead cells. The experiment showed that DCs from the knockout mice have enhanced IL12 secretion, regardless of cell treatment [Fig. 4.5A]. The experiment was then repeated with a subsequent CpG challenge after all treatments [Fig. 4.5B]. The experiment shows an AC-specific significant suppression of CpG-induced IL12 secretion from 4690pg/ml to 3360pg/ml ($p<0.05$, one-way ANOVA). Similar suppression is seen in the identical treatments of DCs from IL10-knockout mice, from 4800pg/ml to 3390pg/ml ($p<0.05$, one-way ANOVA). CpG/dead cell response by DCs was not affected by the Wt/IL10ko background of the mice ($p>0.05$, two-way ANOVA). This indicates that AC-induced secretion of IL10 is not essential for AC-mediated suppression of IL12 secretion by DCs.

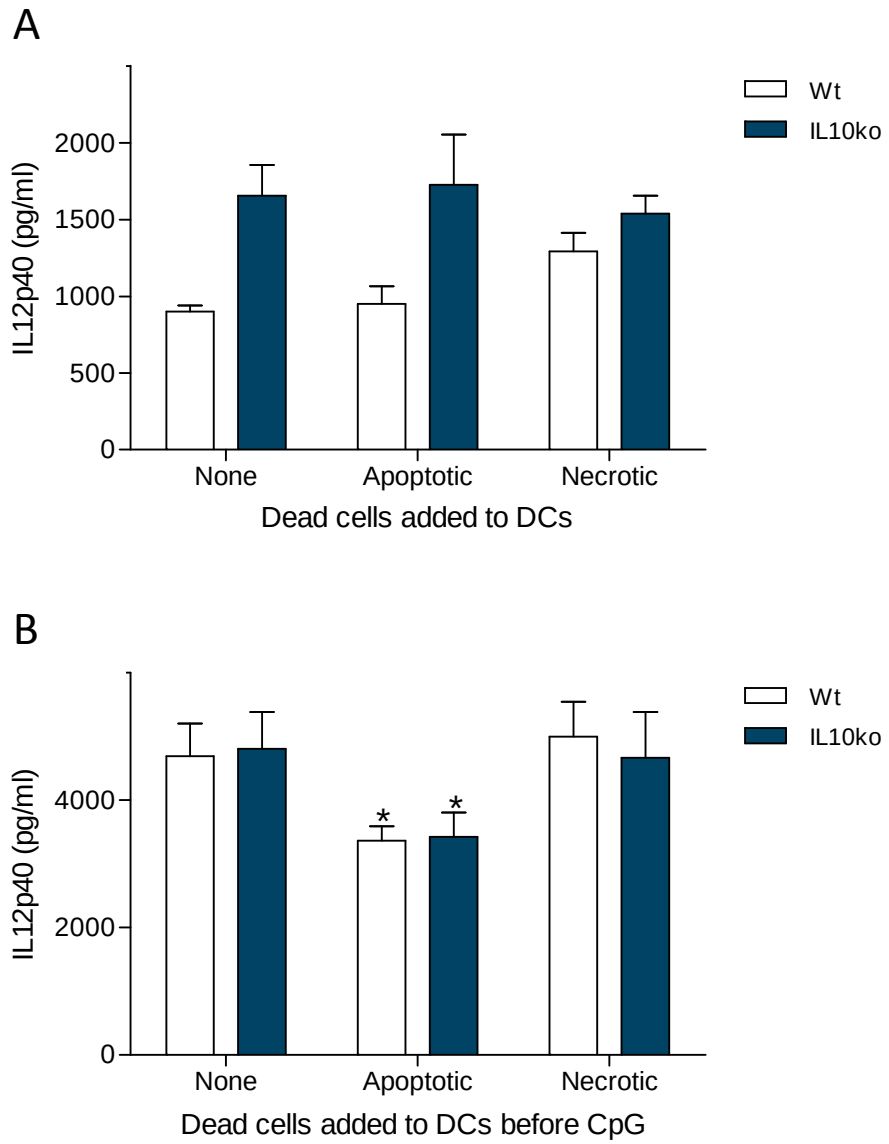


Figure 4.5 Apoptotic cell suppression of IL12p40 production by CpG-stimulated DCs is not IL10-dependent. DCs from wildtype ('Wt') and IL10$^{-/-}$ ('IL10ko') mice were incubated with or without apoptotic cells or necrotic cells for 24hrs (**A**), and treated with CpG for the last 12hrs (**B**). The wildtype mice demonstrated apoptotic cell-specific significant suppression of IL12p40 secretion into the culture supernatant. This significant suppression was also demonstrated by IL10ko mice. * = $p<0.05$, referring to significant difference between the indicated and the corresponding untreated DCs ('None'), one-way ANOVA. Data is from 3 separate experiments, bars indicate SD.

4.2.6 IFNγ production by mitogen-stimulated T cells is suppressed by the CM of apoptotic cell-treated DCs, and enhanced by the CM of necrotic cell-treated DCs

Murine splenocytes were isolated and cultured with 0-10μg/ml concanavalin A (conA) for three days in the CM of untreated DC, AC-treated DC or NC-treated DC. Treatment with conA induced dose-dependent and significant secretion of IFNγ by splenocytes regardless of the type of CM in which they had been cultured [Fig. 4.6]. However, the CM of AC-treated DCs (AC-CM) and that of NC-treated DCs (NC-CM) have significant ($p<0.001$, two-way ANOVA [asterisks mark significance at each conA concentration in comparison to the CM of untreated DCs, one-way ANOVA], but opposite, effects on IFNγ production in response to conA, compared with the CM of untreated DCs. AC-CM significantly abrogates overall IFNγ production whereas NC-CM enhances it. NC-CM alone is sufficient to double mitogen-independent IFNγ production from a baseline of 4100pg/ml by splenocytes treated with CM of untreated DCs, to 9170pg/ml, which is similar to the level of IFNγ production by splenocytes treated with CM of untreated DCs plus a conA dose of 1.25μg/ml, or IFNγ production by splenocytes treated with apoptotic CM plus a conA dose of 10μg/ml.

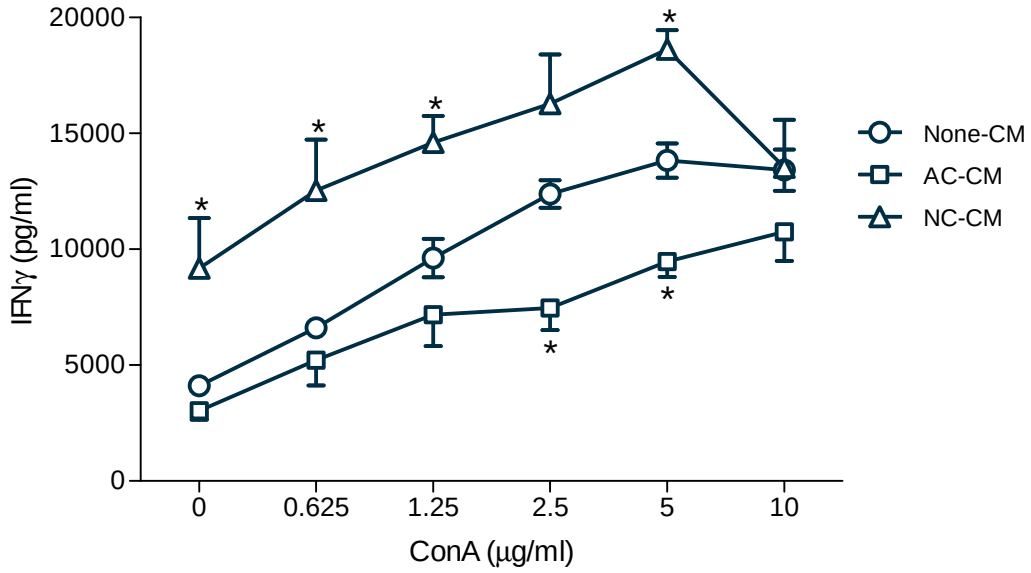


Figure 4.6 IFNγ production by mitogen-stimulated T cells is suppressed by the CM of apoptotic cell-treated DCs, and enhanced by the CM of necrotic cell-treated DCs. Two-way ANOVA confirms that the CM of apoptotic cell-treated DCs ('AC-CM') and the CM of necrotic cell-treated DCs ('NC-CM') both have a significant effect on the overall dose-dependent conA-induced increase of IFNγ secretion by T cells, compared to the CM of untreated DCs ('None-CM'). Specifically, AC-CM suppresses IFNγ secretion and NC-CM enhances IFNγ secretion. * = $p<0.05$, referring to significant difference between the indicated and the corresponding None-CM value, one-way ANOVA. Data is from 3 separate experiments or more, bars indicate SD.

4.2.7 The CM of LPS-treated DCs enhances IFNγ production by mitogen-stimulated T cells, and neither pre-treatment of DC with apoptotic cells nor pre-treatment with necrotic cells affects this enhancement

Splenocytes were cultured with the CM from DCs that were treated with dead cells and LPS or the CM of DC treated with LPS alone. LPS-treatment of the DCs had a significant effect on the dose-dependent influence of CM in the splenocyte culture, resulting in enhanced IFNγ production in response to conA [Fig. 4.7], compared to when CM of untreated DCs is used [Fig. 4.6] ($p<0.001$, two-way ANOVA). Neither the CM of DCs treated with ACs then LPS, nor the CM of DCs treated with NCs then LPS, caused conA-response curves that were statistically different to that caused by the CM of DCs treated with LPS alone ($p>0.05$, two-way ANOVA), demonstrating that pre-treatment of DCs with ACs or NCs did not affect the overall capacity of LPS-matured DCs to enhance IFNγ secretion by T cells. However, one-way ANOVA of separate conA doses revealed that pre-treatment with ACs does have a slight but significant inhibitory effect on IFNγ production by T cells treated at a dose of 5μg/ml conA.

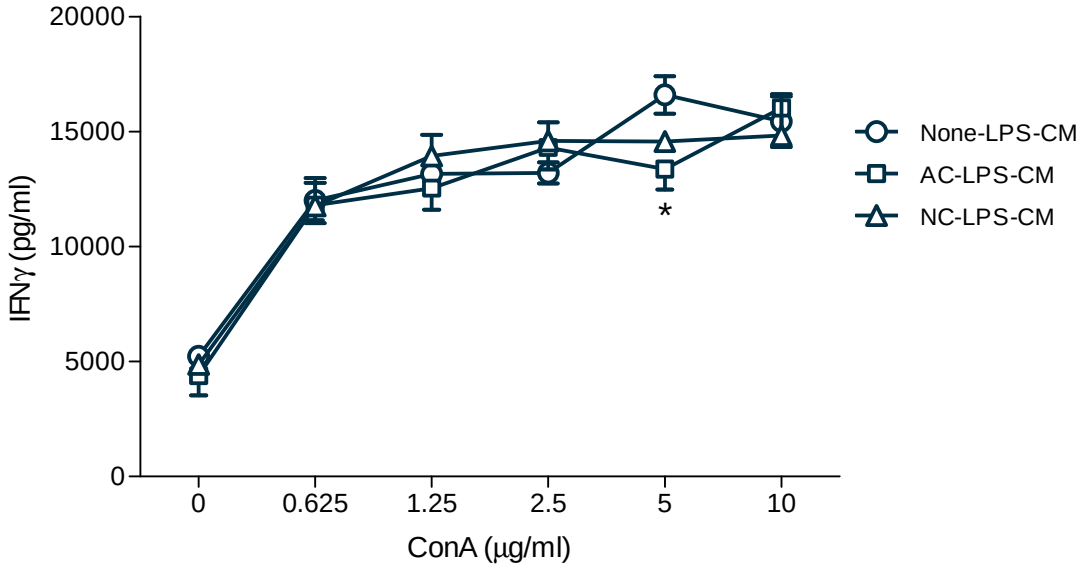


Figure 4.7 The CM of LPS-treated DCs enhances IFNγ production by mitogen-stimulated T cells, and neither pre-treatment of DCs with apoptotic cells nor pre-treatment with necrotic cells affects this enhancement. Two-way ANOVA revealed that pre-treatment with either apoptotic or necrotic cells does not affect the capacity of LPS-treated DC CM to enhance secretion of IFNγ by conA-stimulated T cells. That is, None-LPS-CM, AC-LPS-CM and NC-LPS-CM have no difference in their effect on T cell IFNγ production. * = $p<0.05$, referring to significant difference between the indicated and the corresponding None-LPS-CM value, one-way ANOVA. Data is from 3 separate experiments or more, bars indicate SD.

4.2.8 The CM of CpG-treated DCs enhances IFNγ production by mitogen-stimulated T cells, and pre-treatment of DCs with apoptotic cells, but not with necrotic cells, suppresses this enhancement

Splenocytes were cultured with the CM from DCs that were treated with dead cells and CpG, or the CM of DCs treated with CpG alone. CpG-treatment of the DCs had a significant effect on the dose-dependent influence of CM in the splenocyte culture, resulting in enhanced IFNγ production in response to conA [Fig. 4.8], compared to when CM of untreated DCs is used [Fig. 4.6] ($p<0.001$, two-way ANOVA). The CM of DCs treated with NCs then CpG did not cause conA-response curves that were statistically different to that caused by the CM of DCs treated with CpG alone ($p>0.05$, two-way ANOVA), demonstrating that pre-treatment of DCs with NCs did not affect the capacity of CpG-matured DCs to enhance IFNγ secretion by conA-stimulated T cells. However, the CM of DCs treated with ACs then CpG caused a conA-response curve that was significantly lower to that caused by the CM of DCs treated with CpG alone ($p<0.001$, two-way ANOVA), demonstrating that pre-treatment of DCs with ACs abrogates the capacity of CpG-matured DCs to enhance IFNγ secretion by conA-stimulated T cells.

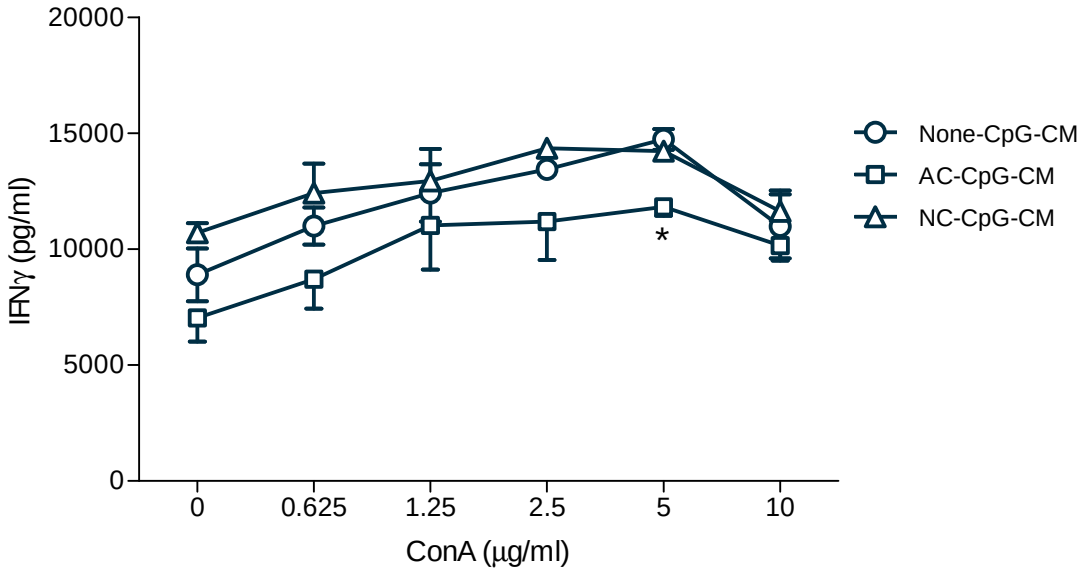


Figure 4.8 The CM of CpG-treated DCs enhances IFNγ production by mitogen-stimulated T cells, and pre-treatment of DCs with apoptotic cells, but not with necrotic cells, suppresses this enhancement. Two-way ANOVA revealed that None-CpG-CM and NC-CpG-CM were not significantly different, i.e. pre-treatment with necrotic cells did not affect the capacity of CpG-treated DC CM to enhance secretion of IFNγ by conA-stimulated T cells. Two-way ANOVA revealed that AC-CpG-CM was significantly lower IFNγ secretion than either None-CpG-CM or NC-CpG-CM, i.e. pre-treatment with apoptotic cells reduced the capacity of CpG-treated DC CM to enhance secretion of IFNγ by conA-stimulated T cells. * = $p<0.05$, referring to significant difference between the indicated and the corresponding None-CM value, one-way ANOVA. Data is from 3 separate experiments or more, bars indicate SD.

4.2.9 The CM of DCs treated with necrotic cells, but not the CM of DCs treated with apoptotic cells, increases secretion of IL17 by mitogen-stimulated T cells

Mitogenic stimulation of splenocytes from this stage in the project was limited to a single dose of 5μg/ml conA only, for conservation of resources (mice, media, etc). This concentration of conA produced the most significant differences in IFNγ between different DC treatments thus far in the study. Splenocytes cultured in CM from untreated DCs secreted 270pg/ml IL17 in response to 5μg/ml conA [Fig. 4.9]. This concentration was enhanced by culturing splenocytes in CM from DCs that had been incubated with NCs, increasing IL17 secretion significantly to 580pg/ml, whereas CM from DC incubated with ACs caused an insignificant increase to 300pg/ml [Fig. 4.9].

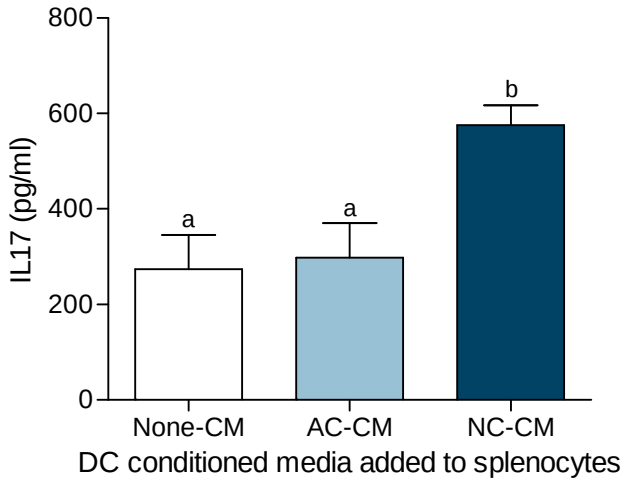


Figure 4.9 The CM of DCs treated with necrotic cells, but not the CM of DCs treated with apoptotic cells, increases secretion of IL17 by mitogen-stimulated T cells. Incubating DCs with necrotic cells generated CM (NC-CM) that induced significantly higher IL17 production by conA-stimulated T cells than was induced by the CM of untreated DCs (None-CM) or apoptotic cell-treated DCs (AC-CM). 'a' and 'b' signify significantly different means, one-way ANOVA. Data is from 3 separate experiments or more, bars indicate SD.

4.2.10 Pre-treatment of DC with apoptotic cells, but not pre-treatment with necrotic cells, suppresses the capacity of CpG-treated DC CM to enhance IL17 secretion by mitogen-stimulated T cells

The CM of CpG-treated DCs (None-CM + CpG) significantly enhances IL17 secretion by 5μg/ml conA-stimulated T cells to 1020pg/ml, compared to the 270pg/ml induced by the CM of untreated DCs (None-CM) ($p<0.01$) [Fig. 4.10]. Pre-treatment of CpG-treated DCs with ACs completely suppressed this enhancement, such that IL17 secretion by conA-stimulated T cells was limited to 260pg/ml ($p<0.01$), which is similar to the measure of IL17 secreted after incubation with the CM of untreated (that is, no CpG or dead cells) DCs ($p>0.05$). Pre-treatment of CpG-treated DCs with NCs had no statistical effect on stimulation of IL17 secretion.

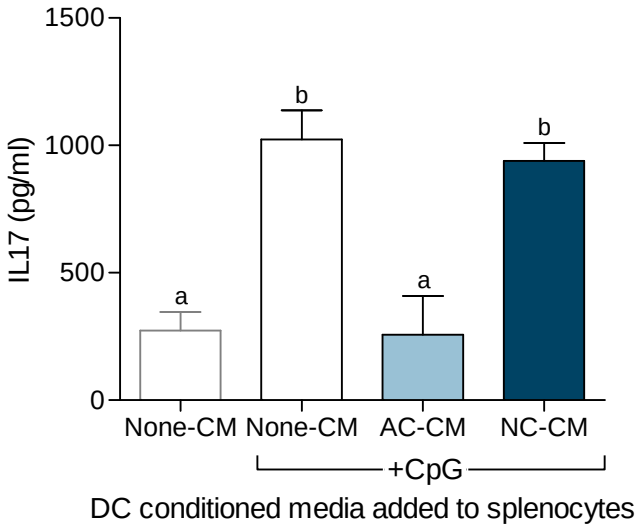


Figure 4.10 Pre-treatment of DC with apoptotic cells, but not pre-treatment with necrotic cells, suppressed the capacity of CpG-treated DC CM to enhanced IL17 secretion by mitogen-stimulated T cells. Using the CM of DCs treated with CpG ('None-CM + CpG') to culture splenocytes significantly enhanced T cell secretion of IL17 in response to ConA ('None-CM'). Pre-treating CpG-treated DCs with apoptotic cells ('AC-CM') significantly suppressed CpG-treated DC capacity to enhance IL17 secretion by conA-stimulated T cells ('None-CM + CpG'). Pre-treating with necrotic cells did not affect the capacity to enhance IL17 secretion. 'a' and 'b' signify significantly different means, one-way ANOVA. Data is from 3 separate experiments or more, bars indicate SD.

4.2.11 DCs treated with apoptotic cells, but not DCs treated with necrotic cells, suppress IL2 secretion by mitogen-stimulated T cells

5μg/ml conA-stimulated T cells cultured in the CM of untreated DCs secreted 530pg/ml IL2. Pre-treatment of the DCs with ACs caused IL2 secretion to be significantly less, at 240pg/ml ($p<0.05$). Pre-treatment of DCs with NCs induced an increase in IL2 secretion to 610pg/ml, which was not significant [Fig. 4.11A].

CpG-treatment of DCs enhanced DC induction of IL2 secretion by conA-stimulated T cells from 530pg/ml to 1500pg/ml [Fig. 4.11B] ($p<0.05$). This enhancement is decreased by pre-treating DCs with ACs, but not significantly, to 930pg/ml. The enhancement is increased by pre-treating DCs with NCs, but again not significantly, to 2060pg/ml. Pre-treatment with NCs results in significantly higher induction of IL2 secretion than does pre-treatment with ACs ($p<0.05$).

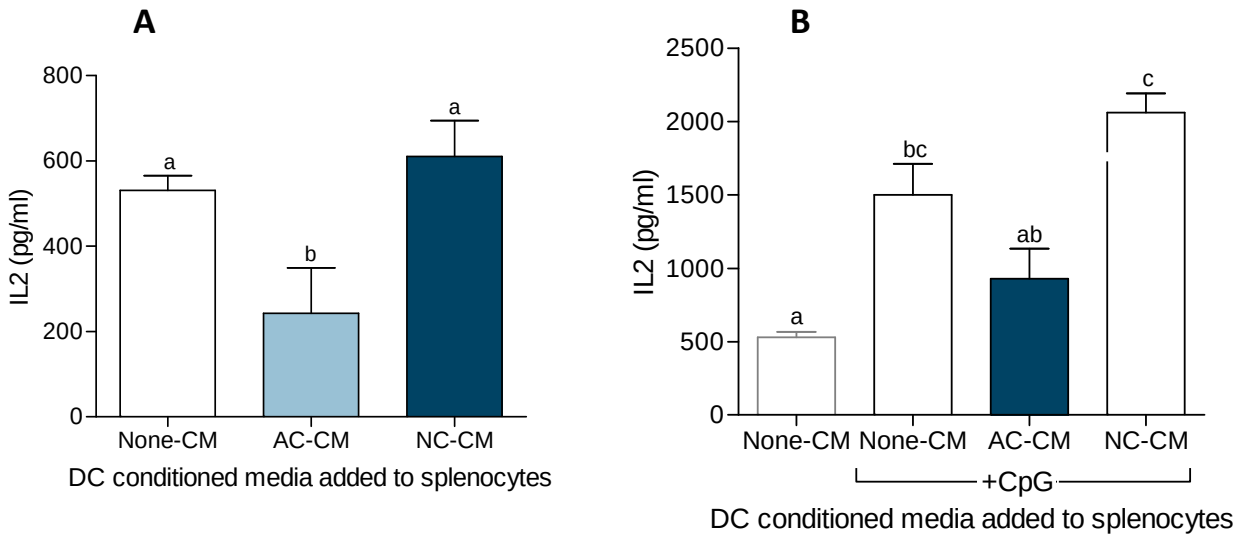


Figure 4.11 A. The CM of DCs treated with apoptotic cells, but not the CM of DCs treated with necrotic cells, inhibits secretion of IL2 by mitogen-stimulated T cells. Incubating DCs with apoptotic cells generated CM (AC-CM) that induced significantly lower IL2 production by conA-stimulated T cells than was induced by the CM of untreated DCs (None-CM) or necrotic cell-treated DCs (NC-CM). 'a' and 'b' signify significantly different means, one-way ANOVA. **B.** Using the CM of DCs treated with CpG ('None-CM + CpG') to culture splenocytes significantly enhances T cell secretion of IL2 in response to ConA (compared to 'None-CM'). Pre-treating CpG-treated DCs with apoptotic cells ('AC-CM') suppresses CpG-treated DC capacity to enhance IL2 secretion by conA-stimulated T cells ('None-CM + CpG'), but not significantly. Pre-treating with necrotic cells does not affect the capacity to enhance IL2 secretion. 'a', 'b' and 'c' signify significantly different means, one-way ANOVA. Data is from 3 separate experiments or more, bars indicate SD.

4.2.12 DCs treated with apoptotic cells, but not DCs treated with necrotic cells, suppress proliferation of mitogen-stimulated T cells

We determined the ability of variously treated DCs to induce immune responses, by using the CM to culture splenocytes with 0-10μg/ml conA and measuring the T cell proliferation. Proliferation was measured by incubating cells with WST-1 and measuring population-correlated absorbance with a spectrophotometer. WST-1 is a tetrazolium salt cleaved by mitochondrial dehydrogenases into a formazan dye: the greater the number of cells, the greater the mitochondrial metabolic activity and the more dye that is formed. Splenocytes cultured in the CM of untreated DCs demonstrate a significant dose-response curve to conA (two-way ANOVA, $p<0.001$). CM from DCs that have been treated with NCs significantly enhances the T cell proliferation curve in response to conA ($p<0.001$, two-way ANOVA) [Fig.4.12], consistent with data above that NC-treated DCs enhance T cell secretion of IL2 [Fig. 4.11]. CM from DCs that have been treated with ACs significantly suppress the T cell proliferation curve in response to conA ($p<0.001$, two-way ANOVA). NC- and AC-treatment of DCs have significantly different effects on the T cell response to conA ($p<0.05$, two-way ANOVA).

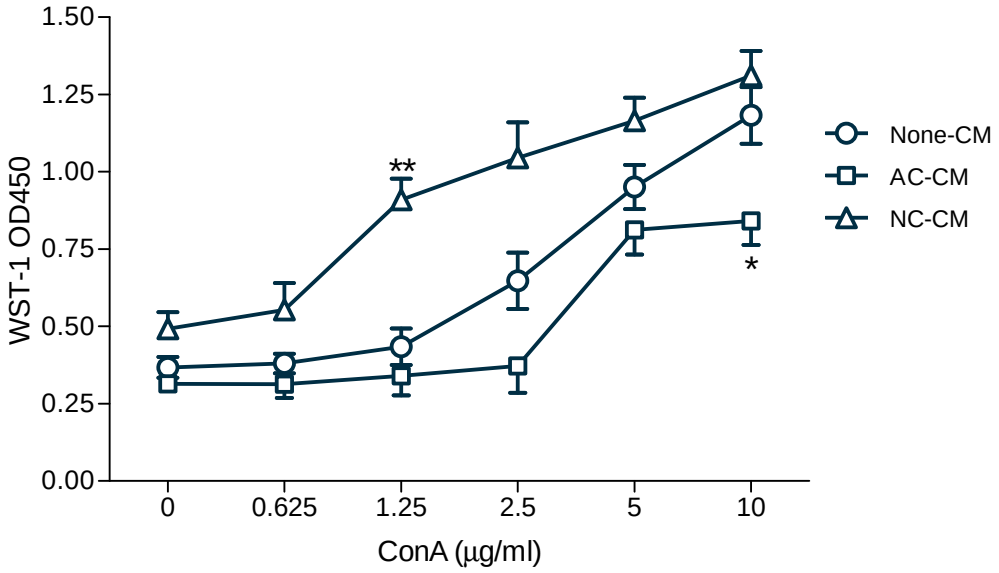


Figure 4.12 DCs treated with apoptotic cells, but not DCs treated with necrotic cells, suppress proliferation of mitogen-stimulated T cells. The CM of necrotic cell-treated DCs (NC-CM) significantly enhances proliferation of T cells in response to conA compared to the CM of untreated DCs (None-CM) ($p<0.001$, two-way ANOVA). The CM of apoptotic cell-treated DCs (AC-CM) suppresses proliferation of T cells in response to conA compared to the CM of untreated DCs (None-CM) ($p<0.001$, two-way ANOVA). * = $p<0.05$, ** = $p<0.01$, referring to significant difference between the indicated and the corresponding None-CM value, one-way ANOVA. Data is from 3 separate experiments or more, bars indicate SD.

4.2.13 Apoptotic cells enhance, and necrotic cells abrogate, DC ability to induce generation of regulatory T cells

One of the key mechanisms for the induction and maintenance of tolerance is the generation of regulatory T cells (Tregs). We examined how ACs and NCs influenced DC capacity to induce Treg differentiation from splenocytes. Splenocytes were treated for 5 days with 5μg/ml conA and various concentrations of CM from untreated and dead cell-treated DCs, and were supplemented on d3 with IL2. Splenocytes were then stained for CD25, CD4 and Foxp3, of which the expression of all three is indicative of a Treg. The CM of untreated DCs induced significant generation of Tregs in correlation to the concentration of the CM, with a maximum Treg count of 15.3% of the total cell population [Fig. 4.13]. Pre-treatment of the DCs with ACs significantly enhanced generation of Tregs ($p<0.001$, two-way ANOVA), with observable differences from as little as 1/32 of the culture media being CM. Pre-treatment with ACs did not significantly affect the maximum Treg population (16.7%) but decreased the concentration of CM at which the maximum, and other percentages, of the Treg population was induced. Pre-treatment of the DCs with NCs significantly abrogated generation of Tregs ($p<0.01$, two-way ANOVA), such that the maximum Treg population induced was 9.6% of the total cell population. Differences between untreated DC CM and NC-treated DC CM were only observable from 1/4 of the culture media being CM and upwards. Interestingly, for all treatments, the percentage Treg population began to plateau or decline from 50% (1/2) CM upwards, most notably in the NC treatment.

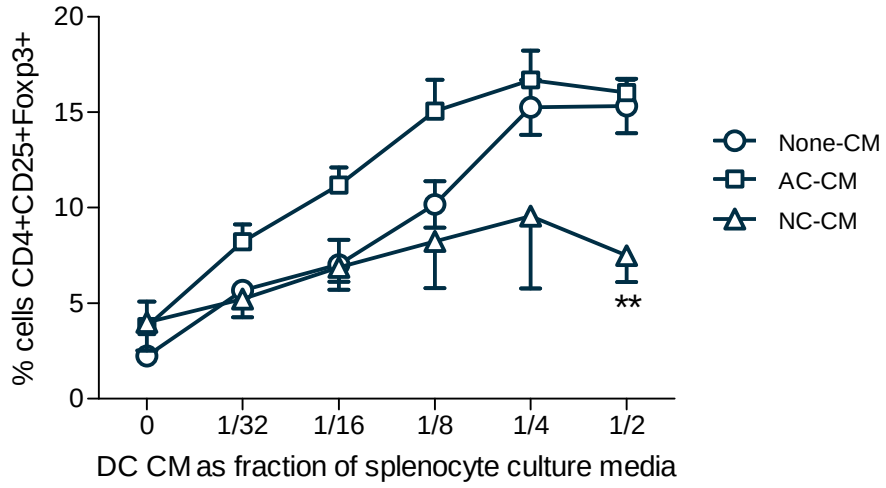


Figure 4.13 Apoptotic cells enhance, and necrotic cells abrogate, DC ability to induce generation of regulatory T cells. Splenocytes were incubated for 5 days with various concentrations of the CM from untreated DCs ('None-CM'), apoptotic cell-treated DCs ('AC-CM') or necrotic cell-treated DCs ('NC-CM'). AC-CM induced generation of significantly more Tregs than did None-CM ($p<0.001$, two-way ANOVA). NC-CM induced significantly fewer Tregs than did None-CM ($p<0.01$, two-way ANOVA). ** = $p<0.01$, referring to significant difference between the indicated and the corresponding None-CM value, one-way ANOVA. Data is from 3 separate experiments, bars indicate SD.

4.2.14 Apoptotic cells partially prevent the CpG-induced reduction of DC capacity to induce generation of regulatory T cells

The CM of DCs that have been treated with CpG [Fig. 4.14] generated significantly less Tregs, than did the CM of untreated DCs [Fig. 4.13] ($p<0.001$, two-way ANOVA), producing a maximum Treg population of just 2.6% of the total cell population. However, pre-treatment with ACs slightly abrogated this reduction by enabling CpG-treated DCs to produce CM that induces significantly more Tregs than the CM from DCs treated with CpG alone ($p<0.01$, two-way ANOVA) [Fig. 4.14], including a maximum of 6.5%. This amount was, however, still significantly lower than the generation of Tregs by DCs treated with ACs alone [Fig. 4.13] ($p<0.001$, two-way ANOVA). Pre-treatment with NCs did not affect the generation of Tregs induced by the CM of CpG-treated DC ($p>0.05$, two-way ANOVA).

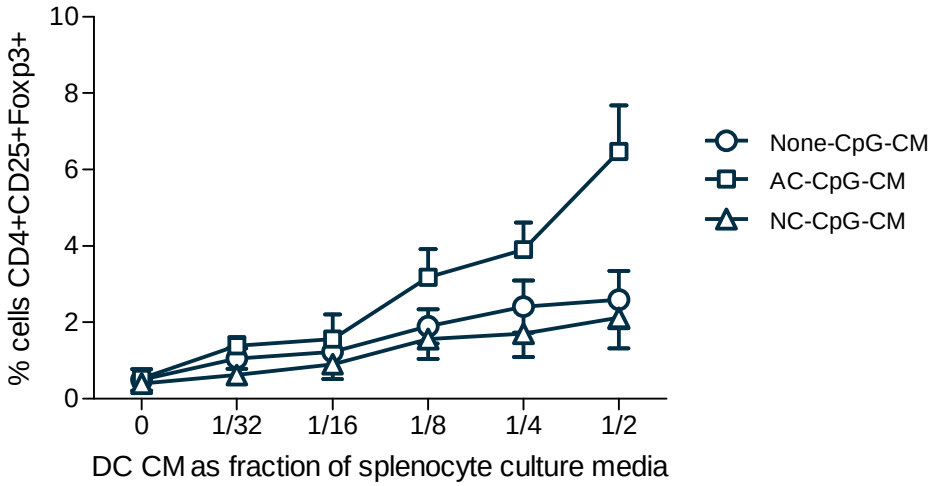


Figure 4.14 Apoptotic cells partially prevent the CpG-induced reduction of DC capacity to induce generation of regulatory T cells. Splenocytes were incubated for 5 days with various concentrations of the CM from CpG-treated DCs ('None-CpG-CM'), or CpG-treated DCs that had been pre-incubated with apoptotic cells ('AC-CpG-CM') or pre-incubated with necrotic cells ('NC-CpG-CM'). None-CpG-CM induced generation of significantly less Tregs than the CM of untreated DCs (None-CM of Fig. 4.13) ($p<0.001$, two-way ANOVA). Pre-treating DCs with ACs slightly inhibited this decrease, as AC-CpG-CM induced the generation of significantly more Tregs than did None-CpG-CM ($p<0.001$, two-way ANOVA). Pre-treating DCs with NCs slightly enhanced this decrease, as NC-CpG-CM induced significantly fewer Tregs than did None-CpG-CM ($p<0.01$, two-way ANOVA). Data is from 3 separate experiments, bars indicate SD.

4.3 Discussion

4.3.1 The apoptotic-cell induced cytokine profile in resting and activated DCs

It is important to note that it is only *secretion* of cytokines that is represented in these results – the assays employed here detect cytokines released into the culture media, and as such make no distinction between cytokines released from pre-formed vesicles and cytokines arising from *de novo* synthesis. Apoptotic cells had no significant effect on secretion of the proinflammatory cytokines IL12p40 or IL6 by resting DCs. ACs significantly increased secretion of the anti-inflammatory cytokines IL10 (almost 8-fold) and increased TGFβ (more than two-fold).

These *in vitro* results correlate well with *in/ex vivo* cytokine measurements by Kushwah et al (2009). They examined the cytokine positivity of DCs taken from lymph nodes local to recent injections of apoptotic cells or necrotic cells. It was found that apoptotic cells did not induce IL12 in DCs (5% of DCs were IL12-positive compared to 30-35% after LPS injections). Contrary to the current findings, necrotic cells also induced no increase in IL12. However, apoptotic cells, but not necrotic cells, induced an increase of TGFβ (198), similar to the findings of the current study.

Apoptotic cells suppressed LPS-induced L12 and IL6 secretion in this investigation, consistent with the effect observed by Williams et al (2008), where apoptotic cells also suppressed TNFα and DC-produced IFNγ (93), indicating that apoptotic cells in the current study may also suppress a wider range of proinflammatory cytokines than just the two examined. DC-produced IFNγ was not studied here, but is implicated in DC expression of IDO, in turn implicated in $CD4^+$ T cell suppression (93). Curiously, though, IL6 has also been acknowledged in a tolerogenic role (247), but this is not supported by the evidence from the current study. The inhibition of IL12 secretion in response to CpG echoes, but is not as dramatic as, landmark findings by Stuart et al (2002)

whereby ACs completely inhibited the IL12 response to substantial doses of LPS (14). This partial abrogation of IL12 secretion is consistent with (and may be due to) earlier findings of the current investigation where up-regulation of some, but not all, co-stimulatory molecules in response to CpG were inhibited.

IL10 secretion after LPS treatment in the current study is similar to that induced by similar concentrations of LPS in other studies e.g. (231) (anti-inflammatory cytokines are induced by co-inflammatory stimuli, as essential mediators of resolution, like co-inhibitory molecules). The apoptotic cell-mediated suppression of IL12 production was observed in wildtype and $IL10^{-/-}$ mice, demonstrating that IL10 is not essential for apoptotic cell suppression of IL12 production. IL10 may indeed cause suppression of DC in this system, either directly on IL12 or otherwise, but this data suggests that if this is a mechanism of suppression it is a redundant one. In this case, TGFβ may be a more important cytokine for tolerance, though some reports indicate that neither IL10 nor TGFβ are required for apoptotic cell-mediated suppression of IL12 production (14). However, this is in contrast with growing evidence that IL10 is pivotal in apoptotic cell-mediated immune suppression (248). Clearly, this requires further investigation.

4.3.2 The apoptotic cell influence on the DC mediation of T cell function

As discussed, the cytokine profile, and the changes in the CpG-induced cytokine profile, induced in DCs by apoptotic cells, is consistent with an anti-inflammatory role and consistent with other studies that use non-tumourous apoptotic cells generated with minimal cell stress. This profile was also consistent with the downstream effects, such as suppressed production of the key Th1 cytokine IFNγ by T cells treated with the conditioned media (CM) of DCs treated with apoptotic cells, compared to production by T cells treated with the CM of untreated DCs. However, apoptotic cell treatment did not suppress the capacity of the CM of LPS-treated DCs to induce

IFNγ production by T cells. This may be due to there still being sufficient concentrations of IL12 to allow T cell activation, or connected to the possibility of IL12-independent activation of T cells as executed by some DC subsets (249).This is consistent with reports that IL12 is not solely responsible for activation of T cells. It is regrettable that there was no time for a thorough investigation of dead cell effects on LPS-induced maturation, including determining the effect of apoptotic cells on cytokine production by LPS-stimulated DCs, in order to compare the findings with those of others. The lack of apoptotic cell-mediated suppression of LPS-enhanced IFNγ production is yet reconcilable with reports of apoptotic cell-mediated suppression of LPS-induced immunity, as IFNγ can elicit the production of IDO (250). Indeed, suppression of LPS-induced immune responses in vitro by apoptotic cells has been demonstrated to occur via an IFNγ-IDO-dependent mechanism (93).

Apoptotic cells did not suppress the capacity of DCs to induce IL17 production by T cells, though nor did they increase IL17 production from the baseline level induced by untreated DCs. However, apoptotic cells induced a complete abrogation of the ability of CpG-treated DCs to induce IL17 production by T cells. This is consistent with suppression of DC IL6 production (IL6 in combination with TGFβ induces Th17 (251)) and suggests that apoptotic cells are efficient at suppressing CpG-induced Th17 immune responses.

Interestingly, T cell assays in this investigation required CM, rather than modified (but possibly no longer actively secretive) DCs themselves. This suggests that the cytokine milieu induced by dead cells is necessary and sufficient, without the presence of directly dead cell-influenced DCs, to modify downstream cellular responses. Whether or not this is sufficient to influence an autoimmune response *in vivo* cannot be answered here, though injections of CM in the autoimmune diabetes model described in this study would provide answers – could CpG-treated, gp33-loaded DCs be prevented from inducing diabetes by administering them simultaneously with

media conditioned by apoptotic cell-treated DCs? This presents implications for both the initiation – and treatment – of autoimmune disorders.

Certainly, targeting cytokines alone is sufficient to allay the symptoms of autoimmune disorders. Current biologics in development for the treatment of rheumatoid arthritis include the JAK (Janus kinase) inhibitors, which specifically inhibit the production of TNFα, IL2 and IL6 among other cytokines. It is not yet known if this novel class of treatment is disease-modifying, but Phase 3 clinical trials have shown great success in improving symptoms of the disease, such that clinical studies have also moved into exploring the benefits of the drugs in other indications, including psoriatic arthritis, psoriasis, and kidney allograft rejection. The question remains – could administration of AC-induced DC cytokines effectively suppress autoimmune inflammation? The evidence provided in this study supports the notion that it could. Could such treatment exact a sustained reduction in inflammation? It is unlikely, if not administered at the time where autoimmunity began, in order to prevent the initiation of autoimmunity at the outset, as in our murine model (See Chapter 5). Of course, this is of little practical use – immunosuppressive cytokine therapy would have too great a risk of adverse effects (for example, opportunistic infections) to be of any use as a constant prophylactic. However, the slight reduction of CpG-mediated inhibition of Treg induction suggests that induction of Tregs by AC-treated DC cytokines may prevail even within the throes of inflammation. Indeed, such mechanisms are important for the resolution of inflammation throughout and subsequent to immune responses. If such a mechanism can have a sufficiently deleterious effect on effector T cells, then there may exist the opportunity to maintain tolerance and induce remission in autoimmune disease.

4.3.3 The necrotic cell-induced cytokine profile and its effect on T cell function

Necrotic cells induced significant levels of IL12 and IL6, such that each equated to as much as half of the corresponding CpG-induced levels. Furthermore, necrotic cells enhanced the CpG-induced secretion of IL12. Also, unlike apoptotic cells, no IL10 or TGFβ was induced. This is highly suggestive of a proinflammatory response. Although the increases in proinflammatory cytokines are smaller than those induced by CpG, they nonetheless translated into enhanced secretion of IFNγ by T cells. In fact, the CM of necrotic cell-treated DCs induced IFNγ production that is significantly greater than that induced by the CM of CpG- or LPS-treated cells. Curiously, the enhancement by necrotic cells of CpG-induced DC IL12 production does not equate to enhancement of IFNγ production by T cells. IFNγ secretion confirms that necrotic cells induce inflammation, as IFNγ is a key cytokine of the Th1 immune response.

Necrotic cell-treated DC CM enhanced IL17 production by T cells. This provides an interesting connection between the association of autoimmunity with both IL17 and secondary necrosis-induced inflammation in autoimmune disorders such as SLE. Curiously, however, these results do not support findings that IFNγ suppresses IL17 and Th17 differentiation (252).

4.3.4 The effects of apoptotic cells and necrotic cells on induction of the generation of regulatory T cells

Untreated DC CM induces dose-dependent generation of Tregs, therefore immature DC appear to constitutively promote tolerance. This has also been reported elsewhere in the constitutive generation of $CD8^+$ T cells (253). Apoptotic cell-treatment enhanced the induction of Tregs, which, considered with the DC cytokine profile induced by apoptotic cells, is consistent with the

conventional understanding of Treg development by means of IL10 and TGFβ from DCs (91). Further, Tregs are well established as mediators of tolerance and prevention of autoimmunity, indicating that apoptotic cells have an active role in the maintenance of peripheral tolerance. This matches recent similar reports (198).

CpG treatment of DCs abolished their ability to induce Tregs, consistent with the cytokine profile of CpG-treated DCs. Interestingly, CpG treatment of human plasmacytoid DCs has been reported to actually promote generation of $CD4^+CD25^+Foxp3^+$ Treg (254). Apoptotic cell-treatment of DCs prior to CpG-treatment helped to partially prevent reduction of Tregs, demonstrating an ability of apoptotic cells to protect against the effects of subsequently encountered pro-inflammatory mediators. This in turn suggests that the effect of apoptotic cells persists and is powerful enough to limit the inflammatory effects of as-yet-unphagocytosed apoptotic cells that progress to secondary necrosis.

Because the gp33 peptide is only 9 amino acids in length, it cannot bind to, and therefore cannot be presented on, MHCII. Therefore, gp33 can only be presented on MHCI to $CD8^+$ T cells, and so the $CD4^+$ Tregs produced in the assay described are polyclonal and not gp33-specific. Jonuleit et al (2000) induced $CD4^+CD25^+$ Tregs by repeatedly stimulating $CD4^+$ T cells (from human cord blood) with immature DCs (255). These Tregs were capable of suppressing Th1 cells in an antigen-specific manner. In addition, this was as a result of direct Treg-Th1 cell-cell contact, independent of IL-10 or DC-Th1 contact. It is possible to speculate that the $CD4^+$ Tregs in this study are behaving similarly *in vitro*, and could have a similar influence in the *in vivo* setting of the diabetes model described later.

4.3.5 Summary

Apoptotic cells induce secretion of the anti-inflammatory cytokines IL10 and TGFβ by DCs. Apoptotic cells also have the power to partially suppress the secretion of inflammatory cytokines secreted in response to CpG. However, IL10 may be redundant in this suppression. This is consistent with reports of a non-exclusive role for IL10 in suppression of autoimmunity in a murine model of EAE (191). We may speculate that IL10 and TGFβ act synergistically in the suppression of DC, and that TGFβ may be a non-redundant mechanism. It appears likely that IL10 plays a very minor role in the instigation of apoptotic cell-mediated suppression. However, IL10 may still be important in the longer term inhibition of immune responses. These effects of apoptotic cells describe the conditions necessary for the increased induction of $CD4^+CD25^+Foxp3^+$ Tregs by apoptotic cell-treated DCs, and explain the ability of apoptotic cells to partially prevent the reduced Treg induction by DCs that is caused by CpG.

A more extensive examination of cytokine production by apoptotic cell- and necrotic cell-treated DCs was regrettably not undertaken due to time and resource constraints, but such an extension is much required for a thorough understanding of the effects of dead cells on DCs. Additionally to IL12p40, IL6, IL10 and TGFβ, it is highly recommended that production/secretion of the cytokines IL1α, IL1β, TNFα, IL23 and prostaglandins are assessed, in order to fully ascertain the mechanisms of possible AC-induced tolerance, NC-induced inflammation, and any other peripheral events that may be incurred as a result. It would also be interesting to examine more Th2 cytokines (IL10 can promote Th2 immunity, as well suppress Th1 immunity) and see if humoral immunity is at all affected, to complement the surmounting information acquired regarding Th1 and Th17 immunity: IL2 and IFNγ are consistent with the Th1 cytokine profile that mediates cell-mediated immunity, and Th17 is central to the Th17 immune response, therefore, the results are indicative of apoptotic cells ability to suppress Th1 and Th17 immune responses.

Cytokine immunology is complicated by the apparent dual roles of many cytokines. In this respect, the current findings can support or contrast with various reports of downstream events. This investigation demonstrates anti-inflammatory effects of apoptotic cells on DCs, associated with increased IL10 and TGFβ secretion, and reduced IL12. Consequently, apoptotic cells reduce the capacity of DCs to elicit Th1 responses, as corroborated by reduced T cell production of IFNγ and IL2, and reduce the capacity to elicit Th17 responses as seen in reduced IL17 production. Overall T cell proliferation induced by conA is also reduced, indicating an inhibitory capacity of apoptotic cell-treated DCs, whilst necrotic cells enhance.

CHAPTER 5 – The effect of apoptotic cells and necrotic cells on the adaptive immune response *in vivo*

5.1 Introduction

Dendritic cell expression of co-stimulatory and co-inhibitory receptor ligands, production and inhibition of cytokines, and interaction with T cells, all culminate *in vivo* in what is dichotomised into either the initiation of an adaptive immune response or the induction of tolerance. Apoptotic and necrotic cells have been associated in various ways to immune tolerance and autoimmunity. To fully examine the effect of dead cells on DCs, therefore, it was important to determine their influence on DC capacity to generate an autoimmune response in vivo. To this end, RIP-GP transgenic mice (first described by Ohashi et el (3)) were bred in order to adapt an established model of inducible antigen-specific autoimmune diabetes. RIP-GP mice contain the gene for the lymphocytic choriomeningitis virus (LCMV) glycoprotein under the control of the rat insulin promoter (RIP). These mice express the glycoprotein (GP) on the insulin-producing islet β cells of the pancreas. Thus, a GP-specific immune response causes destruction of the islet β cells and results in diabetes.

Here, a new model of inducing such an immune response and inducing diabetes in RIP-GP mice is described. This model is novel in the use of CpG to activate DCs that are then pulsed with gp33 peptide – the immunodominant MHCI epitope (33-41) of the LCMV GP [Fig. 5.1] – before being administered according to a specific schedule [Figure 5.2]. Our results showed that DCs that are treated in this way and then injected into RIP-GP mice induce an autoimmune reaction that results in diabetes. Finally, these DCs were pre-treated with apoptotic and necrotic cells (after dead cell-

DC coincubation DCs were isolated from non-phagocytosed dead cells by using CD11c-specific magnetic beads for MACS) in order to investigate the effect of dead cells on DC-induced autoimmunity. We hypothesised that the apoptotic cell-mediated suppression of CpG-induced DC maturation, as observed in the *in vitro* assessment of surface phenotype and cytokine profile, would elicit gp33-specific tolerance and inhibit the onset of autoimmune diabetes [Fig. 5.3]

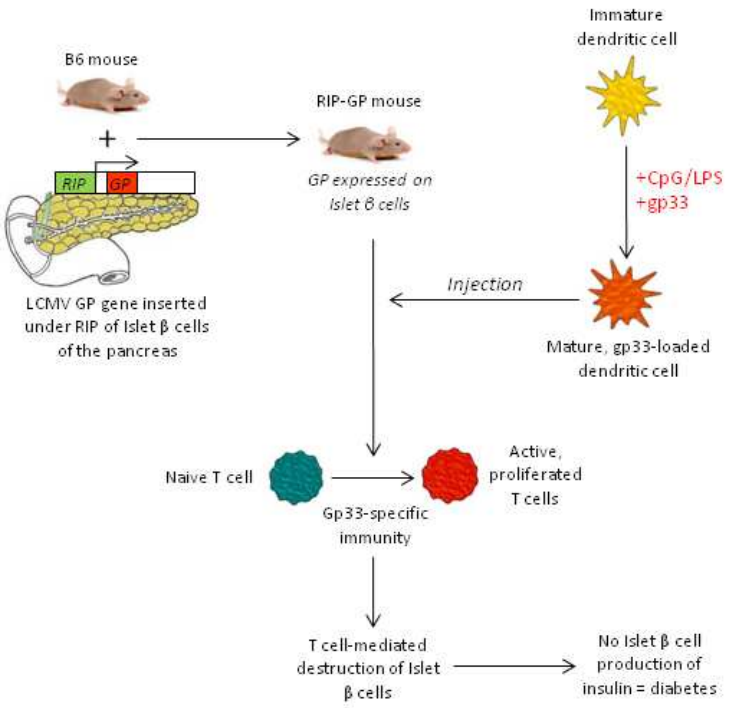


Figure 5.1 Proposed RIP-GP model of DC-induced diabetes. RIP-GP mice are from a C57Bl/6J (B6) background, with the LCMV glycoprotein gene expressed under the control of the rat insulin promoter gene, driving expression of the glycoprotein on insulin-producing Islet β cells. Mature (CpG/LPS-activated) DCs that are pulsed with gp33 (the immunodominant epitope of the LCMV glycoprotein) are injected into RIP-GP mice. These DCs activate any T cells that recognise gp33, which proliferate and execute gp33-specific immunity by destroying the gp33-expressing Islet β cells, resulting in diabetes.

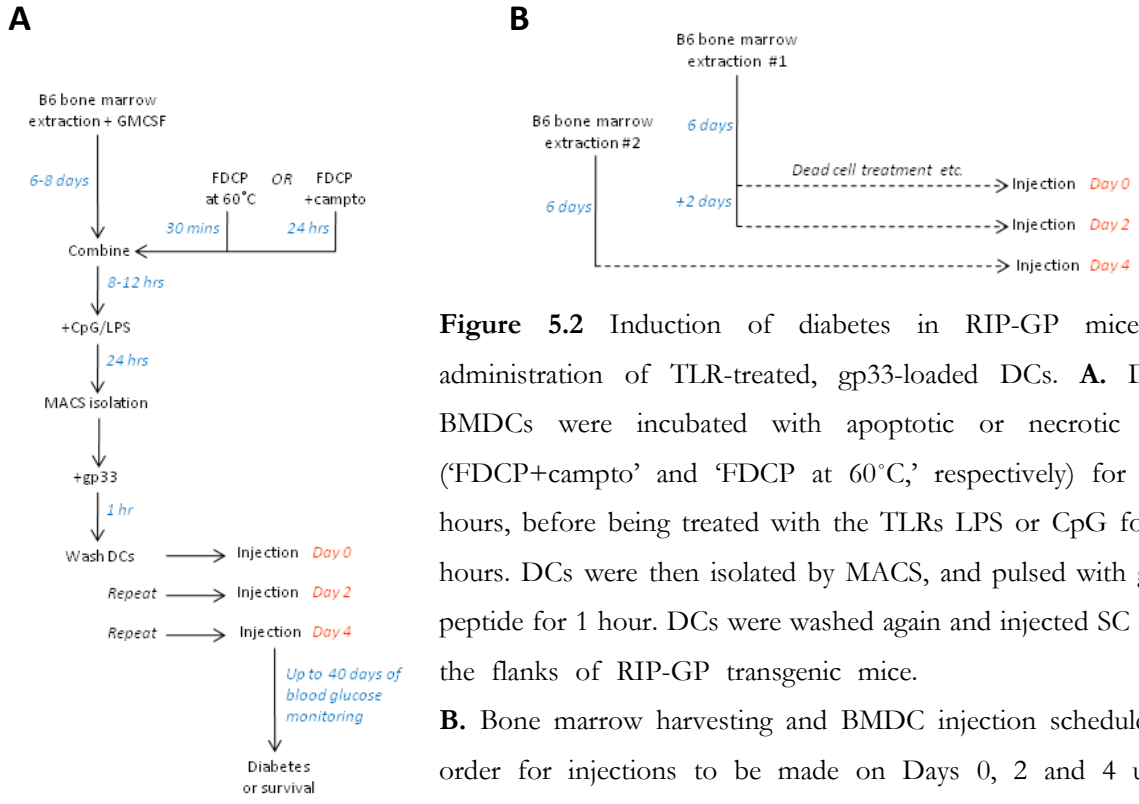


Figure 5.2 Induction of diabetes in RIP-GP mice by administration of TLR-treated, gp33-loaded DCs. **A.** D6/8 BMDCs were incubated with apoptotic or necrotic cells ('FDCP+campto' and 'FDCP at 60°C,' respectively) for 8-12 hours, before being treated with the TLRs LPS or CpG for 24 hours. DCs were then isolated by MACS, and pulsed with gp33 peptide for 1 hour. DCs were washed again and injected SC in to the flanks of RIP-GP transgenic mice.

B. Bone marrow harvesting and BMDC injection schedule: In order for injections to be made on Days 0, 2 and 4 using appropriately aged (hence differentiated) BMDCs, bone marrow was harvested from two separate mice 4 days apart, such that BMDCs injected on Day 0 were 8 days old (6 days culture, two days of treatments), those on Day 2 were 10 days old (8 days culture, 2 days of treatments), and those on Day 4 were 8 days old (second BM harvest, 6 days of culture, 2 days of treatment).

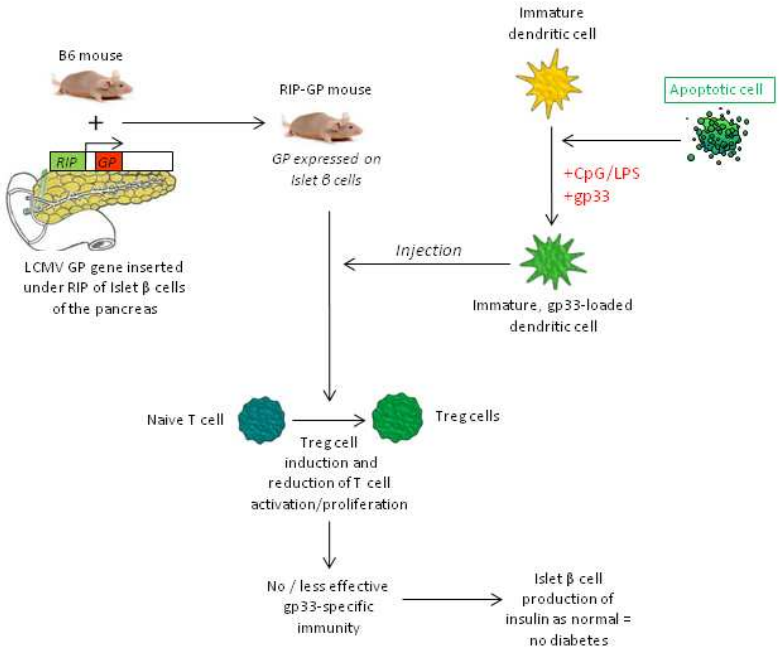


Figure 5.3 Hypothesised model of apoptotic cell-mediated suppression of DC-induced diabetes in the RIP-GP model. Pre-treating DCs with apoptotic cells inhibits CpG-induced maturation, such that gp33 is presented to T cells by immature, tolerogenic DCs. Gp33-specific T cells are induced to undergo deletion, anergy or conversion to Tregs. Gp33-specific tolerance ensues, ensuring that Islet β cells are safe from immune destruction and no diabetes is induced.

5.2 Results

5.2.1 Self-antigen- and maturation-dependent DC immunisation induces diabetes in RIP-GP transgenic mice

DCs were treated with CpG/LPS/neither, then pulsed with gp33 peptide. They were then injected subcutaneously into RIP-GP mice on day 0, day 2 and day 4, at 2-5x10^6 DCs per injection [Figs. 5.1 and 5.2]. Blood glucose levels of the mice were measured one day prior to the first injection then three times a week until the end of the experiment or death, for indication of onset of diabetes. Diabetes was defined, as according to veterinary standards, as three consecutive measurements of blood glucose concentrations of 15mM or above.

Injections of DCs that had been treated with gp33 alone or CpG alone failed to induce diabetes as indicated by blood glucose concentrations of all mice in these groups remaining below 15mM throughout the experiment [Figs. 5.4A & Fig. 5.4B, respectively].

Injections of DCs that had been treated with both CpG and gp33 induced diabetes rapidly in five of six mice [Fig. 5.4C], which is a significant increase in diabetes incidence compared to control injections (no CpG treatment) ($p<0.05$). The earliest blood glucose measurement of >15mM occurred on Day 9, in one mouse, 5 days after the final injection. The first measurement of >15mM (1°15mM) occurred in mice most commonly on Day 11, whilst the third measurement of >15mM (3°15mM) (that is, confirmed diabetes) occurred most commonly on Day 16.

Other routes of administration were explored, and mice administered with CpG-treated, gp33-pulsed DCs by means of peritoneal injection also induced diabetes, in two of three mice [Fig. 5.4D]. 1°15mM and 3°15mM occurred on Day 7 and 11, respectively, for one mouse and Day 9 and 14, respectively, for the other.

Administration of only two SC injections of CpG-treated, gp33-pulsed DCs was not sufficient to induce diabetes in any mice [Fig. 5.4E]. Three injections of LPS-treated, gp33-pulsed DC induced diabetes in only one of three mice [Fig. 5.4F], where 1°15mM and 3°15mM occurred on Day 9 and 14, respectively. 1°15mM was achieved with a 'borderline' measurement of 15.3, therefore measurements were continued until a fourth reading of >15mM before euthanasia.

A regimen of three SC injections of CpG-matured, gp33-loaded DCs efficiently induces a high incidence of diabetes in RIP-GP mice. In this RIP-GP model of diabetes, it is a necessary condition for the DCs to be both mature and gp33-loaded, and to be administered on no less than three consecutive occasions. Diabetes can also be induced with LPS-matured, gp33-loaded DCs, though seemingly less effectively.

Figure 5.4 (Below) CpG-treated DCs that have been pulsed with gp33 peptide induce diabetes in RIP-GP mice. RIP-GP mice were injected (orange arrows) with variously-treated DCs and the blood glucose concentrations were recorded. Three consecutive readings of blood glucose above 15mM, or until the need for euthanasia, were considered indicative of diabetes. **A.** Three subcutaneous injections of gp33-pulsed DCs did not induce diabetes in any mice. **B.** Three subcutaneous injections of CpG-matured DCs did not induce diabetes in any mice. **C.** Three subcutaneous injections of CpG-matured, gp33-pulsed DCs induced diabetes in five of six mice. **D.** Three intraperitoneal injections of CpG-matured, gp-33 pulsed DCs induced diabetes in two of three mice. **E.** Two subcutaneous injections of CpG-matured, gp33-pulsed DCs did not induce diabetes. **F.** Three subcutaneous injections of LPS-matured, gp33-pulsed DCs induced diabetes in one of three mice.

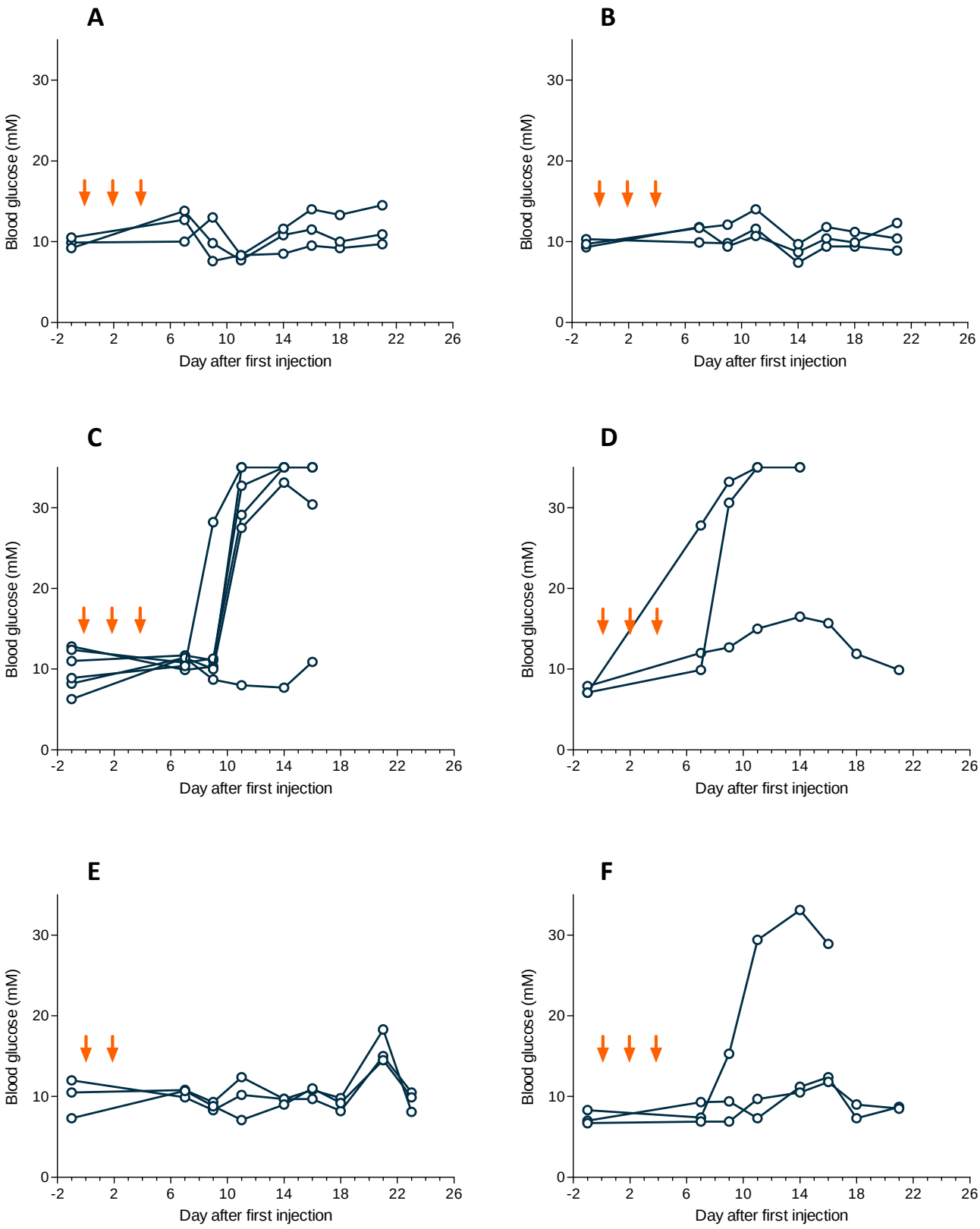


Figure 5.4 See p149.

5.2.2 Self-antigen- and maturation-dependent DC immunisation requires sufficient activation of DCs by CpG, in order to induce diabetes in RIP-GP transgenic mice

The minimum stimulation required to confer on self-antigen-loaded DCs a capacity to induce diabetes was determined, in order to maximise the possibility of suppression by ACs. As established in 5.2.1, a treatment of 1μg/ml CpG for 24hrs was sufficient to activate DCs enough that, after being pulsed with gp33 and injected according to the regimen described in 5.2.1, they induced diabetes in five of six mice [Fig. 5.4]. 100ng/ml CpG for 24 hours was tested next and found to also be sufficient to activate DCs enough that diabetes was induced in three of three mice [Fig. 5.5A]. 50ng/ml for 24 hours was sufficient for induction of diabetes in two of three mice [Fig. 5.5B]. 20ng/ml for 24 hours was insufficient for induction of diabetes in any of three mice [Fig. 5.5C].

Shorter CpG treatment times were then tested, where 1μg/ml and 100ng/ml for 8 hours were sufficient to cause diabetes in two of three mice each [Figs. 5.5D and 5.5E, respectively]. 8 hours of 50ng/ml CpG was sufficient to cause diabetes in just one of three mice [Fig. 5.5F]. 8 hours of 100ng/ml CpG was deemed close to minimum stimulation to infer diabetes-inducing capabilities upon DC, and was therefore the treatment used subsequently in all diabetes experiments.

Figure 5.5 (Below) Self-antigen and maturation-dependent DC immunisation requires a minimum activation of DCs, in order to induce diabetes in RIP-GP transgenic mice. RIP-GP mice were injected (orange arrows) with DCs that had been incubated with various CpG treatments then pulsed with gp33. Three consecutive readings of blood glucose above 15mM, or until the need for euthanasia, were considered indicative of diabetes. **A.** 100ng/ml CpG for 24 hours was sufficient to activate gp33-loaded DCs enough that diabetes was induced in three of three mice. **B.** 50ng/ml for 24 hours was sufficient DC-activation for induction of diabetes in two of three mice. **C.** 20ng/ml for 24 hours was insufficient DC-activation for induction of diabetes in any of three mice. **D.** 1μg/ml for 8 hours was sufficient DC-activation for induction of diabetes in two of three mice each. **E.** 100ng/ml for 8 hours was sufficient DC-activation for induction of diabetes in two of three mice. **F.** 8 hours of 50ng/ml CpG was sufficient DC-activation to cause diabetes in just one of three mice.

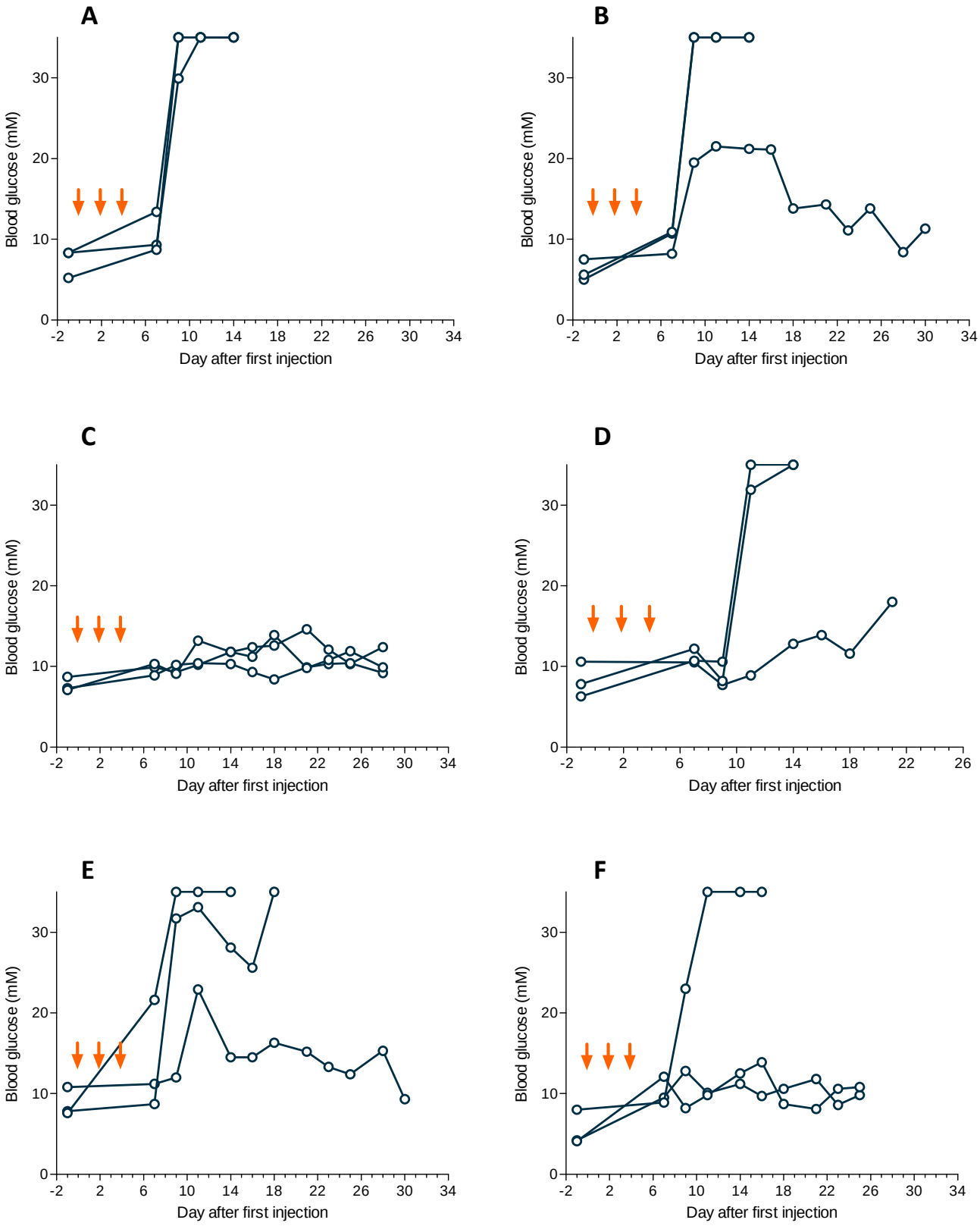


Figure 5.5 See p151.

5.2.3 CpG/gp33-DC immunisation causes an adaptive immune response that is localised to the pancreas

To confirm that CpG/gp33-DC immunisation induced diabetes via an adaptive immune response, DC and T cell populations of the pancreatic draining lymph nodes (PDLN), the inguinal lymph nodes (ILN) (for comparison) and the spleen (representing circulating T cells in the blood) were examined. The ILNs, PDLNs and spleens were removed on Day 10 (six days after final injection of DCs treated with 8hr 100ng/ml CpG and pulsed with gp33).

Diabetic mice (i.e. injected three times with DCs treated with 100μg/ml CpG for 24 hours, plus gp33 peptide) and pre-diabetic mice (injected similarly, but as demonstrated earlier, not all diabetic mice are symptomatic by Day 10), both referred to from herein as diabetic mice, were compared with control i.e. non-diabetic mice (mice injected with DCs that had been pulsed with gp33 alone). Diabetic mice had significantly more CD40-high, i.e. mature, DCs in the PDLN and spleen ($p<0.01$ and $p<0.05$, respectively) [Fig. 5.6C], compared with the non-diabetic mice. There was no significant difference between the ILN DC populations of each group. Diabetic mice had significantly more active CD4+ T cells than non-diabetic mice, as indicated by high expression of CD44, in the spleen ($p<0.05$) [Fig. 5.6A]. Diabetic mice also had significantly more active CD8+ T cells in the PDLN and spleen than non-diabetic mice ($p<0.05$ for both PDLN and spleen) [Fig. 5.6B]. There was no significant difference in populations of activated T cells in the ILNs of diabetic mice compared with the ILNs of non-diabetic mice. Together, these results indicate that gp33-loaded mature DCs initiate an adaptive immune response that localises to the pancreas.

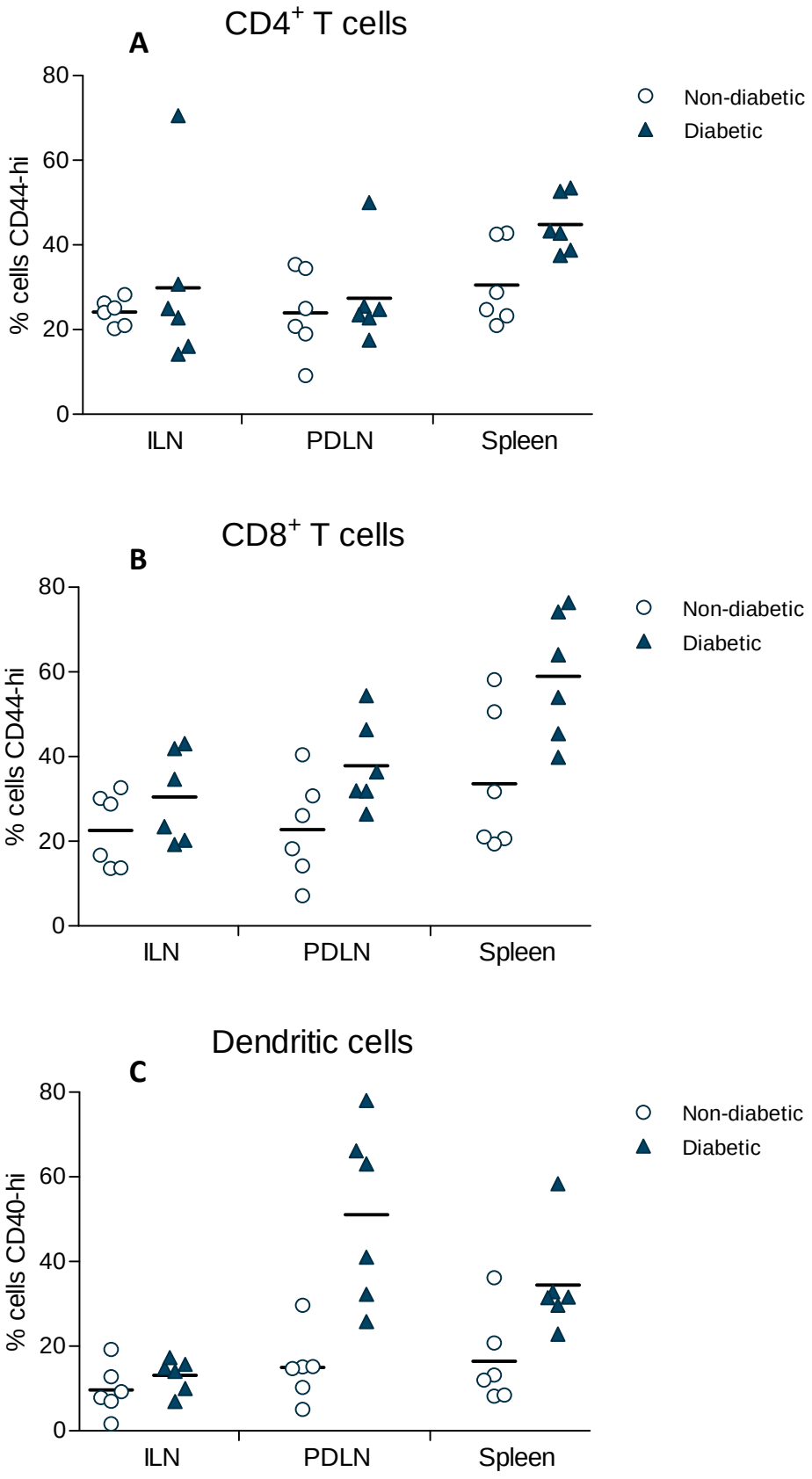


Figure 5.6 See p155.

Figure 5.6 (p154) CpG/gp33-DC immunisation causes a pancreas-specific adaptive immune response. Inguinal lymph nodes (ILN), pancreatic draining lymph nodes (PDLN) and spleens were removed on Day 10 from mice injected with gp33-loaded DCs (Non-diabetic) and mice injected with CpG-treated, gp33-loaded DCs (Diabetic), according to the regimen described in 5.2.1. **A.** Diabetic and non-diabetic mice have similarly low numbers of activated (CD44-hi) CD4+ T cells in the ILN. Both sets of mice have similarly low numbers of CD4+ T cells in the PDLN. Diabetic mice have significantly more activated CD4+ T cells in the spleen than do non-diabetic mice ($p<0.05$). **B.** Diabetic and non-diabetic mice have similarly low numbers of activated (CD44-hi) CD8+ T cells in the ILN. Diabetic mice have significantly more activated CD8+ T cells in the PDLN than do non-diabetic mice ($p<0.05$). Diabetic mice have significantly more activated CD8+ T cells in the spleen than do non-diabetic mice ($p<0.05$). **C.** Diabetic and non-diabetic mice have similarly low numbers of activated (CD40-hi) DCs in the ILN. Diabetic mice have significantly more activated DCs in the PDLN than do non-diabetic mice ($p<0.01$). Diabetic mice have significantly more activated DCs in the spleen than do non-diabetic mice ($p<0.05$). Significance by unpaired T test. Each open circle represents the average reading of triplicate samples of a single mouse, as does each closed triangle.

5.2.4 The capacity of CpG-activated, gp33-loaded DC to induce diabetes in RIP-GP mice is suppressed by apoptotic cells, and also (non-significantly) by necrotic cells

Once established, the CpG-RIP-GP model of diabetes was utilized to assess the effect of dead cells on DC function in the context of autoimmunity and tolerance *in vivo*. Diabetes-inducing DCs were treated with ACs or NCs then injected according to the regimen described in 5.2.1. When not pre-treated with dead cells, CpG-treated gp33-loaded (CpG-gp33) DCs induced diabetes in eight of nine mice (89% incidence) [Fig. 5.7A]. CpG-gp33 DCs that were pre-treated with ACs induced diabetes in just two of seven mice (29% incidence) [Fig. 5.7B], significantly less than DC that were not pre-treated ($p<0.05$). Surprisingly, pre-treatment of DCs with NCs also decreased diabetes induction, though not to a statistically significant extent, as these DCs induced diabetes in just three of six mice (50%) [Fig. 5.7C]. The incidence rate of mice injected with CpG-gp33 DCs is significantly different to the incidence rate of mice injected with AC-pre-treated CpG-gp33 DCs [Fig. 5.7D] ($p<0.05$, log-rank (Mantel-Cox) test), whereas the incidence rate of mice injected with NC-pre-treated CpG-gp33 DCs is not significantly different to either rate.

Figure 5.7 (p157) The capacity of CpG-activated, gp33-loaded DC to induce diabetes in RIP-GP mice is suppressed by apoptotic cells, and also (non-significantly) by necrotic cells. Mice were injected three times (Days 0, 2 and 4, orange arrows) with DCs that had been treated with CpG and pulsed with gp33 peptide. Blood glucose was monitored for elevations indicative of diabetes. **A.** This treatment induced diabetes in 89% of mice. **B.** When the DCs were incubated with ACs before CpG and gp33 treatment, diabetes incidence fell significantly to 28% ($p<0.05$). **C.** DCs incubated with NCs before CpG and gp33 treatment also induced a lower incidence of diabetes than DCs treated with CpG and gp33 alone, though not significantly. **D.** Diabetes incidence graph compares the three treatments directly. For graphs A, B and C, each line represents the blood glucose concentrations of a single mouse.

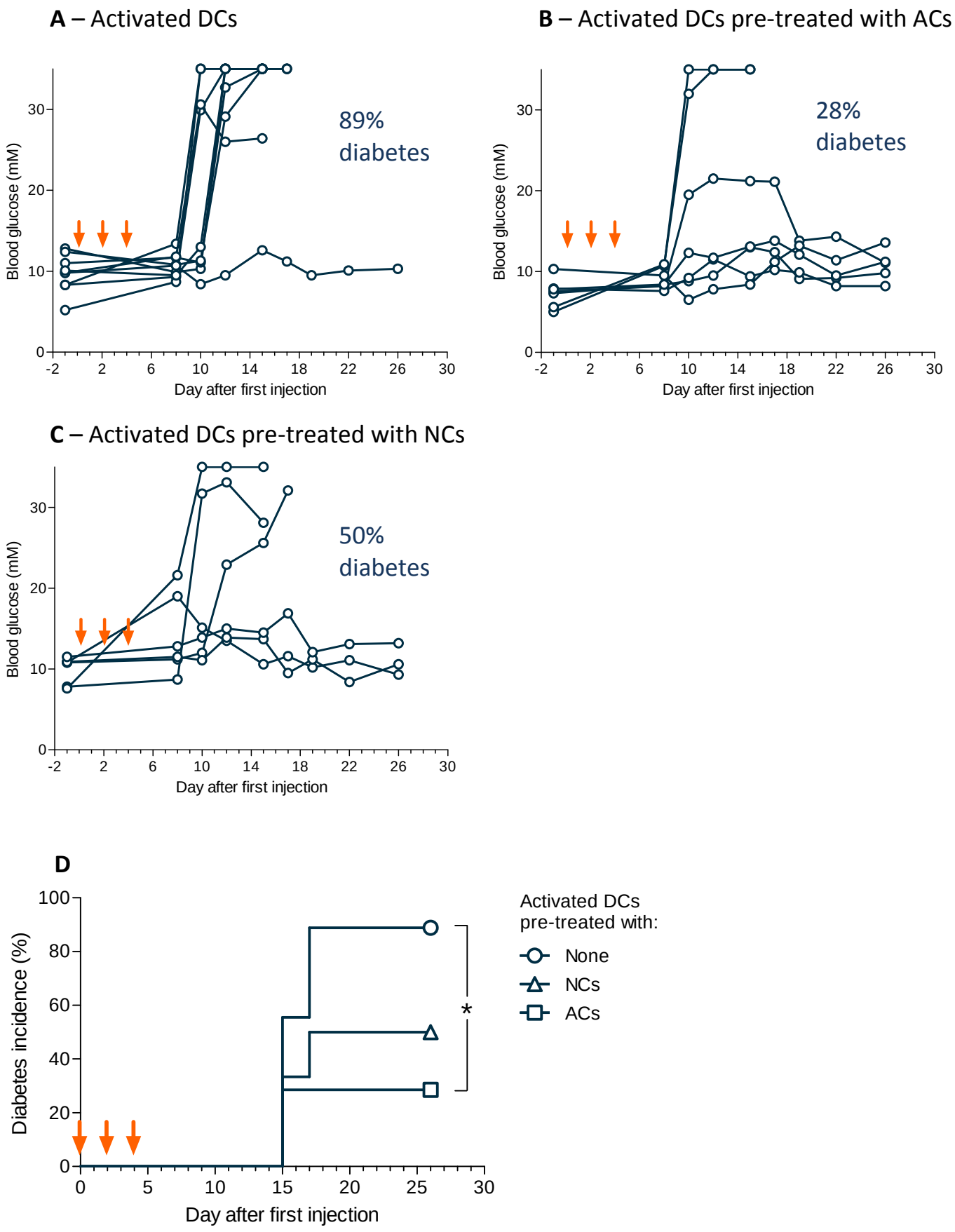


Figure 5.7 See p156.

5.2.5 Necrotic cells, but not apoptotic cells, induce DC expression of TRAIL, CD69 and CD95

The unexpected decrease in the incidence of diabetes by way of pre-treating DCs with necrotic cells was, though not statistically significant, of huge interest. Necrotic cells, but not apoptotic cells, increased number of DCs expressing high levels of TNFα-related apoptosis-inducing ligand (TRAIL) from 20% to 37% ($p<0.05$, one-way ANOVA), increased the number of DCs expressing high levels of CD69 from 17% to 39% ($p<0.05$), one-way ANOVA), and increased the number of DCs expressing high levels of CD95 (Fas) from 5% to 13% [Fig. 5.8 left-hand side column]. CpG-treatment did not affect expression of TRAIL, CD69 or CD95 or their upregulation induced by necrotic cells [Fig. 5.8 right-hand side column].

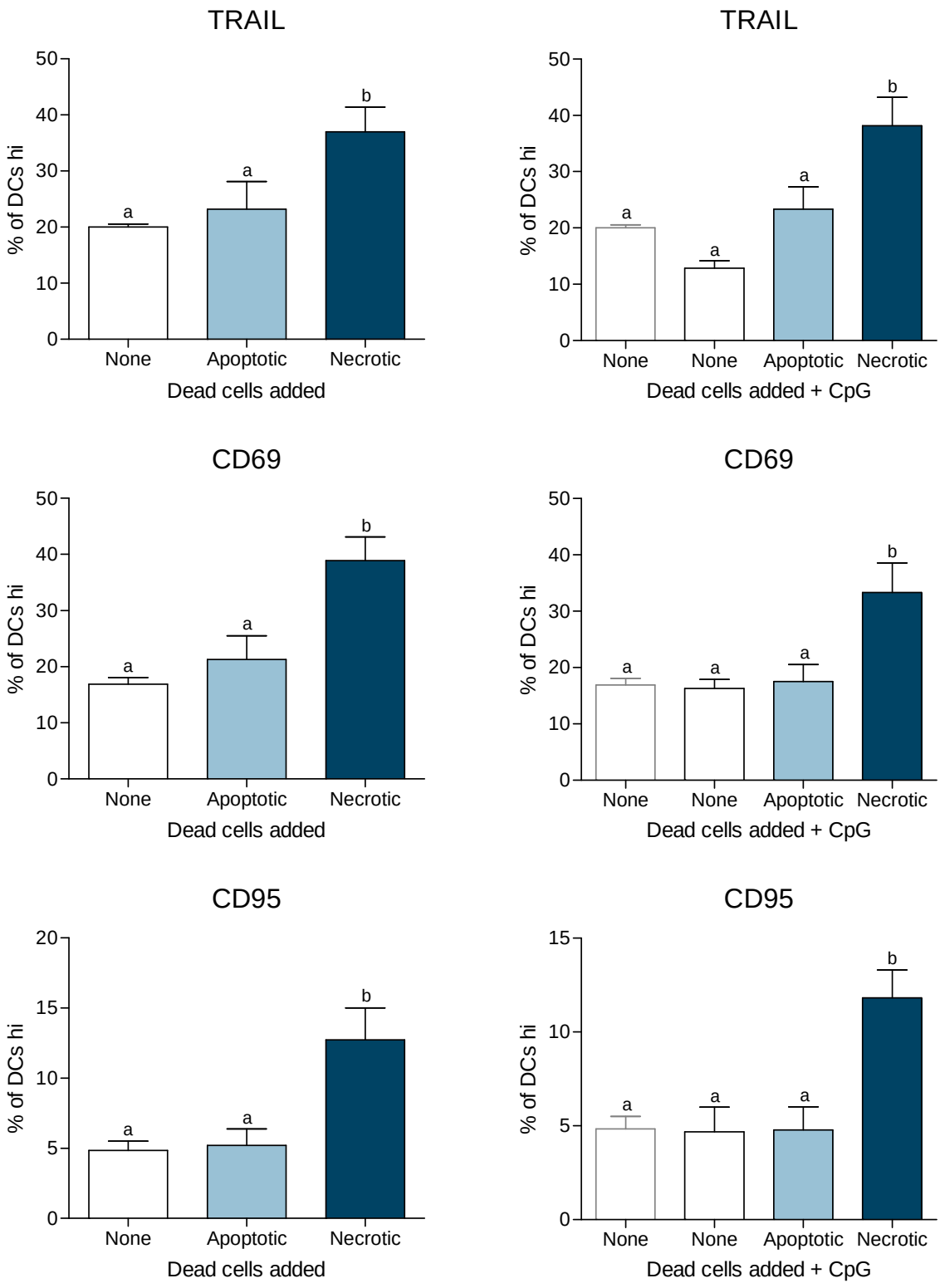


Figure 5.8 Necrotic cells, but not apoptotic cells, induce DC expression of TRAIL, CD69 and CD95. Necrotic cells induced DC upregulation of TRAIL, CD69 and CD95 (Fas) (all $p<0.05$). CpG treatment did not affect expression of either the resting level expression of the molecules or the necrotic cell-induced upregulation. 'a,' and 'b' signify significantly different means, one-way ANOVA. Data for each molecule is from three separate experiments or more, bars indicate SD.

5.3 Discussion

5.3.1 Diabetes induction by treated DCs

This investigation demonstrated diabetes induction by means of treating DCs with the TLR-ligand CpG and pulsing them with gp33 peptide before injecting them into RIP-GP mice. Surprisingly, LPS, appearing in *in vitro* experiments to be the less acquiescent to AC-induced suppression, was less effective at inducing diabetes. Induction with CpG-treated gp33-loaded DCs required three injections with doses of approximately 2-5x10^6 DCs per injection. This regime and dosage correlates well with a previous report of diabetes induced in the RIP-GP model by transgenic, LCMV-GP-expressing DCs (256). In the same study, single injections failed to induce diabetes, but 50% of mice injected with three doses of 10^5 DCs in 6-day intervals, or four doses of 10^4 DCs in 2-day intervals, developed diabetes between Days 10-14. A further 40% developed hyperglycaemia between Days 18-21.

The requirement to pulse DCs with gp33 peptide in order for them to induce diabetes was indicative of gp33-specific immunity. Diabetes can be induced in mouse models by non-immune methods. For example, very high doses of streptozotocin can rapidly induce the disease by direct, chemical destruction of islet β cells, rather than β cell-specific killing by immune cells (257). To confirm that CpG/gp33-DC immunisation induced diabetes via an adaptive immune response, DC and T cell populations of the pancreatic draining lymph nodes (PDLN) were compared with those of the inguinal lymph nodes (ILN), and populations in the spleen (representing circulating T cells in the blood) were also examined. The ILNs, PDLNs and spleens were removed on Day 10 (six days after final injection of DCs treated with 8hr 100ng/ml CpG and pulsed with gp33), which, according to initial tests demonstrating symptomatic diabetes by Day 10 in most mice, would be the optimum time for an immune reaction to be confirmed by the detection of activated

DCs and T cells in the PDLN and in the blood (spleen). Diabetic mice had significantly more CD40-high DCs and CD44-high CD8 T cells in the spleen and PDLN than in the ILN, whereas non-diabetic mice did not. Diabetic mice also had significantly more CD44-high CD4 T cells in the spleen than in the ILN, unlike non-diabetic mice. The presence of activated DCs and T cells in the PDLN and not the ILN demonstrated that a systemic immune response occurred that was locally active in the pancreas. This response no doubt initiated in the draining lymph nodes at the injection site, where naive T cells were primed, entered the blood circulation (hence high numbers in the spleen) and then the pancreas. This would have been followed by a second wave of T cell activation and proliferation in the pancreatic lymph node induced by the initial β cell destruction.

5.3.2 Apoptotic cell-mediated suppression of diabetes

Apoptotic cells were effective at inhibiting the ability of CpG-treated gp33-loaded DCs to induce diabetes in the RIP-GP model. Although this was accurately hypothesised due to the suppressive effects seen in the *in vitro* stages of the investigation, it was no less encouraging to observe the effects in a complete immune response *in vivo*. This is a novel demonstration of apoptotic cell-mediated suppression of autoimmune diabetes, and a novel demonstration of apoptotic cell-mediated suppression of a CpG-induced immune response. The latter findings are supported by a demonstration of PS-mediated inhibition of CpG-induced immunity *in vivo* (258). The confirmation of the tolerogenicity of apoptotic cells has substantial implications both for practical applications and for the direction of future research, as is discussed in the Concluding Discussion.

Whereas we cannot be sure of the AC-treated DC-mediated generation of gp33-specific CD8+ Tregs and their role in the suppression of diabetes in this model (though they are almost certain to play a role), we have described in Chapter 4 the induction of CD4+CD25+ Tregs by AC-treated DCs. We may speculate as to how they suppress CD8+ T cell immunity, the primary mediator of

islet β cell damage. CD4+C25+ Tregs can suppress polyclonal CD4+ T cells (259), which of course has an impact on T cell-mediated immunity because CD4+ T cells are required for a full and efficient CD8+ T cell response (260). Also, CD4+C25+Foxp3 Tregs have been shown to directly suppress CD8+ T cells by cell-bound TGFβ-mediated suppression (261) or by inducing Fas-mediated apoptosis (262). In addition, polyclonal CD4+C25+ Tregs that are not specific to gp33 in the *in vivo* model here may nonetheless induce immunosuppression specific to a range of islet β cell antigens. In a mechanism known as 'infectious tolerance,' Tregs induce tolerance not only to a specific antigen but also to others co-expressed by the same APC, generating new Tregs that will do likewise to create an increasing tolerance repertoire (263). In the 'bystander suppression' mechanism, a common APC is not even required, as soluble factors mediate the induction of tolerance (264). The CD4+CD25+ Tregs described n Chapter 4 therefore have a potentially key role in the suppression of diabetes observed in the *in vivo* model. Just how essential that role is, though, could be determined in future experiments by selective deletion of CD4+ Tregs in the tolerance model – failure for tolerance to be achieved would indicate an indispensable function.

The accomplishment of apoptotic cells in generating successfully tolerogenic DCs in this model transforms some findings from earlier in the investigation into more noteworthy results. Firstly, it can now be deduced that total inhibition of co-stimulatory molecules is not necessary for deletion of or induction of anergy in autoreactive T cells. Suppressed induction of diabetes by pre-treating CpG-treated DCs with apoptotic cells, despite some co-stimulatory markers remaining highly up-regulated, is consistent with reports that DCs can direct Treg expansion regardless of their maturation state (265). Once again, the cytokine profile appears to be of key significance in driving tolerance.

5.3.3 Reduction of diabetes incidence by necrotic cells

The decrease in the incidence of diabetes by pre-treating DCs with necrotic cells was very unexpected. Srivastava and Basu et al (2000) postulated that necrotic cell-induced maturation of DCs may be a slower, much more regulated form of maturation than that induced by LPS and other PAMPS (113). Involvement of this as-yet hypothetical pathway in this case may have interrupted the CpG-mediated pathway of maturation and prevented DCs from reaching their T cell-stimulatory potential.

Although not statistically significant, the necrotic cell-induced reduction was of huge interest and prompted closer inspection of necrotic cell-treated DCs. Because the in vitro evidence accumulated thus far in the investigation suggested that necrotic cell-treated DCs have a high capacity to stimulate T cells, especially by way of pro-inflammatory cytokines, it was hypothesised that necrotic cell-treated DCs inhibit immune responses by direct inhibition of (already activated) effector cells. Although necrotic cells induced upregulation of co-inhibitory molecules, they also induced upregulation of co-stimulatory molecules, unlike apoptotic cells, so suppressive effects by these means seemed implausible. Other surface molecules were examined and it was found that molecules involved in cell death were upregulated by necrotic cells but not apoptotic cells. Necrotic cells induced significant upregulation of the death inducer TRAIL, the death receptor CD95 (Fas), and the T cell activation marker CD69.

CD69 is fairly ubiquitous in lymphocytes but it is chiefly associated with being a T cell activation marker involved in promoting early T cell proliferation (266). However, CD69 also appears to have a regulatory role in reducing infection-induced immunopathology, as its absence is associated with inflammatory diseases subsequent to Listeria monocytogenes infection (267). Furthermore, diminished CD69 has been associated with compromised Treg function and subsequent systemic sclerosis (268), and exacerbated disease in collagen-induced arthritis (269).

TRAIL, an apoptosis inducing molecule, is constitutively expressed on many cell types. Over-expression of TRAIL on DCs can help protect mice from GVHD and leukaemia relapse (270).

TRAIL-upregulation may be the pathway responsible for findings observed by Liang et al (2006) whereby DCs can be mature but nonetheless tolerogenic – by inducing T cell death (271). Furthermore, TRAIL expression has been observed to induce Tregs (272), though necrotic cell treatment of DCs is not associated with an increase in Tregs in this study.

Increased Fas expression increases the potential for apoptosis induced by Fas-ligand (FasL)-bearing cells or soluble FasL. Necrotic cell-upregulated expression of death receptors on DCs warrants that the DC death studies performed earlier in the investigation [Section 3.2.2.2] be extended to longer periods of time. This may indicate the promotion of death in activated, self-Ag-carrying DCs, though the real-life, functional implications of this degree of Fas up-regulation requires further study, particularly *in vivo*. It is tempting to speculate that the potential promotion of death in necrotic cell-treated DCs represents a simple immunoregulatory mechanism that ensures such DCs do not survive long enough to induce serious autoimmunological consequences.

Confusingly, these three molecules are involved in three different mechanisms – direction of T cell responses, inducing apoptosis in others, and inducing its own apoptosis. However, with some conjecture, all can be associated with a diminished immune response: Modulation of T cell behaviour towards anergy or Treg conversion; induction of apoptosis in activated T cells that do not respond to the first option; or, failing the first two options, self-destruction in order to prevent prolonged release of proinflammatory cytokines (indeed, the initial secretion of proinflammatory mediators may be important for tolerance by recruiting professional phagocytes to clear a site of extensive necrotic damage).

5.3.4 Summary

This investigation has demonstrated a novel method of inducing diabetes in a modified version of an established experimental system. Confirmed autoimmune diabetes was induced by way of a

reliable method and a well characterised immune response. The DC-mediated induction of diabetes in this model allowed for well-controlled modifications of DCs *in vitro* to be examined in the context of full immune responses *in vivo*. As such, the full implications of the distinct differences between apoptotic cells and necrotic cells in their effects on DCs could be observed.

Apoptotic cells accurately conveyed their *in vitro* tolerogenic characteristics in their suppression of the DC-induced onset of diabetes. Interestingly, necrotic cells, which in vitro displayed distinct pro-inflammatory effects, partially (not significantly) suppressed the onset of diabetes. Upon closer inspection of surface molecules, it is possible to hypothesise that upregulation of Fas, CD69 and TRAIL represents a comprehensive immunoregulatory mechanism involving immediate recruitment of immune cells (by pro-inflammatory cytokine secretion), deletion and/or anergy in self-reactive T cells, and death.

CHAPTER 6 – General Discussion

6.1 Key findings

In this investigation, various methods of inducing cell death were evaluated. Reliable methods of generating homogenous populations of well-characterised apoptotic cells or necrotic cells, namely 24-hour camptothecin-treated FDCP and 60˚C incubation for 30 minutes, respectively, were developed. These methods were employed to show effects of apoptotic and necrotic cells on dendritic cells and *in vitro* and *in vivo* immune responses for the first time.

In vitro findings support previous reports of the anti-inflammatory response of DCs to apoptotic cells, and the inflammatory response of DCs to necrotic cells. The previously-reported inhibitory effect of apoptotic cells on the LPS-induced Th1 response is supported here, such as the production of IL10 and TGFβ and suppression of IL12. However, the previously-reported inhibitory effect of apoptotic cells on LPS-induced upregulation of co-stimulatory molecules is contested. Apoptotic cells, but not necrotic cells, are shown for the first time to have a suppressive effect on CpG-induced upregulation of co-stimulatory molecules and pro-inflammatory cytokines. Novel observations of the upregulation of DC expression of co-inhibitory molecules B7DC and B7H1-4 induced by both apoptotic cells and necrotic cells have been described here.

Apoptotic cells suppressed the capacity of untreated and CpG-treated, but not LPS-treated, DCs to elicit IFNγ production by T cells. Apoptotic cells, but not necrotic cells, suppressed CpG-induced DC capacity to enhance T cell proliferation and production of IL2 and IL17. Apoptotic cells, but not necrotic cells, induced regulatory T cells and partially restored the induction that was suppressed by CpG-treated DCs. Finally, apoptotic cell-modulation of DCs inhibited the induction

of autoimmunity in a novel modification of an *in vivo* model of diabetes. Interestingly, the proinflammatory phenotype of necrotic cell-treated DCs did not translate into enhanced immunity *in vivo*. Instead, a tolerogenic outcome occurs, that the data indicates is not mediated by CD4+CD25+Foxp3+ Tregs. Novel evidence for the possibility of necrotic cell-induced tolerance by a variety of means, including direct T cell killing, has been described.

6.2 Uptake of apoptotic cells

Since earlier experiments show that a significant proportion of DCs do not phagocytose ACs, it may be argued that DCs that have not phagocytosed dead ACs respond to LPS in the normal manner and inhibit or subdue (presumably by the action of cytokines) the response of those that have phagocytosed ACs. That is, ACs *would* inhibit PAMP-induced CD40 (etc) up-regulation were it not for the presence of other, matured, DCs. This would suggest that AC-suppressed DCs may be subservient to the influence of mature DCs. It also raises the question of whether DCs require direct interaction with ACs in order to become tolerogenic, or can become suppressed by association (directly or otherwise) with other, AC-suppressed DCs.

However, we have demonstrated that these populations of AC-treated DCs, of which less than 25% have phagocytosed ACs [Fig. 3.7], incur suppression of proinflammatory cytokines, up-regulation of anti-inflammatory cytokines, and suppression of capacity to induce diabetes *in vivo*. This would suggest that inhibition of up-regulation of co-stimulatory molecules is not essential for the tolerogenic (or at least, reduced inflammatory) function of DCs. Without further experimentation, it is difficult to confirm if a) cytokine and functional changes occur in only those DCs that have phagocytosed ACs, in which case the influence of these tolerogenic DCs is

sufficient to negate that of non-tolerogenic DCs that presumably respond to PAMPs in the normal manner (i.e. there is a *net* tolerogenic effect), or b) DCs that have phagocytosed ACs confer tolerogenic status upon those DCs that have not phagocytosed ACs (i.e. there is a *total* tolerogenic effect). The lack of PAMP-induced proinflammatory cytokines in cultures of DCs with ACs does not corroborate the case for either situation, considering that the methods of tolerogenesis by DCs are not fully known. For example, in situation a, tolerogenic DCs may absorb, destroy or otherwise nullify proinflammatory cytokines nearby (or counter them by induction of IL10, TGFβ and/or IDO). Or, in situation b, there is simply no inflammatory response because effectively all DCs become tolerogenic. Further experiments that may help clarify, including the use of cytokine-permeable membranes, whereby a cytokine-permeable membrane separates two identical populations of DCs, to only one of which are added ACs, could be very informative.

6.3 Co-inhibitory molecules

Although the immune effects of B7H2 and B7H3 are very much contested in the literature, the findings of this investigation support the notion of an inhibitory role in the context of DC responses to CpG. B7DC, B7H1 and B7H4 are also associated with tolerance in this experimental system. It is tempting to speculate that certain molecules are prominent or more influential in the behavioural switch from immunogenic to tolerogenic DC. For example, B7H3 has the greatest relative increase in expression after AC-treatment both in comparison to untreated DCs and in comparison to NC-treated DCs, in no-PAMP, and LPS- and CpG-treated DCs. However, the co-stimulation interaction between DCs and T cells is complex, and the balance between co-stimulation and co-inhibition is not yet fully understood. It would be up to future studies to report the relative influence of each co-stimulatory and co-inhibitory molecule in the DC functional outcome, through the use of Ab-blocking and knockouts. However, B7DC and B7H1 have been

directly linked to reducing DC capacity to stimulate T cells (68) and anti-inflammatory B7DC/B7H1-PD-1 signalling is thought to specifically interfere with pro-inflammatory CD80/CD83-CD28 signalling. Although using the mean fluorescence intensity of each treatment would probably provide a more accurate assessment, the percentage of DCs expressing high levels of CD80, CD86, B7DC and B7H1 in response to various treatments may be of some use in predicting ensuing T cell responses. For each treatment combination of DCs ± none/ACs/NCs ± none/LPS/CpG , below is calculated the ratio of:

(%CD80-high DCs + %CD86-high DCs) / (%B7DC-high DCs + %B7H1 DCs)

Ratio of CD80 and CD86 expression to B7DC and B7H1 expression, after various treatments:

		Dead cell type added		
		None	AC	NC
TLR added	No TLR	0.44	0.44	0.57
	LPS	0.76	0.78	0.87
	CpG	0.75	0.51	0.81

The relative values of the ratios correlate well with the average IFNγ secretion by T cells cultured with the CM of the corresponding DC treatment (see below). Between the two sets of data can be observed the following parallels: The increase of the LPS and CpG treatments compared to No TLR; the necrotic-cell induced increase compared to None, in the No TLR treatment; the high values across all LPS treatments; the apoptotic cell-induced decrease in the CpG treatment.

IFNγ secretion (mean, across all tested doses of ConA, pg/ml to nearest hundred):

		Dead cell type added		
		None	AC	NC
TLR added	No TLR	11200	8000	15100
	LPS	14100	13600	13900
	CpG	12500	10600	13100

Of course, more robust statistical analysis would be necessary, and the MFI would no doubt be a more accurate factor, but the statistical relationship between the co-stimulatory molecules CD80 and CD86 and the co-inhibitory molecules B7DC and B7H1 could prove useful as a predictive model of immunological outcome. If proved accurate, the time and resources saved by removing

the need for T cell assays could be valuable for clinical or commercial diagnostic purposes. The method could even prove useful for predicting the effectiveness of tumour vaccines in dendritic-cell based immunotherapies.

6.4 Recommended future work

The confirmation of the influence of the apoptotic cells generated in this investigation corroborates the earlier *in vitro* findings. In confirming their influence, camptothecin-treated FDCPs can now be considered as an ideal model of tolerogenic apoptotic cell. The novel model of suppression of autoimmunity in vivo has enormous use for its ability to distinguish between tolerogenic and immunogenic dendritic cells and provide a clear readout.

The model of apoptotic cell-mediated suppression of autoimmunity can be used in future work to address several questions. Firstly, it is important to ascertain if the Treg-induced mechanism of tolerance suggested by the *in vitro* results of this investigation is implicated in the *in vivo* model. To this end, it is proposed that the blood and spleen of non-diabetic mice in this model is sampled for circulating Tregs – in particular, gp33-specifc Tregs. This would determine whether apoptotic cell modulation in this model merely inhibits immunity or it actively induces tolerance towards the apoptotic cell-expressed autoantigens. Further to ascertaining Treg development, the roles of IL10 and TGFβ in suppression of diabetes in the present model must be clarified. IL10/TGFβ blocking *in vivo* or the use of transgenic knockout mice could help to discover the mechanism of suppression.

DCs are a crucial part of maintenance of peripheral tolerance, but other mechanisms are also important, e.g. B cell autoantibodies etc. It would be useful to observe how AC-induced suppression interacts with these pathways.

The distinction between suppression of LPS and CpG immune responses could have significant implications, and also prove a useful tool in narrowing down the possible mediators of tolerance. Between CpG and LPS it was somewhat surprising that CpG, appearing to be the more acquiescent of the two to AC-induced suppression *in vitro*, was the more effective at inducing diabetes. And indeed it was unanticipated that LPS was as ineffectively inhibited as it was, given the weight of previous findings.

Several distinctions between LPS and CpG signalling may be responsible for the difference between their respective potentials for suppression by apoptotic cells. These distinctions may help to elucidate the mechanisms of immune response regulation by endogenous dead cells. Factors that are worthy of consideration include: TLR9s role in antiviral immunity versus the antibacterial immunity elicited by TLR4, the LPS receptor; the internal location of TLR9 versus the cell surface location of TLR4; the role of each TLR in dead cell clearance (TLR9 also recognises mammalian DNA); any other functions of each TLR, for example CpG encourages macrophage phagocytosis of apoptotic neutrophils (273).

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