The Role of Oxidative Stress in the Regulation of Dendritic Cell Function

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Charles Alderman

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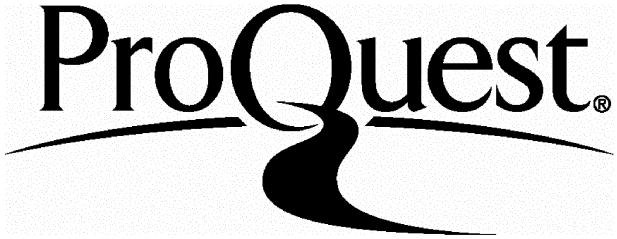
Department of Pharmacology
University College London
Gower Street
London WC1E 6BT



Abstract

The archetypal view of the immune system is that it has evolved primarily to distinguish between non-infectious "self" and infectious "non-self". However, this view has been superseded by the paradigm that the immune system is based on the recognition of a "danger" signal. The key to this signal is thought to be activation of dendritic cells (DC), which is a critical step in the initiation of adaptive immune responses. However, there is little experimental evidence to support the role of "danger" signals in the activation of DC. Perhaps the most noteworthy hypothesis is that oxidative stress may constitute a common denominator pathway for the activation of DC. This hypothesis has yet to be investigated in detail, and is the subject of this thesis.

Human DC were differentiated from monocytes by seven days of culture in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4. When supplemented to purified cell populations, non-toxic concentrations of reactive oxygen species (ROS) were unable to induce the maturation of DC. In addition, ROS were found not to represent prerequisite activation signals for DC *in vitro*.

It was hypothesised subsequently that the effects of oxidative stress on DC may be mediated indirectly *via* the actions of oxidised protein and lipid derivatives. Thus, either advanced oxidation protein products (AOPP) or oxidised low-density lipoproteins (LDL) could be representative of the fundamental mechanism for the identification of damaged-"self" by DC.

AOPP were found to increase the T-cell stimulatory capacity of DC (as assessed by oxidative mitogenesis assays) without inducing their conventional maturation, whilst oxidised LDL were shown to cause both phenotypic and functional maturation of DC. Furthermore, native LDL caused DC aggregation, and oxidised LDL caused DC apoptosis. In conclusion, oxidised derivatives of "self" may play an important contribution to physiological and pathophysiological responses involving DC maturation.

To my parents, with love

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Chapter 1

General Introduction

1.1 Introduction to Dendritic cells

The first member of the dendritic cell (DC) family to be identified was the Langerhans' cell in 1868. Paul Langerhans concluded that these cells were of neuronal origin, which remained the dogma until 1968 when Breathnach *et al.*, finally ruled out the possibility of a neuronal crest origin for these cells.

In 1973 Steinman and Cohn described a novel cell population found within murine spleen that contained distinctive cytoplasmic extensions, which resembled those seen arising from nerve cells. Therefore, they called the processes "dendrites" and the cells "*dendritic* cells". It has subsequently been shown that, in lymphoid tissue, dendrites are not unique to DC as they can also be seen on the surface of macrophages and B cells, but despite this the terminology has persisted (Wright-Browne *et al.*, 1997).

1.1.1 Overview of DC functions

Whilst several lineages of DC are known to exist, it is generally accepted that they are of bone marrow origin. With the exception of the cornea, they have been isolated from all human organs (Steinman, 1991). It is generally accepted that upon activation, DC migrate from peripheral tissues, through the afferent lymph and blood vessels, to localised T cell areas within secondary lymphoid organs (i.e. lymph nodes and spleen). This migration is associated with the uptake, processing and presentation of peripheral antigens within lymphoid organs.

Upon binding to mature DC, antigen specific $CD4^+$ T cells may differentiate into either a Th1 or Th2 phenotype. In the presence of interleukin (IL)-12, the Th1 phenotype is established, leading to the production of interferon (IFN)-γ, which is known to activate macrophages. In conjunction with IL-12, IFN-γ also promotes the differentiation of $CD8^+$ T cells. In the presence of IL-4, mature DC promote the differentiation of $CD4^+$ T cells into a Th2 phenotype which release

IL-4 and IL-5, leading to the activation of eosinophils and the secretion of antibodies by B cells.

1.1.1.1 DC and B cell interactions

DC are most frequently discussed in terms of their specialised ability to induce T cell-mediated immunity. However, one should also consider the fact that DC are required for the indirect stimulation of B cell growth and antibody production both *via* the activation of Th cells, and more directly through their release of IL-12 (Banchereau & Steinman, 1998).

In addition to these pan-B cell effects, DC may promote immunoglobulin class-switching and, in the presence of IL-10, secretion of IgA (Fayette *et al.*, 1997).

1.1.1.2 The role of DC in tolerance

DC are also implicated in the induction and maintenance of tolerance: this may be mediated through a distinct subset of DC. A candidate cell that may induce tolerance is the lymphoid DC, which is known to restrict $CD4^+$ and $CD8^+$ T-cell responses, possibly through reduced production of IL-2 (Süss & Shortman, 1995; Kronin *et al.*, 1996). Alternatively, a single population of DC may retain the capacity to induce an immune response or tolerance depending on additional signals such as the degree of costimulation or the local cytokine environment (Sallusto & Lanzavecchia, 1999).

1.1.2 Initiation of Adaptive Immunity

1.1.2.1 Before Janeway

For many years immunological research centred on the role of lymphocytes in host defence, more specifically on the mechanisms guiding clonal selection. It was concluded from this work that the adaptive immune system develops

through the random rearrangement of gene families, with the formation of clonally distributed receptors. But this did not answer, or perhaps more importantly, ask the question; what initiates an adaptive immune response?

Adaptive lymphocyte specificity is generated randomly and requires additional signals to mount an immune response. B cells receive these signals mainly through the recognition of specific $CD4^+$ T cells, whereas T cells require costimulatory signals delivered by antigen presenting cells. Indeed, it has been shown that in the absence of costimulatory signals, antigen presenting cells are unable to stimulate T cells (Jenkins & Schwartz, 1987). Given the dependence of antigen presenting cells on such signals, it has been concluded that the immune system is controlled through the presence or absence of costimulatory molecules. It is thought that the role of antigen presenting cells, in particular the DC family of antigen presenting cells (Ibrahim *et al.*, 1995), is to combine costimulation with the detection of the presence of pathogenic organisms and thus, signal the need to mount an adaptive immune response, as opposed to the induction of tolerance.

1.1.2.2 The "Janeway Hypothesis"

Recognition of the importance of pathogenic organisms in the initiation of adaptive immunity is a relatively recent event. Janeway and colleagues first hypothesised that known activators of the innate immune system may signal the presence of an infectious organism and thus trigger an adaptive immune response. They envisaged that primordial receptors (pattern recognition receptors) may recognise conserved microbial structures that are integral to pathogenesis or the structure of the microbe (Janeway, 1992). These receptors would recognise a broad-spectrum of microbial structures not expressed by the host. An advantage of this hypothesis is that microbes could not avoid immune recognition through simple mutations, since these would alter the pathogenesis or structure of the microbe. Such conserved structures could include teichoic acids and lipopolysaccharide (LPS) as common components on the surface of $Gram^+$ and $Gram^-$ bacteria, respectively, mannans within yeast cell walls and double-

stranded RNA as found in several groups of viruses (Medzhitov & Janeway, 1997).

This model, however, implies that activation of the adaptive immune system is critically dependent upon the presence and recognition of a foreign conserved microbial structure. Thus, it fails to explain the fact that many viruses do not bind pattern recognition receptors, but are known to induce an adaptive response. Additionally, rejection of allotransplants could only be explained by the premise that the healthy organs are all infected before transplantation; this is unlikely to be the case.

1.1.2.3 The importance of danger

1.1.2.3.1 The Danger theory

Since adaptive immune responses cannot be primed in the absence of antigen presenting cells (APC) activation, and conserved microbial structures are alone insufficient to account for APC activation, it has seen suggested that the adaptive immune system must respond to, or more importantly, be activated by the recognition of a stress or danger signal (Matzinger, 1994). Thus transplant rejection would be explained by damage induced by the surgical procedure and immune responses against viruses would be initiated by viral induced tissue damage or a product of virally infected cells such as IFN-α or heat shock proteins.

This hypothesis is supported by the knowledge that cellular responses to stress have been reported (Yoshimura, 1998), but, it remains to be resolved whether or not DC could be activated in a similar manner.

1.1.2.3.2 Danger signal

A suitable signal must be present only at times of danger or host damage, and must, therefore, exclude physiological cell turnover. In addition, the danger sensitive APC must express a suitable receptor mechanism to identify the potential hazard. Potential danger signals fall into three main categories: i) intracellular constituents which are only located extracellularly upon cell necrosis, (e.g. DNA, RNA and mitochondria); ii) signals produced by cells under stress, (e.g. heat shock proteins are induced by heat, irradiation, viral infections and toxins); iii) pro-inflammatory products, including cytokines (e.g. TNF-α and IL-1) and free radicals (Ibrahim *et al.*, 1995).

Early support for the danger hypothesis came from the finding that the pro-inflammatory cytokines, TNF-α and IL-1, are able to mature DC *in vitro*. However, there is scant evidence for the direct activation of DC by products of necrotic cell death. And, indeed, where this is found to be the case (Gallucci *et al.*, 1999; Sauter *et al.*, 2000) the effect appears to be restricted to cell lines, suggesting that the more likely explanation is mycoplasma infection, which has been shown recently to activate DC (Salio *et al.*, 2000).

Regardless of how attactive the danger model is, two further caveats should be noted. Firstly, the rapid rejection of long-term allotransplants upon cessation of immunosuppressive drugs (Newstead *et al.*, 1998) may only be explained as a side effect of the drug treatment that prevents the induction of peripheral tolerance (Matzinger, 1998). Secondly, it has been shown that, in the absence of a danger signal, the adoptive transfer of T cells or the reconstitution of T cells through bone marrow transplantation can lead to rejection of well-healed allotransplants (Bingaman *et al.*, 2000).

1.1.3 Characterisation of DC

1.1.3.1 DC sub-popuiations

1.1.3.1.1 Conventional DC

A variety of APC, including B cells, B lymphoblasts, and peritoneal macrophages, are able to activate sensitised T cells. However, one of the key features of the "Steinman" DC was their unique ability to induce the proliferation of resting T cells in a primary allogeneic mixed leucocyte reaction (Steinman, 1978). Thus, DC is, to a certain extent, a generic term used to describe cell populations that lack a unique differentiation marker and can be defined best by functional properties.

Human DC are large cells with an irregularly shaped nucleus, which may contain multiple nucleoli. The surface of DC contain multiple protrusions including spiny dendrites, bulbous pseudopods and large lamellipodiae, which continually extend and retract. At the phenotypic level DC express high levels of MHC class I and II, in addition to the common leukocyte antigen CD45 but do not express classical T, B, NK or monocyte markers (Wright-Bowne *et al.*, 1997). It may be because of this lack of positive markers that an increasing number of DC subpopulations, derived from both myeloid and lymphoid precursors, have been described. The observation that DC express GM-CSF receptors, but, as opposed to all other mononuclear phagocytes, do not bind M-CSF (Kämpgen *et al.*, 1994) may prove to be an important observation in this field.

Interactions between mature DC and lymphocytes are aided by their high surface expression of intercellular adhesion molecules such as ICAM-1 (CD54), ICAM-3 (CD50), LFA-3 (CD58), and costimulatory molecules CD40, B7-1 (CD80) and B7-2 (CD86) (Stockwin *et al.*, 2000). An additional DC marker is CD83, thought to be present on most human DC (Zhou *et al.*, 1992). Receptors for vitamin D and retinoic acid have also been detected on DC (Fritsche *et al.*, 2000).

Early investigations into DC ontology demonstrated that single $CD34^+$ haemopoietic progenitor cells are capable of forming either pure monocyte populations or a mixture of monocytes and DC (Reid *et al.*, 1992; Szabolcs *et al.*, 1995; Caux *et al.*, 1992) but never DC alone. This suggests that DC are a derivative of monocytes with a common myeloid origin.

DC have been isolated from human peripheral blood, which express low levels of CD14, lack characteristic T, B and NK cell markers with low expression of costimulatory molecules (Thomas & Lipsky, 1993). These immature DC may exist as a population of proliferating committed progenitor cells (Romani *et al.*, 1994) derived from DC colony forming units in the bone marrow (Young *et al.*, 1995). Two further DC subsets have been identified in peripheral blood based on their expression of CD11c (O'Doherty *et al.*, 1994).

An additional population of DC may exist which does not express the common myeloid markers CD13 and CD33, indicating a possible kinship of DC, T, B and NK cells, (Galy *et al.*, 1995) with a common ($CD34^+/CD10^+$) lymphoid progenitor. Consistent with this is the finding that these cells express CD4 and have a plasma cell morphology (Ardavin *et al.*, 1993).

A separate class of DC known as the dendritic epidermal cell expresses the T cell glycoprotein Thy-1. These cells also express γδ T cell receptors (TCR) and may have a functional role similar to that of NK cells. Although the origins of these cells are unknown they are not thought to derive from the monocyte-macrophage lineage.

Recently, two distinct subsets of DC have been characterised based on their ability to influence the development of Th1 and Th2-type immune responses (Rissoan *et al.*, 1999) and are known as DC1 and DC2 respectively.

1.1.3.1.2 Follicular DC

Follicular DC reside in the germinal centres of lymph nodes and are directly involved in their organisation. They express a unique set of surface molecules but lack the common leukocyte antigen CD45 (Schriever *et al.*, 1991), and are not thought to be bone marrow-derived (Liu *et al.*, 1996). Follicular DC display antigen-antibody complexes that are captured through Fc binding, but not processed antigen. These immune complexes are thought to function mainly in the maintenance of long-term B cell memory (Tew *et al.*, 1997).

Naïve B cells that recognise specific antigen on the surface of the follicular DC, process this antigen and present the peptide-MHC complex, which may in turn be recognised by specific T cells. These cells clearly differ markedly from conventional DC. They have been the subject of a recent review, and will not be discussed further here (Tew *et al.*, 1997).

1.1.3.1.3 Langerhans' cells

Langerhans' cells are probably the best characterised DC. They are bone marrow derived (Frelinger *et al.*, 1979; Katz *et al.*, 1979) and can be identified on the basis of unique intracytoplasmic organelles known as Birbeck granules.

Langerhans' cells express cutaneous lymphocyte-associated antigen (CLA), the ligand for E-selectin, which promotes their migration to the skin (Strunk *et al.* 1997). As yet, *in vitro* studies have been unable to induce the expression of CD14 on Langerhans' cell progenitors. This is in contrast to $CD34^+$ interstitial DC progenitors, which can readily form monocytes/macrophages and DC, given the appropriate cytokine stimuli (Caux *et al.*, 1996). A clear distinction between interstitial DC and Langerhans' cells is the finding that naïve B cells are unresponsive to Langerhans' cells (Banchereau & Steinman, 1998). Langerhans' cells will not be discussed further within this introduction.

1.1.3.2 Antigen uptake

As sentinels of the immune system, immature DC are specialised in their ability to sample and internalise foreign antigens. Upon receiving a maturation signal DC increase their antigen-uptake capacity within 1-2 hours. This decreases as the cells mature (Rescigno *et al.*, 1999). This pattern of antigen uptake by DC contrasts with that of macrophages, which have a greater uptake capacity that decreases with antigen loading but not stimulation. Antigen uptake by DC is achieved through a number of specific and non-specific mechanisms.

The major non-specific uptake mechanism is macropinocytosis. This is an efficient mechanism for sampling the extracellular milieu through the uptake of fluid into pinocytic vesicles, and then concentrating the sample by the expulsion of excess water (Norbury *et al.*, 1997). Down-regulation of macropinocytosis, upon maturation, may be a direct consequence of the loss of integrity of polymerised actin filaments, which are disassembled following maturation (Winzler *et al.*, 1997).

Specific uptake mechanisms include phagocytosis, the main route by which bacteria enter DC (Moll *et al.*, 1993), and receptor-mediated uptake of antigen. These mechanisms may be stimulated through the binding of Fc receptors [e.g. FcγRI (CD64), FcγRII (CD32) and FcεR1 (CD23)] (Fanger *et al.*, 1996; Maurer *et al.*, 1998) and complement receptors [e.g. C3 bi (CD11b/CD18)] (Green *et al.*, 1980), although they are thought to be less important in DC than macrophages. Recent reports have also described the importance of C-type lectin receptors, including the macrophage mannose receptor (Sallusto *et al.*, 1995), in the specific uptake of antigens by DC.

Maturation induces the phenotype of DC to change from that of an antigen-capturing mode to one associated with their messenger and presentation functions, thus fulfilling the characeristics of a sentinal.

1.1.3.3 DC migration

The recruitment of DC has been demonstrated to occur into airway epithelium upon inhalation of bacteria and viruses (McWilliam *et al.*, 1996). LPS has been shown to induce the migration of DC from peripheral tissues (Roake *et al.*, 1995) to T-cell areas, within draining lymph nodes (De Smedt *et al.*, 1996). The interpretation of this is that chemotactic factors must regulate both the recruitment of immature or precursor DC and, upon maturation, their emigration from peripheral tissues to secondary lymphoid organs. This is achieved through distinct subsets of chemokines and the regulation of chemokine receptor expression. It should also be noted that DC also migrate to lymph nodes at a steady rate in the absence of stimulation (Drexhage *et al.*, 1979).

The initial stage of DC recruitment is the tethering and rolling of peripheral blood cells, either DC, or their precursors, to the endothelium. This has been shown to occur continuously *in vivo*, on non-inflamed dermal endothelium and is mediated through the binding of a glycosylated form of P-selectin glycoprotein ligand (PSGL)-1, on the surface of DC, to P- and E-selectin, which are constitutively expressed at low levels on endothelial cells but are upregulated dramatically by inflammatory stimuli (Robert *et al.*, 1999).

Inflammatory stimuli have been shown to stimulate the expression of adhesion molecules on DC and endothelial cells, such as intercellular cell adhesion molecule (ICAM)-1 (CD54) and vascular cell adhesion molecule (VCAM)-1 (CD106) which promote firm adhesion and extravasation of DC (Rescigno *et al.*, 1999). The recruitment of DC to a site of inflammation is further aided by a variety of inflammatory chemokines released from maturing DC themselves (Sallusto *et al.*, 1998).

The best documented pathway for this is that pro-inflammatory stimuli, including LPS and TNF-α, induce endothelial cells and monocytes to release macrophage inflammatory protein (MIP)-3-α, which attracts DC from peripheral blood through the binding of (CC chemokine receptor) CCR6. MIP-1-α, MIP-1-β and

RANTES (regulated upon activation, normal T-cell expressed and secreted) are also thought to induce the recruitment of immature DC, acting through the ligation of the chemokine receptor CCR5. Upon maturation the expression of CCR5 and of CCR6 is downregulated which allows the DC to escape the influence of this local chemokine environment following maturation.

Maturation of DC is also associated with an increase in their expression of CCR7; this allows responsiveness and attraction to the local gradient of the chemokine MIP-3-β (Dieu *et al.*, 1998). MIP-3-β is restricted to lymphoid organs and released from scattered cells within T cell-rich areas (Rossi *et al.*, 1997). CCR7 is also the receptor for secondary lymphoid-tissue chemokine (SLC) which is produced by mature DC in T-cell areas (Gunn *et al.*, 1998) and is thus thought to be a key signal for the emigration of DC towards T-cell areas of secondary lymphoid organs. Migration of mature DC is also aided by the CXC chemokine stromal-derived factor (SDF)-1-α through the increased expression of (CXC chemokine receptor) CXCR4 (Lin *et al.*, 1998).

Similar responsiveness profiles have also been shown in an *in vitro* model of transendothelial migration (Lin *et al.*, 1998).

DC are found in afferent but not efferent lymph, indicating that after migration to the lymph node they are either trapped in this site or, more likely, die and are cleared (Hart, 1997). The clearance of activated DC occurs within 10 days of their arrival to the lymph node (Kurtz *et al.*, 1996), and their removal could be either a direct consequence of their activation (De Smedt *et al.*, 1996) or mediated through cognate antigen interactions (Rathmell *et al.*, 1995). The clearance of DC is thought to be the mechanism by which activated T cells disengage from mature DC, allowing them (the T cells) to proliferate unhindered and migrate to sites of inflammation (Ingulli *et al.*, 1997). The dissemination of the T cells eliminates the focus of T-cell activation and may, thereby, represent a mechanism by which T cell-mediated immunity can be terminated (Ingulli *et al.*, '97).

1.1.3.4 Antigen presentation

1.1.3.4.1 Conventional antigen presentation

Immature DC constantly synthesise new MHC class II molecules. These molecules are assembled in the endoplasmic reticulum (ER) in conjunction with invariant chain (Ii), which prevents peptide loading of the MHC II (Mellman *et al.*, 1998). These complexes are then stored in specialised compartments known as MHC II enriched compartments (MIIC), that accumulate in immature DC. In contrast to murine *ex vivo* DC models, human DC transiently express peptide-MHC complexes on their surface, these are rapidly degraded allowing turnover of class II molecules. There is also a constant turnover of MHC II-Ii in MIIC vesicles (Pierre *et al.*, 1997).

During DC maturation, MIIC compartments are loaded with antigen, convert into non-lysosomal vesicles and display their MHC-peptide complexes on their surface. The increase in expression of MHC II is also associated with a transient increases in the rate of MHC I and II synthesis (Cella *et al.*, 1997), which acts to maximise the potential for surface peptide expression and hence function.

Fully mature DC express virtually all their MHC II on their surface. This is seen in both murine and human DC models (Watts, 1997). Maturation is associated with a down-regulation of endocytosis and consequently the half-life of peptide-MHC II is increased from about 10 hours to over 100 hours (Cella *et al.*, 1997).

1.1.3.4.2 Cross-presentation of antigens to $CD8^+$ lymphocytes

Immature DC have the unique ability to cross-present antigens derived from apoptotic cells in the form of MHC I-peptide complexes. This is in contrast to macrophages which can efficiently phagocytose apoptotic cells but degrade rather than cross-present this material. This provides a mechanism by which DC can either stimulate or induce tolerance to $CD8^+$ T cells, in the absence of being

infected themselves (Brossart & Bevan, 1997). These actions are refered to as cross-priming and cross-tolerance respectively. The integrin receptor $\alpha_v\beta_5$ is expressed at high levels on the surface of immature DC and may be responsible directly for the trafficking of apoptotic cells (Albert, 1998).

It remains to be resolved how DC express extracellular derived peptides in the context of MHC class I, since, in theory, these have no access to the cytosol. An obvious exception to this general rule occurs when the DC are infected themselves.

1.1.3.5 Maturation of DC

1.1.3.5.1 Maturation signals for DC

1.1.3.5.1.1 Maturation of DC by pro-inflammatory agents

Although the maturation of DC is central to the role they play in initiating immune responses, the complete array of maturational signals has yet to be fully elucidated. To date, DC are known to be activated by a number of pro-inflammatory agents including cytokines and microbial constituents, but there is no definitive evidence to support the hypothesis that constituents of tissue damage can directly activate DC.

It is suggested frequently that IL-1-β is able to mature DC. However this may be dependent upon the autocrine release of TNF-α, as has been shown for Langerhans' cells (Winzler *et al.*, 1997). Stimulation of DC through the paracrine production of TNF-α may also aid in the maturation of DC in response to $Gram^+$ and $Gram^-$ bacteria (Riva *et al.*, 1996). *In vitro*, TNF-α alone is capable of maturing human DC derived from monocytes (Sallusto & Lanzavecchia, 1994) and bone marrow (Young *et al.*, 1995), and Langerhans' cells (Caux *et al.*, 1997), and may be sufficient to induce complete maturation of DC, but only in the presence of foetal calf serum (Jonuleit *et al.*, 1997).

Unmethlyated CpG motifs are a common component of bacterial DNA and have been shown to activate DC (Sparwasser *et al.*, 1998) by a mechanism that is dependent upon activation of the stress kinase p38 and cellular upake *via* endocytosis (Häcker *et al.*, 1998). The receptor for this process has yet to be identified.

Langerhans' cells can be activated by a variety of chemical stimuli, and are known to be involved in contact sensitivity. Recently it has also been shown that monocyte-derived DC (MDDC) are responsive to a variety of chemical stimuli. The responses are typical of maturation or the induction of apoptosis, depending on the chemical (Manome *et al.*, 1999).

Clinical trials frequently rely on the supernatant of cultured monocytes (monocyte-conditioned medium) to mature DC. However, in addition to stimulatory components, this supernatant contains unknown factors, some of which may be inhibitory either of DC or their precursors. It has, therefore, been suggested that a cocktail containing TNF-α, IL-1-β, IL-6 and prostaglandin (PG) E_2 (Jonuleit *et al.*, 1997; Neumann *et al.*, 2000) would replace this.

1.1.3.5.1.2 DC-T interdependence and mutual activation

In vivo the interdependence of DC and T cells can readily be demonstrated: i) migration of DC from the blood to the spleen is disturbed in nude mice but can be reconstituted by injecting T cells (Kupiec-Weglinski *et al.*, 1988); ii) the antigen presentation capacity of Langerhans' cells in athymic nude mice is reduced but can be restored by thymus transplantation (Grabbe *et al.*, 1993); iii) ablation of DC causes developmental defects of thymocytes leading to severe thymic atrophy (Solomon *et al.*, 1994). These symbiotic interactions are further illustrated in the maturation of cognate DC and T cells.

Recirculating T cells migrate between the T-cell zones of secondary lymphoid organs (Howard *et al.*, 1972). In the absence of specific antigen recognition, $CD4^+$ T cells are thought to interact transiently with DC leading to colocalisation but not cluster formation (Ingulli *et al.*, 1997). However, in the presence of a specific antigen interaction, T cells up-regulate their expression of adhesion molecules, leading to stable binding and sustained TCR signalling (Young *et al.*, 1992).

T cells are found to cluster around DC in all primary responses, and this is an essential process for the induction of T-cell proliferation (Inaba & Steinman, 1986) preceding the activation of T cells (Austyn *et al.*, 1988). The clustering of DC is aided by their relative lack of CD43 expression. In this manner, DC are distinct from monocytes and macrophages which express this highly negatively charged molecule, thus inhibiting cluster formation through the increased electrostatic repulsion of their membranes (Enger *et al.*, 1992). Clusters of proliferating T cells are found in proximity to DC within the T-cell zones of secondary lymphoid organs, following injection of superantigens or allogeneic cells (Luther *et al.*, 1997; Kudo *et al.*, 1997).

DC induce the expression of IL-2R on T cells, and are thus capable of controlling the proliferation of the T cells through their release of, and responsiveness to IL-2 (Austyn *et al.*, 1983). In addition, DC can induce T cells to release B-cell-stimulating factors by a mechanism that is dependent upon IL-2 (Inaba *et al.*, 1983). *In vivo* cluster formation coincides with IL-2 production leading to the differentiation of T cells into effector cells and the induction of T cell proliferation (Ingulli *et al.*, 1997).

Naïve T cells are dependent upon co-stimulation through CD28 for activation (Harding *et al.*, 1992), and in the course of activation transiently express CD40L (CD154). CD40-CD40L crosslinking further promotes maturation of DC until these interactions are firmly established (Caux *et al.*, 1994).

Activated and memory T cells promote the survival of DC by interactions between TNF-related activation-induced cytokine (TRANCE) receptors on the surface of the DC and members of the TNF family of proteins on the surface of the T cells (Wong *et al.*, 1997). TRANCE-receptor expression is restricted to mature DC where it is highly expressed. TRANCE induces DC to form densely packed clusters, and has been shown to enhance survival of a murine DC model and of human monocyte-derived DC (MDDC) (Wong *et al.*, 1997).

In the absence of antigen recognition mature DC would not receive survival signals and would, therefore, apoptose by default. TRANCE and CD40L promote the survival of mature DC through increased expression of Bcl-x_L (Wong *et al.*, 1997). CD40L also up-regulates Bcl-2 in human DC derived from $CD34^+$ progenitors and concomitantly, renders the DC virtually resistant to Fas-induced apoptosis (Bjorck *et al.*, 1997).

Production of IL-12 by DC appears to be dependent selectively upon CD40L (Cella *et al.*, 1996). It may, therefore, be possible for T cells that express CD40L to stimulate the activity of neighbouring T cells through the local activation of DC (Cella et al '96). Ligation of MHC II can also directly signal the production of IL-12 by DC (Koch *et al.*, 1996).

1.1.3.5.2 Inhibition of DC Activation

In addition to these putative activation signals, it is becoming increasingly clear that a number of different mechanisms may exist that are able to either counteract or inhibit the activation of DC. Examples of these include the recent suggestions that vaccinia virus (Engelmayer *et al.*, 1999) and IL-10 (Buelens *et al.*, 1997) may exert immunosuppressive effects by inhibiting the maturation of DC.

1.1.3.5.2.1 NK cells as inhibitors of DC maturation

Immature MDDC are particularly susceptible to lysis by NK cells, but the molecular mechanisms involved in this interaction have yet to be resolved (Wilson *et al.*, 1999). It has been speculated that NK cells and DC could converse in an opposing fashion to either initiate or prevent an immune response (Shah *et al.*, 1985). Certainly, NK cells are known to affect the outcome of adaptive immune responses, and their role in this has been the subject of a recent review (Kos, 1998). It is also clear that DC are able to enhance the cytotoxicity and production of IFN-γ by NK cells (Kitamura *et al.*, 1999).

1.1.3.5.2.2 Glucocorticoids as inhibitors of DC activation

Glucocorticoids are known to modulate a variety of immune responses. However, their precise mode of action has yet to be resolved. The main glucocorticoid in human plasma is cortisol, secretion of which is rapidly increased by almost any stress, whether physical or neurogenic and acts to reduce inflammatory responses. Glucocorticoids are also widely given as anti-inflammatory and immunosuppressive agents, the beneficial effects of which are traditionally attributed to down-regulation of cytokine expression by monocytes/macrophages and T cells (Boumpas *et al.*, 1991).

It has recently been suggested that the immunosuppressive actions of glucocorticoids may be through the inhibition of DC maturation. Indeed, it has been demonstrated glucocorticoids can inhibit the maturation of DC by TNF-α and CD40L (Piemonti *et al.*, 1999). A possible mechanism for the inhibition could be the increased synthesis of IκBα, and thus decreasing the translocation of NF-κB to the nucleus (Auphan *et al.*, 1995). It has also been shown that the glucocorticoid, dexamethasone, can inhibit the development of splenic DC in mice (Moser *et al.*, 1995).

1.1.3.6 Signalling mechanisms in DC

1.1.3.6.1 Role of NF-κB pathway in DC

Once the DC have been activated, the transcription factor that has been examined in most detail in DC is nuclear factor (NF)-κB. This is ubiquitous in eukaryotic cells and activated as part of the cellular response to oxidative stresses (Remacle *et al.*, 1995), as well as to many pathogenic and pro-inflammatory stimuli including viral infections, LPS, ionising radiation, IL-1 and TNF-α (Baeuerle, 1991). The NF-κB complex is composed of two proteins, one member of which is derived from the NFKB-1/-2 family (also known as p50/p52), and the other from the REL/RELA/RELB family (also known as p65). NF-κB is transcriptionally active as either a heterodimer or a p50 homodimer (Nabel & Verma, 1993).

In the inactive state NF-κB is retained in the cytosol bound to an inhibitory protein, IκB, which is phosphorylated, cleaved and degraded upon stimulation. NF-κB then rapidly translocates to the nucleus where p50 binds to the κB motif of promotors and enhancers, initiating transcription (Powis *et al.*, 1997). Blocking the translocation of NF-κB to the nucleus has been shown to inhibit DC maturation by LPS (Rescigno *et al.*, 1998). NF-κB plays a central role in inflammation and acute-phase responses (Schreck & Baeuerle, 1991): serving not only as a responder to a variety of stimuli, but also to induce the transcription of genes encoding a variety of cytokines, cytokine-receptors, cell adhesion molecules, acute-phase proteins and growth factors (Sen & Packer, 1996). The nature of the response varies depending on the cell type investigated but is always part of a protective response (Remacle *et al.*, 1995).

DC contain high levels of all known Rel family members (Granelli-Piperno *et al.*, 1995). During the differentiation of monocytes to DC, RelB is the only member of the Rel family of proteins that is strongly up regulated; however, this

also occurs in the development of macrophages and is, therefore, not a unique feature of DC (Neumann *et al.*, 2000).

RelB deficient mice show a selective loss of myeloid DC with a lack of mature DC in lymphoid organs but still develop Langerhans' cells, and lymphoid DC within the thymus (Wu *et al.*, 1998). This emphasises the notion that the predominant role of NF-κB may be related to maturation and not generation of DC (Burkly *et al.*, 1995). It has further been suggested that the activation of NF-κB may represent a general mechanism for DC maturation (Rescigno *et al.*, 1998).

However, the role of NF-κB in DC is more complex than this. For example, Bcl-3 is an inhibitor of NF-κB, and has also been shown to be up-regulated upon maturation of DC, suggesting a role in either the maturation process or the function of mature DC (Neumann *et al.*, 2000). Furthermore, a low level of active nuclear NF-κB, has been suggested as a possible mechanism for the growth factor-dependent survival of these cells.

These observations may prove to be important in the future if inhibitors of NF-κB are given as immunosuppressive agents.

1.1.3.6.2 Role of MAP Kinases in DC

A group of enzymes known as the mitogen activated protein (MAP) kinases, or stress kinases, play an important contribution to DC maturation. MAP kinases are divided into three groups, namely extracellular signal regulated kinase (ERK), Jun NH_2 terminal kinase (JNK) and p38. They can be stimulated by a number of extracellular ligands as well as cellular stress. LPS is known to activate all three MAP kinases in murine macrophages (Hambleton *et al.*, 1996; Han *et al.*, 1994; Weinstein *et al.*, 1992) but predominantly activates ERK in murine models of immature DC (Rescigno *et al.*, 1998). Inhibitors of ERK are unable to prevent the maturation of DC induced by LPS, but lead to the premature apoptosis of mature DC. It is thus suggested that maturation signals

act through at least two distinct pathways to regulate the maturation (*via* NF-κB) and survival (*via* ERK) of DC (Rescigno *et al.*, 1998).

It has been shown that DC may be dependent partially upon p38 activation for their production of IL-12 (Lu *et al.*, 1999) and the expression of CD80, CD83 and CD86 (Ardeshna *et al.*, 2000). However, the increased expression of CD40 and HLA-DR on DC, following treatment with LPS, may be independent of p38 activation (Ardeshna *et al.*, 2000). The potential functions of JNK in DC have yet to be investigated specifically.

1.1.4 Role of DC in chronic inflammation

Much of the emphasis in most DC studies has been upon the acute inflammatory system. However, it is important to recognise that these cells are implicated in the formation of chronic inflammatory milieus. Thus, at sites of chronic inflammation, TNF-α and lymphotoxin frequently lead to ectopic lymphoid neogenesis, and result in localised areas of activated T cells (Zinkernagel, 1996). DC have been shown to be involved in this type of lymphoid neogenesis and in the maintenance of chronic inflammation (Ludewig *et al.*, 1998).

It has been speculated that one way to maintain chronic inflammation would be if mature DC fail to emigrate from their sites of activation. The mechanism proposed relies on the release of chemokines from mature DC, such as Epstein-Barr virus-induced molecule 1 ligand chemokine (ELC), thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC), which are known to attract activated T cells and DC through CCR7 and CCR4. DC could subsequently receive additional maturation and survival signals through the actions of CD40L and TRANCE. In addition, resting T cells could be activated, in the absence of a specific antigen, by a combination of cytokines, thus perpetuating the inflammatory process (Sallusto & Lanzavecchia, 1999).

1.1.5 DC-based immunotherapy

Interest in DC-based immunotherapy was stimulated by the realisation that this may prove particularly useful in the treatment of many cancers. This suggestion is, in part, derived from the finding that many tumours avoid recognition through inhibiting the maturation of DC (Hart *et al.*, 1999). In addition, it is known that many human tumours express tumour-associated antigens (TAA), which may be recognised by host lymphocytes and, therefore, potentially stimulated by activated DC.

There are essentially two different approaches that may prove to be of use in DC-based immunotherapy. The first, and perhaps the simplest, is to administer factors that promote the differentiation of DC *in vivo*; in effect to increase antigen surveillance and presentation. The most promising data indicating the potential of this approach has come from the finding that the haemopoietic growth factor Flt-3 increases the production of functionally active DC in mice (Shurin *et al.*, 1997). Flt-3 has further been shown to promote tumour regression *in vivo* (Lynch *et al.*, 1997). The second, and more complicated approach, is to culture DC *ex vivo*, load them with specific antigens and then infuse these back into the patient, with the aim of initiating specific immune responses.

Animal models have shown that, in the presence of costimulation, tumour specific DC can indeed reverse T-cell anergy and induce tumour rejection (Avigan, 1999). Moreover, early clinical trials have shown that DC based vaccines can stimulate anti-tumour immune responses, with tumour regression being observed in patients with non-Hodgkin's lymphoma and melanoma (Timmerman & Levy, 1999).

DC-based immunotherapy may also prove useful in the treatment of persistent viral diseases, such as viral hepatitis (hepatitis B virus and hepatitis C virus), and in the maintenance of tolerance, for the treatment of autoimmune diseases and allograft rejection (Stockwin *et al.*, 2000).

1.2 Introduction to Oxidative Stress

Thus far, the main emphasis of DC research has been on the physiology of immature and mature DC, and on their responses to cytokines and LPS, whilst relatively little attention has focused on the range of chemical and micro-environmental stimuli that may activate DC. Perhaps the most noteworthy of such possible research is based on the hypothesis that DC might be activated by free radicals (Ibrahim *et al.*, 1995) and that this may be a common denominator pathway for other signals. It is thus suggested that "oxidative stress" may constitute the key "danger" signal to influence DC.

1.2.1 Terminology of "oxidative stress"

The term, "redox state," is used as a convenient and arbitrary indication of the degree to which a system is oxidised or reduced. Equally the term, "oxidative stress," can be defined as an imbalance between the pro- and anti-oxidant influences at a particular site (McQuaid & Keenan, 1997).

Free radicals are defined as molecular species that contain at least one free unpaired electron. Naturally occurring free radicals are typically oxygen or nitrogen based (Lander, 1997). Owing to the reactivity of these species and the readiness with which their precise nature interchanges, the terminology reactive oxygen species (ROS) and reactive nitrogen species (RNS) are employed, depending on the origin of the species, irrespective of whether they are radicals or even derived from radicals.

Free radicals can be electrically neutral, or retain a positive or negative charge. They can be formed by three different mechanisms: i) homolytic fission of a covalent bond; ii) the addition of a single electron; iii) loss of a single electron.

Two of the electrons in molecular oxygen are unpaired; thus, oxygen may be considered a di-radical. Reactions are, therefore, promoted between oxygen and other free radicals. In the context of free radical biology this concept is usually

ignored and oxygen is considered as a normal molecule with an increased susceptibility to accept single electrons or add to free radicals (Deby & Goutier, 1990).

RNS are thought to regulate a number of important physiological functions, as recently reviewed (Bogdan *et al.*, 2000), and will not form part of this discussion.

1.2.2 Oxidative Stress pathways

The origin of oxidative stress can be traced back to about 2 billion years ago. At this time molecular oxygen began to accumulate in the atmosphere as a waste product of photosynthesis by cyanobacteria. This resulted in the adaptation of electron-transport chains allowing the transfer of electrons from NADH to O_2, thus forming efficient aerobic metabolism based on a readily available substrate (Alberts, 1994). However, a consequence of this was the release of superoxide radicals (O_2^-) from the flavoprotein, NADH dehydrogenase and ubiquinone-cytochrome c reductase regions of the mitochondrial respiratory chain (Turrens & Boveris, 1980). Evolution has, therefore, accepted the potential consequences of radical production in exchange for aerobic respiration.

To defend against the adverse effects of ROS, a complex network of anti-oxidants has evolved, both to eliminate ROS and to prevent their formation. Evolution has also sought beneficial ways of utilising the toxic effects of ROS to protect host organisms from infectious agents and malignant tumours. The most well known and characterised of these mechanisms is the respiratory burst, whereby phagocytic cells release superoxide, through activation of the enzyme NADPH oxidase, located on their plasma membrane. Phagocytic cells release ROS as part of a co-ordinated host-defence mechanism. However, other cells have also been shown constitutively to release superoxide and hydrogen peroxide (Burdon, 1995), raising the question of a potential physiological significance of these species: this has yet to be demonstrated and identified.

A number of fungi, protozoa and bacteria have evolved anti-oxidant defences to counter these oxidative killing mechanisms (Mehlotra, 1996). For example: *Mycobacterium tuberculosis* express a low level of catalase which strongly protects the bacilli against oxidative killing by H_2O_2 (Manca *et al.*, 1999). *Salmonella typhimurium* expresses periplasmic copper/zinc superoxide dismutase (SOD), thus protecting against oxidative attack by superoxide (De Goote *et al.*, 1997); and exposure of *Escherichia coli* to superoxide induces genes encoding Mn-SOD and the oxidative DNA repair enzyme endonuclease IV. The bacterial responses to oxidative stress may be selective, with different genes activated upon exposure to H_2O_2 and O_2^- (Demple, 1997).

1.2.3 Detrimental actions of ROS

Louis Pasteur first illustrated the toxicity of molecular oxygen in the 19th century. This was demonstrated by the rapid rate at which many anaerobic bacteria are killed upon exposure to air and is a direct consequence of random oxidation of biological molecules induced by the presence of oxygen (Alberts *et al.*, 1994).

The most vulnerable components of a cell to oxidative stress are, unsaturated fatty acids, proteins and DNA (Kehrer, 1993). One consequence of the oxidation of unsaturated fatty acids is the formation of lipid peroxides (peroxidation reaction), which in turn reduces their hydrophobicity and, therefore, the fluidity and integrity of membranes. Oxidation of proteins increases their rate of catabolism and, therefore, owing to the central role of proteins in virtually all biological processes, has major effects on normal cellular functions. Finally, oxidation of bases within DNA can result in point mutations, mutant protein formation (Kehrer, 1993) and mutagenic lesions (Demple & Harrison, 1994).

Numerous repair mechanisms have evolved to prevent the accumulation of damaged cellular products. Such as specific enzymes that repair oxidatively damaged nucleic acids, and proteolytic enzymes that remove damaged proteins. Oxidised lipids are acted upon by lipases, peroxidases and acyl transferases, which aid in their elimination.

Most cellular lipids are closely packed thus promoting molecular interactions and the propagation of chain reactions. The rate of lipid peroxidation increases with both the content of polyunsaturated lipids and the degree of fatty acyl unsaturation (North *et al.*, 1994). Lipid peroxidation is particularly detrimental owing to perpetuation of this chain reaction and the production of reactive aldehydes, which can lead to the indirect damage of other cell components at distant sites (Comporti, 1989).

It should be noted that much of the tissue damage associated with inflammation can be regarded as a direct consequence of oxidative stress, that can be prevented by anti-oxidants.

1.2.4 Production of ROS

Cellular production of free radicals is usually as a result of electron transfer reactions, but may also result from other factors such as ionising radiation.

The best-characterised enzyme capable of producing ROS upon ligand stimulation is NADPH oxidase. This enzyme complex relies on NADPH as the electron donor and acts to convert molecular oxygen to superoxide.

$$NADPH + H^+ + 2O_2 \rightarrow NADP^+ + 2H^+ + 2O_2^-$$

In addition to phagocytic cells, NADPH oxidase has also been identified in smooth muscle cells (Griendling *et al.*, 1994), chondrocytes (Lo & Cruz, 1995), and kidney epithelial cells (Cui & Donglas, 1997).

A ubiquitous eukaryotic source of ROS is the electron transport chain where it is estimated that 2-4% of the oxygen consumed is reduced to superoxide and not water (Boveris & Chance, 1997). Superoxide is also released from the endoplasmic reticulum (ER) where electrons are found to leak from NADPH cytochrome P450 reductase (Cross & Jones, 1991). Additional sources of ROS

include hypoxanthane/xanthane oxidase, lipoxygenase, cycloxygenase, and peroxisomal oxidases (Egan *et al.*, 1976; Boveris *et al.*, 1972).

1.2.5 Chemistry of ROS

Supeoxide is produced by the single electron reduction of oxygen.

$$O_2 + e^- \rightarrow O_2^-$$

However, molecular oxygen may also undergo two-electron reduction, through the action of oxidases, to liberate H_2O_2. Examples of such enzymes are xanthane and glucose oxidase (Favier *et al.*, 1995).

$$O_2 + 2e^- + 2H^+ \rightarrow H_2O_2$$

Hydrogen peroxide may also be formed from water, by the energy from ionising radiation, or by the reduction of superoxide anions, in the presence of metal ions.

$$O_2^- + Fe^{2+} + 2H^+ \rightarrow H_2O_2 + Fe^{3+}$$

In vivo, H_2O_2 is predominantly formed from the reduction of O_2^- catalysed by superoxide dismutase (SOD). This involves one-electron reduction and represents a defensive mechanism against O_2^--induced tissue damage. Hydrogen peroxide may, in turn, be reduced to form hydroxyl radicals in the presence of u.v. light or when catalysed by metal ions, as given by the Fenton reaction.

$$O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO^- + HO^\bullet \quad \text{(Fenton)}$$

$$O_2^- + H_2O_2 \rightarrow HO^- + HO^\bullet + O_2 \quad \text{(Haber-Weiss)}$$

(Favier *et al.*, 1995)

The rate constant of the Harber-Weiss reaction is much lower than that of the dismutation reaction, implying that this reaction does not play an important

contribution to oxidative stress *in vivo*. The Fenton reaction, which is based on the redox cycling ability of iron, is, therefore, the predominant reaction *in vivo* (Trenam *et al.*, 1992).

Superoxide is a poor oxidising agent, the main direct targets of which are proteins containing iron-sulphur clusters (Fridovich, 1986). However, superoxide reacts rapidly with nitric oxide to yield peroxynitrite ($ONOO^-$) and peroxynitrous acid (ONOOH), the latter being a strong oxidant with nitrating properties. Peroxynitrous acid is released from activated macrophages, neutrophils and endothelial cells (Ischiropoulos *et al.*, 1992).

Hydrogen peroxide reacts directly with very few targets. For example, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) is the only enzyme of the glycolytic pathway oxidised by H_2O_2 (Schräufstatter *et al.*, 1990). However, in the presence of transition metals, H_2O_2 may form hydroxyl radicals ($OH^\bullet$) the reactivity of which causes proteolysis and alteration to both the secondary and tertiary structure of proteins: this also correlates with increased protein hydrophobicity (Pacifici & Davies, 1990).

Most of the detrimental effects associated with oxidative stress, *in vivo*, are believed to result ultimately from the production of hydroxyl radicals (Nakamura *et al.*, 1997). Hydroxyl radicals are indiscriminate oxidants, reacting almost instantaneously at a rate that approaches their diffusion limit (Saran & Boveris, 1994).

1.2.6 Defence mechanisms against oxidative stress

1.2.6.1 Anti-oxidants

To defend against the adverse effects of ROS, cells utilise a number of different mechanisms to both eliminate ROS once they are formed and prevent their formation. Examples of the former include a number of enzymes (SOD, catalase and glutathione peroxidase) (Chance *et al.*, 1979) and small molecules

(glutathione, and vitamins A, C, and E). Whilst examples of the latter include transferrin and caeruloplasmin, which bind to transition metals thereby preventing catalysis by the Fenton reaction. The importance of exogenous anti-oxidants in the health of old age is becoming increasingly evident (Halliwell, 1996a). In addition to these specialised systems, albumin is also able to chelate copper ions (Halliwell, 1988) and has been shown to have anti-oxidant properties.

Despite the fact that catalase and SOD are localised to the sites of production of the ROS that they have evolved to eliminate, cells are, nevertheless, found to have a resting concentration of O_2^- and H_2O_2. In the cytosol these are 10^{-12}-10^{-11}M and 10^{-9}-10^{-7}M respectively (Chance *et al.*, 1979). However, upon stimulation, the concentration of H_2O_2 may reach 10^{-4}M at the surface of macrophages (Dröge *et al.*, 1994).

SOD and catalase form consecutive reactions with a number of similarities. For example they both involve oxidation and reduction of transition metals (although in different orders), an intermediate product and, show a linear increase in enzyme activity over a wide range of substrate concentrations.

1.2.6.1.1 Superoxide dismutase

SOD is the only enzyme with a free radical as a substrate. The catalysed reaction is known as dismutation since free radical reductants produce non-radical products. Thus, the stability of O_2^- is increased through generation of its dismutated product (H_2O_2).

Copper and zinc containing forms of SOD are found in the cytosol and mitochondrial intermembrane space of eukaryotic cells. These are sensitive to high concentrations of cyanide. A manganese containing form of SOD, which is insensitive to cyanide, is located in the matrix space of mitochondria.

Spontaneous dismutation of superoxide occurs 10^4 fold slower at physiological pH as a result of the low H^+ concentration (Marin & Rodriguez-Martinez, 1995). This is, therefore, not thought to play a major contribution *in vivo*. The consecutive reactions of the copper containing form of SOD are shown below.

$$SOD\text{-}Cu^{2+} + O_2^- \rightarrow SOD\text{-}Cu^{+} + O_2$$

$$SOD\text{-}Cu^{+} + O_2^- + 2H^+ \rightarrow SOD\text{-}Cu^{2+} + H_2O_2$$

Thus, the overall reaction is:

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Despite the fact that SOD acts to increase the concentration of H_2O_2, it also inhibits the release of Fe^{2+} from ferritin (Deby & Goutier, 1990). The expected increase in production of hydroxyl radicals is thereby prevented, since the predominant physiological sources of iron ions are ferritin and haemoglobin.

1.2.6.1.2 Catalase

Catalase is compartmentalised in peroxisomes where most oxidases are located, thus limiting the release of H_2O_2 from these sites. Peroxisomes do not possess any SOD activity and are thought to represent a vestigial mechanism for the elimination of oxygen from primitive eukaryotic cells. The evolution of mitochondria, and their ability to couple this reaction with oxidative phosphorylation, is thought to have left the function of the peroxisomes simply to fulfil those not taken over by the mitochondria.

Catalase is involved in two reaction pathways that have completely different kinetic characteristics. These are the catalytic and peroxidatic pathways. The catalytic pathway results in the rapid reduction of H_2O_2 only, whilst the peroxidative pathway proceeds at a slower rate but is important in the

detoxification of various substances (including phenols and alcohol) by the liver and kidneys. This pathway also has an essential role in the synthesis of thyroid hormones (Chance *et al.*, 1979). An additional reducing substrate is required for the peroxidative pathway and does not result in the formation of molecular oxygen (Marin & Rodriguez-Martinez, 1995).

Both pathways require the formation of an intermediate oxidation product, known as compound I.

$$\text{catalase-Fe}^{3+} + H_2O_2 \rightarrow \text{compound I}$$

$$\text{compound I} + H_2O_2 \rightarrow \text{catalase-Fe}^{3+} + 2H_2O + O_2$$

$$\text{compound I} + AH_2 \rightarrow \text{catalase-Fe}^{3+} + 2H_2O + A$$

The protective effect of catalase, on oxidative stress, is thought to be an indirect result of a lower $OH^{\bullet}$ concentration (Chance *et al.*, 1979).

1.2.6.1.3 GSH

GSH (L-γ-glutamyl-L-cysteinyl-glycine) is ubiquitous in eukaryotic cells. It is the substrate for the reduction of peroxides by glutathione peroxidase, but may also spontaneously oxidise and thereby detoxify H_2O_2 (Marin & Rodriguez-Martinez, 1995).

The sulphydryl residue of cysteine within glutathione is responsible for most of the actions of this molecule. Oxidation of this residue results in the production of glutathione disulphide (GSSG), which may be reduced back to GSH through the action of glutathione reductase. The intracellular concentration of GSH is between 1 and 10nM. This is considerably greater than that of GSSG, thus maintaining the intracellular environment in a highly reduced state, compared to the extracellular milieu. The ratio of GSH:GSSG varies between different

intracellular compartments being highest in the nucleus (100:1) and lowest in the endoplasmic reticulum.

A sequence of ATP-dependent reactions catalysed by γ-glutamylcysteine synthetase and GSH synthetase are required for the synthesis of GSH. GSH is a negative feedback inhibitor of this process.

Most cells rely on the availability of L-cysteine to determine the intracellular level of GSH. L-cysteine is, in turn, derived from L-cystine, a disulphide-linked oxidised dimer found within blood plasma. L-cystine is transported into the cell and accounts for 90% of extracellular cysteine (Sen, 1998). It is of interest to note that rate of transport of L-cystine into T cells is lower than other cells (Gmünder *et al.*, 1991), thus decreasing their supply of GSH and consequently increasing their susceptibility to changes in redox state.

1.2.6.1.4 Thioredoxin

Thioredoxin catalyses the reduction of disulphide bonds, and thus, determines the oxidation state of protein thiols. The catalytic site of this protein contains the conserved sequence Try-**Cys**-Gly-Pro-**Cys**-Lys. These cysteine residues protrude from the protein, thus promoting their reversible oxidation *via* the formation, and reduction, of disulphide bonds. The reduction of these bonds is dependent upon NADPH and the selenoenzyme thioredoxin reductase (Sen, 1998). The human form of thioredoxin is also known as adult T-cell leukemia-derived factor (ADF) (Nakamura *et al.*, 1997) and, among its multiple functions, may represent an important costimulus for the immune system (Schenk *et al.*, 1996).

Thioredoxin has been shown to protect endothelial cells against oxidative stress induced by the activation of neutrophils (Nakamura *et al.*, 1994). It acts directly to detoxify H_2O_2 (Spector *et al.*, 1988) and as a free radical scavenger (Schallreuter & Wood, 1986). Moreover, extracellular thioredoxin stimulates the uptake of cystine, a glutathione precursor, into cells, thus potentiating its own

anti-oxidant actions. In contrast to these anti-inflammatory functions, thioredoxin is a chemoattractant for neutrophils, monocytes, and T cells (Bertini *et al.*, 1999).

Oxidative stresses promptly induce the translocation of thioredoxin into the nucleus, thus creating favourable conditions for the binding of NF-κB and AP-1 to DNA (Nakamura *et al.*, 1997). This translocation represents a possible means by which signals may be sent to the nucleus and may also be involved directly in the translocation of NF-κB into the nucleus (Hayashi *et al.*, 1993). It is interesting to note that the levels of thioredoxin increase after exposure to H_2O_2 and u.v. light, which are associated with increased NF-κB activity (Sachi *et al.*, 1995).

Within the nucleus thioredoxin associates and regulates the nuclear redox factor Ref-1. This in turn reduces specific cysteine residues within c-jun and c-fos, thus promoting their binding to DNA (Xanthoudakis & Curran, 1992). Thus, despite the fact that oxidation is required for the initial activation, the reduced state is required for their binding to DNA.

In the absence of Ref-1, HeLa cells are hypersensitive to killing by a variety of oxidants indicating that Ref-1 may represent a fundamental link between oxidative stress and the regulation of transcription (Dalton *et al.*, 1999). In addition, Ref-1 plays an important role in DNA repair *via* its apurinic/apyrimidinic endonuclease activity (Nakamura *et al.*, 1997).

A number of potentially important findings indicate a link between thioredoxin and the competence of the immune system. For example, the immunosuppressants cyclosporin A and FK506 are found to reduce the levels of thioredoxin (Furuke *et al.*, 1995). Thioredoxin levels are also significantly lower in DC and activated macrophages of lymph nodes (where thioredoxin is usually highly expressed) in patients with AIDS and AIDS-related complex (Masutani *et al.*, 1992). Finally, overproduction of thioredoxin in human T lymphotropic virus (HTLV)-1 transformed T cell lines is thought to cause the constitutive activation

of NF-κB (Sen & Packer, 1996), which may be mediated through the induction of IL-2R expression (Teshigawara *et al.*, 1985).

1.2.6.1.5 Small anti-oxidants

In addition to the above anti-oxidants, a number of smaller molecules are found to exert anti-oxidant properties.

α-tocopherol (vitamin E) is probably the most important free radical scavenger in cell membranes. However, the relative physiological importance of additional lipid soluble anti-oxidants, e.g. ubiquinol, have yet to be resolved.

Ascorbic acid (vitamin C) is an important water-soluble free radical scavenger that acts both intracellularly and within plasma. Ascorbic acid has been shown to confer part of its protective response by sparing the endogenous cellular anti-oxidant capacity (Siow *et al.*, 1998). Plasma also contains various additional anti-oxidants, including uric acid.

1.2.6.2 Repair Mechanisms

In the absence of specific radical scavengers, many cells are capable of excising lipid hydroperoxides (LOOH), by a mechanism that has yet to be fully characterised. To date, three enzymes have been shown to have peroxidatic activity and are thus implicated in the repair of LOOH lesions. These are glutathione-peroxidase (GPX), phospholipid hydroperoxide glutathione peroxidase (PHGPX), and non-seleno GSH-S-transferase type α (GSTα). GPX and PHGPX both contain selenocysteine active sites that are involved in two-electron reduction of peroxides to redox-inert alcohols. The finding that Se-deficiency leads to the accumulation of LOOH, and accelerated cell death following an oxidative stress, highlights the importance of these enzymes.

Under physiological conditions, GPX is thought to act primarily on polar oxidants e.g., H_2O_2 and fatty acid hydroperoxides, whilst PGHPX acts on a broad

range of lower polarity substrates. PGHPX is, thus, more efficient than GPX at removing phospholipid, cholesterol, and cholesterol ester hydroperoxides (Thomas *et al.*, 1990a; Thomas *et al.*, 1990b).

Many oxidising agents are known to induce apoptosis in a variety of cell types. The involvement of lipid peroxidation in many of these stress-induced signalling pathways is inferred by the finding that peroxidases are able to suppress the induction of apoptosis (Ramakrishnan *et al.*, 1996).

1.2.7 The putative role of ROS in signalling

It has also been shown recently that H_2O_2 can confer a cellular protective response against further oxidative stress, provided that the initial level of stress is below the threshold capacity of the cells (Gardner *et al.*, 1997); beyond which cellular death ensues. The protective response is mediated by a compensatory elevation in the production of anti-oxidants. Stress may also follow normal metabolic reactions when an insufficient oxygen supply leads to the formation of oxygen free radicals. It is, therefore, evident that oxidative stress can have a positive influence on cellular processes.

In addition, accumulating evidence suggests that ROS may represent a novel mechanism of cellular regulation, as hypothesised by Saran and Bors (1989). The physiological basis of this mechanism is thought to be the strategic exposure (Lander *et al.*, 1996), or sequence (Dröge *et al.*, 1994), of redox sensitive cysteine residues, thus provoking their reversible oxidation. Therefore, the emerging concept is that ROS are not solely to be considered as cell adversaries, but may have an essential physiological role as signalling molecules.

The actions of ROS may be mediated directly through alterations in protein conformation, as in their action on kinases and transcription factors, which frequently dimerise or form multimers, resulting in neutralisation of their function (Adler *et al.*, 1999a). Alternatively, ROS may reversibly oxidise redox-sensitive proteins through cysteine rich regions.

This putative role of the redox state in regulation of cellular functions is analogous to phosphorylation except that the modification occurs through the oxidation of cysteine residues, as opposed to phosphorylation of specific serine or tyrosine residues (Finkl, 1998). Such regulation occurs at concentrations well below that required to inflict oxidative damage.

Following oxidation of cysteine residues, the formation of disulphide bonds may lead to the exposure of hydrophobic domains and protein destabilisation. This mechanism has been proposed to account for the heat shock response to oxidative stress (McDuffee *et al.*, 1997). However, the formation of intra- and intermolecular disulphide bonds is not the only mechanism by which oxidative signals may be transduced. The binding of Jun and Fos proteins to DNA requires a single conserved cysteins residue (Lys-Cys-Arg) to be in the reduced state. Thus, oxidative regulation is thought to result from the reversible oxidation of this cysteine residue to either a sulfenic (RSOH) or sulfinic (RSO_2H) acid (Abate *et al.*, 1990).

Oxidative stress may also induce indirect effects on cellular function *via* the action of oxidised lipids. An example of this is the increase in synthesis of glutathione by human vascular smooth muscle cells following exposure to oxidised LDL (Siow *et al.*, 1998).

1.2.7.1 The putative role of ROS as second messengers

Over the past decade, numerous reports have suggested that ROS may function as intracellular second messengers. Such a conclusion is based on the observation that a diverse range of anti-oxidants have been reported to inhibit the activation of NF-κB by an equally diverse set of stimuli, such as phorbol esters, TNF-α and H_2O_2. It has been suggested that a single ROS dependent step is involved in the activation of this transcription factor, and thus, that ROS may act as specific signalling molecules in the form of second messengers (Nakamura *et al.*, 1997).

If one assumes that second messengers have, by nature, a set of characteristic properties, then the feasibility of ROS as second messengers can be examined by comparison to known second messengers, such as Ca^{2+} and cAMP. Analysis of the basic properties of second messengers reveals that they are all ubiquitous, small, highly diffusible molecules that can readily be synthesised and degraded. These are consistent with the properties of ROS.

In addition, if one examines the production of Ca^{2+} or cAMP, then it becomes apparent that a highly efficient mechanism must exist for the generation of second messengers. It has been suggested that NADPH oxidase may be such an enzyme, since it catalyses the formation of O_2^-; has been identified in a number of cell types (not only phagocytic cells); and increases the intracellular ROS concentration upon activation.

Further, the activation of second messengers by extracellular signals is found to be a complex process, often involving guanine nucleotide binding proteins, as in the case of adenylate cyclase activation. The proposed mechanism for the activation of NADPH oxidase is equally complex involving the translocation of several cytosolic proteins (i.e. $gp40^{phox}$, $gp47^{phox}$, $gp67^{phox}$ and Rac2) to a membrane complex (comprising of $gp91^{phox}$, $gp22^{phox}$ and Rap1a).

However, this is not the whole answer since NADPH oxidase is not ubiquitous. In addition, this enzyme is under the control of protein kinase (PK) C. Thus phorbol esters (e.g. PMA, phorbol 12-myristate 13-acetate) can activate NADPH oxidase *via* PKC and result in the production of O_2^-; but despite this TNF-α can activate NF-κB by a mechanism that is independent of PKC (Meichle *et al.*, 1990).

It has also been suggested that signals may exists between the plasma membrane and either the mitochondria or peroxisomes, to promote alterations in the redox state. Such a pathway has been shown to exist in some but not all cell types. For example, TNF-α can stimulate the production of superoxide from the mitochondria of L-929 cells, but this does not occur in BHK-12 or HeLa cells

(Hennet *et al.*, 1993). However, lack of a functional mitochondrial electron transport chain, as a consequence of either drug treatment or organelle depletion, results in a significant suppression in the activity of NF-κB (Schulze-Osthoff *et al.*, 1993).

Alternatively, stimuli may increase the level of ROS through inhibition of anti-oxidant enzymes. Evidence for such pathway is derived from the fact that the anti-oxidant, N-acetylcysteine, can inhibit activation of NF-κB induced by protein synthesis inhibitors (Schreck *et al.*, 1991).

To ensure adequate regulation of second messengers, not only is it important to regulate the initiation of the signal, but also its abrogation. In the cases of cAMP and Ca^{2+} efficient mechanisms have evolved for their elimination, such as cAMP phosphodiesterase and Ca^{2+} ATPase pumps respectively. The presence of radical scavenging systems, in addition to the fact that ROS are highly reactive, results in their short half-life, thus, providing a means by which ROS may be eliminated.

The putative mechanism of action of ROS as second messengers is significantly different from that of its counterparts. Traditional second messengers act by means of noncovalent interactions with their targets, whereby, a simple equilibrium is established allowing the degree of activation to be dependent upon the concentration of the second messenger. However, ROS act by oxidising their target proteins. Therefore, to return the system back to its original state ROS, must not only be removed, but the target protein must also be reduced.

Finally, despite the usual specificity of enzyme systems, all known second messengers have a number of different substrates and, thereby, regulate multiple cellular functions. It has been suggested that ROS may act on redox sensitive transcription factors, thus modifying the pattern of gene expression upon activation. This is not to say that ROS only, or even primarily, act on transcription factors to elicit their response, but this may represent one of many putative modes of action.

It can, therefore, be concluded that ROS satisfy many of the criteria required to constitute a novel group of second messengers.

1.2.7.2 Redox Regulation of Gene Transcription

Almost every gene implicated in the cellular response to stress can be regulated through redox-sensitive proteins (Adler *et al.*, 1999a). In addition, several transcription factors are reported to be directly regulated by the cellular redox state. The best characterised of these are NF-κB and AP-1, although other trenscription factors, such as Maf and Nrl, may also be regulate by a similar mechanism (Kerppola & Curran, 1994).

1.2.7.2.1 NF-κB

NF-κB is known to regulate a number of important functions in DC and other cells, as detailed previously. It has been reported that H_2O_2, and other peroxides, are able directly to activate NF-κB and, in this manner, may account for the indirect effects of numerous stimuli. Activation under these circumstances is thought to be specific for peroxides and not mediated through the action of O_2^- (Schmidt *et al.*, 1996).

Proteins of the NF-κB family bind to DNA through their Rel homology domain. Despite the fact that NF-κB is activated by pro-oxidative conditions, the initial sequence of this domain contains a critical cysteine residue that must be in the reduced state to bind DNA (Matthews *et al.*, 1992). The reduced state is maintained by a number of reducing systems including thioredoxin and Ref-1.

Activation of NF-κB is inhibited by both high and low levels of GSSG suggesting that optimal activation occurs at intermediate concentrations of this oxidation product (Dröge *et al.*, 1994).

IκB

There are three known isoforms of IκB, entitled α, β, and γ. IκBα and β target p65 whilst IκBγ may bind to either subunit. The initiating and prerequisite signal for the activation of NF-κB is the phosphorylation of IκB, leading to its subsequant cleavage and allowing translocation of NF-κB into the nucleus (Sen & Packer, 1996).

N-acetylcysteine and the metal ion chelator pyrrolidinedithiocarbamate (PDTC) are able to block the cleavage of IκB induced by H_2O_2, leading to the retention of NF-κB in the cytoplasm. This suggests that the main action of the oxidants may be on the inhibitory subunit (Schreck & Baeuerle, 1991). It is theoretically possible that IκB could selectively be damaged by oxidative stress, subsequently resulting in its release and activation of the transcription factor. Alternatively, IκB may be cleaved by proteases either activated by an oxidant or specific for the oxidised IκB subunit.

Several compounds, including PMA, TNF and LPS (all of which have been implicated in DC activation), have been shown to activate NF-κB by phosphorylating serine residues 32 and 36 of IκB. This implies that all these pathways may converge on a single kinase (Baldwin, 1996) that has not as yet been identified.

1.2.7.2.2 AP-1

AP-1 refers to a family of basic domain/leucine zipper transcription factors formed by the dimerisation of products of the FOS and JUN immediate-early response gene families. These may be transcriptionally active in the Jun-Jun or Jun-Fos forms (Forrest & Curran, 1992). Binding of these complexes to specific cis-acting transcriptional control DNA elements, known as the 12-O-tetradecanoyl phorbol-13-acetate (TPA) response elements (TRE), allows them to regulate the expression of a variety of genes, including many cytokines.

To activate AP-1, Jun and Fos must be phosphorylated. JNK and ERK, respectively (Karin, 1995) mediate this. AP-1 then translocates to the nucleus where a reduced environment aids its binding to DNA. Substitution of critical cysteine residues in both FOS and JUN proteins (cys-154 and cys-272, respectively), for serine results in loss of redox regulation and increased DNA binding (Abate *et al.*, 1990).

Oxidants may activate AP-1 indirectly through the activation of kinases, as reviewed recently (Dalton *et al.*, 1999), or by a mechanism that is dependent on PKC but not oxidative stress (Schreck & Baeuerle, 1991). Oxidant-dependent activation of AP-1 relies on transient increases in intracellular calcium. However, the role of calcium and the radical responsible for this mobilisation remains elusive (Dalton *et al.*, 1999).

Little is currently known about the expression and function of this trancription factor in DC.

1.2.7.3 The regulation of stress kinases by ROS

Oxidation of cysteine residues within proteins induces the formation of intra- and inter-molecular disulphide bonds, resulting in alterations of protein structure and their association with other cellular proteins. The best characterised of such proteins are thioredoxin and glutathione S-transferase (GST), which are known to regulate several stress responsive proteins (Adler *et al.*, 1999a). In the absence of oxidative stress, the stress kinases apoptosis signal-regulated kinase (ASK) 1 and JNK are inhibited through their assosiation with thioredoxin and GSTp, respectively. The inhibitory actions of GSTp on JNK account for its low kinase activity in non-stressed cells, since in the absence of GSTp, cells have a higher resting level of JNK activity (Adler *et al.*, 1999b). Activation of JNK leads to an increase in the expression of GSTp, *via* the phosphorylation of c-jun, thus providing a negative feedback mechanism for the activation of JNK (Ainbinder *et al.*, 1997).

Oxidative stress induces the dissociation of thioredoxin and GSTp from their respective ligands, resulting in their homotypic dimerisation and allowing kinase activity to ensue. ASK1 activates p38 and JNK; substrates of which are key components of the cellular response involved in protection against further oxidative stress.

Oxidative stress has also been shown to activate ERK and p38. This is, in part, thought to be an indirect effect of the inhibition of tyrosine phosphatases (Gabbita *et al.*, 2000).

1.2.7.4 The regulation of tyrosine phosphatases by ROS

Tyrosine phosphatases have been highlighted recently as a potential means by which oxidative stress may influence cellular functions. They are between one and three orders of magnitude more active than tyrosine kinases and share the motif Cys-X_5-Arg in their active site (Fauman & Saper, 1996). The cysteine residue must be in the reduced state to allow enzyme activity, thus providing a mechanism for the modulation of tyrosine phosphorylation by the redox state. Oxidative stress induces the oxidation of these critical cysteine residues leading to inactivation of tyrosine phosphatase activity and allowing the action of tyrosine kinases to transiently predominate. It is interesting to note that this is a selective tyrosine phosphatase response and is not seen with serine/threonine protein phosphatases.

1.2.8 Putative role of ROS in disease pathogenesis

Elevated levels of ROS are implicated in over one hundred diseases. However, free radicals are often associated with disease pathogenesis simply because no other mechanism has yet been elucidated. In many of these conditions, the presence of free radicals may represent a secondary event following trauma or the presence of toxins (Halliwell *et al.*, 1992), rather than a primary event. Notable exceptions are rheumatoid disease (Halliwell *et al.*, 1995) and

inflammatory bowel disease (Grisham *et al.*, 1994), both of which are associated with inappropriate phagocyte activation.

A variety of DNA and RNA viruses are known to be associated with increased formation of ROS (Schwarz, 1996). Such a phenomenon has been studied in most detail with respect to human immunodeficiency virus (HIV)-1, where the virus is thought to increase the rate of apoptosis in T cells. This is preceded by an increase in the release of free radicals by their mitochondria, and consequently their depletion of GSH and activation of caspase 3. The release of ROS by mitochondria is largely controlled by the supply of NADPH, which is, in turn, regulated by the enzyme trans-aldolase. Indeed, the level of apoptosis in HIV infected cells can be regulated by manipulating the expression of trans-aldolase (Banki *et al.*, 1998). This suggests that the degree of apoptosis in HIV-1 infection may be regulated through oxidative stress. This is also supported by the observation that patients with HIV have very high levels of circulating oxidised protein derivatives (Witko-Sarsat *et al.*, 98).

Given the preceding discussion, one would intuitively assume that excessive oxidative stress is best avoided, as it may cause disease states. However, there are several paradoxes that suggest that this may be an oversimplification. For example, given that polyunsaturated fatty acids have a greater susceptibility to oxidative attack than unsaturated fatty acids, how is it possible to explain the beneficial effects of polyunsaturated fatty acids on the cardiovascular system?

1.2.9 Problems with the current theory

Over recent years, research in the field of free radical biology has gained a great deal of momentum, with several journals dedicated entirely to this subject. However, it worth noting that several concepts within this field of research are not universally accepted.

It has been proposed that H_2O_2 may represent a common signal for the activation of NF-κB. However, the data to support this view is inconsistent and

controversial. The activation of NF-κB by peroxides does not occur in all cell types (Das *et al.*, 1995), or even subclones of particular cell lines. For example, H_2O_2 only activates NF-κB in subclone JR of Jurkat T cells and not other subclones or T-cell lines (Anderson *et al.*, 1994; Brennan & O'Neill, 1995). This alone is compelling evidence to suggest that ROS are not ubiquitous second messengers.

Furthermore, in a cellular system unresponsive to the effects of H_2O_2, anti-oxidants were still found to suppress the action of known NF-κB stimuli (Israel *et al.*, 1992). Also, known activators of NF-κB in COS-1 cells are unresponsive to the actions of catalase (Suzuki *et al.*, 1995).

Anti-oxidants and chelators are often employed to highlight the role of oxidants. However, many of these agents are known to act on a variety of cellular components distinct from ROS (Sukuzi & Packer, 1993), and may disrupt synergism between oxidants and additional agents. Millimolar concentrations of anti-oxidants are frequently required to inhibit the activation of NF-κB by micromolar levels of ROS (Ginn-Pease *et al.*, 1998).

The activation of cellular functions by ROS are often demonstrated under reducing conditions. This removes cells from their physiological redox homeostasis, leaving the results uninterpretable. In support of this, it has been shown that a variety of reducing substances, and particularily those affecting cysteine residues, can increase the binding of NF-κB to DNA (Ginn-Pease *et al.*, 1998). GSH depletion has been shown to suppress activation of NF-κB by H_2O_2 thus indicating that GSH is required for optimal activation of NF-κB (Ginn-Pease & Whisler, 1996).

ROS have not been demonstrated to represent an integral signal transduction component within any single pathway (Ginn-Pease *et al.*, 1998). Further, transcription factors exclusively activated by ROS have not been found and probably do not exist (Dalton *et al.*, 1999). It must, therefore, be suggested that

ROS may be mediators of certain signalling pathways but not ubiquitous second messengers.

Recent evidence suggests that cellular injury may be a synergistic effect of ROS and additional agents, including proteinases, cationic proteins, hemolysins, phospholipases, etc. The effects of multicomponent systems may, therefore, provide a more representative example of the *in vivo* milieu associated with inflammatory and infectious sites (Ginsburg, 1998). It must, therefore, be suggested that factors such as intracellular pH, ion fluxes and calcium homeostasis may be important when considering the characteristics of inflammatory and infectious processes. Therefore, using single agonists to represent the inflammatory milieu is, at best, a questionable approach (Ginsburg, 1998). Furthermore, isolated cell systems are often exposed to unphysiological levels of oxygen, in addition to the complex redox reactions that involve various constituents of culture media. With these concepts in mind, it has been suggested that the field of redox biology would be best served by moving away from *in vitro* studies and concentrating upon *in vivo* models (Halliwell, 1996b).

1.3 Oxidation modifications of proteins

Oxidative stress is known to be associated with sites of inflammation, and must, therefore, be considered as a potential direct activator of DC. It is possible to extend the "danger" hypothesis to include secondary products associated with sites of tissue damage (this suggestion will be examined further in Chapter 5). If the route of oxidative injury is indirect, it must clearly include selective protein derivatives that are produced as a result of tissue damage.

Activation of neutrophils induces their release of myeloperoxidase (MPO), along with serine proteases, from azurophilic granules. MPO utilises H_2O_2, the dismutated product of O_2^- produced by the respiratory burst, and ubiquitous chloride ions, to form a number of oxidants. Hypochlorous acid (HOCl) is the strongest of these oxidants produced in appreciable amounts, and accounts for at least 25% of the H_2O_2 released (Foote *et al.*, 1983). HOCl is an indiscriminate oxidant (Albrich *et al.*, 1981), local concentration of which may reach 100μM (Schraufstätter *et al.*, 1990).

HOCl can oxidise and chlorinate proteins, and has more recently been implicated in the oxidation of LDL (Daugherty *et al.*, 1994). MPO is the only human enzyme capable of producing HOCl (Thomas & Fishman, 1986).

The biological functions of proteins can be lost through oxidation and chlorination, especially following gross modifications. However, less pronounced modifications have been shown, *in vitro*, to enhanced the immunogenic properties of proteins (Marcinkiewicz *et al.*, 1991).

It is suggested that the decrease in the ability of microsomes and mitochondria to regulate intracellular calcium levels, upon oxidative stress, may be due to the loss of function of proteins that are essential for the transport of calcium across cellular membranes. The loss of protein function may be a direct effect of the oxidation of protein thiols (Comporti, 1989).

1.3.1 Characterisation of oxidative damage to proteins

Oxidation of proteins can be broadly segregated into subtle and gross patterns of modification, depending on whether or not the oxidation is reversible.

1.3.1.1 Subtle oxidative modification of proteins

Low levels of oxidative stress can inflict a number of subtle modifications to proteins. The two amino acids residues most sensitive to oxidation are cysteine and methionine; these are also the only residues in which oxidative modifications can be repaired. Based on this, it has been suggested that these residues may represent a ROS scavenger system that protects against irreversible oxidative damage (Berlett & Stadtman, 1997). It is of interest to note that the major anti-oxidant capacity of normal human plasma, towards H_2O_2 and HOCl, can be accounted for by the numerous thiol groups on albumin (van der Vliet *et al.*, 1994).

In addition, several enzymes are known to be sensitive to ambient peroxides, referred to as the "peroxide tone." An example of this is cyclooxygenase, which is dependent on low levels of lipid hydroperoxides for optimal activity and is thereby regulated by the redox state (Cleland, 1984).

1.3.1.2 Gross oxidative modification of proteins

More extensive oxidation of proteins results in various gross modifications that cannot be repaired. Such oxidation may be "site-specific" if transition metals are sequestered adjacent to the proteins, such as the binding of copper to histidine residues. Transition metals are then able to generate hydroxyl radicals, through the Fenton reaction, which are known to act close to their site of formation.

Aromatic amino acids are also among the preferred targets for ROS attack (Berlett & Stadtman, 1997). Exposure of these amino acids to oxidising agents

often results in the formation of irreversible intermolecular covalent bonds (Davies, 1987). An example of this is free radical attack on tyrosine residues, which may result in the formation of tyrosyl radicals. These radicals may then react with other tyrosyl radicals, or with tyrosine residues, to form stable biphenolic compounds. 2,2, biphenol bityrosine is the major product of this type of reaction (Davies, 1987). Dityrosine can readily be detected *in vitro* by virtue of the intense fluorescence of biphenolic compounds, and it is also protease-resistant and may be used as a marker for phagocyte-induced oxidative damage *in vivo* (Heinecke *et al.*, 1993).

Sulphydryl (-SH) groups are also highly susceptible to oxidative attack. They are present in non-protein thiols such as free cysteine and glutathione, and protein thiols, where they play important roles in a number of protein functions, including conformational structure, binding of substrate and protection against free radical attack. Oxidation of cysteine residues can result in the formation of inter- and intra-molecular disulphide bonds. More extensive oxidation induces the cleavage of disulphide bonds and the conversion of cysteine to cysteic acid residues (Carrasco-Marin *et al.,* 1998).

HOCl is also reported to induce chloramine formation on NH_2-terminal amino acids and lysine residues (Stelmaszynska & Zgliczynski, 1978). This may then lead to deamination and fragmentation with the subsequent formation of protein carbonyls (-C=O) (Schraufstätter *et al.*, 1990).

Chloramines are long-lived protein species, which have a half-life of approximately 18 hours and, thus, retain the oxidising capacity of HOCl (Weiss *et al.*, 1983). A degree of protein oxidation by HOCl may, therefore, be a result of secondary oxidation by chloramines (Thomas *et al.*, 1982). Under physiological conditions, proteins chlorinated by HOCl, or chloramines, can slowly be transformed into aggregated final products during contact with a second oxidant e.g. O_2^- or H_2O_2 (Olszowski *et al.*, 1996).

1.3.2 Detection of protein oxidation *in vivo*

Despite the major anti-oxidant capacity of normal human plasma, oxidised protein derivatives are found to occur *in vivo*. Oxidation of globular proteins yields a higher percentage of products from surface residues than the hydrophobic residues, such as leucines and valines. Hydroxylysines and particularly 3-hydroxylysine may, therefore, represent a sensitive and useful marker of radical-mediated protein oxidation *in vivo* (Morin *et al.*, 1998).

Another useful marker of oxidative stress *in vivo* is the production of chlorotyrosine. This is produced by the reaction of HOCl and tyrosyl residues (Domigan *et al.*, 1995). Although this is a minor reaction product, accounting for less than 3% of the hypochlorous acid that reacts with human serum albumin (HSA), it is the only modification of proteins, identified to date, that is unique to HOCl (Kettle *et al.*, 1995).

1.3.3 Immunogenicity of oxidised and chiorinated proteins

It can clearly be seen that chlorination of proteins is a physiological process. It has further been shown that low levels of chlorination facilitate antigen presentation. This may be mediated through improved processing or by a mechanism that prepares the antigen for presentation (Marcinkiewicz *et al.*, 1991). However, excessive oxidation reduces antigenicity, which may be related to the susceptibility of the chlorinated protein to proteolysis (Carrasco-Marin *et al.*, 1998).

1.3.4 Modified protein products

1.3.4.1 Characterisation of advanced glycation end products

In addition to oxidation, proteins may also be modified through glycation to form advanced glycation end products (AGE). AGE are predominantly formed by reactions involving the side-chains of lysine, arginine and histidine, and reducing

sugars (including, glucose, fructose, hexose phosphates, trioses, and triose phosphates). This is termed "glycation" or the Maillard reaction, the products of which are reversible Shiff-base adducts. These subsequently yield AGE through a series of rearrangements, dehydration and oxidation reactions (Münch *et al.*, 1998).

AGE accumulate on long-lived proteins such as collagen and basement membrane, as a function of glycaemic levels and time. In diabetic blood vessels, increased formation of glucose-derived AGE appears to contribute to the process of vascular occlusion by chemically trapping LDL and plasma proteins that leak out of the circulation (Vlassara & Bucala, 1996). AGE cross-linked peptides and protein products are protease-resistant, and must be cleared through specific cell surface receptors on macrophages (Vlassara *et al.*, 1985).

Degradation products of AGE-modified proteins are found in the circulation as low molecular weight, highly reactive AGE peptides. These products are normally cleared by the kidneys but may react with serum proteins, and have been shown to be immunogenic *in vitro* (Vlassara *et al.*, 1992).

N^{ε}-(carboxymethyl) lysine (CML) and pentosidine are examples of AGE. The formation of CML is inhibited by the absence of oxygen or presence of oxidant scavengers (Baynes, 1991), thus indicating a link between the Maillard reaction and oxidative stress.

CML has been shown to accumulate during the oxidation of LDL, without an associated increase in the concentration of its putative precursor. This indicates that CML results from both carbohydrate and lipid oxidation and may, thereby, be considered a biomarker of general oxidative stress (Requena *et al.*, 1996).

1.3.4.2 Receptors for advanced glycation end products

For AGE to mediate an effector action, they must bind receptors on the surface responsive cells. The two receptor classes known to mediate the uptake of AGE-modified proteins, are the class A scavenger receptor (El-Khoury *et al.*, 1994) and a 35kDa protein, known as the receptor for AGE (RAGE) (Neeper *et al.*, 1992), which is member of the immunoglobulin superfamily.

In healthy individuals, RAGE is usually expressed at low levels on human endothelial cells (Ritthaler *et al.*, 1995). However, patients with a range of peripheral occlusive diseases (with or without diabetes) show enhanced endothelial RAGE expression. The expression of RAGE also increases in vasculature affected by inflammatory disorders and murine atherosclerotic plaques (Park *et al.*, 1996). It is likely that RAGE has ligands other than AGE, and that multiple factors other than AGE impact on its expression (Ritthaler *et al.*, 1995). For example, TNF has been shown to increase the expression of RAGE on cultured endothelial cells (Ritthaler *et al.*, 1995) and to increase the binding of AGE-albumin to mononuclear phagocytes (Vlassara *et al.*, 1988a): this increase in the expression of RAGE is dependent upon activation of NF-κB (Li & Schmidt, 1997).

It is, therefore, suggested that AGE and RAGE may have a potential role in the inflammatory response. Such a view is further supported by the finding that heterogeneous AGE have been shown to bind to RAGE on the surface of endothelial cells, mononuclear phagocytes and vascular smooth muscle cells. Moreover, they have been shown to signal through ERK, p38 (McDonald *et al.*, 1998) and NF-κB (Huttunen *et al.*, 1999), providing further evidence that these products may constitute a "danger" signal.

1.3.4.3 Characterisation of advanced oxidation protein products

Recently, a heterogeneous population of oxidised protein derivatives derived from gross oxidative stress has been defined as advanced oxidation protein products (AOPP). AOPP are defined as dityrosine-containing cross-linked protein products (Witko-Sarsat *et al.*, 1996). The definition is important since it excludes protein aggregates that form as a result of disulphide links following a subtle oxidative stress (Vissers & Winterbourn, 1991). The terms "advanced oxidation" and "dityrosine containing" are, to a certain extent, synonymous as indications of gross modifications.

The levels of AOPP *in vivo* correlate with plasma concentrations of dityrosine and AGE-pentosidine, as indices of oxidant-mediated protein damage, but not with thiobarbituric acid-reactive substances (TBARS), as a marker of lipid peroxidation (Witko-Sarsat *et al.*, 1996). This correlation suggests that AOPP are more accurate markers of oxidative stress than products of lipid peroxidation. Moreover, levels of AOPP also correlate closely with those of neopterin, the monocyte activation marker. It has, therefore, been suggested that AOPP may represent a novel class of pro-inflammatory mediator.

AOPP may be formed from oxidised plasma proteins (especially albumin) but do not themselves have oxidant properties (Witko-Sarsat *et al.*, 1996). They can, therefore, not be reduced by anti-oxidants, which are known to increase following a mild oxidative stress. The formation of AOPP may provide a mechanism for the accumulation of a "danger" signal following successive oxidative stresses. The potential to elicit an adaptive immune response would, therefore, not be prevented by the protective mechanisms that ensue following mild oxidative stresses (see section 1.2.7).

Furthermore, given the close structural resemblance of AOPP and AGE, it has been suggested that AOPP may be cleared, and indeed act, by similar receptors that are implicated in the removal of AGE. It is, therefore, possible that RAGE and scavenger receptors may play an important role in the actions of AOPP.

1.3.4.4 Formation of AOPP *in vivo*

AOPP has been isolated from human peripheral blood, suggesting that they are formed *in vivo*. However, given the anti-oxidant capacity of normal human serum, it is unlikely that albumin would be oxidised to this extent within the peripheral circulation. It is, therefore, suggested that the formation of AOPP is probably preceded by the diffusion of native albumin into sites of oxidative stress, AOPP-albumin may then leak from these sites back into the peripheral circulation, and may also act on surrounding cells.

It is known that at sites of inflammation, much of the tissue damage is caused by oxidants, which are released primarily by activated neutrophils (Weiss, 1989). These oxidants, including ROS (e.g. O_2^-, H_2O_2 and $OH^\bullet$) and chlorinated oxidants (e.g. HOCl), have the ability to react with a wide range of biological molecules. It is also reported that, HOCl is extremely efficient at inducing dityrosine formation, and the production of irreversible protein cross-links (Vissers & Winterbourn, 1991). Indeed, it has been suggested that the activity of myeloperoxidase (Witko-Sarsat *et al.*, 1998), and thus HOCl, may initiate the formation of AOPP, *in vivo*. Furthermore, chlorotyrosine, a specific marker of proteins oxidised with HOCl, is found in atherosclerotic plaques that also contain high levels of dityrosine (Fu *et al.*, 1998).

AOPP have been isolated from the plasma of patients undergoing maintenance haemodialysis. It has been well documented that in this procedure, the constantly renewed activation of neutrophils, following blood interaction with bioincompatible membranes, results in the generation of large amounts of oxidants, including those described above. The increased release of oxidants may lead to the depletion of anti-oxidants, thus promoting the formation of AOPP, which have been implicated in atherosclerosis (Witko-Sarsat *et al.*, 1996). Indeed, those patients undergoing maintenance haemodialysis are known to have an increased risk of developing atherosclerosis (Oishi *et al.*, 2000).

Although not defined as AOPP, dityrosine-linked proteins have been identified in large quantities in the intima of atherosclerotic plaques, but not in normal arteries (Fu *et al.*, 1998). In a separate study, dityrosine levels in low density lipoproteins (LDL) isolated from atherosclerotic plaques were 100-fold higher than those in circulating LDL (Leeuwenburgh *et al.*, 1997). It is also of interest to note that mature DC have been found within atherosclerotic plaques (Bobryshev *et al.*, 1996) and several inflammatory sites, such as the rheumatoid joint (Thomas *et al.*, 1994a). Although the connection may initially seem unlikely, they are all sites of oxidative stress and are infiltrated by high numbers of recently activated T cells. There have been many studies of the DC within the rheumatiod joint and recent studies have reported that DC within the atherosclerotic plaque form multiple contacts with T cells (Bobryshev *et al.*, 1997). These contacts promote the survival of the T cells, suggesting that DC may contribute to atherosclerotic lesion formation.

The presence of dityrosine-containing proteins at sites of inflammation is of particular interest since these sites show strikingly high expression of RAGE (Park *et al.*, 1996), which may bind AOPP. However, the expression of RAGE on DC has yet to be investigated specifically.

1.3.4.5 Effects of AOPP and AGE on cellular functions

Since AOPP have only recently been characterised, the actions of these compounds have scarcely been investigated. However, given the similarities of AOPP and AGE, is has been suggested that they may have similar actions *in vivo* (Witko-Sarsat *et al.*, 1998). Therefore, it is possible to predict the effects that AOPP may have by analogy to the putative actions of AGE.

AGE modified proteins have been shown to stimulate a number of pro-inflammatory responses. These responses include transendothelial chemotaxis of human monocytes (Kirstein *et al.*, 1990) with their release TNF-α and IL-1-β (Vlassara *et al.*, 1988b). Upon exposure to AGE, endothelial cells show accelerated cell growth, enhanced thrombogenicity and increased

permeability (Esposito *et al.*, 1989), which may be mediated through activation of NF-κB (Yan *et al.*, 1994); further suggesting that AGE, and indeed AOPP, may act as general pro-inflammatory mediators.

1.4 Oxidation modification of lipids

The suggestion that oxidative derivatives of proteins and lipids may have an important effect in the regulation of DC function has not been made previously and has not been investigated specifically. However, lipids too are known to be targets of oxidative stress and must, therefore, be considered as a potential means by which oxidative stress may act indirectly upon DC. This concept will be examined and discussed further in Chapter 6.

1.4.1 Classification of plasma lipids

Plasma lipids can be segregated into four categories based on their chemical structure.

Fatty acids are straight-chain compounds that may be either saturated or unsaturated. In this form the chemicals are also known as non-esterified or free fatty acids. Fatty acids are transported in the blood bound to albumin and represent an essential source of energy during the fasting state, following depletion of carbohydrate levels.

Triglycerides are based on glycerol that is esterified with three fatty acids. This is the major form in which fatty acids are stored and accounts for about 95% of adipose tissue lipids.

Phospholipids are chemically similar to triglycerides but contain a water-soluble phosphate and nitrogenous base. Phospholipids are synthesised by all cells. They constitute an integral part of cell membranes and also serve as a substrate for phospholipase, which catalyses the formation of arachidonic acid.

Cholesterol has a steroid structure that may be esterified to form cholesterol esters or remain as free cholesterol. Cholesterol is known to be important in

maintaining the plasticity of cell membranes and is the precursor from which steroid hormones are synthesised.

1.4.2 Lipoproteins

As a result of the insolubility of lipids in water, they are transported throughout the bloodstream as macromolecular complexes (lipoproteins) comprising a core of non-polar lipids (triglycerides, cholesterol and cholesterol esters) surrounded by a coat of polar phospholipids. The stability of lipoproteins is increased by the presence of proteins (known as apoproteins) within their surface. The lipoproteins also allow receptor-mediated recognition of the lipoprotein particles.

Lipoproteins can be segregated into five structurally different classes with differing densities. The lipoproteins with the lowest density are those with the greatest triglyceride content.

Chylomicrons are synthesised in the small intestine and principally contain triglycerides. They are required for the absorption of dietary lipids and initially contain the apoproteins B-48, A-I and A-II, but later acquire apo C-II and E from HDL within the bloodstream. Apo C-II is the cofactor for the enzyme lipoprotein lipase, which is located on endothelial cells and removes triglyceride from these particals. The chylomicron remnant contains most of the original dietary cholesterol and is taken up by the liver.

Very low-density lipoproteins (VLDL) are synthesised by the liver for the transport of endogenously synthesised triglycerides and cholesterol to adipose tissue. These particles initially contain apo B-100 but also acquire apo C and E from HDL. Triglycerides are depleted through the action of lipoprotein lipase leaving intermediate-density lipoproteins (IDL).

IDL bind to hepatocytes through apo E. They may then either be catabolised or lose futher triglyceride to form low-density lipoproteins (LDL).

LDL particles are the main carriers of cholesterol to peripheral tissues; they contain apo E and a single apo B_{100}, which binds to LDL receptors. Upon binding of LDL to the apoprotein receptor they are endocytosed. The receptor is recycled back to the membrane and the LDL is degraded in lysosomes. Thus, intracellular cholesterol levels are controlled through the synthesis and expression of the LDL apoprotein receptor.

High-density lipoproteins (HDL) contain the lowest ratio of lipids to proteins. They are, therefore, denser than other lipoproteins. HDL are formed by both the liver and intestine and contain apo A, which activates the enzyme LCAT (lecithin-cholesterol acetyltransferase). LCAT is able to esterify sequestered cholesterol allowing HDL to transport excess cholesterol from the periphery to the liver.

1.4.3 Oxidised LDL

Interest in the function of oxidised LDL (oxLDL) was triggered by the observation that macrophages rapidly take up oxLDL, resulting in the formation of lipid-loaded foam cells. This observation initiated a vast degree of interest in the physiological conditions under which LDL are oxidised, the mechanisms involved, and the pathological consequences of this oxidation.

Most of the interest surrounding oxidised lipoproteins is based on LDL. It should be noted that other lipoproteins may also be oxidised. However, the rate of endocytosis of these particles is not comparable to that of oxLDL (Parthasarathy *et al.*, 1990) and, therefore, they have stimulated less interest, and will not be discussed in further detail below.

1.4.3.1 LDL subfractions

LDL are not a discrete entity. Rather, they comprise a heterogeneous population with differing densities. The small dense LDL subfractions are more susceptible to oxidation than the larger less dense fractions (de Graaf *et al.*, 1991). Two

distinct LDL phenotypes have been defined. These are, A and B, based on the predominance of either large buoyant or small dense LDL particles respectively. Increased levels of these smaller fractions are associated with an increase in the risk of developing coronary heart disease (CHD). Phenotype B is inherited in a dominant manner, the penetrance of which is dependent upon hormonal factors, and rarely seen in young males or premenopausal women. It should be noted that the proportion of LDL subfractions can be modulated by a variety of pharmacological and behavioural (diet and exercise) factors (Williams *et al.*, 1990).

1.4.3.2 Oxidation of LDL

The susceptibility of LDL to oxidation depends on their content of lipid hydroperoxides and their proportions of saturated, monounsaturated and polyunsaturated fatty acids (Bonanome *et al.*, 1992; Kleinveld *et al.*, 1993).

In vitro, LDL oxidation can be induced by a number of different cell types and by cell-free oxidising systems such as, lipoxygenase, myeloperoxidase, oxygen radicals, u.v.-light, γ-irradiation, haem, copper ions and hypochlorous acid. *In vivo* oxidation of LDL is thought to occur within the arterial wall, where proteoglycans and other constituents of extracellular matrix sequester LDL, thus promoting their oxidation. Native LDL entering a lesion are rapidly modified, in part by oxLDL. This prevents their escape or drainage by the lymphatics (Yang *et al.*, 1996).

At sites of inflammation, the oxidation of LDL is promoted by a number of factors. These include a decreased pH and an increase in the formation of free radicals, which is a result of the release of iron following the intracellular degradation of ferritin by the autophagic vacuolar apparatus of macrophages.

At sites of low pH, which may reach 3.6 at the surface of activated macrophages (Silver *et al.*, 1988), superoxide radicals are protonated to form hydroperoxyl

radicals ($HO_2^{\bullet}$). These are potent oxidants that have been shown to consume α-tocopherol and induce lipid peroxidation (Bedwell *et al.*,1989).

Oxidation of LDL is essentially a lipid peroxidation chain reaction. However, this is complicated by interactions between different components of the LDL. Peroxidation of unsaturated lipids may be initiated by both free radicals (e.g. peroxy radicals and hydroxyl radicals) and non-radical species (eg. singlet oxygen, ozone and peroxynitrite) (Girotti, 1998). For example O_2 can react directly with unsaturated fatty acyl groups to give LOOH. However, the majority of lipid peroxidation reactions are initiated by free radical-mediated abstraction of a hydrogen atom from an unsaturated lipid. This is followed by a sequence of propagation reactions, yielding various products including alkanals, alkenals, hydroxyalkenals, ketones and alkanes.

1.4.3.3 Lipid Peroxidation

Hydroxyl radicals initiate lipid peroxidation by the abstraction of allylic hydrogens from unsaturated lipids (LH) to yield lipid radicals ($L^{\bullet}$). The abstracted hydrogens are typically derived from the *sn*-2 fatty acyl groups of phospholipids or the C-7 hydrogen of cholesterol (Girotti, 1998). Lipid radicals may then react rapidly with molecular oxygen to leave peroxyl radical intermediates ($LOO^{\bullet}$), which may propagate chain reactions by abstracting a hydrogen atom from an adjacent lipid. The resulting products are a lipid hydroperoxide (LOOH) and a new lipid radical. Lipid hydroperoxides are more stable than their radical intermediates but have a greater polarity than their precursor lipids.

Lipid hydroperoxides may induce detrimental effects directly on lipid membranes or undergo one- or two-electron reduction. One-electron reduction of hydroperoxides induces the detrimental effects associated with chain peroxidation (see section 1.2.3), thus exacerbating the oxidative damage, whilst two-electron reduction is involved in detoxifying the hydroperoxides (Girotti *et*

al., 1998). The oxidation of LDL is an example of lipid peroxidation induced by one-electron lipid hydroperoxide turnover.

One-electron reduction of lipid hydroperoxides is favoured under conditions in which Fe^{2+} ions are chelated in close proximity to the lipid hydroperoxides. The reaction product is an oxyl radical ($LO^{\bullet}$), which predominately form epoxyallylic peroxyl radicals ($OLOO^{\bullet}$) following rearrangement and oxygenation. Oxyl radicals are also capable of abstracting hydrogen atoms, thus initiating peroxidation, or undergoing β-scission forming an aldehyde and an alkyl radical. The formation of epoxyallylic peroxyl radicals will lead to further rounds of lipid peroxidation with the formation of further lipid hydroperoxides (Wilcox & Marnett, 1993).

Breakdown of lipid hydroperoxides always results in the production of aldehydes, many of which are biologically active, the best characterised of these are malondialdehyde (MDA) and 4-hydroxynonenal (HNE). During the oxidation of LDL, the aldehydes produced may form covalent bonds with lysine residues on apo B (Steinbrecher, 1987). The formation of these bonds reduces the charge of the particles and allows their recognition by scavenger receptors (Hoff *et al.*, 1989).

Intermediates of lipid peroxidation may also induce the fragmentation of apo B_{100}, through non-enzymic oxidative cleavage, producing lipoxidation products, defined as proteins modified by intermediates of lipid peroxidation, of molecular mass 14-200 kDa (Fong *et al.*, 1987). Some of these lipoxidation products represent new immunogenic epitopes and have been shown to induce both humoral and cellular immune responses (Witztum, 1997). Indeed, in addition to the increased proportion of activated T cells within atherosclerotic lesions, up to 10% of these cells may be specific for oxLDL (Stemme *et al.*, 1995). Furthermore, presentation of these antigenic epitopes to B cells is known to lead to the formation of anti-oxLDL antibodies.

1.4.3.4 Inhibitors of lipid peroxidation

The best known inhibitors of lipid peroxidation are the chain-breaking anti-oxidants. These anti-oxidants act by competing with unsaturated lipids for peroxyl radicals and thus reduce the rate of lipid hydroperoxide formation; examples of these reagents include α-tocopherol (vitamin E) and butylated hydroxytoluene (BHT) (Girotti, 1998). $^{\bullet}NO$ is also reported to react with peroxyl radicals and must, therefore, be considered as a chain breaking lipid anti-oxidant (O'Donnell *et al.*, 1997).

The anti-oxidant action of α-tocopherol results in the formation of a tocopheroxyl radical, that has a very low reactivity and rarely propagates chain reactions, but may also terminate chain reactions by binding other radicals.

In addition to specific anti-oxidant mechanisms, there are a number of indiscriminate mechanisms that may be employed to remove oxidants such as $^1O_2$, O_3 and $OH^{\bullet}$ (Buettner, 1993).

An increasing appreciation of the role of oxidative mechanisms in the promotion of a variety of disease states has initiated a vast degree of interest in the potential benefits of anti-oxidant vitamin supplements. Vitamin E supplements have consistently been shown to decrease the susceptibility of LDL to oxidation, but the epidemiological data to support an associated reduction in cardiovascular death is inconsistent (Stampfer *et al.*, 1993; Hense *et al.*, 1993).

It should be noted that although oxidative mechanisms are known to play an important role in the clinical manifestations of a number of diseases, the potential therapeutic benefits of anti-oxidants are not necessarily the result of their anti-oxidant actions. It has, for example, been shown that vitamin E alters the lipid composition of atherosclerotic plaques, aggregation of platelets, membrane fluidity and intracellular calcium mobilisation (Ferns *et al.*, 1993). It has been shown recently that vitamin E may also induce the downregulation of scavenger

receptor genes and thereby prevent oxLDL-mediated apoptosis (Ricciarelli *et al.*, 2000).

An additional important chain breaking anti-oxidant is ascorbic acid (vitamin C). Ascorbic acid is water-soluble and has been shown, at physiological concentrations, to inhibit the oxidation of LDL (Jialal *et al.*, 1990) and the cytotoxic actions of moderately oxidised LDL on human vascular smooth muscle cells (Siow *et al.*, 1999).

1.4.4 Oxidation of LDL *in vivo*

Oxidation of LDL is not simply an *in vitro* phenomenon since atherosclerotic lesions have been shown to contain antigenic epitopes that resemble *in vitro* oxLDL. In addition, auto-antibodies to oxLDL have also been detected *in vivo* (Horkko *et al.*, 1996) and are frequently used as a marker for the association of oxLDL with specific diseases. Moreover, LDL isolated from peripheral blood have been shown to contain trace amounts of peroxidised lipids (Thomas *et al.*, 1994b). However, more extensively oxidised LDL are rapidly cleared from the circulation by the liver (Ling *et al.*, 1997).

The majority of *in vitro* studies are conducted on LDL oxidised in the presence of metal ions. It is, however, unlikely that this process occurs in the circulation, since free copper and iron ions are not generally found in plasma and the presence of even minute amounts of serum completely prevent the oxidation of LDL *in vitro* (Parthasarathy *et al.*, 1998). Moreover, diseases associated with elevated levels of copper and iron in the plasma and tissue, such as Wilson's disease and haemochromatosis, respectively, are not associated with an increased risk of atherosclerosis (Hamilton, 1997), which is known to be associated with oxLDL formation.

1.4.4.1 The presence of oxLDL at sites of tissue damage

Inflammation is known to be associated with a pro-oxidant local milieu. Such milieu would be expected to promote the oxidation of infiltrating LDL. However, there are additional factors that would also encourage this oxidation. These factors include the entrapment and retention of lipoproteins by the extracellular matrix, thus, promoting their oxidation by lipoxygenases, reactive oxygen species, peroxynitrate and myeloperoxidase (Hajjar *et al.*, 1997). In addition, glycoaminoglycans are negatively charged and may attract hydrogen ions through electrostatic attraction, creating a localised area of reduced pH, thus also encouraging LDL oxidation.

It has been shown that acidic conditions increase the rate of oxidation of LDL by iron ions *in vitro*: this suggests that the interstitial fluid within a cluster of activated macrophages may provide the ideal conditions for LDL oxidation to occur (Morgan & Leake, 1995). A reduction in pH also induces the release of transition metal ions from their bound proteins (eg. transferrin and caeruloplasmin); these may then catalyse the oxidation of LDL (Lamb & Leake, 1994) through the Fenton reaction.

The potential importance of a reduced pH in promoting the oxidation of LDL *in vivo* is exemplified by rheumatoid disease. The synovial fluid within the inflammed joints of these patients has been shown to have a pH value of 7.2 compared to 7.4 in normal subjects (Farr *et al.*, 1985). In addition to the generalised oxidative stress that these patients are found to have (Lunec *et al.*, 1981), the LDL at these sites show evidence that they have been subjected to greater oxidative stress than plasma controls (James *et al.*, 1998).

Moreover, oxLDL have been shown to increase the rate of native LDL modification. Therefore, native LDL entering a lesion that is occupied by oxLDL may be rapidly modified before they have the chance to return to the blood stream or be drained by the lymphatic system (Yang *et al.*, 1996).

Finally, it should be noted that relatively high concentrations of LDL are found at sites of atherosclerotic lesions. The normal concentration of circulating LDL is 0.69 ± 0.23mg LDL protein/ml (Miller, 1984), but this can increase to 1.39 ± 0.34mg LDL protein/ml in the subendothelial space (Smith & Staples, 1980). This known site of LDL oxidation, therefore, coincides with a key migratory location of DC, thus promoting their interaction.

1.4.4.2 Cell-mediated oxidation of LDL

The current consensus is that cell-mediated oxidation of LDL requires binding of LDL to their apoprotein receptors and is dependent upon the sustained presence of the cells (Aviram & Rosenblat, 1994). Therefore, it may be possible for cells to confer selectivity to oxidation through their expression of LDL apoprotein receptors.

The first cell type shown to mediate the oxidation of LDL was a rabbit aortic endothelial cell line (Henriksen *et al.*, 1981). It has since been shown that numerous human cell types are also capable of promoting the *in vitro* oxidation of LDL, including aortic endothelial cells (van Hinsbergh *et al.*, 1986), smooth muscle cells (Heinecke *et al.*, 1986), $CD4^+$ lymphocytes (Lamb & Leake, 1993), neutrophils and macrophages (Katsura *et al.*, 1994).

Micro-molar concentrations of redox-active metal ions appear to be an absolute requirement for *in vitro* cell-mediated oxidation of LDL. This is reflected by the fact that most of these *in vitro* investigations have been conducted in media containing a high concentration of divalent ions. The composition of different media must, therefore, be considered as an important factor when examining the ability of cells to oxidise LDL.

The most frequently used media to determine the ability of cells to oxidise LDL is Ham's F-10. This contains both copper and iron, and is known to support the oxidation of LDL by various cell types. The ability of RPMI-1640 to support cell-mediated oxidation of LDL remains controversial, since this media does not

contain any added metals. However, it is probable that metal contaminants may be derived from reagents or water used to make the media, or from the cells themselves. Media also differ in their content of cysteine and/or cystine and anti-oxidants, such as lipoic acid and phenol red (Rice-Evans & Bruckdorfer, 1995).

The oxidative modifications of LDL have mainly been investigated under cell-free conditions. However, a variety of comparative studies have shown that that copper- and cell-mediated oxLDL are to a large extent homologous; and, more important, appear to function in a similar manner. However, some minor differences have been reported, one of which is a possible difference in their content of cholesterol-α-epoxide (Bhadra *et al.*, 1991).

1.4.4.3 Mechanisms of cell-mediated oxidation of LDL *in vivo*

The mechanism(s) employed by cells to mediate the oxidation of LDL have yet to be resolved. A variety of mechanisms have been proposed to account for the initiation or propagation of oxidation. These include:

1) The cellular release of ROS.
2) The release of substrates for the generation of ROS.
3) Peroxidised lipids may transfer directly from the cells to the LDL or may propagate lipid peroxidation reactions through the release of peroxidases.
4) Cellular enzymes may oxidise lipids within the LDL.

Alternatively, cells may not induce peroxidation of unsaturated fatty acids directly, but reduce the anti-oxidant potential of conjugated anti-oxidants. This could be mediated through the consumption of α-tocopherol (Rice-Evans & Bruckdorfer, 1995), thus increasing the susceptibility of LDL to oxidation.

1.4.4.4 The role of lipoxygenase enzymes in the oxidation of LDL *in vivo*

Lipoxygenase enzymes are capable of oxidising LDL and have been detected within atherosclerotic lesions. For these reasons they have been proposed to account for the oxidation of LDL *in vivo*. However, 15-lipoxygenase oxidises polyunsaturated fatty acids in a stereospecific manner, and whilst such products have been isolated from early lesions (Folcik *et al.*, 1995), they are not common in more advanced atherosclerotic plaques (Kuhn *et al.*, 1994). Thus indicating that this is not the predominant mechanism by which LDL are oxidised *in vivo* and highlighting the importance of metal catalysed oxidation. The main effects of 15-lipoxygenase are likely to be the result of the production of eicosanoids.

1.4.4.5 The role of myeloperoxidase in the oxidation of LDL oxidation *in vivo*

Hypochlorite is a potent oxidising agent that has also been shown to modify LDL such that they may bind to scavenger receptors. In this case, the main target of modification is the protein moiety of the LDL, $apoB_{100}$. Myeloperoxidase is located within neutrophils and monocytes and has thus been identified within atherosclerotic lesions (Daugherty *et al.*, 1994). Antihypochlorite-modified protein antibodies have illustrated that proteins oxidised by the myeloperoxidase system are found in advanced atherosclerotic lesions. It is, however, unknown whether this is solely a product of advanced atherosclerotic lesions, or may have a role in atherosclerotic lesion formation.

1.4.5 Cellular actions of oxLDL

Seemingly independent of the mechanism of oxidation, the core observation about the role of oxLDL is that they are generally pro-atherogenic, acting as chemoattractants for circulating monocytes and T cells, whilst inhibiting the motility of macrophages (Terkeltaub *et al.*, 1994; Steinberg, 1997). In this

context, the cellular actions of native LDL are generally less pronounced than those of oxLDL.

Oxidised LDL can induce apoptosis in a variety cell types, including PC12 cells (Draczynska-Lusiak *et al.*, 1998), smooth muscle cells (Nishio *et al.*, 1996), human macrophages (Wintergerst *et al.*, 2000), and human coronary artery endothelial cells (Li *et al.*, 1998).

Oxidised LDL have multiple effects on human endothelial cells including inducing their expression of ICAM-1, leading to monocyte adhesion (Kamanna *et al.*, 1999), their formation of actin stress fibers, and the production of intercellular gaps, allowing increased endothelial permeability (Essler *et al.*, 1999).

It is interesting to note that HDL may inhibit the cellular actions of oxLDL, such as the expression of VCAM-1 on endothelial cells (Calabresi *et al.*, 1997) and synthesis of platelet activating factor (PAF) (Sugatani *et al.*, 1996).

1.4.5.1 Acute *in vitro* exposure to LDL

A variety of cell types have shown an immediate increase in intracellular calcium upon exposure to both native and oxidised LDL. This response was consistently greater for oxidised as opposed to native LDL. Intracellular changes in pH have also been shown to occur within the first two minutes of the addition of LDL to cells. Both of the above effects have been demonstrated in normal fibroblasts, in addition to fibroblasts that do not express the LDL apoprotein receptor, implicating a mechanism that is independent of the LDL receptor (Hamilton, 1997). A similar mechanism has been implicated in the proliferative response of vascular smooth muscle cells to native LDL, which is dependent upon the activation of PKC and tyrosine kinases (Metzler *et al.*, 1999).

In contrast to the effects of prolonged culture, prostacyclin release is stimulated upon acute exposure to oxLDL (Triau *et al.*, 1988).

1.4.5.2 Prolonged *in vitro* exposure to LDL

Native and oxidised LDL have been shown to be incorporated into cell membranes following prolonged culture. Since cholesterol is an integral component of cell membranes, it is not surprising to find that a number of cellular functions are altered following inclusion of additional lipids. The main effects noted are alterations in membrane fluidity and the activities of membrane proteins.

Phosphatidylinositol metabolism and inhibition of prostacyclin release are also observed following prolonged culture in the presence of native LDL. This observation may be significant in relation to atherosclerosis since prostacyclin inhibits adhesion of monocytes and platelets to the endothelial wall (Hamilton, 1997).

1.4.6 Signalling mechanisms induced by oxLDL

OxLDL have been shown to activate numerous signalling mechanisms including protein kinases (mainly PKC) (Li *et al.*, 1998), mitogen-activated protein kinases (MAPKs), hydrolases (such as phospholipase C and Ca^{2+}-activated phospholipase A_2), Rho and its effector Rho kinase (Essler *et al.*, 1999), and to down-regulate cyclic adenosine monophosphate (AMP)- and cyclic guanosine monophosphate (GMP)-mediated responses. Furthermore, oxLDL may lead to a further oxidative stress through the production of intracellular free radicals, as shown in human endothelial cells (Maziere *et al.*, 1999).

OxLDL are also reported to activate transcription through the increased expression of c-myc and c-fos (demonstrated in human vascular smooth muscle cells) (Scott-Burden *et al.*, 1989), enhanced binding of STAT1 and STAT3 (signal transducers and activators of transcription) to their respective consensus binding sites (demonstrated in human endothelial cells) (Maziere *et al.*, 1999), and activation of NF-κB (demonstrated in a variety of cells, including PC12 cells) (Draczynska-Lusiak *et al.*, 1998). These actions are of particular interest

given the key role of STATs and NF-κB in the signal transduction and transcription of pro-inflammatory mediators.

Moreover, there is an increasing realisation that lipid hydroperoxides, in addition to other products of oxidative stress, may mimic natural agonists such as TNF-α (Girotti, 1998). Of particular interest is the finding that Cu^{2+}-induced oxidised LDL contain phospholipid derivatives with platelet activating factor-like activity (Heery *et al.*, 1995). Thus, lipid products of oxidative stress may act directly as second messengers.

Lipid hydroperoxides may also activate phospholipase (PL)A_2. Since this enzyme is able to liberate arachidonate from phospholipids, it is suggested that lipid hydroperoxides could influence the production of eicosaniods (Girotti, 1998). It is also known that lipid hydroperoxides are able to modulate prostaglandin and leukotriene synthesis, *in vivo*, by means of activating lipoxygenase and cyclooxygenase at low concentrations and inactivating these enzymes at higher concentrations (Halliwell & Chirico, 1993).

Recently, it has been suggested that LDL may also function as carriers of (as yet unidentified) bioactive phospholipids (Sachinidis *et al.*, 1999) thereby providing an additional means by which they may stimulate cell signalling.

1.4.6 The effects of LDL and oxLDL on endocytosis and exocytosis

Cellular uptake mechanisms are dependent not only upon different stimuli but also the lipid composition of the membrane (Schroit & Gallily, 1979) and a threshold of total intracellular vesicular size, beyond which uptake of further ligand is impaired (Bolton *et al.*, 1997). Furthermore, high concentrations of oxLDL have been shown to inhibit pinocytosis by a mechanism that is independent of cell death (Borsum *et al.*, 1985).

ApoB is found, *in vitro*, to accumulate in lysosomes of macrophages, implying that oxLDL are poorly catabolised (Mander *et al.*, 1994).

1.4.7 Scavenger Receptors

Since oxLDL are capable of mediating several of their effects independently of LDL apoprotein receptor ligation (see section 1.4.5.1), a class of receptors has been defined by their ability to bind modified low-density lipoproteins; these are the scavenger receptors. There are presently six classes of scavenger receptors (termed A to F), each of which have a broad binding specificity, as reviewed recently by Dhaliwal & Steinbrecher (1999).

Scavenger receptors are expressed on the surface of many cells, including monocytes and macrophages, and may also recognise both microbial surface constituents and intact microbes, implying that they may have a role in innate immunity.

The current hypothesis is that native LDL are recognised by LDL apoprotein receptors, whereas, oxLDL bind scavenger receptors. This switch is partially the result of a decreased positive charge on the surface of the LDL, mediated by intermediate products of lipid peroxidation that bind to lysine residues within apo B_{100}.

It is of interest to note that pro-inflammatory stimuli, including LPS, up-regulate the expression of scavenger receptors on macrophages [SR-AI/II (class A), CD 36 (class C) and CD 68 (class D)] (Fitzgerald *et al.*, 2000) and that this may, in turn, regulate oxLDL influence *in vivo*.

Monocyte-derived DC have been shown to express a number of scavenger receptors including CD36 (Pimpinelli *et al.*, 1991) and CD68 (Palucka *et al.*, 1998). In addition to this, it is possible that oxLDL may be internalised *via* Fc receptors following the formation of autoantibodies against oxLDL (Salonen *et al.*, 1992), which are known to occur *in vivo*. Alternatively, oxLDL may act independently of a receptor-mediated process.

1.5 Aims

DC represent a fundamental cell type involved in the initiation and regulation of adaptive immune responses. However, the known activators of DC are unable to account for the initiation of many adaptive immune responses. It is, therefore, suspected that sites of acute and, indeed, chronic tissue damage must contain an elusive signal that is capable of maturing DC. The "danger" hypothesis proposes that products derived from tissue damage may be the initiating factor that stimulates DC maturation and, thus, adaptive immune responses. Indeed, tissue damage, inflammation and oxidative stress are intimately related.

The aim of this thesis was to examine the hypothesis that DC may be induced to mature upon recognition of oxidative stresses.

Fresh human DC are difficult to isolate, they were, therefore, differentiated from peripheral blood monocytes cultured in the presence of GM-CSF/IL-4, which is currently the most efficient *in vitro* model system in human studies. However, since this is a relatively novel model, it will be necessary initially to validate the isolation of DC and to characterise a suitable protocol for examining the effects of various stress stimuli on DC *in vitro*.

Following elucidation of the direct effects of oxidative stress on DC, the potential that ROS may constitute a common denominator pathway for the maturation of DC will then be investigated. The hypothesis that oxidised derivatives of "self" may represent fundamental signals for the maturation of DC will subsequently be detailed and investigated. Perhaps the most noteworthy of such oxidised protein and lipid products are a novel class of oxidised protein derivatives (AOPP) and oxidised LDL, respectively. These potential oxidative stress messengers will be isolated and characterised before their abilities to induce the phenotypic and functional maturation of DC are explored.

Chapter 2

Materials & Methods

2.1 Materials

All reagents were of the highest grade commercially available. Unless otherwise stated all chemicals and reagents were obtained from Sigma (UK) Ltd. (Poole, Dorset, UK). Disposable pipettes were from Philip Harris (Leicestershire, UK). Pipette tips were from Elkay (Hampshire, UK).

2.2 Cell and Tissue Culture

2.2.1 Safety and sterile procedure

All manipulations involving human tissues were performed in a M.D.H., InterMed class 2 safety cabinet, using sterile techniques. All short and long term cell cultures were carried out at a temperature of 37°C in an atmosphere comprising 95% air and 5% carbon dioxide.

2.2.2 Culture medium

Unless otherwise stated, all cell cultures were conducted in Roswell Park Memorial Institute medium-1640 (RPMI-1640) (Gibco BRL, Paisley, UK) that was supplemented with 100U/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine (all from Clare Hall Laboratories, Imperial Cancer Research Fund, London, UK) and 10% v/v foetal calf serum (heat inactivated at 56°C for 30 minutes). The combined medium was denoted as complete medium.

2.2.3 Cell counting

All cell samples were counted using a Neubauer haemocytometer. 20μl of cell suspension was mixed thoroughly with an equal volume of 0.04% trypan blue stain (phosphate-buffered saline). This mixture was then used to count the cell density. This technique allows a rapid assessment of cell viability, since trypan

blue is excluded from live cells. Samples were only used if they were greater than 90% viable.

2.2.4 Primary cells

2.2.4.1 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers by density gradient centrifugation, according to the method of Romani *et al.*, 1994. Peripheral blood (120ml) was obtained by venesection using a 19/21g butterfly needle into a heparinized (100U/ml) syringe. This was diluted into a freshly prepared solution of 120ml phosphate buffered saline (Clare Hall Laboratories) supplemented with 4ml 0.1% ethylene diamine tetra-acetic acid (EDTA). 30ml of this diluted solution were carefully layered over 17.5ml of Lymphoprep 1077 (Nycomed Pharma, Oslo, Norway) (50ml tissue culture tube; Falcon), and centrifuged for 30minutes at 600g (brake off), at room temperature. The interface over the Lymphoprep 1077 (Nycomed Pharma), which contained the PBMC, was aspirated and washed three times with Hanks' balanced salt solution (HBSS) (Gibco BRL).

2.2.4.2 Derivation of monocyte-derived dendritic cells

The total PBMC fraction from 120ml of fresh peripheral blood was resuspended in 36ml of complete medium and incubated in 6-well (Falcon) tissue culture plates (3ml per well) for 2 hours. Non-adherent cells were then removed and adherent cells cultured in fresh complete medium supplemented with 100ng/ml human recombinant granulocyte macrophage-colony stimulating factor (GM-CSF) (kindly provided by Schering-Plough, Kenilworth, New Jersey, USA) and 50ng/ml human recombinant interleukin-4 (IL-4) (kindly provided by Schering-Plough).

Monocyte-derived DC were purified from non-adherent cells (see section 2.2.4.4.1). According to the conventional protocol (Woodhead *et al.*, 1998), non-adherent cells were removed following seven days of uninterrupted culture (see Table 2.1). However, according to the modified protocol, after three days of culture, non-adherent cells were removed, purified to isolate the partially differentiated monocyte-derived dendritic cells (MDDC), and recultured in fresh complete medium supplemented with GM-CSF/IL-4 for an additional four days (see Table 2.1) (at a concentration of 5×10^5 cells/ml; 3ml per well).

Table 2.1 Protocol for DC assays

Day	Conventional protocol	Modified protocol
1	Isolation of PBMC	
2		
3		
4		Purification of MDDC
5		
6		
7	Purification of MDDC Stimulate MDDC	Stimulate MDDC
8	Phenotype MDDC and prepare proliferation assays	

2.2.4.3 Preparation of T cells

Following 2-hour of culture, non-adherent PBMC were aspirated and resuspended at approximately 5 x 10^6 cells/ml in freezing medium at 4°C (foetal calf serum containing 10% dimethyl sulphoxide (DMSO)). Samples were then transferred to 1ml cryotubes (Nunc) and frozen at -85°C in a polystyrene freezing box, until required for use.

When fresh T cells were required for use in proliferation assays, they were purified directly from the non-adherent PBMC and not frozen.

To recover the non-adherent PBMC fraction, frozen vials were thawed rapidly in a water bath at 37°C and then washed twice in HBSS before being purified.

2.2.4.4 Cell purification

Mixed cell populations were resuspended in 2ml of ice cold complete medium and incubated on ice for 45 minutes with occasional agitation, in the presence of mouse anti-human antibodies that recognised the major contaminating cell populations, as indicated in the respective sections. Samples were then washed twice in ice cold HBSS and then resuspended in ice cold complete medium (2ml). 5µl of immunomagnetic beads coated with sheep anti-mouse Ig (Dynal, Merseyside, UK) (washed twice in ice cold HBSS) were then added per 10^6 cells. Samples containing the beads were then rotated for 45 minutes to allow the beads time to bind the contaminating cells. The magnetic beads and contaminating cells were then removed by placing sample tubes adjacent to a magnet for 2 minutes. Supernatants were then transferred to fresh tubes and placed adjacent to a magnet for a further 2 minutes. Supernatants were then aspirated and contained the purified cells.

2.2.4.4.1 Purification of monocyte-derived DC

Non-adherent monocyte-derived DC were aspirated from 6-well plates. 6ml of these samples were gently layered over 8ml Lymphoprep 1077. Cells were then centrifuged at 600g for 30 minutes at room temperature (brake off) to remove dead cells and debris. Cells were removed from the Lymphoprep 1077 interface and washed three times in HBSS before being further purified to remove contaminating lymphocytes.

2.2.4.4.2 Removal of contaminating lymphocytes

Cells were counted and resuspended in 2ml of complete medium (4°C). The contaminating cells were then removed by negative immunomagnetic depletion (see section 2.2.4.4). The monoclonal antibodies added were anti-CD2 (to remove NK cells and T cells; final concentration 2µg/ml) anti-CD3 (to remove T

cells; supernatant) and anti-CD19 (to remove B cells; supernatant) (see Table 2.2).

2.2.4.4.3 Purification of fresh monocytes

Fresh monocytes were purified from 2 hour adherent PBMC. Wells were washed with HBSS (without Ca^{2+} and Mg^{2+}) and then resuspended in 3ml HBSS supplemented with 3mM EDTA. Samples were then incubated for 45 minutes at 37°C to detach the adherent monocytes. Samples were then vigorously resuspended to dislodge the cells.

Purified monocytes were then obtained by bead depletion as for monocyte-derived DC (see section 2.2.4.4.1).

2.2.4.4.4 Purification of T cells

Cells were counted and resuspended in 4ml of complete medium (4°C). The contaminating cells were then removed by negative immunomagnetic depletion (see section 2.2.4.4). The monoclonal antibodies added were anti-HLA-DR (to remove monocytes, DC, B cells and activated T cells; supernatant), anti-CD14 (to remove monocytes; supernatant) and anti-CD19 (to remove B cells; supernatant) (see Table 2.2).

2.2.4.5 Stimulation of monocyte-derived DC

Unless otherwise stated, monocyte-derived DC were stimulated to mature by the addition of the given potential maturation agents for the final 24 hours of culture (see Table 2.1). Control samples were prepared by adding of an equal volume of control solvent. Unless otherwise stated, each stimulus was added from a stock solution at 100 times the final concentration.

Throughout this project, lipopolysaccharide (LPS from Salmonella minnesota; Sigma) was stored frozen at –20°C from a single stock solution that was diluted in complete medium.

Human recombinant TNF-α (R&D Systems, Minneapolis, USA) was stored at a stock concentration of 100μg/ml in PBS at –20°C.

2.2.4.6 Dendritic cell phenotypic analysis

2.2.4.6.1 Immunofluoresence staining

Samples containing 2 x 10^5 – 5 x 10^5 cell/stain were harvested and resuspended in 50μl of blocking buffer (HBSS containing 10% rabbit serum (Gibco) and 0.1% NaN_3) for 15 minutes at 4°C. 50μl of primary mAb (supernatants or when known 5μg/ml) (Table 2.2) were then added to the cell suspension in a 96-well round-bottom plate (Falcon), and incubated for a further 30 minutes on ice. Cells were washed twice with blocking buffer, to remove any unbound antibody, and then incubated for a further 30 minutes with the secondary antibody, a fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse IgG (Dako, Glostrup, Denmark) diluted 1:20 in blocking buffer. Finally, cells were washed twice in HBSS containing 0.1% NaN_3, agitated, and fixed by adding 50μl of HBSS containing 0.1% NaN_3, followed by 100μl of HBSS containing 3.7% formaldehyde. Samples were stored at 4°C in the dark and analysed within five days of staining.

2.2.4.6.2 Flow cytometry

Stained cells were analysed on a FACScan (Beckton-Dickinson, Mountain View, CA, USA) using WinMDI software. For each sample not less than 5000 events were acquired. The data was examined relative to a negative control sample with no primary antibody (FITC) or relative to an irrelevant primary antibody control,

as given. Cells with the characteristic size and granularity of DC were selected for analysis (polygonal gate), and the expression of various surface molecules on this population was determined. A marker was set such that <2% of the negative control cells gave a fluorescence signal beyond this level. The percentage fluorescence (% + ve) refers to the percentage of cells with fluorescence above this marker, whilst the mean fluorescence intensity (MFI) gives a measure of the amount of fluorescent probe that is bound to each cell. Values of mean fluorescence intensities are presented as linear units.

Table 2.2 Antibodies used for cell surface phenotyping

Reagent	Clone	Isotype	Source
CD1a	NA1/34	IgG2a	A gift from Prof. A. McMichael, John Radcliffe Hospital, Oxford, UK
CD2	XIX.8	IgG2b	Harlan Sera-lab, Crawley Down, UK
CD3	UCH-T1	IgG1	A gift from Prof. P. C. L. Beverley, The Edward Jenner Institute for Vaccine Research, Newbury, UK
CD11a	5E6	IgG1	A gift from Andre van Agthoven, University of Michigan Medical centre, Ann Arbor, MI, USA
CD13	MCS-2	IgG1	A gift fromProf. K. Sagawa, Kurume University School of Medicine, Kurume, Japan
CD14	HB246	IgG2b	A gift from Prof. P. C. L. Beverley
CD19	BU12	IgG1	A gift from D. Hardie, Birmingham Medical School, Birmingham, UK
CD40	MAB89	IgG1	Immunotec, Luton, Bedsfordshire, UK
CD45	SN 130	IgG1	A gift from Prof. P. C. L. Beverley
CD86	BU63	IgG1	A gift from D. Hardie, Birmingham Medical School, Birmingham, UK
CD87	3B10	IgM	A gift from Prof. V. Magdolen, Technische Universitat Dresden, Germany
CD98	J1 E1B	IgG1	A gift from Prof. K. Skubitz, University of Minnesota Medical School, Minneapolis, USA
CD147	H84	IgG2b	A gift from Prof. K. Sagawa
CD148	143.41	IgG1	A gift from Prof. R. Vilella and Dr. A. Gaya, Servei d'Immumologia, Hospital Clinic, Barcelona, Spain
Anti-collagen (type II)	C1	IgG2a	A gift from Prof. R. Holmdahl, University of Lund, Sweden

Anti-HLA-class I	W6/32	IgG2a	Harlan Sera-lab, Crawley Down, UK
Anti-HLA-DQ	Ia3	IgG2a	A gift from Prof. R. Winchester, New York University School of Medicine, NY, USA
Anti-HLA-DR	L243	IgG2a	A gift from Prof. P. C. L. Beverley,

2.2.4.7 Proliferation assays

Prior to conducting the proliferation assays, all cell samples were washed twice in complete medium. All proliferation assays were conducted using a constant number (10^5) of purified T cells and incubated with increasing numbers of purified monocytes or monocyte-derived DC. Experiments were performed in 96-well flat bottom plates (Nunc). Quantification of cell proliferation was by means of [methyl-^{3}H] thymidine (ICN Pharmaceuticals Inc., CA, USA) incorporation. Cells were pulsed with 10μl of 100μCi/ml [methyl-^{3}H] thymidine for the final 16 hours of incubation (1μCi/well). Assays were performed in triplicate.

Cells were transferred from wells on to glass fibre filters (Wallac, Turku, Finland) using a Tomtec cell harvester. "Melt-on" scintillant (Wallac) was added to the filters, and incorporation of radiolabel into cells was quantified using a 1450 Microbeta liquid scintillant counter (Wallac).

2.2.4.7.1 Autologous CD 3-dependent assay

Monocyte-derived DC and T cells, which were obtained from the same donor, were incubated together for 2 hours prior to the addition of CD 3 monoclonal antibody (final concentration 0.1μg/ml). Samples were incubated for 48 hours and then [methyl-^{3}H] thymidine was added for a further 16 hours.

2.2.4.7.2 Mixed leukocyte assay

Monocyte-derived DC and T cells, which were obtained from different donors, were incubated together for 120 hours before [methyl-^{3}H] thymidine was added for a further 16 hours.

2.2.4.7.3 Autologous oxidative mitogenesis assay

Purified T cells were resuspended in a 2mM solution of sodium periodate (made up in PBS and sterile filtered using a 0.2μm filter; Acrodisc®, MI, USA) at a concentration of 5×10^6 cell/ml, and incubated at 4°C for 30 minutes. Cells were then washed twice in complete medium and added to monocytes and monocyte-derived DC from the same donor. Samples were incubated for 48 hours and then [methyl-^{3}H] thymidine was added for a further 16 hours.

2.2.4.8 Assessment of cell viability

2.2.4.8.1 Reduction of MTT

The reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by cells was initially thought to give an indication of mitochondrial activity (Slater *et al.*, 1963). However, it has recently been shown that cellular membranes are impermeable to MTT and that the reduction of MTT by cells is dependent upon vesicular trafficking. Indeed, it has been shown that the formation of insoluble extracellular formazan crystals does not reduce the integrity of cell membranes (Abe & Saito, 1998); suggesting that these are secreted rather than penetrate cell membranes. Furthermore, increased rates of exocytosis decrease the amount of MTT reduced (Liu & Schubert, 1997). It has also be shown that inhibition of the mitochondrial enzyme succinate dehydrogenase only partially inhibits the reduction of MTT by cells (Abe & Saito, 1998). It has, therefore, been concluded that the reduction of MTT by cells

gives an indication of cellular activity rather than viability, as originally assumed (Mosmann, 1983).

100µl of 5 x 10^5 purified DC/ml were incubated with the given agents for 24 hours in 96-well flat bottom plates (Nunc). 20µl of filtered (0.2µm filter; Acrodisc®) MTT (final concentration 1mg/ml) in PBS was added to triplicate samples for the final 4 hours of culture. The reduction of MTT was stopped by the addition of 100µl of 10% w/v sodium dodecyl sulphate (SDS) in 0.01M HCl. Absorbance was measured after a further 18 hours incubation in the dark. Data is presented as % cell activity relative to control samples. This was calculated according to the following formula.

$$\% \text{ activity} = [(A_{570nm}-A_{630})_{sample}/(A_{570nm}-A_{630})_{control}] \times 100$$

2.2.4.8.2 Propidium iodide inclusion

Propidium iodide (P.I.) is only able to enter cells once the integrity of cellular membranes are compromised. Therefore, positive staining gives an indication of cell viability. P.I. uptake was analysed in the second fluorescence channel of a FACScan (Beckton-Dickinson) and using WinMDI software.

Prior to testing the inclusion of P.I., crossover between P.I. (red) and FITC (green) was compensated for using the respective single colour stains.

2.2.4.8.3 Detection of apoptosis

Apoptosis was detected by using TACS™ Annexin V-FITC apoptosis detection kit (R&D Systems) and conducted according to the manufactures instructions. Compensation was set as detailed in section 2.2.4.8.2.

2.2.4.9 Flow cytometric assay of ROS formation using DCF

Samples were washed twice in PBS supplemented with 0.4mM calcium and 0.8mM magnesium before being resuspended to a concentration of 5 x 10^5 DC/ml in the same medium. 10µl/ml of 1mM 2'7'-dichlorofluoresceine diacetate (DCF-DA) (stock concentration10mM; final concentration 10µM) (Molecular Probes, Cambridge, UK) was then added. Samples were then separated equally into 1ml aliquots (5.4ml polypropylene tubes; Elkay) and incubated in a water bath at 37°C for 15 minutes to allow inclusion of the dye into the cells.

The initial (baseline fluorescence) was measured after 10 minutes, stimuli were added after a further 5 minutes (10µl of 100 times final concentration) and fluorescence detected every 5 minutes thereafter, for a total of 60 minutes. Fluorescence was analysed using a FACScan (Beckton-Dickinson) and WinMDI software. 5000 individual data points were collected for each sample.

Control samples were supplemented with an equal volume of PBS. Mean fluorescence intensity values were subtracted from control values at each time point, in order to compensate for spontaneous ROS fluxes and passive diffusion of the fluorescent dye from the cells.

2.2.5 Isolation of bacterial DNA

Escherishia Coli (Strain B) was cultured in LB medium and incubated overnight at 37°C with vigorous shaking. The culture was heat killed in a water bath at 80°C for 1 hour. The sample was then centrifuged (10000g, 2 minutes) and the supernatant discarded. The remaining pellet was resuspended into 500µl TE buffer (see Section 2.8) and incubated with 50µl of 10mg/ml lysozyme for 1 hour at 37°C. 70µl of 10% w/v SDS and 6µl of 10mg/ml proteinase K were then added and incubated for 20 minutes at 65°C. 100µl of 5M NaCl were then added and mixed thoroughly. 80µl of CTAB/NaCl (see Section 2.8) were then added

and the sample incubated for 10 minutes at 65°C. 700µl of chloroform/isoamyl alcohol, at a ratio of 24:1, were then added and the sample and centrifuged for 5 minutes at 10000g to remove CTAB-protein/polysaccharide complexes. The aqueous supernatant was then transferred to a new eppendorf tube and 420µl of 100% isopropanol added to precipitate the DNA. The DNA was then centrifuged for 5 minutes at 10000g and the pellet washed in 70% ethanol to remove residual CTAB.

To confirm the isolation of DNA, the sample was run on a 1% agarose gel (supplemented with 0.1µg/ml ethidium bromide) in TAE buffer.

2.3 Protein oxidation and analysis

0.05M phosphate buffered saline (PBS) was freshly prepared using reagents from Sigma. Spectrophotometric measurements were carried out with a Hitachi U-1500 UV/Vis Spectrophotometer (Tokyo, Japan). Fluorescence spectra were recorded with a Perkin-Elmer 3000 fluorescence spectrophotometer (Beaconsfield, UK).

2.3.1 Oxidation of BSA with hypochlorous acid

Various concentrations of NaOCl were added to a solution of 40mg/ml bovine serum albumin in 0.05M PBS, pH 7.4, and incubated for 30 minutes at room temperature. 40mg/ml bovine serum was used as it is close to the physiological albumin concentration. Similarly, pH 7.4 is close to that at which myeloperoxidase, and thus HOCl, would act extracellularly.

The concentration of the NaOCl stock solution was measured spectrophotometrically assuming a molar extinction coefficient of $350M^{-1}cm^{-1}$ at 290nm at pH 12. At pH 7.4, approximately 50% of OCl^- ions are protonated to HOCl (Morris, 1966). In this report, the term HOCl will refer to the sum of both species.

After oxidation, excess thiosulphate at a thiosulphate-HOCl molar ratio of 2:1 was added to the samples to remove HOCl excess. The thiosulphate and its oxidation products were then removed by dialysis against 0.05M phosphate buffer at 4°C, for 24 hours. It is important to note that the control BSA (i.e. non-oxidised) was treated in exactly the same way as the oxidised protein, except that the initial 30 minutes of incubation was carried out using 0.05M PBS in place of the oxidising agent (NaOCl).

2.3.2 Quantification of advanced oxidation protein products

Oxidation of bovine serum with HOCl results in chlorination of amine groups. The formation of advanced oxidation protein products (AOPP) was quantified as described previously by others (Witko-Sarsat *et al.*, 1996). The samples of oxidised protein were analysed spectrophotometrically, and calibrated with chloramine-T solutions, which in the presence of potassium iodide absorb at 340nm. Chloramine-T standard curve (Figure 2.2) was constructed by adding 50µl of 1.16M potassium iodide to 1ml of chloramine-T solution (0-100µmol/litre), followed by 100µl acetic acid (BDH Chemicals, Poole, UK). The solution was mixed, and the absorbance immediately read at 340nm against a blank containing the same solutions, but with the chloramine-T replaced by 1ml of 0.05M PBS. The same method was employed for the test samples, but with the chloramine-T replaced with 1ml of oxidised bovine serum, at a concentration of 1mg/ml. AOPP formation was expressed as µmol/litre of chloramine-T equivalents.

2.3.3Analysis of AOPP

2.3.3.1 Measurement of dityrosine

The formation of dityrosine residues is a defining feature of AOPP, and it can be used as an index of protein cross-linking and aggregation following oxidative stress (Witko-Sarsat *et al.*, 1996). The formation of dityrosine within oxidised samples of bovine serum albumin (1mg/ml) was calculated assuming the extinction coefficient ε_{315}=32.8$mM^{-1}cm^{-1}$ (Olszowski *et al.*, 1996).

2.3.3.2 Measurement of free amino groups

The TNBS assay (Spadaro *et al.*, 1979) gives an indication of the number of free amino (-NH_2) groups. Test samples containing 0.6mg of bovine serum albumin and 0.6mM 2,4,6-trinitrobenzensulfonic acid (TNBS) in 0.8ml of 2.5mM sodium borate buffer, pH 9.2, were incubated for 30 minutes at 37°C. Next, 0.8ml of 0.2M NaH_2PO_4 containing 18mM Na_2SO_3, and 0.25ml of 2.5% sodium dodecyl sulphate (SDS) were added, and the complete reaction mixture incubated at room temperature for 2 hours. Absorbance was measured at 420nm against a blank containing 0.05M phosphate-buffered saline instead of the protein sample. The concentration of $-NH_2$ groups was then calculated assuming the extinction coefficient of ε_{420}=13$mM^{-1}cm^{-1}$.

2.3.3.3 Measurement of sulphydryl levels

Sulfhydryl (-SH) groups were determined by using DTNB (3-carboxy-4-nitrophenyl disulphide, Ellman's reagent). A 16.6µl volume of protein sample (25mg protein/ml), was pipetted into 1ml of freshly prepared 2M guanidine thiocyanate, 500mM Tris, 10mM EDTA, pH 7.6, and 100µM DTNB. The reaction mixture was incubated in the dark for 25 minutes and the absorbance at

412nm was measured, against a blank that contained 0.05M phosphate-buffered saline in place of the protein sample. The –SH concentration was calculated assuming the extinction coefficient ε_{412}=13,600$M^{-1}cm^{-1}$ (Thannhauser *et al.*, 1987). The determination of sulfhydryl groups by this method is based on the theory that –SH groups reduce DTNB to TNB, which has a yellow colour.

2.3.3.4 Determination of tryptophan fluorescence

The number of tryptophan residues was calculated by measuring the relative fluorescence intensities of the samples of oxidised bovine serum albumin (1mg/ml) at 275nm excitation and 334nm emission (Vissers & Winterbourn, 1991). The results were expressed as % control (i.e. non-oxidised BSA) fluorescence.

2.3.3.5 Polyacrylamide gel electrophoresis (PAGE)

Gels of 7.5% acrylamide (National Diagnostics, Atlanta, USA) were prepared and run using the mini-protein electrophoresis procedure (Bio-Rad Laboratories, Hercules, USA). Protein concentrations were adjusted to have 10μg per well in a sample buffer containing 60mM Tris-HCl pH 6.8, 10% glycerol, 0.05% bromophenol blue and 2% SDS. Protein samples were boiled for 5 minutes in order to denature the protein and then run along with a molecular weight marker. Gels were developed with either Coomassie blue (BDH) or silver staining procedures (Bio-Rad Laboratories).

2.3.4 Detection of lipopolysaccharide (LPS)

The *Limulus amebocyte* lysate assay (E-Toxate®; Sigma) was used for the detection and semiquantitation of LPS. The test was carried out according to manufacturers instructions.

2.4 Isolation and Oxidation of LDL

2.4.1 Preparation of native LDL

40ml of venous blood was withdrawn, from healthy fasted donors, into 2ml 0.1M EDTA and centrifuged immediately (800g, 4°C, 20minutes). The plasma was aspirated and placed in a 50ml tissue culture tube (Falcon). The density of the plasma was measured using a specific gravity refractometer and adjusted to 1.21g/ml by the addition of KBr.

The weigh of KBr required was calculated using the following equation.

$$KBr(g) = \text{volume of plasma} \times \frac{(\text{density required} - \text{present density})}{1 - (\text{PSV KBr} \times \text{density required})}$$

PSV = Partial Specific Volume

PSV KBr = 0.309

2.7ml of density adjusted plasma was loaded into 8.9ml Beckman Optiseal polyallomer disposable centrifuge tubes and overlaid with a solution of density 1.006g/ml. Samples were placed into pre-cooled titanium, fixed-angle rotor, type 70.1 Ti and centrifuged (170,000g, 4°C, 3 hours).

Following centrifugation an orange band appeared, about ¾ from the base of the tube, representing endogenous carotenoids bound to native LDL. The band was removed using a syringe. The density of the band was measured to confirm that this was between 1.019-1.063g/ml, i.e. the density of LDL. The density of the LDL was then adjusted, by the addition of a high density solution, to a density of 1.15g/ml.

The volume of high density solution (HDS) required was calculated using the following equation.

$$V_{HDS}(ml) = \text{volume of LDL} \times \frac{(\text{density required} - \text{present density})}{(\text{density HDS} - \text{density required})}$$

density of HDS = 1.33g/ml

3ml of density adjusted native LDL was overlaid with a solution of density 1.063g/ml and centrifuged in 8ml open-top thick-walled Beckman polycarbonate ultracentrifuge tubes (170,000g, 4°C, 16 hours) (titanium, fixed-angle rotor, type 70.1 Ti). This spin acts as a wash spin to remove albumin and to concentrate the native LDL. Since, by definition, LDL have a density of less than 1.063g/ml they floated to the top of the tube where they were carefully removed with a pipette.

Native LDL were then run down a series of two PD10 columns (Pharmacia, Herts, UK). Prior to use, the columns were pre-equilibrated with 25ml of low-phosphate buffer treated with washed Chelex-100 (1mg/ml) and supplemented with 10μM EDTA.

The protein concentration was quantified by a modified Lowry assay according to the method of Wang and Smith, 1975. Protein standard was prepared using bovine serum albumin. A representative example is shown in Figure 2.1.

2.4.2 Oxidation of LDL

Native LDL were diluted in low-phosphate buffer (without $Ca^{2+}Mg^{2+}$, pH 7.4) to a concentration of 100μg protein/ml. The concentration of EDTA within this solution will then be calculated from the dilution ratio, given that the initial concentration of EDTA was 10μM. EDTA (final concentration 100μM, stock concentration 0.15M) was then added to a fifth of the LDL solution to give the native LDL sample. The remaining solution was divided into four universal

containers and a final concentration of 5μM $CuSO_4$ (after accounting for the remaining EDTA) was added to each (stock solution, 20mM). The time course of diene formation was then monitored spectrophotometrically (A_{234}) (37°C), against a blank of low-phosphate buffer, for one sample that was not sterile. The oxidation of sterile LDL samples was arrested at the states of mild, moderate and maximal oxidation, as defined in Chapter 6, by the addition of EDTA (final concentration 100μM, stock concentration 0.15M).

3ml of native and oxidised LDL samples were dialysed against three changes of 120ml RPMI-1640 for 24 hours before being added to the DC. It can, therefore, be calculated that the final concentration of EDTA within each of the LDL samples was 1.6nM. Following dialysis, samples were filter sterilised using a 0.2μm filter (Sartorius, Göttingen, Germany).

All samples of LDL were used within one week of their isolation from peripheral blood.

2.4.3 Analysis of LDL and oxidised LDL

2.4.3.1 Measurement of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) assay gives a measure of breakdown products of lipid peroxidation, such as malondialdehyde, which react with thiobarbituric acid when heated at low pH (Esterbauer & Cheeseman, 1990).

The content of TBARS within LDL samples was assayed by the method of Callaway et al., 1998. 100μl of 100μg/ml LDL samples were added to 100μl of 8.1% w/v sodium dodecyl sulphate and 750μl of 20% v/v acetic acid (adjusted to pH 3.5 with NaOH). Samples were centrifuged in eppendorf tubes (10000g, 15 minutes), 500μl of the supernatant was aspirated, and this was added to 500μl of

0.8% w/v thiobarbituric acid. The 1ml volume was then heated for 30 minutes at 100°C before being cooled on ice. The absorbance of the samples was measured at 532nm against a blank sample that contained an equal quantity of low-phosphate buffer in place of the LDL sample. Samples were run in duplicate and mean values compared to a standard curve of 1,1,3,3-tetramethoxypropane. A representative example of the standard curve is given in Figure 2.3.

2.4.3.2 Agarose gel electrophoresis of LDL

The electrophoretic migration of LDL through an agarose gel was used to give an indication of the surface charge of LDL as they were oxidised. Agarose gel electrophoresis of LDL was also essential to ensure that a single class of lipoprotein was isolated.

LDL were run in gels comprising an agarose-agar mixture, according to the method of Noble, 1968.

2.4.3.3 Lipid Hydroperoxide assay

Lipid hydroperoxides were quantified spectrophotometrically based on the method of El-Saadani *et al.*, 1989. Samples were run in duplicate and mean values compared to a standard curve of H_2O_2. A representative example of the standard curve is given in Figure 2.4.

2.5 Preparation of dialysis tubing

Cellulose dialysis tubing (Fisher Scientific, UK) was cut into strips and prepared by boiling in EDTA.Na_2 (0.38g/l) for 5 minutes. The dialysis tubing was then washed in distilled water and boiled for a further 5 minutes in EDTA.Na_2 (0.38g/l). The samples were subsequently washed in distilled water and stored in distilled water at 4°C until required.

2.6 Photography

All photographs were taken using a COHU high performance CCD camera (Diavert) and analysed using NIH image 1.61/ppc.

2.7 Statistical analysis

The Student's *t*-test, paired or unpaired as appropriate, was used to compare the data.

2.8 List of Buffers and Density Solutions

TE buffer – 10mM Tris/HCl and 1mM EDTA.Na_2 – adjusted to pH 8.0

TAE buffer – 40mM Tris-acetate and 1mM EDTA – adjusted to pH 8.0

LB medium – 10g Bacto-tryptone, 5g Bacto-yeast extract and 10g NaCl – adjusted to a volume of 1l with distilled water, and pH 7.5 with NaOH

CTAB/NaCl – 10g CTAB (N-cetyl-N,N,N-trimethyl ammonium bromide; Merck) and 4.1g NaCl – adjusted to a volume of 100ml with distilled water

Low phosphate buffer – 0.14M NaCl, 1.90mM NaH_2PO_4 and 8.1mM Na_2HPO_4 – adjusted to pH 7.4

Low density solution – approximate density 1.006g/ml – 0.15M NaCl and 297μM EDTA.Na_2 – adjusted to pH7.4

High density solution – approximate density 1.33g/ml – 2.62M NaCl, 2.98M KBr and 297μM EDTA.Na_2 – adjusted to pH7.4

1.063g/ml density solution – this was prepared by mixing the low and high density solutions above.

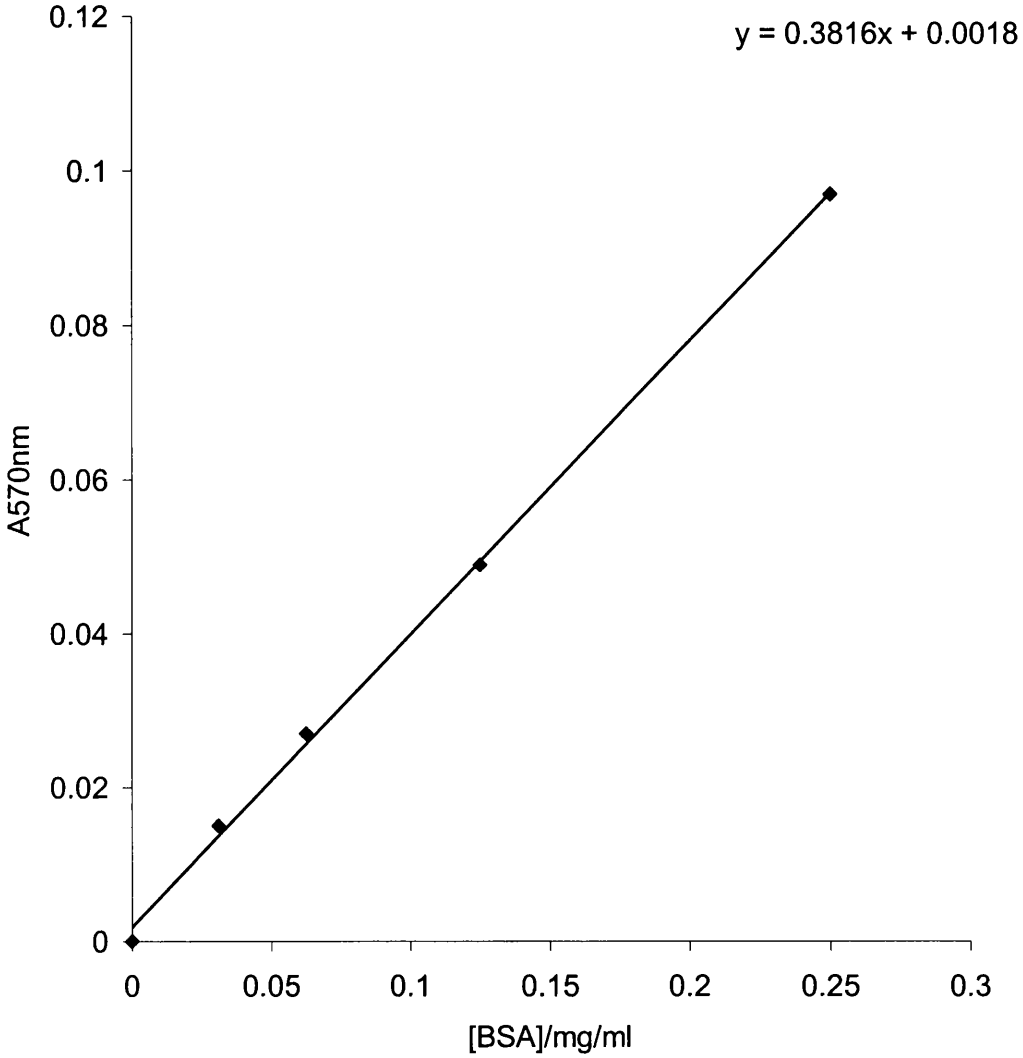


Figure 2.1 A standard curve displaying the relationship between protein concentration and absorbency. Samples were assayed according to the protocol described in section 2.4.1 and the mean of duplicate samples calculated. A standard curve was prepared freshly for each assay. One representative example of such an experiment is shown.

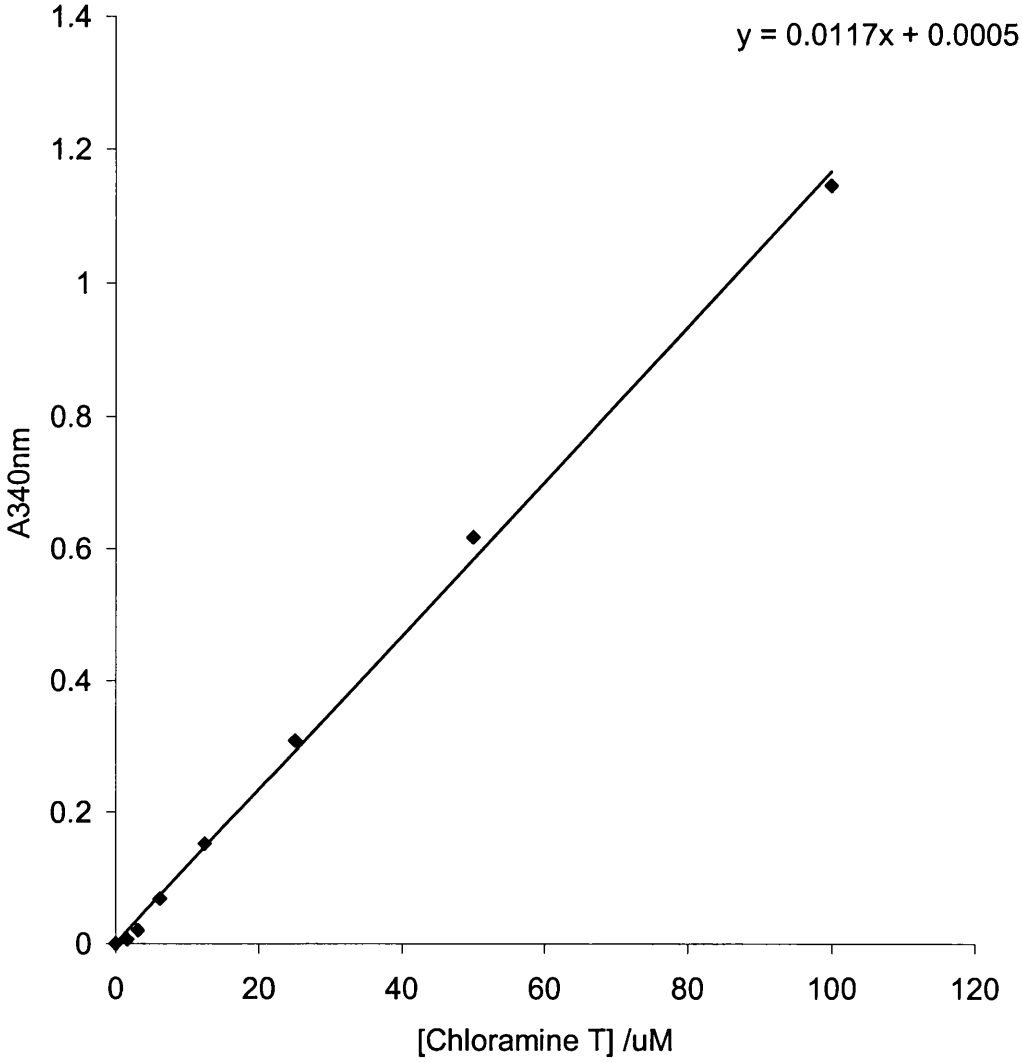


Figure 2.2 A standard curve displaying the relationship between Chloramine T concentration and absorbency. Samples were assayed according to the protocol described in section 2.3.2 and the mean of duplicate samples calculated. A standard curve was prepared freshly for each assay. One representative example of such an experiment is shown.

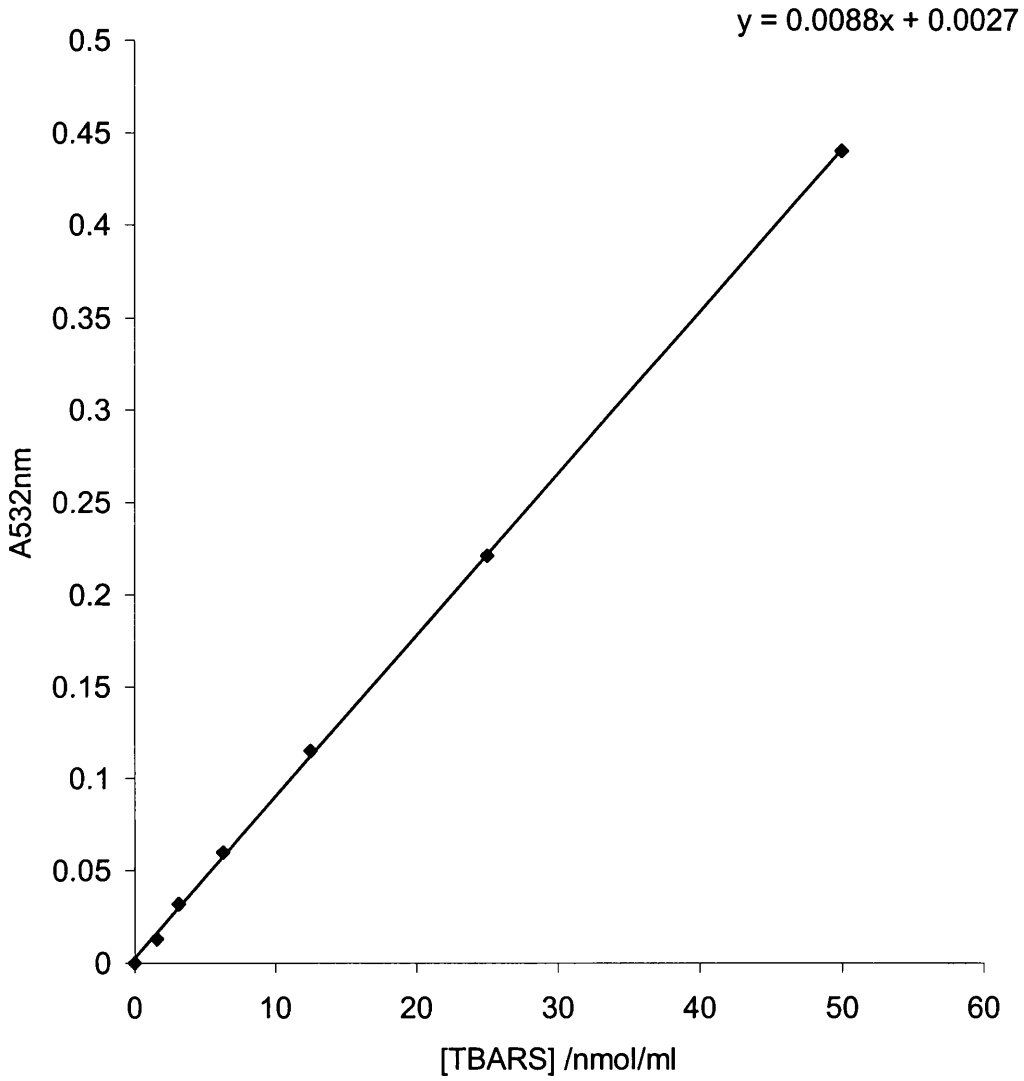


Figure 2.3 A standard curve displaying the relationship between thiobarbituric acid reactive substances (TBARS) concentration and absorbency. Samples were assayed according to the protocol described in section 2.4.3.1 and the mean of duplicate samples calculated. A standard curve was prepared freshly for each assay. One representative example of such an experiment is shown.

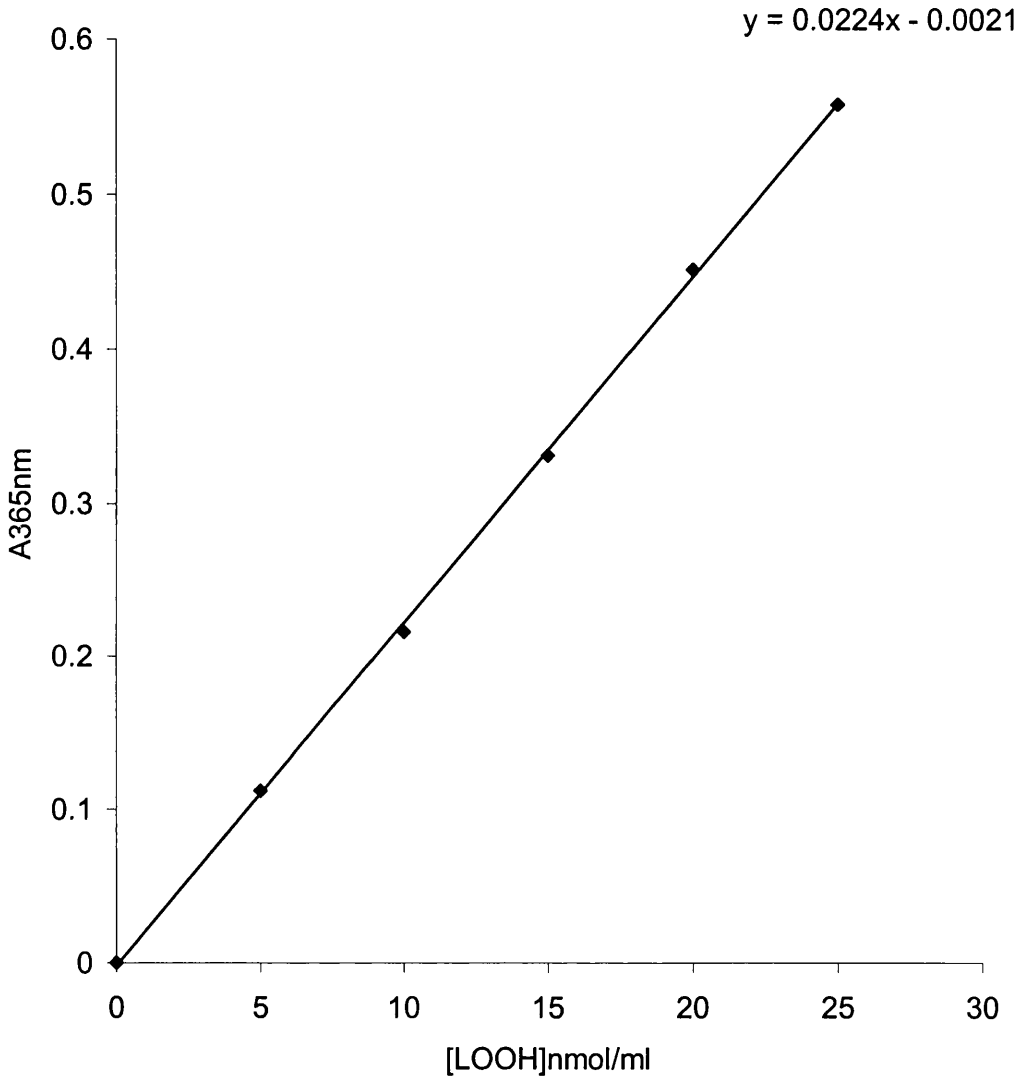


Figure 2.4 A standard curve displaying the relationship between lipid hydroperoxides (LOOH) concentration and absorbency. Samples were assayed according to the protocol described in section 2.4.3.3 and the mean of duplicate samples calculated. A standard curve was prepared freshly for each assay. One representative example of such an experiment is shown.

Chapter 3

Isolation & Characterisation of Dendritic cells

3.1 Introduction

DC are generally considered to be the most efficient stimulators of T-cell-mediated immune responses. However, research in the field of DC immunobiology has been hampered by a number of factors. Perhaps the most important of these are · lack of a unique differentiation marker and the difficulties involved in their isolation. Now that substantial numbers of DC can be obtained *in vitro*, full appreciation of their functional role and therapeutic potential is rapidly being realised.

3.1.1 Preparation of DC *in vitro*

It has been known for over a decade that DC can be isolated from human peripheral blood but constitute less than 1% of mononuclear cells. Therefore, without using extreme measures, relatively few DC can be obtained by this method. These DC were further shown to consist of distinct subsets (Thomas & Lipsky, 1994).

The quest to isolate an increased proportion of DC then progressed to the use of $CD34^+$ haematopoitic progenitor cells. Reid *et al.*, (1990) found that haematopoitic progenitor cells derived from either bone marrow or the peripheral circulation could be induced to differentiate into DC in the presence of GM-CSF and TNF-α. Prior administration of G-CSF increases the mobilisation of haematopoitic progenitor cells and was found to increase the yield of DC obtained from the circulation.

Following *in vitro* differentiation these samples contained a mixed population of macrophages and DC. Moreover, it was found that the DC were heterogeneous in terms of size and staining behaviour (Bernhard *et al.*, 1995; Pickl *et al.*, 1996) and therefore constituted an inappropriate experimental model.

In 1994, Sallusto and Lanzaveccia reported that a homogeneous cell population, not too dissimilar from immature DC, could be derived from monocyte-enriched peripheral blood mononuclear cells (PBMC), when cultured with GM-CSF and IL-4 for 7 days. This built on the prior knowledge that GM-CSF is required to maintain the viability of murine Langerhans' cells (Witmer-Pack *et al.*, 1987) and that IL-4 suppresses monocyte development (Jansen *et al.*, 1989). Thus, it was predicted that these conditions might allow DC differentiation to ensue.

An additional advantage of this protocol was that these DC were retained in an immature state. These cells were shown to undergo a maturation step when cultured in the presence of either TNF-α or CD40L. It was subsequently shown that virtually all typical ($CD14^+$) monocytes could be induced to differentiate into DC without concomitant proliferation (Pickl *et al.*, 1996). These DC are, therefore, not derived from a small subsets of proliferating DC progenitors thought to be present within PBMC fractions (Romani *et al.*, 1994).

It has since been shown that there is a considerable degree of plasticity between monocyte-derived DC (MDDC), monocytes and macrophages, driven by the prevailing cytokine environment (Paluka *et al.*, 1998). Maturation of DC leads to their terminal differentiation and consequently abrogation of this plasticity: this is in part a result of down-regulation of M-CSF receptor expression (Akagawa *et al.*, 1996).

Based on these findings, it has been hypothesised that in the event of an inadequate innate immune response, macrophages may differentiate into DC, migrate to secondary lymphoid organs and initiate an adaptive immune response (Paluka *et al.*, 1998). In support of this, it has been found that following transendothelial migration, monocytes can differentiate into DC within two days (Randolf *et al.*, 1998). This may explain the rapid recruitment of DC into airway epithelium upon inhalation of bacteria and viruses (McWilliams *et al.*, 1996).

To date, monocytes represent our most readily available source of DC and are currently the focus of much of today's research in this field.

3.1.2 Determination of the functional capacity of APC

Although not specifically investigated, one would predict that the DC best suited for use as therapeutic agents would be those with the maximum functional responses. Owing to the central role that DC play in orchestrating T-cell responses, one would also predict that, for use as adjuvants of the immune system, these functional responses would be best assessed in terms of their ability to act as accessory cells in T-cell proliferation assays.

Accessory cell function is assayed most commonly by the mixed leukocyte reaction (MLR). In this, the accessory cells and T cells are derived from different donors, representing a model of allogenic transplantation. However, functional responses can also be assessed in autologous assays, whereby in the absence of a specific antigen an additional signal is required. Such a signal may be provided by an anti-CD3 antibody, or alternatively, a covalent bond between the accessory and T cells, as in the oxidative mitogenesis assay.

The addition of anti-CD3 mAb directly cross-links the TCR providing a primary signal to the T-cell. This assay is, therefore, independent of the level of MHC expression and solely gives an indication of the costimmulatory capacity of the accessory cells (Johnson & Jenkins, 1994).

The oxidative mitogenesis assay is thought to be an *in vitro* representation of a physiological event. It has been known for many years that functional responses to a specific antigen can be inhibited by some monosaccharides. The inhibition could not be accounted for by inhibitory actions at the levels of antigen processing or presentation, but was subsequently shown to be dependent upon a common aldehyde group on these inhibitory monosaccharides.

It has further been shown that these aldehyde groups prevent the formation of reversible intercellular covalent bonds. In the presence of a specific antigen, these bonds spontaneously form between ε-amino groups of lysyl residues and

carbonyl groups on the accessory and T cells, thus forming Schiff base interactions. Soluble aldehyde groups are thought to exert their inhibitory action through binding to exposed amino groups and thereby preventing the formation of intercellular Schiff bases (Rhodes, 1989).

In the absence of a cognate antigen, this interaction can be modelled through the formation of aldehydes on membrane glycoproteins. Chemically these can be formed by either oxidation of N-acetylneuraminic acid (NANA) by sodium periodate or by treating cells with neuraminidase to expose galactosyl or N-acetylgalactosaminyl residues, and then oxidising these residues with galactose oxidase to form aldehyde groups. (Rhodes, 1989). As would be predicted, these interactions can be inhibited by the weak reducing agent sodium cyanoborohydride, which is specific for Schiff bases.

Despite the fact that this functional assay is rarely employed, it has been suggested that stimuli of oxidative mitogenesis may represent a novel immunopotentiatory therapeutic tool in the treatment of chronic diseases (Rhodes et al., 1995).

3.2 Results

3.2.1 Comparative phenotype of monocytes and DC

The mandatory starting point for DC studies *in vitro* is to confirm the phenotypic profile of these cells. The tool most frequently used to quantify the surface expression of phenotypic markers is the flow cytometer. Cells were first tagged with specific monoclonal antibodies and then labelled using a dye-coupled anti-immunoglobulin. Flow cytometry can then be used to provide information regarding the size and granularity of cells, and fluorescence emissions, which indicates the degree of surface-molecule expression. The size (forward scatter) and granularity (side scatter) of cell populations can be displayed as two-dimensional scatter diagram, whilst the expression of single surface markers is displayed as a histogram of fluorescence intensity against number of cells (events).

Figure 3.1 gives clear evidence of an increase in both forward (FSC-H) and side (SSC-H) scatter upon the differentiation of monocytes to DC. It can further be seen from Figure 3.1 that immature and mature DC are of similar sizes and granularities. The histograms presented in Figure 3.1 show that, within the gated populations given, fluorescence intensities associated with CD3 and CD19 staining are similar to those observed following staining with an irrelevant antibody (IgG2a). It can, therefore, be concluded that the gated cell populations do not contain cells that express CD3 (T cells) or CD19 (B cells).

Further to characterise the differentiation of monocytes to immature and mature DC, a detailed analysis of their relative surface marker expression (Figure 3.2) was required. The mean results from five such experiments are summarised in Figure 3.3.

The differentiation of monocytes to immature DC was associated with significant increases in their expression of HLA-DQ and –DR, and CD1a. It can be seen that

the purified monocyte population consistently contains greater than 95% $CD14^+$ (typical) monocytes. DC are shown to express CD14, although to a significantly lesser extent than monocytes. Upon maturation, with a known physiological stimulus, DC significantly increase their surface expression of HLA-DQ and –DR. Also the percentage of cells expressing CD86 increased.

3.2.2 Phenotypic instability of DC

It has been shown that DC can differentiate from monocyte-enriched PBMC. However, this consists of a mixed population derived from an adherent cell population that may include other cells as well as monocytes. The "contaminating" cells are not a majority population but, nonetheless, the DC must be purified to maintain the consistency of the population. This modification also ensured that the different test stimuli used later were acting directly on the DC and not through an indirect mechanism.

To document the phenotype of the purified population, DC were cultured for six days, purified, and then cultured for a further 24 hours in the presence of cytokines: this gave very similar results to those shown in Figure 3.3. In the absence of cytokines, significant increases occur in the expression of HLA-class I, and the percentage of cells expressing CD14 and CD86 (Figure 3.4). Since monocytes and mature DC express high levels of CD14 and CD86, respectively, these changes could be interpreted as indicating a possible dedifferentiation (CD14) or maturation (CD86) of immature DC in the absence of cytokines.

3.2.3 Phenotypic and functional effects of re-culturing DC

The purification of DC raises several questions about whether or not the act of replating DC itself results in their phenotypic or functional maturation, as previously suggested (Gallucci *et al.*, 1999). Figure 3.5 shows that, upon replating DC, there are no significant differences in their surface expression of HLA-DQ or –DR, or CD86. It can, therefore, be seen that replating DC does not

induce the phenotypic maturation of DC in a manner that is akin to the maturational effects of LPS, as shown in Figure 3.3.

Further to explore the effects of replating DC, the ability of these cells to stimulate the proliferation of T cells was investigated. Figure 3.6 shows that, when assessed by an autologous CD3-dependent assay, DC stimulate the proliferation of T cells, as measured by ^{3}[H]thymidine incorporation, in a dose-dependent manner. It can further be seen from Figure 3.6 that, compared to control DC, replated DC have a reduced capacity to induce the proliferation of T cells.

The reduction in functional capacity could be a result of direct stress on the DC, or alternatively, a consequence of their transient readherence.

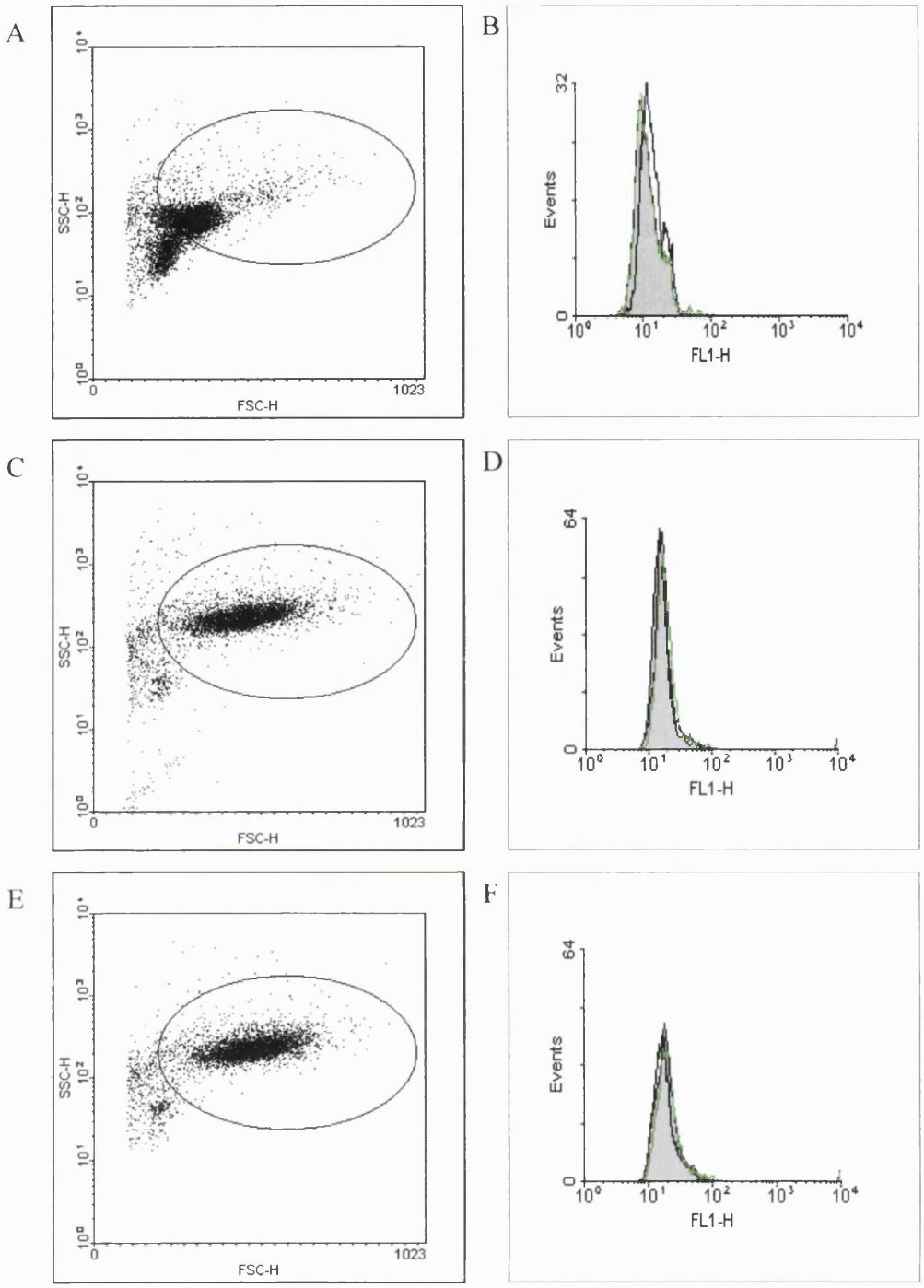


Figure 3.1. Typical FACScan profiles of monocytes and, immature and mature monocyte-derived DC. Scatter diagrams are given displaying cell size (FSC-H) against granularity (SSC-H) (A, C and E), and histograms representing surface staining (B, D and F). Monocyte-derived DC were generated from monocyte-enriched PBMC cultured for 7 days with GM-CSF/IL-4. Maturation was induced by the addition of 100ng/ml LPS for the final 24 hours of culture. Purified populations of monocytes (A and B), and, immature (C and D) and mature monocyte-derived DC (E and F) are given with negative controls representing IgG2a (filled profiles), CD3 (black lines) and CD19 (green lines). One representative of five experiments is shown.

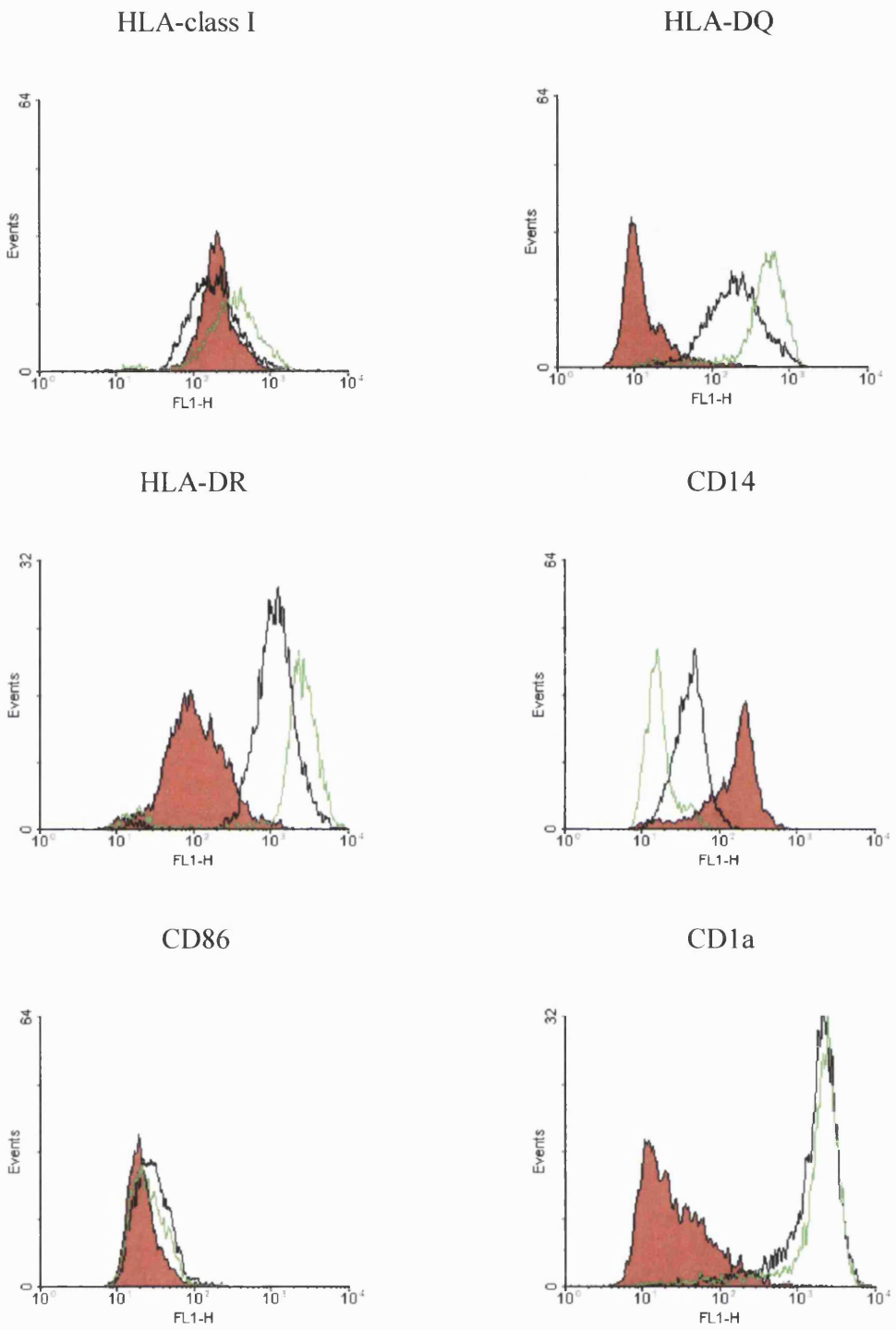


Figure 3.2. Typical histograms representing surface staining of DC differentiation markers on monocytes and, immature and mature monocyte-derived DC. Markers are given for individual plots. Monocyte-derived DC were generated from monocyte-enriched PBMC cultured for 7 days with GM-CSF/IL-4. Maturation was induced by the addition of 100ng/ml LPS for the final 24 hours of culture. Purified populations of monocytes (filled profiles) and, immature (black lines) and mature (green lines) monocyte-derived DC are shown. One representative of five experiments is shown.

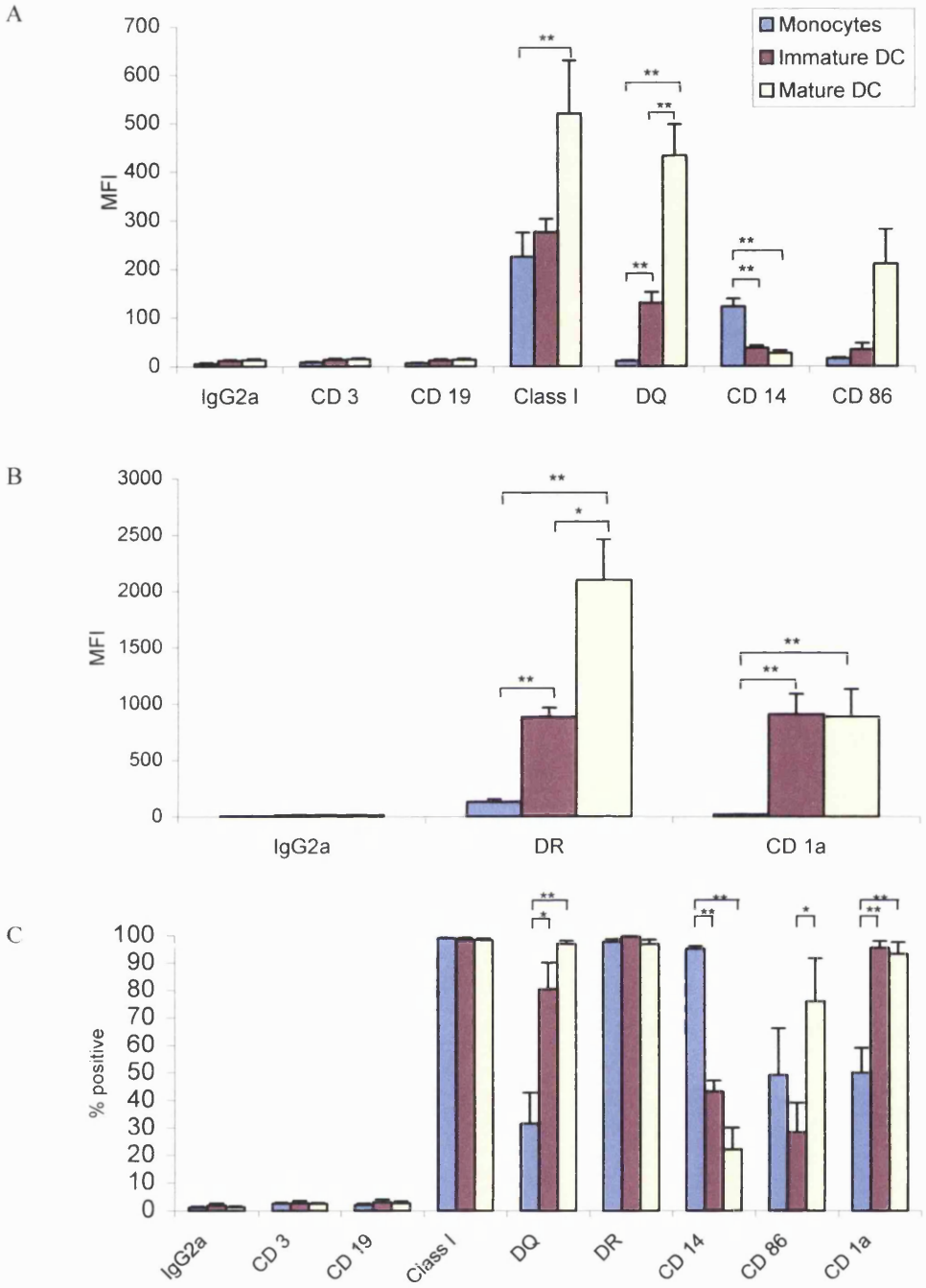


Figure 3.3. Characteristic surface molecule expression of freshly prepared monocytes and, immature and mature monocyte-derived DC. Monocyte-derived DC were generated from monocyte-enriched PBMC cultured for 7 days with GM-CSF/IL-4. Maturation was induced by the addition of 100ng/ml LPS for the final 24 hours of culture. Surface expression was analysed as detailed in Materials and Methods and given as, mean fluorescence intensities (MFI) ± SEM (A and B) and, percentage positive cells ± SD (C). Mean values are given from five matched independent experiments. An irrelevant IgG2a primary mAb is given as control, in addition to CD3 and CD19. *, $p<0.05$; **, $p<0.01$.

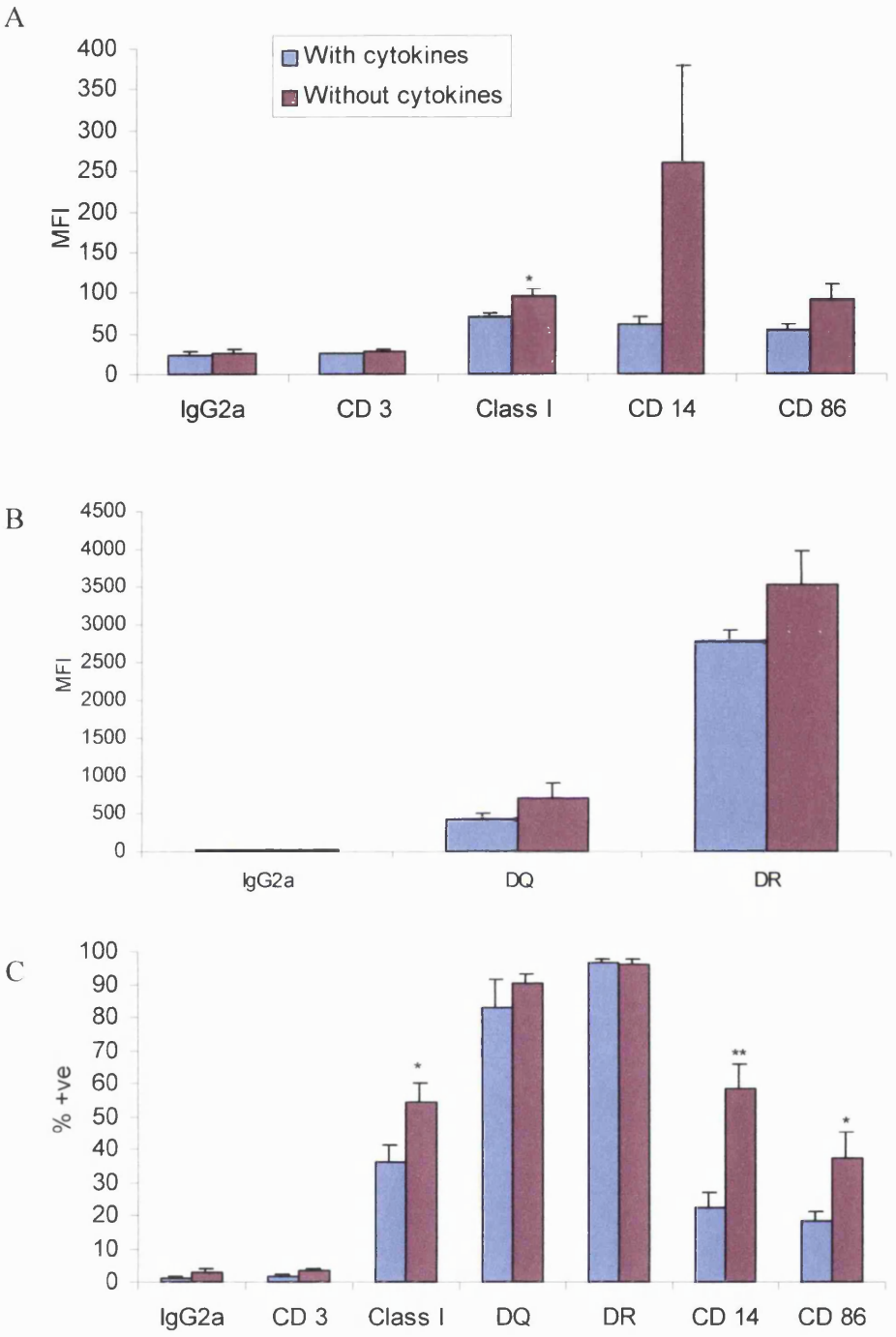


Figure 3.4. Phenotypic instability of monocyte-derived DC. Monocytes were cultured for 6 days in GM-CSF/IL-4. Non-adherent monocyte-derived DC were then purified and replated for a further 24 hours in the presence or absence of GM-CSF/IL-4. Surface expression was analysed as detailed in Materials and Methods and given as, mean fluorescence intensities (MFI) ± SEM (A and B) and percentage positive cells ± SD (C). Mean values are given from 6-8 independent experiments per data point. An irrelevant IgG2a primary mAb is given as control, in addition to CD3. *, $p<0.05$; **, $p<0.01$.

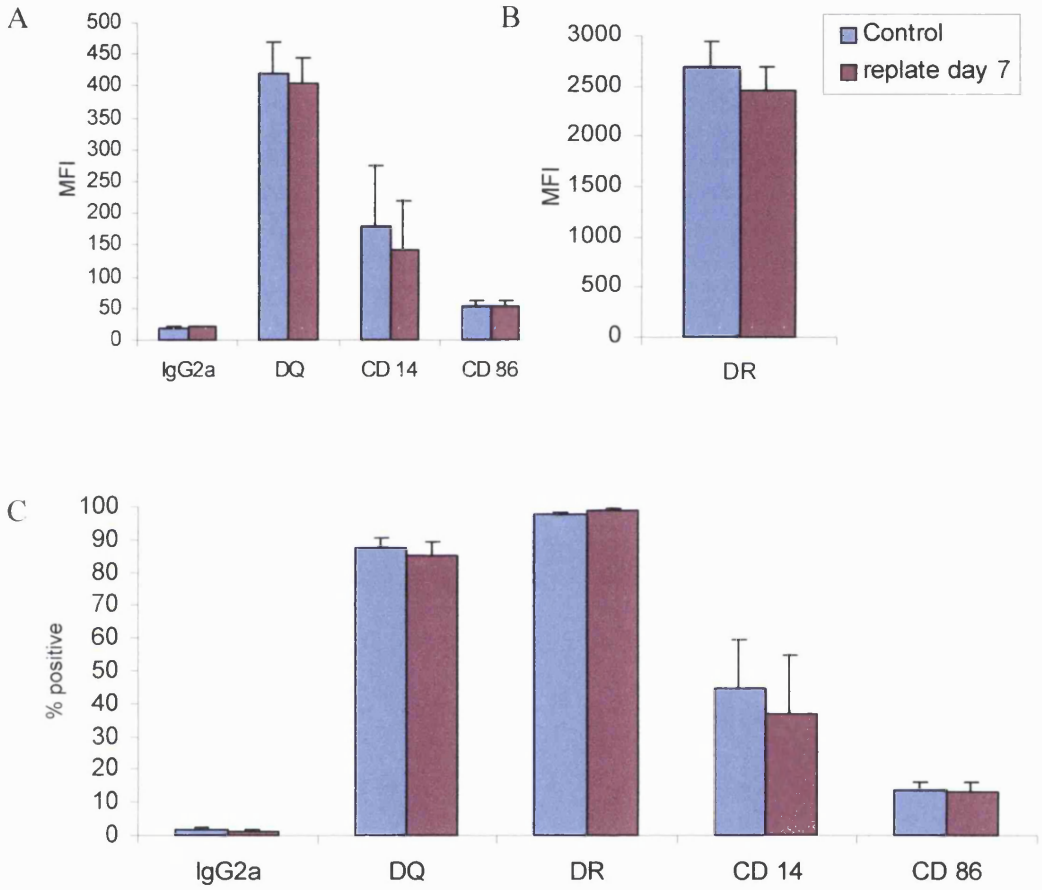


Figure 3.5. Re-culturing monocyte-derived DC is not associated with their phenotypic maturation. DC were generated from monocyte-enriched PBMC cultured with GM-CSF/IL-4 for 7 days. After 6 days, paired samples were transferred to new 6-well plates for the remaining 24 hours of culture. Surface expression was analysed as detailed in Materials and Methods and given as, mean fluorescence intensity (MFI) ± SEM (A and B) and percentage positive cells ± SD (C). Mean values are given from 4 independent experiments. An irrelevant IgG2a primary mAb is given as control. Statistical analysis revealed no significant differences between the two cell populations given.

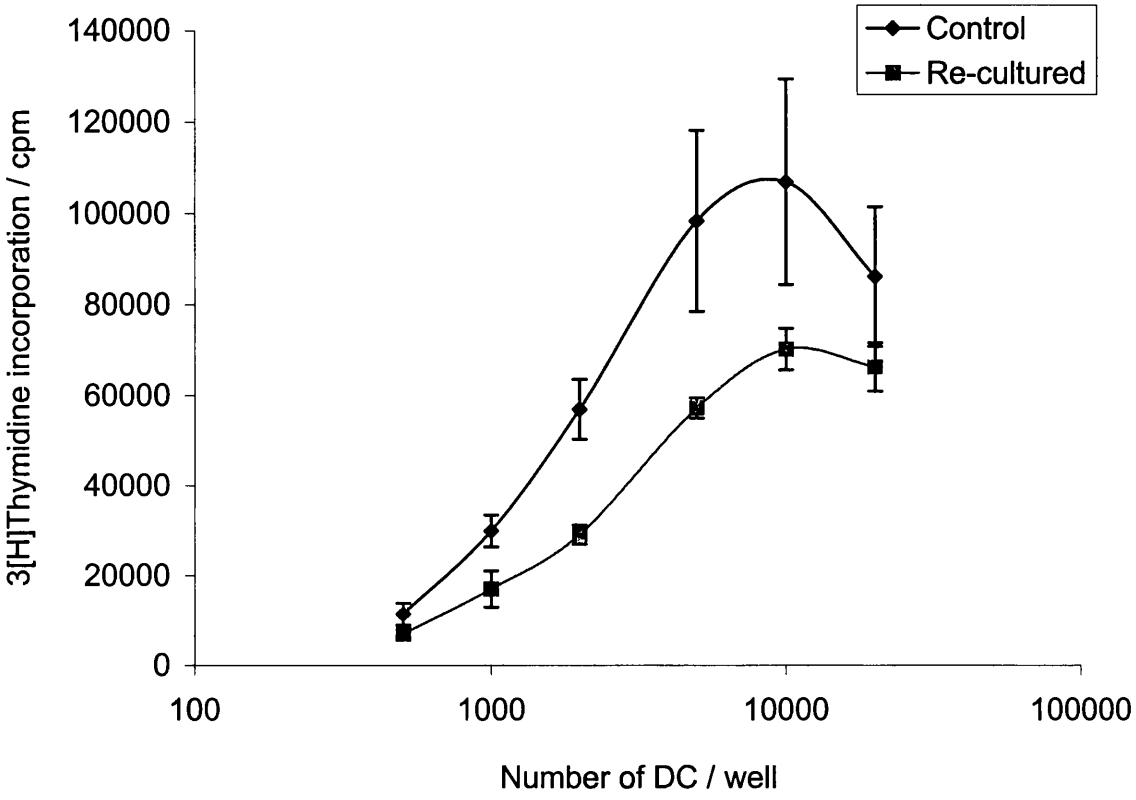


Figure 3.6. Re-culturing monocyte-derived DC is associated with a reduction in their ability to stimulate the proliferation of T-cell; as assessed by an autologous CD3-dependent assay. Monocyte-derived DC were generated from monocyte-enriched PBMC cultured with GM-CSF/IL-4 for 7 days. After 6 days, paired samples were transferred to new 6-well plates for the remaining 24 hours of culture. Monocyte-derived DC were then harvested, purified and added to 10^5 purified autologous T cells for 48 hours in the presence of 0.1μg/ml anti-CD3 antibody. The response was quantified by ^{3}[H]Thymidine incorporation during a further 16 hours of incubation. Samples were run in triplicate and are presented as mean ± SD. One experiment representative of three is shown (n=3; 2000 DC/well; % control response after re-culturing, 59.7 ± 13.7%).

3.2.4 Functional changes associated with maturation of DC

Mature DC are generally regarded as being functionally more active than their immature precursors. However, when the ability of immature and mature DC to stimulate the proliferation of T cells was assessed by a CD3-dependent assay, mature DC were found to be less able to stimulate the proliferation of resting T cells, as measured by ^{3}[H]thymidine incorporation, than immature DC (Figure 3.7).

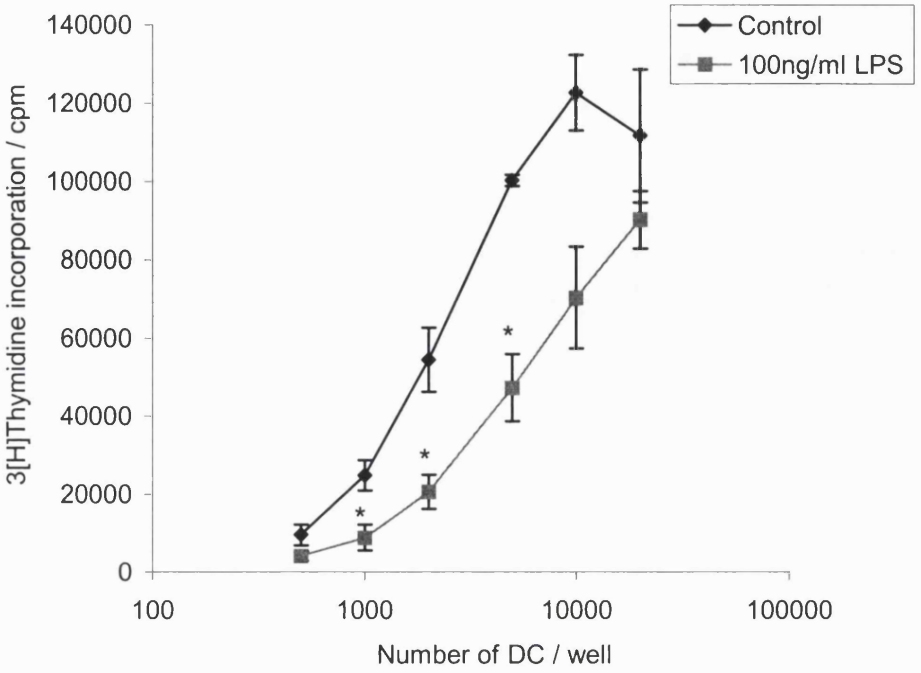
It can further be seen from Figure 3.7 that, when T cells alone were incubated in the presence of anti-CD3, very low levels of ^{3}[H]thymidine incorporation were observed, suggesting that they do not proliferate. Since it is known that DC do not proliferate, it is possible that the ^{3}[H]thymidine incorporation reported, when immature and mature DC were incubated with anti-CD3 (Figure 3.7), may reflect the proliferation of residual contaminating lymphocytes or residual ^{3}[H]thymidine within the DC, which have a much greater volume than T cells.

It should be noted that the CD3-dependent assay relies on a primary signal delivered by a soluble anti-CD3 mAb, as detailed in the Materials and Methods and previous reports (Retault *et al.*, 1998). Since immobilised anti-CD3 mAb is able to activate T cells (Johnson & Jenkins, 1994), this is probably independent of Fc receptors. One possible explanation for the decrease in functional capacity upon DC maturation is that DC are known to mature further upon binding T cells, and maturation is associated with a transient increase in endocytosis (see Section 1.1.3.2), so there may be a decrease in the amount of soluble antibody available to stimulate the T cells.

In contrast to the CD3-dependent assay, maturation of DC is shown to be associated with a significant increase in their ability to induce the proliferation of T cells, as indicated by ^{3}[H]thymidine incorporation, when assessed by a MLR (Figure 3.8). It can further be seen in Figure 3.8 that the statistically significant increases in T-cell proliferation occur at high numbers of DC / well. When high

numbers of the individual cell populations were incubated alone, very low levels of ^{3}[H]thymidine incorporation were observed, suggesting that little proliferation occurred.

Figure 3.9 shows that when the ability of cell populations to stimulate the proliferation of T cells was examined in an autologous sodium periodate mediated oxidative mitogenesis assay, the differentiation of monocytes to immature and then mature DC was associated with a graded stimulatory capacity. It can clearly be seen that the stimulatory effect of immature DC is significantly greater than that of monocytes, and increases further upon maturation. From Figure 3.9 it can further be seen that in the absence of T cells, APC or periodate treatment, relatively low levels of ^{3}[H]thymidine incorporation, and thus T-cell proliferation occurred.



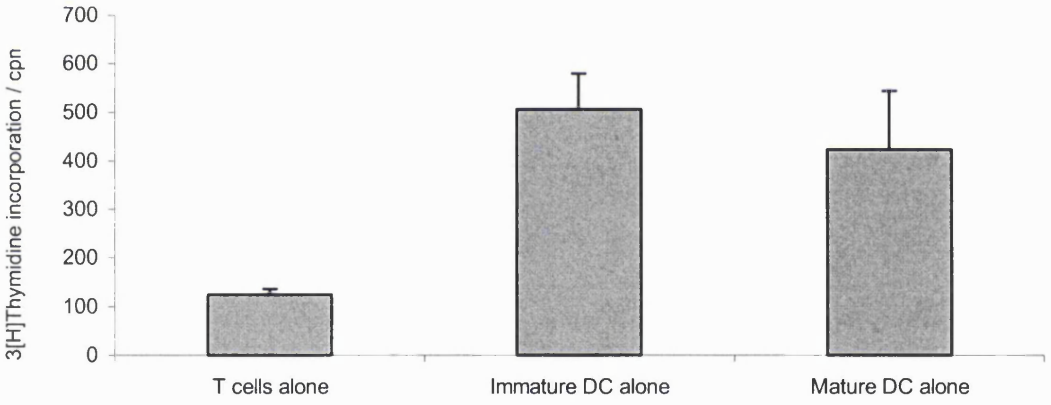


Figure 3.7. Maturation of monocyte-derived DC is associated with a reduction in T-cell activating capacity when assessed by an autologous CD3-dependent assay. DC were generated from monocyte-enriched PBMC cultured with GM-CSF/IL-4 for 7 days. Maturation was induced by the addition of 100ng/ml LPS for the final 24 hours of culture. Monocyte-derived DC were subsequently harvested, purified and added to 10^5 purified autologous T cells for 48 hours in the presence of 0.1μg/ml anti-CD3 antibody. The response was quantified by ^{3}[H]Thymidine incorporation during a further 16 hours of incubation. Samples were run in triplicate. The mean of three independent experiments is shown ± SEM. *, $p<0.05$. Lower graph gives control responses.

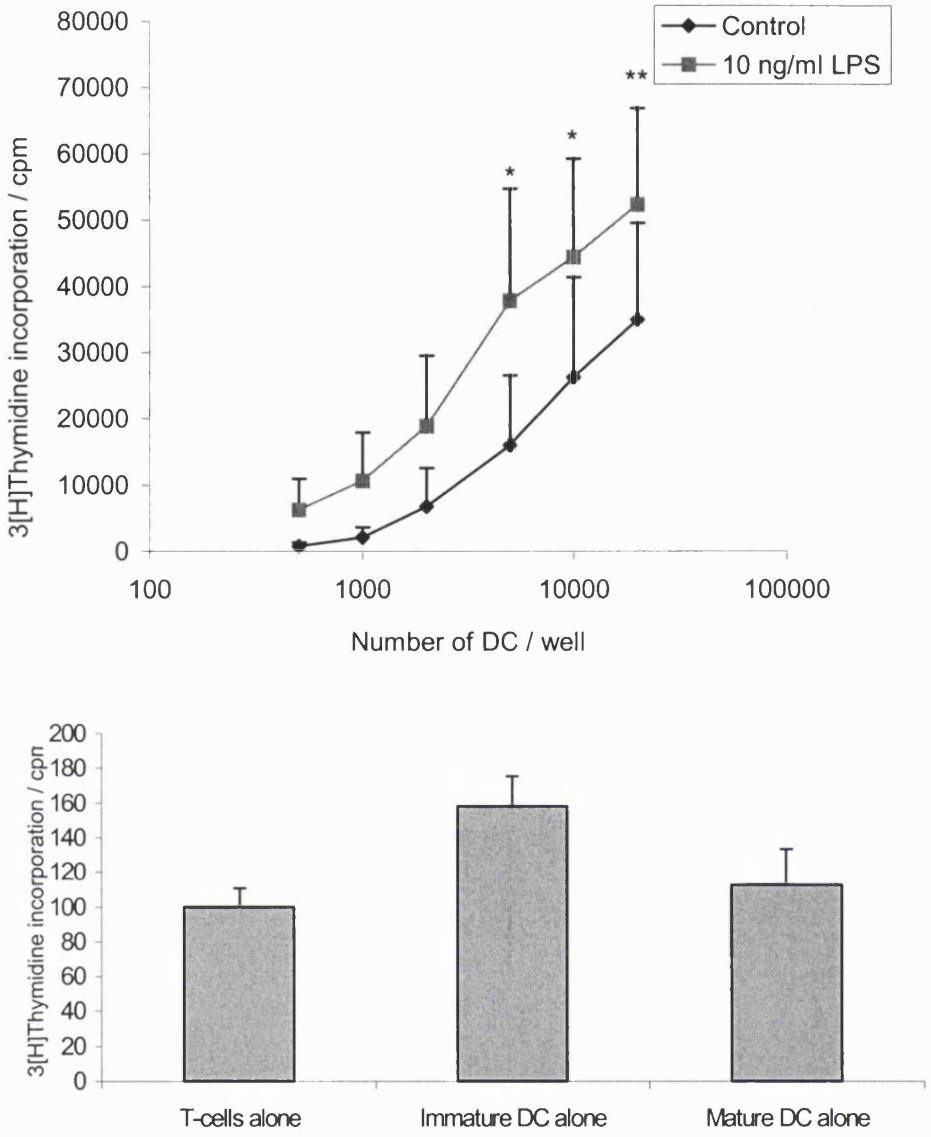
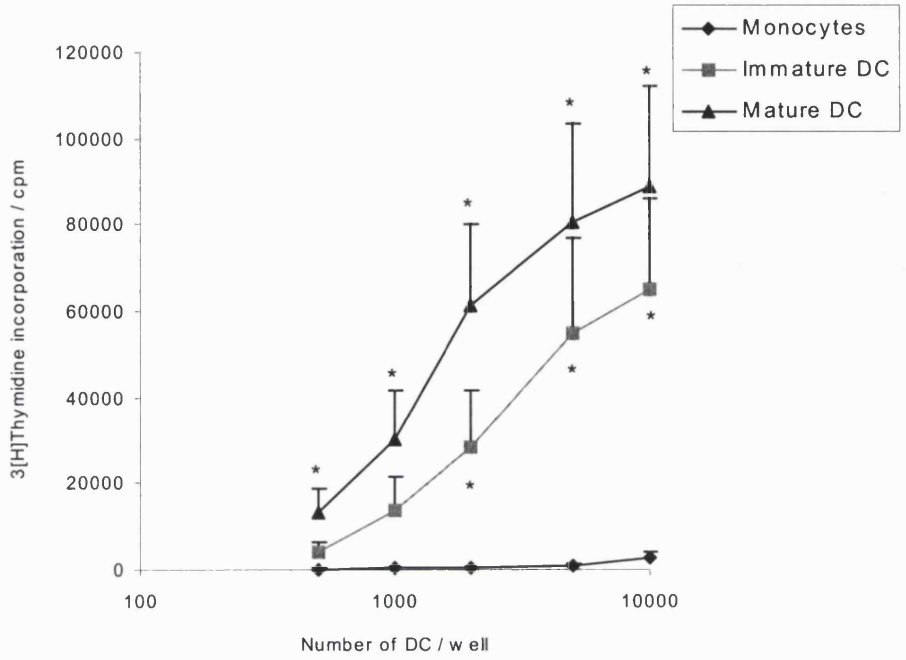


Figure 3.8. Maturation of monocyte-derived DC is associated with an increase in T-cell activating capacity when assessed by a mixed leukocyte reaction. DC were generated from monocyte-enriched PBMC cultured with GM-CSF/IL-4 for 7 days. Maturation was induced by the addition of 100ng/ml LPS for the final 24 hours of culture. Monocyte-derived DC were subsequently harvested, purified and added to 10^5 purified allogeneic T cells for a further five days. The response was quantified by ^{3}[H]Thymidine incorporation during a further 16 hours of incubation. Samples were run in triplicate. The mean of five independent experiments is shown ± SEM. *, $p<0.05$; **, $p<0.01$. Lower graph gives control responses.



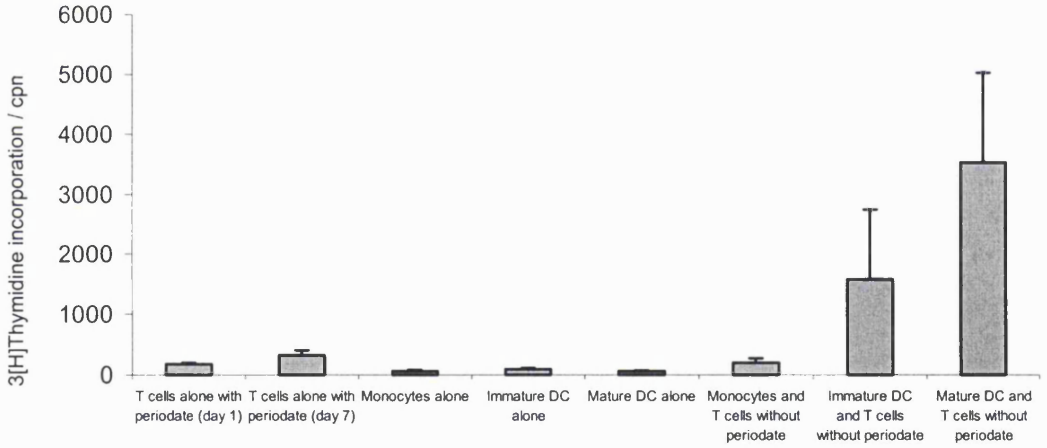


Figure 3.9. Differentiation and maturation of monocyte-derived DC is associated with an increase in T-cell activating capacity when assessed by an autologous oxidative mitogenesis assay. DC were generated from monocyte-enriched PBMC cultured with GM-CSF/IL-4 for 7 days. Maturation was induced by the addition of 100ng/ml LPS for the final 24 hours of culture. Monocyte-derived DC were subsequently harvested, purified and added to 10^5 purified autologous T cells for a further 48 hours. The response was quantified by ^{3}[H]Thymidine incorporation during a further 16 hours of incubation. Samples were run in triplicate. The mean of five independent experiments is shown ± SEM. Statistical analysis for immature DC is given relative to monocytes and for mature DC is given relative to immature DC. *, $p<0.05$. Lower graph gives control responses.

3.2.5 Functional benefits of purifying DC on day 4

Figure 3.10 illustrates that in addition to the aforementioned necessity of purifying DC there are also functional benefits. DC are defined, and indeed were initially isolated, by their ability to stimulate the activation of resting T cells. It can be seen from Figure 3.10 that DC purified halfway through the culture period are able to stimulate significantly more ^{3}[H]thymidine incorporation, and thus T-cell proliferation, than those left for the entire seven days. Figure 3.11 illustrates that purification of DC halfway through their culture are capable of maturing further, as shown by their increased capacity to be activated upon culture for 24 hours in the presence of 10ng/ml LPS.

3.2.6 Mechanism by which DC purified on day 4 are functionally more active

It has been shown that replating DC, or more likely, the transient readherence that is associated with this, alters the functional responses of DC. It is interesting to postulate that this may be the mechanism by which cells, purified halfway through the culture, are functionally more active than those not purified. Figure 3.12 illustrates that replating DC on day 4 does indeed significantly increase the ability of DC to stimulate the proliferation of T cells. However, replating alone is insufficient to account for the total increase in functional responses observed.

Figure 3.13 illustrated that if the cells are washed on day 4 and then re-cultured in the presence of new media and cytokines for the remaining 4 days, then they are functionally as active as those purified on the same day (day 4). It is, therefore, suggested that although replating DC may account partially for the increased functional responses observed, replating DC in fresh media and cytokines may account for the total increase in functional responses.

The advantages of changing the culture media on day 4 can also be seen at the phenotypic level. Figure 3.14 shows that the phenotype of DC purified on day 4

is remarkably similar to that of DC cultured from day 4, without purification, in fresh media and cytokines. It is, therefore, suggested that replacing the media and cytokines on day 4 results in a relatively low level of CD86, HLA-DQ and CD14 expression, and increases the expression of CD1a.

From Figure 3.14 it can be seen that when DC were purified on day 4 and then cultured for the remaining 4 days in media used for the first three days of culture (old media, purify day 4), the phenotype of DC is similar to that found for DC purified on day 8, and DC replated on day 4. It can, therefore, be seen that replating DC on day 4, or any step of the purification process, does not induce the surface expression of CD86, HLA-DQ, CD14 or CD1a to change on DC.

Moreover, from Figure 3.14 it can be seen that DC purified on day 4 express higher levels of CD1a and lower levels of CD14 than those cultured in the conventional way. Figure 3.14 also shows that despite the fact that cells purified on day 4 are functionally more active than those purified on day 8, they have a lower expression of markers usually associated with their maturation (i.e. CD86, and HLA-DQ and –DR).

3.2.7 Extended phenotypic analysis of DC purified on day 4

To characterise further the effects of purifying DC on day 4, an extended phenotypic analysis was conducted. Figure 3.15 illustrates that conventional DC and DC purified on day 4 express similar levels of the novel DC markers CD87, CD98, CD147, CD148 and the costimulatory molecule CD40. They also express similar levels of the myeloid marker CD13 and the common leukocyte antigen CD45.

A representative experiment is shown in Figure 3.17 with negative controls presented in Figure 3.16.

3.2.8 Dose-dependent nature of LPS

The increases in surface expression of HLA-DQ and –DR, and CD86 in response to LPS are shown to be dose-dependent (Figure 3.18), suggesting that maturation of DC is a graded process. It can be seen from Figure 3.18 that the first significant increase in the expression of CD86 occurred following the incubation of DC with 1ng/ml LPS for 24 hours.

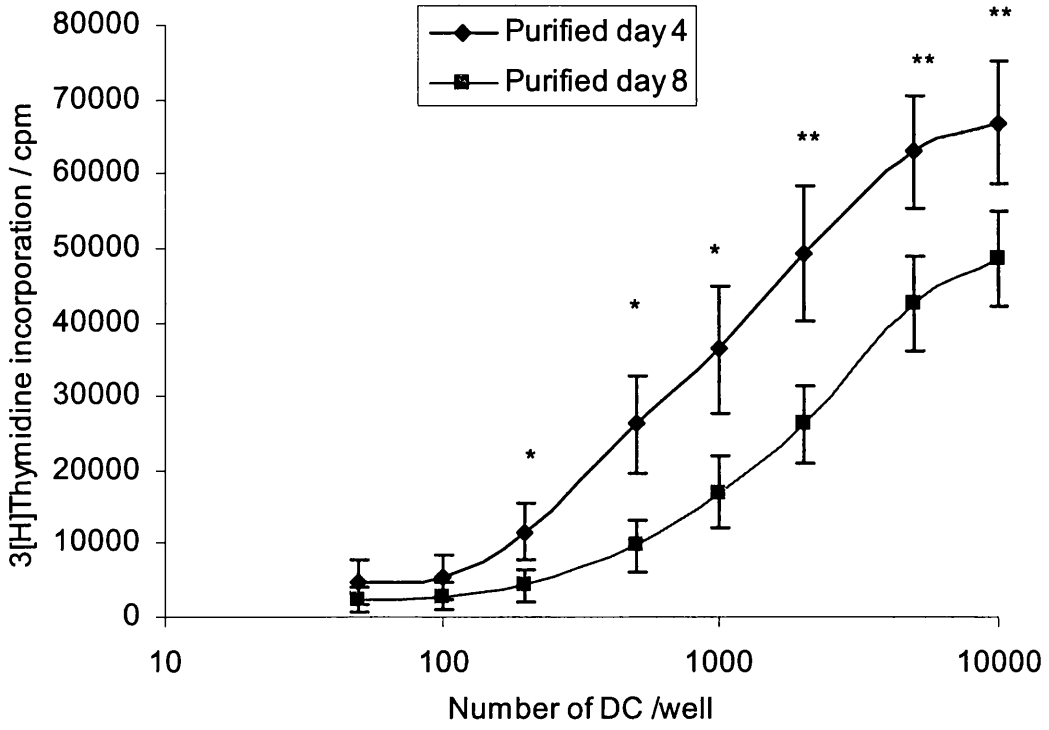


Figure 3.10. Monocyte-derived DC that were purified halfway through their differentiation had a greater T-cell activating capacity than those which were not purified. T-cell activating capacity was assessed in an autologous oxidative mitogenesis assay. DC were generated from monocyte-enriched PBMC cultured with GM-CSF/IL-4. Monocyte-derived DC were subsequently either purified on day 4 and cultured for the remaining four days with fresh cytokines and media at 5 x 10^5 DC/ml, or purified on day 8. Monocyte-derived DC were then added to 10^5 purified autologous T cells for 48 hours and the response quantified by ^{3}[H]Thymidine incorporation during a further 16 hours of incubation. Samples were run in triplicate. The mean ± SEM of 5-6 paired experiments is shown for each concentration of DC. *, $p<0.05$; **, $p<0.01$.

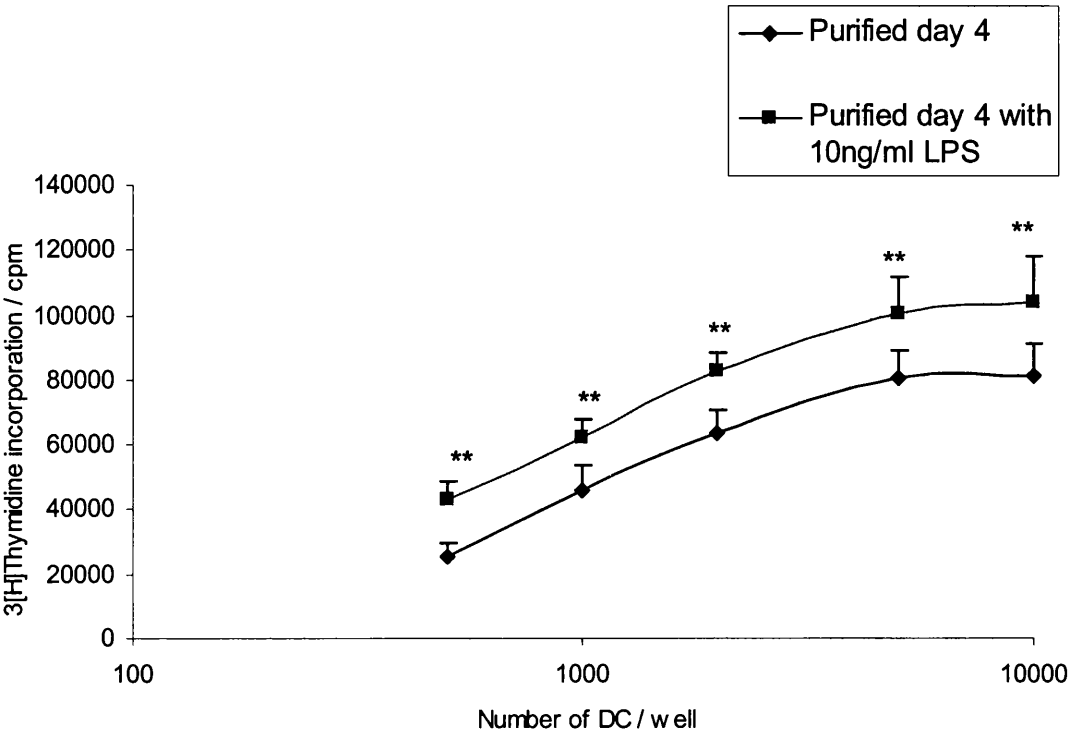


Figure 3.11. Monocyte-derived DC that are purified on day 4 can further increase their T-cell activating capacity upon stimulation with LPS. T-cell activating capacity was assessed in an autologous oxidative mitogenesis assay. Monocyte-derived DC were generated from monocyte-enriched PBMC cultured with GM-CSF/IL-4. Monocyte-derived DC were subsequently purified on day 4, and stimulated on day 7 for a further 24 hours. Monocyte-derived DC were then added to 10^5 purified autologous T cells for 48 hours and functional responses quantified by ^{3}[H]Thymidine incorporation during a further 16 hours of incubation. Samples were run in triplicate. The mean ± SEM of seven paired experiments is shown. **, $p<0.01$.

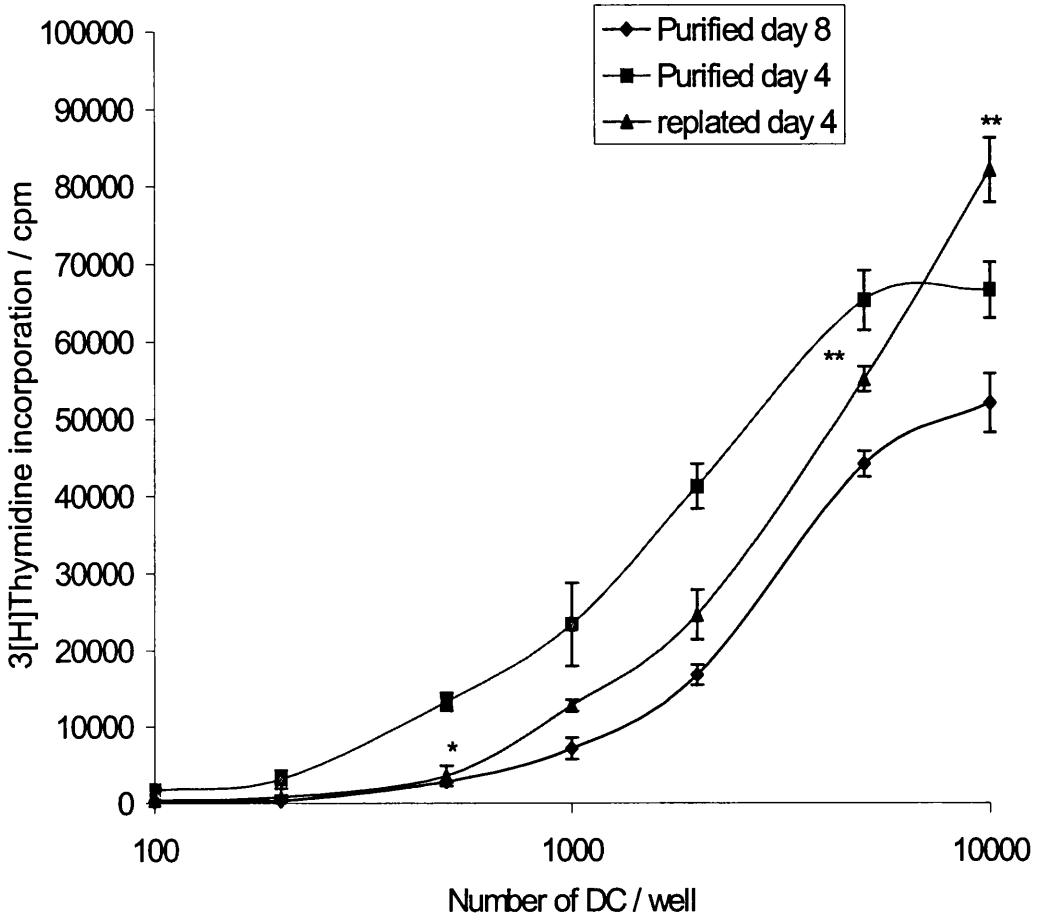


Figure 3.12. Re-plating monocyte-derived DC on day 4 is associated with an increase in T-cell activating capacity when assessed in an autologous oxidative mitogenesis assay. Monocyte-derived DC were generated from monocyte-enriched PBMC cultured with GM-CSF/IL-4 for 7 days. On day 4, paired samples were transferred to new 6-well plates. Monocyte-derived DC were harvested on day 8, purified, and added to 10^5 purified autologous T cells for 48 hours. Functional responses were quantified by ^{3}[H]Thymidine incorporation during a further 16 hours of incubation. Samples were run in triplicate. Mean ± SD of one experiment representative of four is shown (n=4; 5000 DC/well; relative to purified day 4, replate day 4 = 78.4 ± 4.9%, purify day 8 = 63.8 ± 4.4%). Statistical analysis for replated 4 DC is given relative to purified day 4 DC. *, $p<0.05$; **, $p<0.01$.

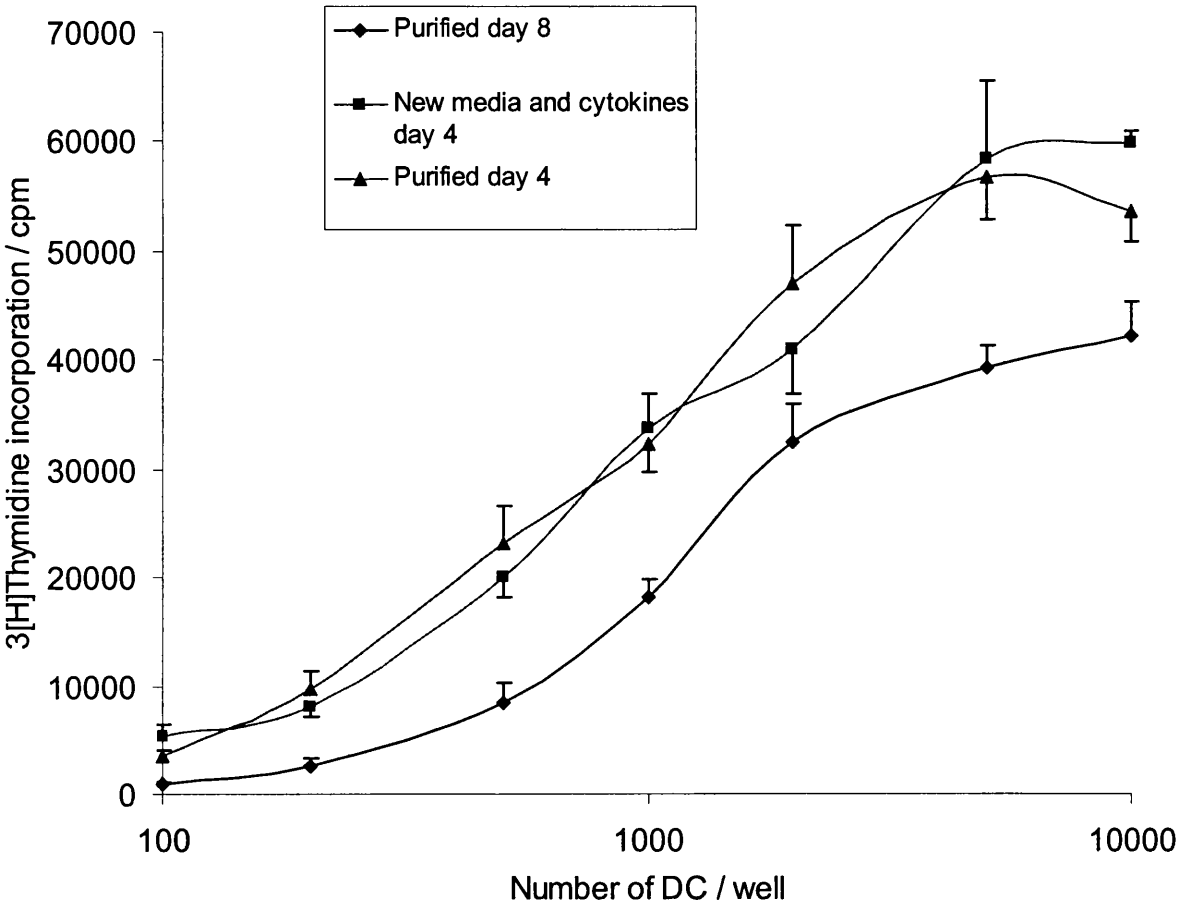


Figure 3.13. Monocyte-derived DC that are purified on day 4 are functionally more active as a result of replenishing their supply of nutrients and cytokines. T-cell activating capacity was assessed in an autologous oxidative mitogenesis assay. Monocyte-derived DC were generated from monocyte-enriched PBMC cultured with GM-CSF/IL-4. On day 4, paired samples were washed and transferred to new 6-well plates. Monocyte-derived DC were harvested on day 8, purified, and added to 10^5 purified autologous T cells for 48 hours. Functional responses were quantified by ^{3}[H]Thymidine incorporation during a further 16 hours of incubation. Samples were run in triplicate. Mean ± SD of one experiment representative of three is shown (n=3; 5000 DC/well; relative to purified day 4, new media & cytokines day 4 = 108 ± 5.3%, purify day 8 = 70 ± 1.6%).

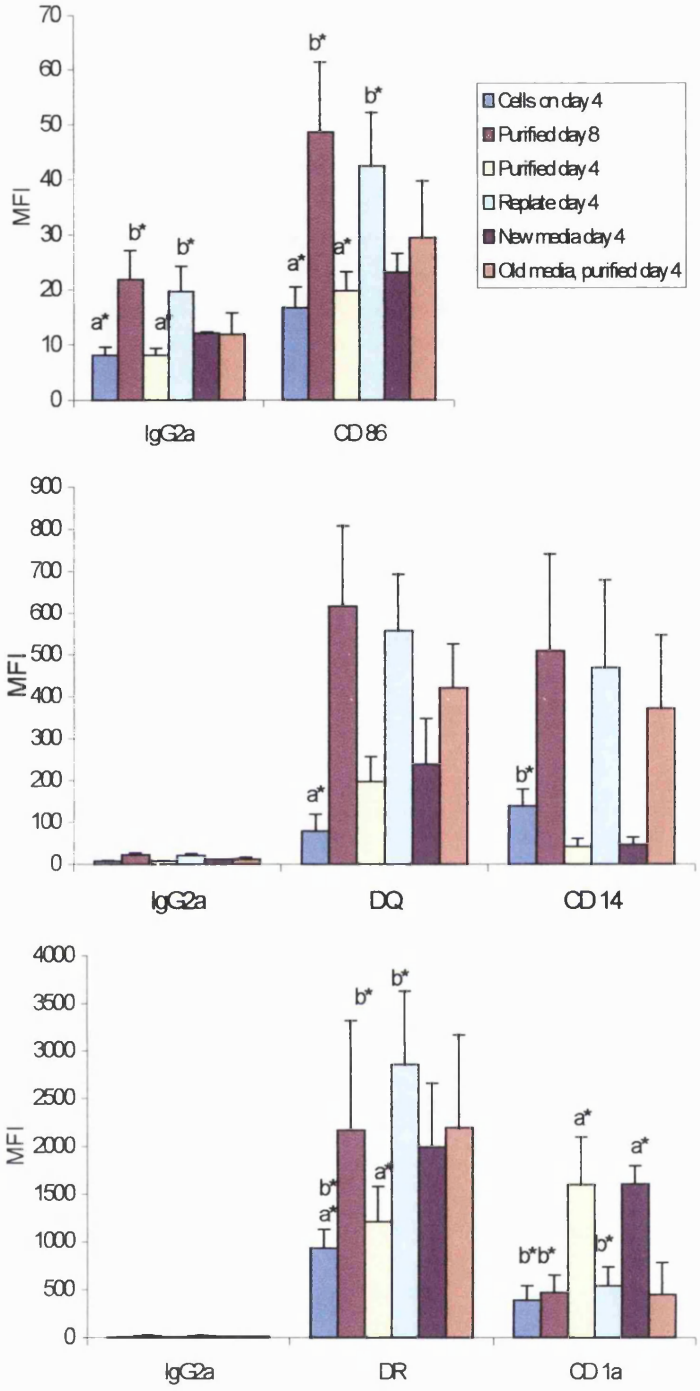


Figure 3.14. Monocyte-derived DC purified on day 4 show a phenotype that is more characteristic of immature DC than those purified on day 8. The immature phenotype of monocyte-derived DC purified on day 4 is consistent with the effects of renewing the media and cytokines on day 4. Monocyte-derived DC were generated from monocyte-enriched PBMC cultured with GM-CSF/IL-4. Samples were then either stained or subject to various conditions, for the remaining culture period. Surface expression was analysed as detailed in Materials and Methods and given as, mean fluorescence intensity (MFI) ± SEM. Mean values are given from 3-5 independent experiments. An irrelevant IgG2a primary mAb is given as control. *, $p<0.05$. a, relative to paired purified day 8 samples; b, relative to paired purified day 4 samples.

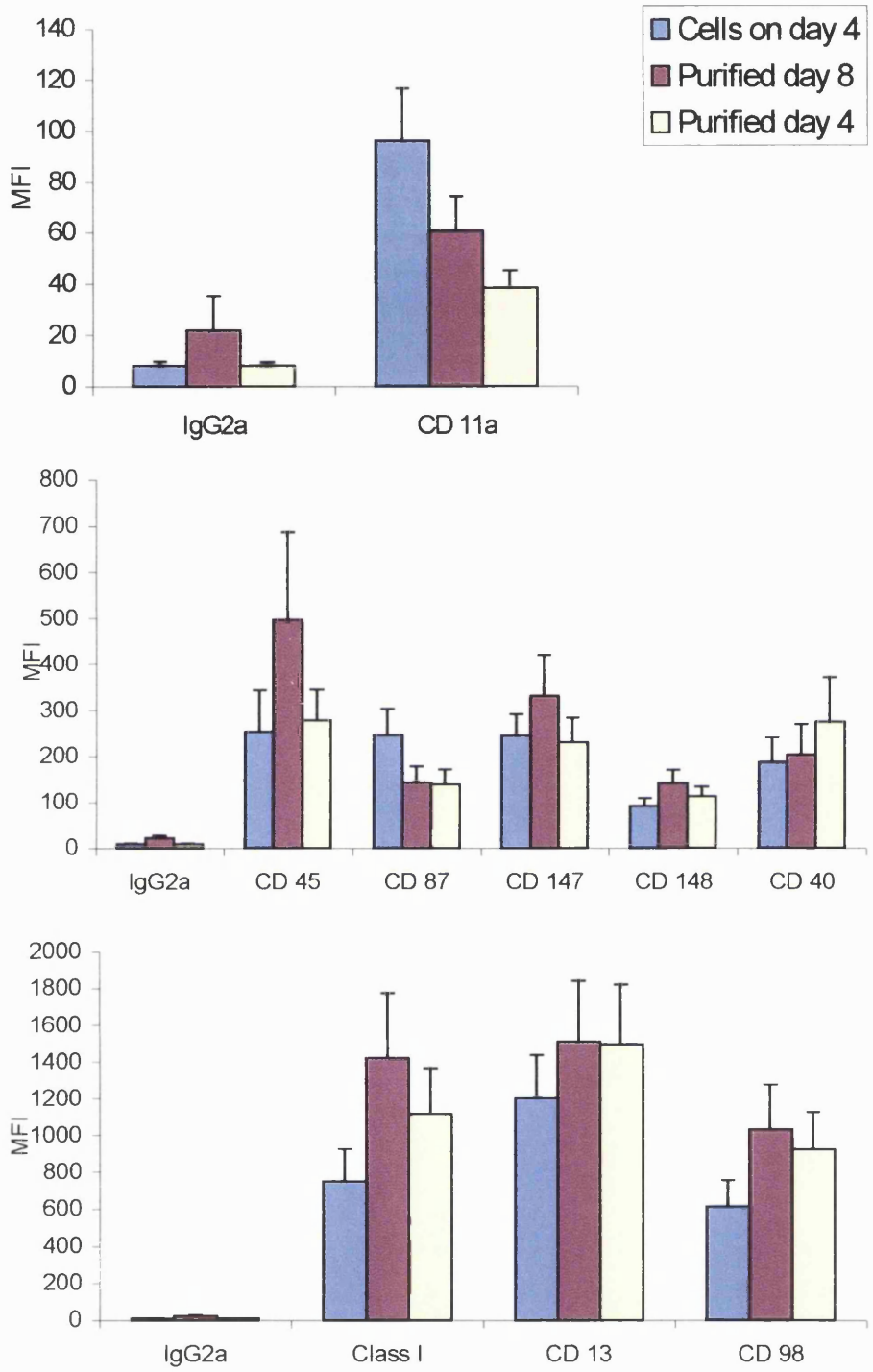


Figure 3.15. Conventional monocyte-derived DC and DC purified on day 4 express similar levels of novel DC markers. Monocyte-derived DC were generated from monocyte-enriched PBMC cultured with GM-CSF/IL-4. Samples were stained, purified on day 4 or left in culture until day 8. Surface expression was analysed as detailed in Materials and Methods and given as, mean fluorescence intensity (MFI) ± SEM. Mean values are given from 4-5 independent experiments. An irrelevant IgG2a primary mAb is given as control. No statistically significant differences were observed between the expression of the given surface markers on monocyte-derived DC purified on day 4 and day 8.

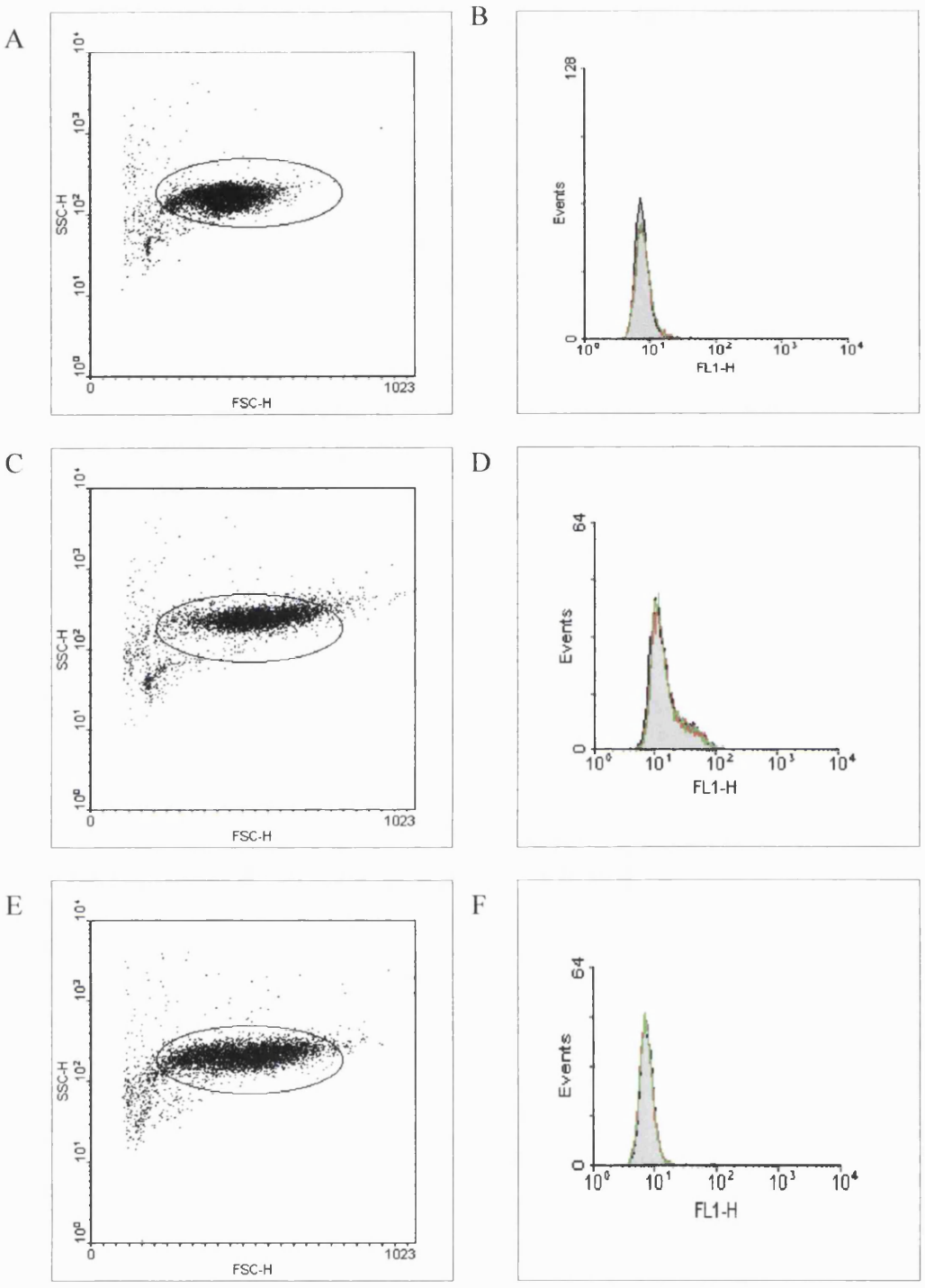


Figure 3.16. Typical FACScan profiles of monocyte-derived DC on day 4 and, samples purified on day 4 and day 8. Scatter diagrams are given displaying cell size (FSC-H) against granularity (SSC-H) (A, C and E), and histograms representing surface staining (B, D and F). Samples were prepared as given in Figure 3.1. Purified populations of monocyte-derived DC on day 4 (A and B), day 8 monocyte-derived DC (C and D) and monocyte-derived DC purified on day 4 (E and F) are given with negative controls representing IgG2a (filled profiles), CD3 (red lines) and CD19 (green lines). One representative of five experiments is given.

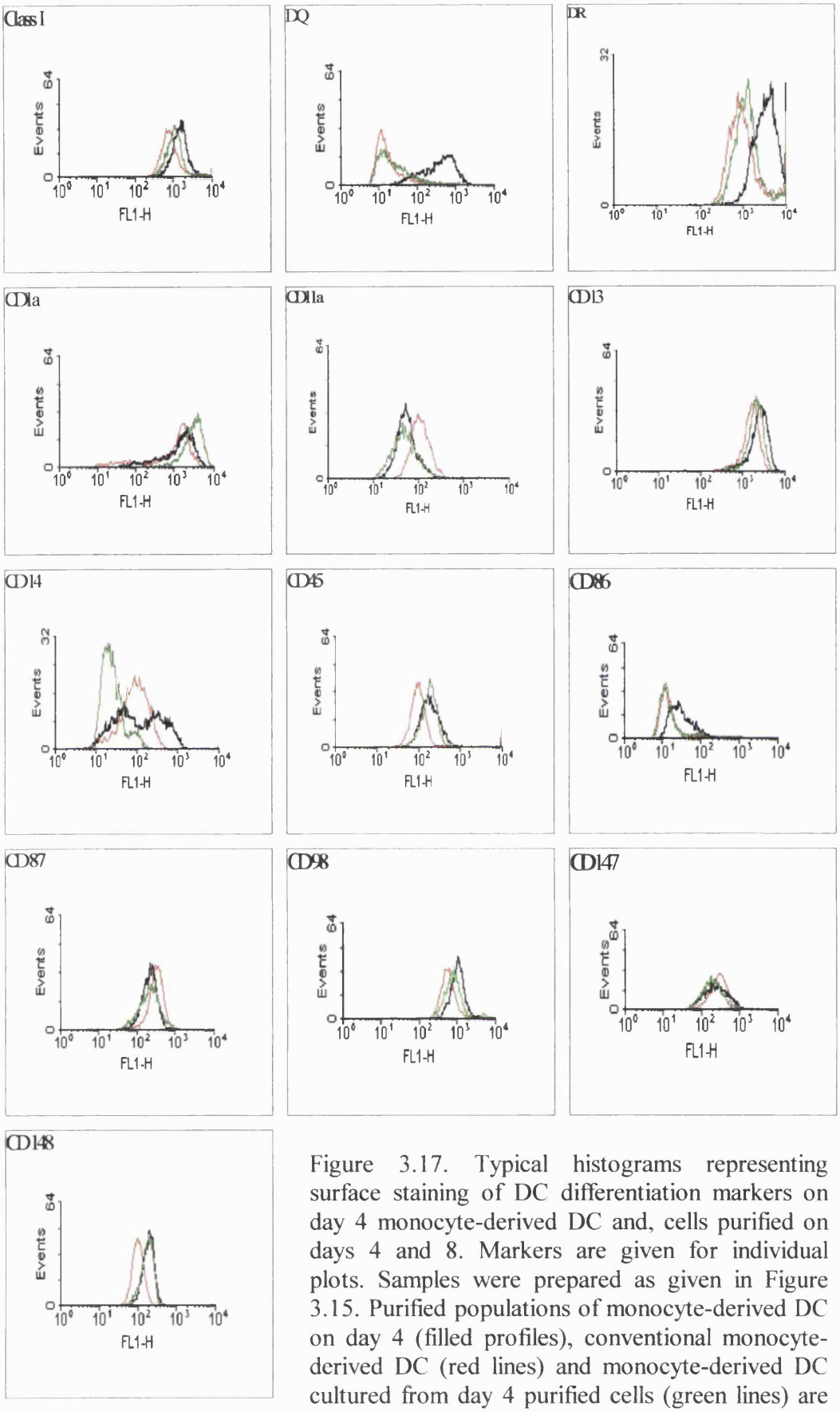


Figure 3.17. Typical histograms representing surface staining of DC differentiation markers on day 4 monocyte-derived DC and, cells purified on days 4 and 8. Markers are given for individual plots. Samples were prepared as given in Figure 3.15. Purified populations of monocyte-derived DC on day 4 (filled profiles), conventional monocyte-derived DC (red lines) and monocyte-derived DC cultured from day 4 purified cells (green lines) are shown. One representative of five experiments is given.

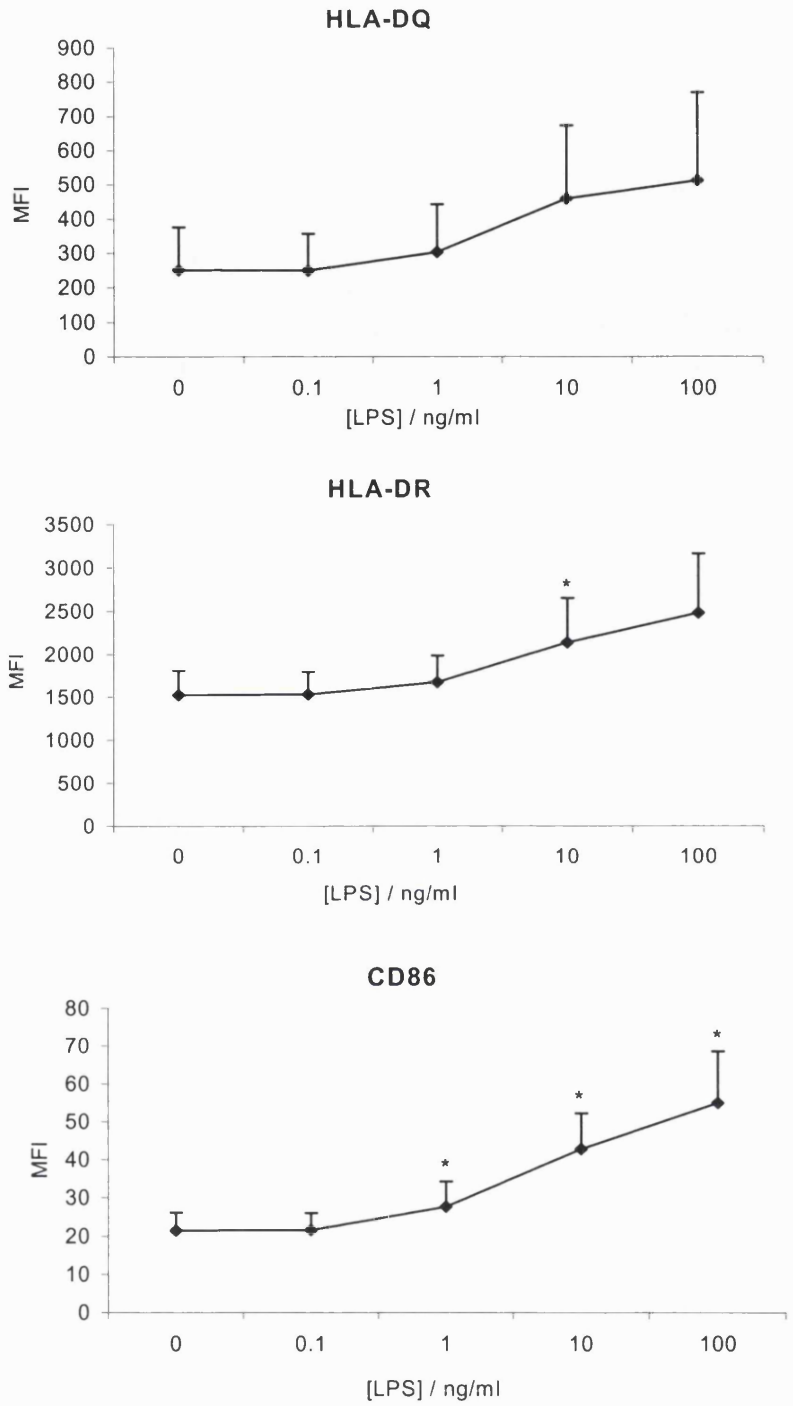


Figure 3.18. Monocyte-derived DC are activated in a dose-dependent manner following incubation with LPS. Monocyte-derived DC were differentiated from monocyte-enriched PBMC cultured with GM-CSF/IL-4. These cells were purified on day 4 and stimulated with LPS on day 7 for 24 hours. Surface expression of HLA-DQ and –DR, and CD86 were then analysed as detailed in Materials and Methods and given as mean fluorescence intensity (MFI) ± SEM. Mean values are given from four independent experiments. Statistical analysis is given relative to control samples (0ng/ml LPS). *, $p<0.05$.

3.3 Discussion

It is imperative to remember that the differentiation and subsequent derivation of DC from monocytes, in the presence of GM-CSF and IL-4, provides us with a convenient model of immature DC, although this derivation can not be assumed without phenotypic analysis of these cells. For this reason it is of utmost importance to establish how the derived cells compare to those reported by other groups in this field, and how they are defined as a distinct cell population.

3.3.1 CD1a/CD14 Expression: defining features of opposing differentiation pathways

One popular DC marker is CD1a, this is expressed by Langerhans' cells and interdigitating cells of lymphoid tissues. It represents a novel family of antigen presenting molecules with a major recognition element for naïve T cells (Porcelli *et al.*, 1995). However, CD1a has not been found on any peripheral blood myeloid cells, indicating that its expression may be dependent upon a local cytokine environment (Thomas & Lipsky, 1994). *In vitro* it has been shown that GM-CSF alone is responsible for the increased expression of CD1 on peripheral blood monocytes as they differentiate into DC (Kasinrerk *et al.*, 1993), although IL-4 stabilises this expression (Pickl *et al.*, 1996). GM-CSF has also been shown to induce the expression of CD1a on macrophages (Kasinrerk *et al.*, 1993), without increasing their stimulatory capacity (Akagawa *et al.*, 1996). However, it is known that DC within the synovial fluid of patients with rheumatoid arthritis are exposed to GM-CSF yet do not express CD1a (Thomas *et al.*, 1994a).

It appears, therefore, that although MDDC are known to express CD1a, this is not a unique differentiation marker of DC prepared from monocytes *in vitro*, and cannot be used as definitive proof of DC differentiation. This is further emphasised by the finding that DC may be derived, *in vitro*, from monocytes following transendothelial migration. This differentiation occurs in the absence

of exogenous cytokines and does not induce the expression CD1a on these cells (Randolf *et al.*, 1998).

Figure 3.3 shows that CD1a is expressed to a significantly greater extent on DC than monocytes. However, since 50% of monocytes are shown to express CD1a, Figure 3.3(C) also suggests that the increase in CD1a expression is a result of up-regulation of expression and not new-expression, as concluded by Pickl *et al.*, (1996). Maturation of DC is reported to reduce the expression of CD1a on MDDC (Zhou & Tedder, 1996). However, no such down-regulation was observed in these studies.

MDDC purified on day 4 show a high level of CD1a expression (Figure 3.14) and are functionally more active than conventional MDDC (Figure 3.10), which have a lower CD1a expression. This is consistent with a previous report (Chapuis *et al.,* 1997) that demonstrated a correlation between the percentage of $CD1a^+$ MDDC and their functional responses.

In contrast to CD1a, the expression of CD14 on MDDC is a contentious issue. CD14 is generally considered to be the receptor for LPS-LPS binding protein complexes but has recently been shown to bind specific ligands on apoptotic cells (Devitt *et al.*, 1998). Indeed, it has been suggested that the binding of apoptotic cells may represent the evolutionary advantage of CD14 and that Gram negative bacteria could have evolved to benefit from binding to CD14 (Matzinger, 1998). In support of this hypothesis, it has been shown that CD14 knockout mice show increased clearance of Gram negative bacteria (Haziot *et al.*, 1996).

CD14 is found in both plasma and located on the membrane of typical monocytes as a glycosylphosphatidylinositol (GPI)-anchored protein (Ziegler-Heitbrock & Ulevitch, 1993). Human peripheral blood DC do not to express CD14 (Freudenthal & Steinman, 1990; Thomas *et al.*, 1993), yet our model of DC relies on differentiation from $CD14^+$ monocytes (Chapuis *et al.*, 1997); so how physiological are these DC in terms of CD14 expression?

It is generally accepted that monocytes cultured in the presence of GM-CSF/TNF-α differentiate into a DC population that expresses CD14 (Rosenzwajg *et al.*, 1996; Bernhard *et al.*, 1995; Mackensen *et al.*, 1995). However, Sallusto and Lanzaveccia (1994), who originally described the differentiation of monocytes to DC, by means of culture with GM-CSF/IL-4, gave the qualitative assurance that, "CD14 was either low or negative," and, therefore, similar to blood DC. This view has been accepted and supported by a number of other studies (Pickl *et al.*, 1996; Verhasselt *et al.,* 1997; Romani *et al.*, 1996; Akagawa *et al.*, 1996) including Chapuis *et al.*, (1997) who state that DC, "lost membrane CD14". On closer inspection however, they found that although the Leu-M3 epitope of CD14 could not be identified, the expression of the My4 epitope was only relatively decreased. In addition, the level of CD14 mRNA is down regulated but not absent in these cells (Pickl *et al.*, 1996). The presence of CD14 on a cell population can, therefore, not automatically define them as not DC. Indeed, expression of CD14 on GM-CSF/IL-4-derived DC is not without support (Chapuis *et al.*, 1997; Zhou & Tedder, 1996).

Figures 3.3 and 3.14 illustrate that after seven days of culture these MDDC express varying degrees of CD14, which is clearly above background expression. It has been suggested that the presence of CD14 may indicate the immaturity of DC (Peters *et al.*, 1996), with the implication being that the rate of the down regulation may simply be insufficient over the time frame studied. However, this appears unlikely since Figure 3.3 shown that these cells are in a state in which they can mature. It can also be seen from Figure 3.14 that CD14 expression increases between days 4 and 8, suggesting that GM-CSF and IL-4 may need to be replenished during the seven days of culture. Since IL-4 has been shown to potentiate the down-regulation of CD14 induced by GM-CSF on monocytes (Ruppert *et al.*, 1993), one would predict the involvement of both of these cytokines.

Indeed, when fresh cytokines and media are supplied to the DC on day 4, they express less CD14 than those cultured without interruption for seven days. This

down-regulation can occur through reduced transcription of CD14 as well as active shedding of membrane CD14 (Ziegler-Heitbrock & Ulevitch, 1993).

It appears, therefore, that CD14 is not expressed to a great extent on DC isolated from peripheral blood, but is expressed to a varying degree on MDDC, with less expression following maturation. It is of interest to note that, as with MDDC purified on day 8, Langerhans' cells within the nasal-associated lymphoid tissue coexpress CD14 and CD1a (Graeme-Cooke *et al.*, 1993).

3.3.2 MHC Class I and Class II expression on DC

Peripheral blood DC express higher levels of MHC Class I than circulating monocytes (Freudenthal & Steinman, 1990). However, Pickl *et al.,* (1996) have found no difference between monocytes and MDDC, with regard to their expression of MHC Class I. Figure 3.3 also shows that monocytes and MDDC have equal expression of MHC Class I, and this is found to increase upon maturation and incubation in the absence of cytokines.

High expression of all MHC class II products (HLA-DP, -DQ, -DR) is the most distinctive feature of DC and helps to explain their potency as antigen presenting cells (Freudenthal & Steinman, 1990). Up-regulation of MHC Class II expression is known to accompany the differentiation of monocytes to DC (Pickl *et al.*, 1996). The up-regulation was found to be significant for both HLA-DQ and -DR (Figure 3.3), which further up-regulate upon maturation.

The low level of expression of HLA-DQ and –DR on DC purified on day 4 is surprising, given the functional responses of these cells. However, the responses demonstrate that antigen presentation is not the key signal that drives the oxidative mitogenesis assay. It must also be suggested that, during the latter half of MDDC differentiation, they may slowly mature as the constituents of the culture media are consumed or degraded.

3.3.3 Costimulatory molecules on DC

The best known costimulators are CD80 and CD86 (B7-1, -2), which provide signals to T cells through their ligation with CD28/CTLA-4. Freshly isolated human peripheral blood DC and Langerhans' cells express neither CD80 nor CD86 (Thomas & Lipsky, 1994). However, CD80 is found on MDDC to varying extents, with a small sub-population of cell highly expressing CD80 after differentiation. The expression of CD86 increases during the first two days of culture and then decreases, finally resulting in a $CD1a^{+}/CD86^{low/-}$ population (Chapuis *et al.,* 1997; Palucka *et al.,* 1998; Pickl *et al.*, 1996): this can be seen in Figures 3.3 and 3.14. The expression of CD86 on MDDC purified on day 4 follows the same pattern as that seen with HLA-DQ and –DR, further suggesting that MDDC may start to mature during the latter half of the differentiation.

Maturation of DC increases their expression of CD86, which is a key component of the increased stimulatory capacity of these cells (Fagnoni *et al.*, 1995). Figure 3.17 show that significant increases in the expression of CD86 first occur at 1ng/ml LPS, which is identical to that reported by Verhasselt *et al.*, (1997).

3.3.4 LPS induction of DC maturation

LPS is known to be a potent physiological stimulus of DC. However, this is paradoxical given the relative lack of CD14 expression on DC. In addition, the CD14 that is expressed may be truncated and functionally altered (Chapuis *et al.*, 1997).

Verhasselt *et al.*, (1997) resolved this paradox by demonstrating that the low doses of LPS (1ng/ml) could be inhibited by a blocking anti-CD14 mAb, serum-free media or soluble CD14-depleted serum: thus implying that a soluble CD14-dependent pathway is essential for the activation of DC by low concentrations of LPS, as previously reported for epithelial and endothelial cells (Pugin *et al.*, 1993). However, at higher concentrations of LPS (1000ng/ml), removal of soluble CD14 was unable to prevent the activation of DC, suggesting the

involvement of other LPS-sensitive receptors, such as CD11c/CD18. Indeed, LPS can activate monocytes in the absence of both membrane CD14 and soluble CD14, and hence is not an absolute requirement for activation (Lynn *et al.*, 1993).

Sensitive responses to LPS have further been shown to require the binding of LPS monomers from aggregates or bacterial membranes to the plasma protein LBP (LPS binding protein) (Hailman *et al.*, 1994). LBP then transfers LPS to binding sites on CD14, which passes LPS into the plasma membrane. The signal transduction pathway for LPS has yet to be fully resolved but is thought to involve members of the Toll-like receptor family, culminating in the activation of NF-κB (Medzhitov *et al.*, 1994).

Toll receptors were first identified in *Drosophila*, where they were shown to participate in dorsoventral pattern formation of the embryo and an antifungal response (Lemaitre *et al.*, 1996). In human, five homologous Toll receptors have been identified and designated Toll-like receptors (TLR) (Rock *et al.*, 1998). The importance of TLR4 was highlighted by the findings that mice with natural mutations in TLR4 are hyporesponsive to LPS and that a constitutively active form of TLR4 activates NF-κB and AP-1 (Wright, 1999).

TLR2 recognises constituents of Gram positive bacteria, and may also be responsive to high concentrations of LPS in the absence of CD14 (Yang *et al.*, 1998). Since the concentration of LPS may reach up to 10μg/ml in abscess cavities and with faecal soiling of the peritoneum (Lynn *et al.*, 1993), these alternative pathways may potentially be of importance.

3.4 Conclusion

DC can be defined by morphological, phenotypic and functional characteristics. Morphological analysis is inadequate in terms of veiled cell formation since M-CSF cultured cells acquire similar DC features but do not express CD1a (Chapuis *et al.*, 1997), form rosettes with resting T cells or stimulate T-cell proliferation (Akagawa *et al.*, 1996). The lack of a unique DC marker makes phenotypic characterisation difficult, although the presence of CD1a and absence of CD14 are often defining features (Chapuis *et al.*, 1997; Pickl *et al.*, 1996), despite the fact that CD1a is not a physiological DC marker (Freudenthal & Steinman, 1990). The present study, however, shows that CD14 may be expressed by functional DC. Since GM-CSF can induce CD1a expression on macrophages (Kasinrerk *et al.*, 1993), phenotypic classification of DC awaits the identification of a specific marker. The functional responses of DC, as assessed by their ability to stimulate T-cell proliferation, is, therefore, the only reliable marker of DC differentiation *in vitro*.

The three stages of DC differentiation can be distinguished by functional studies, as illustrated in Figure 3.9. However, the surprising result obtained in the anti-CD3-dependent assay (Figure 3.7) is confirmed to be spurious by comparison to the MLR (Figure 3.8) and oxidative mitogenesis assays (Figure 3.9). Indeed, it is possible that a decreased functional response in the CD3-dependent assay may reflect the increased maturity of DC (see section 3.2.4). Analysis of the functional capacity of DC by the oxidative mitogenesis assay has been shown to yield a consistent and reliable determination of the ability of DC to induce the proliferation of resting T cells.

Immature MDDC are plastic until their maturation, which is terminal. Therefore, following purification, immature MDDC require the continuous presence of GM-CSF/IL-4 to prevent their dedifferentiation. In addition, MDDC are generally considered to be non-adherent cells but are known to transiently adhere when transferred to new culture plates. Therefore, the only way to stimulate pure MDDC populations, whilst avoiding their phase of transient readherence, is to

purify these cells several days before their stimulation: thus it was decided to purify the cells after three days of culture (on day 4), then allow them to continue their differentiation in the presence fresh media and cytokines. The early purification allows sufficient time for the MDDC to form their characteristic clusters, without altering the total incubation time.

When verifying the phenotypic and functional properties of MDDC that are purified halfway through their culture, it was found that these cells are functionally more active than conventional MDDC and have a phenotype that is more characteristic of immature MDDC. It was also shown that the cells express similar levels of a novel set of DC markers, as described by Woodhead *et al.*, (1998).

These data suggest that purifying MDDC on day 4 may be advantageous in immunotherapy involving DC. However, cells cultured in the presence of FCS are not suitable for clinical use. The optimal clinically suitable media for the culturing of *ex vivo* DC is RPMI-1640 supplemented with 1% autologous plasma, but other media such as X-VIVO or AIM-V are also suitable (Romani *et al.*, 1996). Therefore, to test the potential benefits of early DC purification on what may turn out to be a new era in immunotherapy, one would need to repeat these experiments in the alternative media.

Chapter 4

The Role of ROS in the Regulation of Dendritic cell Function

4.1 Introduction

4.1.1 Hypothesis

Oxidative stress has been implicated in the pathogenesis of a variety of diseases. In many of these, the pathology has been attributed to the activation of specific cells, or even the direct activation of various signalling mechanisms.

In 1995, Ibrahim *et al.*, hypothesised that free radicals could activate DC. The implication of this hypothesis was that oxidative stress may signal the presence of "danger", and thus, the need to initiate an adaptive immune response. Indeed, if this were found to be the case, then this would be the first evidence that products derived from damaged tissues may activate DC and will lead to various suggestions of the potential physiological and pathological significance of this activation.

It has also been suggested that the maturation of DC may be triggered by their detection of a fundamental distinction between apoptosis and necrosis (Ibrahim *et al.*, 1995). Given this suggestion, it is interesting to note that ligation of fas induces the activation of two distinct signalling pathways, which can be segregated depending on the intracellular production of ROS. The pathway that is associated with the production of ROS has been shown to result in necrosis as opposed to apoptosis, which occurs in the absence of ROS (Vercammen *et al.*, 1998). It is, therefore, possible that the detection of ROS by DC may represent the means by which they distinguish between these two forms of cell death, and thus, the presence of a potential "danger".

The potential activation of DC by ROS is supported by the additional suggestion that responses to ROS are most likely to be physiologically relevant in cells of the immune system, since these cells are frequently associated with areas of oxidative stress (Nakamura *et al.*, 1997).

4.1.2 Literature supporting the hypothesis

Previous reports have characterised the activation of DC by H_2O_2 (Rutault *et al.*, 1998). However, these experiments were conducted on a mixed population of DC and lymphocytes. It is, therefore, possible that the DC may have been activated indirectly *via* contaminating lymphocytes. Since T cells may be activated by oxidative stress, this could lead to an increase in their expression of CD40L, which in turn would activate DC.

In a separate report (Verhasselt *et al.*, 1999), the anti-oxidant N-acetylcysteine was shown to inhibit the activation of DC, suggesting that this activation may be dependent upon oxidative signalling mechanisms. However, the excessive concentrations of N-acetylcysteine used during these studies may have exerted toxic effects on the DC, which would also account for the inhibitory effects observed.

There is an accumulating pool of evidence suggesting that ROS may directly activate distinct signalling pathways and thereby various cellular functions. However, much of these data are based on false interpretations and fallible assumptions. Therefore, the aim of this chapter is to investigate whether or not oxidative stress can activate DC directly and therefore, help to clarify this issue.

4.1.3 Cellular oxidative stresses *in vitro*

In vitro, oxidative stress can be induced by a number of techniques. Perhaps the simplest, and most common, of these is by the direct addition of H_2O_2. However, H_2O_2 in known to degrade rapidly upon addition to culture media and may, therefore, provide a poor representation of the prolonged oxidative stress thought to occur *in vivo*. To solve this problem, one can employ enzymatic systems that continuously release ROS throughout their culture.

Alternatively, oxidative stress may be promoted by compounds that deplete anti-oxidants or compounds that undergo redox cycling during their intracellular

metabolism, with the concomitant release of ROS. An example of the former compounds is agents that deplete GSH, thereby acting to increase the propensity of cellular injury through oxidative stress. Perhaps the best known redox cycling agent is vitamin K (menadione).

Menadione (2-methyl-1,4-naphtoquinone) can undergo one- and two-electron reduction reactions. Two-electron reduction of this quinone is catalysed by DT-diaphorase, in the cytosol, and results in the production of the hydroquinone. Two-electron reduction of menadione, therefore, prevents redox cycling and may be considered as a protective mechanism. Several flavoenzymes, most importantly NADPH-cytochrome P-450 reductase, are able to catalyse the one-electron reduction of menadione to the semiquinone free radical. In the presence of molecular oxygen this semiquinone free radical is rapidly oxidised back to menadione, resulting in the production of a superoxide radical. Menadione has been shown to initially deplete intracellular GSH before inducing cytotoxicity (Comporti, 1989).

Despite the fact that menadione is lipid soluble, the toxicity of this compound is thought to result from the production of superoxide and not lipid peroxidation (Comporti, 1989).

4.2 Results

4.2.1 Effects of ROS on DC

Before it was possible to examine the effects of ROS on DC, it was essential to establish the maximum concentration of ROS that could be applied to DC without inducing cell death. The main reasons for studying the effects of oxidative stresses with little or no toxicity were not only to ensure that sufficient numbers of DC survived to allow their phenotypic and functional characterisation, but also to eliminate the possibility that products derived from apoptotic or necrotic cells may interfere with the maturation of DC. The cellular activity of DC, as an indication of their viability, was assessed by examining the effects of ROS on the ability of DC to reduce MTT; this was quantified spectrophotometrically and calculated relative to control samples in the absence of oxidant treatment, as detailed in Materials and Methods.

It can be seen from Figure 4.1 that H_2O_2 reduced the cellular activity of DC in a dose-dependent manner. It was not established whether or not the reduction in the ability of DC to reduce MTT, following the presence of ROS, represented decreased cell survival or an inhibitory effect on the ability of DC to reduce MTT. Figure 4.1 also illustrates that culturing DC for 24 hours with initial concentrations of up to 100μM H_2O_2 did not inhibit significantly the ability of DC to reduce MTT, suggesting that concentration of H_2O_2 below 100μM induce neither significant inhibitory nor toxic effects on DC.

H_2O_2 was applied to the DC at the initial concentrations of 10μM, 30μM and 100μM, since these concentration represent a range of doses of H_2O_2 that did not inhibit significantly the resting activity of DC, as determined by their ability to reduce MTT. Figure 4.2 illustrates the effects of the range of non-toxic concentrations of H_2O_2 on the phenotype of DC. The effects of treating DC for 24 hours in the presence of 10ng/ml LPS are also presented, not only as a positive control, to which the potential effects of H_2O_2 can be compared, but also

to verify the fact that, prior to the addition of H_2O_2, the DC were in an immature state.

It can be seen from Figure 4.2 that the surface expression of HLA-DQ and –DR, and CD86, which are known to increase upon DC maturation, were increased significantly upon incubation of DC for 24 hours in the presence of 10ng/ml LPS. Moreover, from Figure 4.2 it can be seen that non-toxic concentration of H_2O_2 are unable to increase the surface expression of HLA-DQ or –DR, or CD86 on DC. It is, therefore, suggested that if H_2O_2 is able influence the phenotype of DC, this does not occur in a manner akin to the maturational effects induced by LPS. Figure 4.2 B, D and F give representative examples of the effects of non-toxic concentrations of H_2O_2 on the surface expression of HLA-DQ and –DR, and CD86 on DC, following a period of 24 hours culture. The representative examples given illustrate further the consistent lack of any detectable effect of H_2O_2 on the expression of the characteristic maturation markers presented. Despite the fact that a relatively limited phenotypic analysis was conducted, it should be noted that the three markers investigated are all known to indicate the maturation status of DC and, thereby, each support the notion that H_2O_2 is unable to induce the phenotypic maturation of DC.

Further to characterise the effects of non-toxic concentrations of H_2O_2 on DC, the effects of H_2O_2 on the ability of DC to stimulate proliferation of resting T cells was examined. Figure 4.3 illustrates that, when assessed by the oxidative mitogenesis assay (see Materials and Methods), DC were able to induce the proliferation of T cells, as detected by ^{3}[H]thymidine incorporation, in a dose-dependent manner. It can also be seen from Figure 4.3 that incubation of DC for 24 hours in the presence of 10ng/ml LPS, prior to conducting proliferation assay, increased significantly the ability of DC to induce the proliferation of T cells, as determined by the increase in ^{3}[H]thymidine incorporation relative to control samples. Following incubation of DC with a range of non-toxic concentration of H_2O_2, the ability of DC to induce the proliferation of T cells was also examined (Figure 4.3). Figure 4.3 demonstrates that incubation of DC with non-toxic concentration of H_2O_2 neither stimulates nor hinders the ability of DC to induce

the proliferation of resting T cells. Indeed, it can be seen that the responses induced by DC incubated in the presence of H_2O_2 mirrored similar responses induced by DC that had not been subjected to the oxidative stress.

It can, therefore, be seen that, under the conditions of these experiments, concentrations of H_2O_2 with little or no toxicity (Figure 4.1) are unable to induce the phenotypic (Figure 4.2) or functional (Figure 4.3) maturation of DC directly. The data presented in Figures 4.1-4.3 support the initial hypothesis that, in previous reports (Rutault *et al.*, 1999), DC may have been activated indirectly *via* contaminating lymphocytes, although this was not examined further. Alternatively, since the reported data are based on 300μM H_2O_2, it is possible that the level of toxicity may have yielded necrotic products, which may, in theory, activate DC.

It was possible that the lack of effect with H_2O_2 reported here reflected the transitory nature of the compound. It was, therefore, pertinent to investigate the stimulatory effects of a prolonged oxidative stress, and this was initially achieved by the addition of glucose oxidase (GOD). GOD utilises glucose and molecular oxygen as its substrates, and releases H_2O_2 as a by-product. Although the production of H_2O_2 was not demonstrated directly, this was assumed to be the cause of the inhibitory effects of high concentrations of GOD on the cellular activity of DC (Figure 4.4). Following a period of 24 hours culture in the presence of increasing concentrations of GOD, the ability of DC to reduce MTT was examined as an indication of their viability (Figure 4.4). Three concentrations of GOD that did not reduce significantly the cellular activity of DC were then determined and investigated for their potential maturational effects on DC. The concentrations of GOD, and thus presumable of H_2O_2 indirectly, investigated were 10ng/ml, 30ng/ml and 100ng/ml.

Figure 4.5 shows that concentrations of GOD that were unable to inhibit the cellular activity of DC significantly (Figure 4.4) were also unable to induce the phenotypic maturation of DC. The effects of GOD on the ability of DC to stimulate the proliferation of resting T cells, in an oxidative mitogenesis assay,

were then investigated. The maturational effects of 10ng/ml LPS, a known activator of DC, are also presented in Figure 4.5; this again demonstrates that the DC used in this study could be induced to mature during 24 hours of culture in the presence of a maturation stimulus. Figure 4.6 demonstrates that the ability of DC to stimulate the proliferation of resting T cells, as determined by ^{3}[H]thymidine incorporation, was not affected significantly by the prior incubation of DC with concentrations of GOD that did not inhibit their cellular activity.

Further to explore the actions of ROS on DC, a third oxidising agent was investigated. The agent selected was menadione, which continuously releases superoxide through redox cycling, thus also ruling out the possibility that the maturation of DC was dependent upon a selective ROS. Since menadione is not soluble in water, this was initially dissolved in ethanol and then diluted in complete medium, before being added to the DC. Throughout the following experiments, therefore, it was necessary to include additional samples to evaluate the effects of ethanol alone on the maturation of DC. In each case, the concentration of ethanol applied to the DC was the maximum concentration used within that set of experiments. Ethanol alone was unable to inhibit the reduction of MTT by DC.

Three concentrations of menadione that did not inhibit significantly the cellular activity of DC, as assessed by their ability to reduce MTT (Figure 4.7), were then selected and investigated for their potential maturational effects on DC. The concentration of menadione applied to DC, for 24 hours, were, 5μM, 10μM and 15μM. It can be seen in Figure 4.8 that the above concentration of menadione were unable to induce similar phenotypic maturation effects compared to those induced by LPS. The concentration of ethanol applied to DC in these studies was 0.015% v/v, which was identical to the concentration of ethanol within the DC sample containing 15μM menadione; this was also shown not to induce the phenotypic maturation of DC (Figure 4.8).

Menadione, at concentrations that were unable to inhibit the cellular activity of DC, was also examined for its effects on the ability of DC to induce the proliferation of resting T cells, as assessed by ^{3}[H]thymidine incorporation in an oxidative mitogenesis assay. Figure 4.9 demonstrates that 10ng/ml LPS was able consistently to increase the ability of DC to induce the proliferation of resting T cells. Furthermore, it can be seen from Figure 4.9 that culturing DC with menadione induced a greater degree of variability in T-cell proliferation than had been found previously for H_2O_2 and GOD. However, the responses did not deviate significantly from control responses observed in the absence of oxidant treatment.

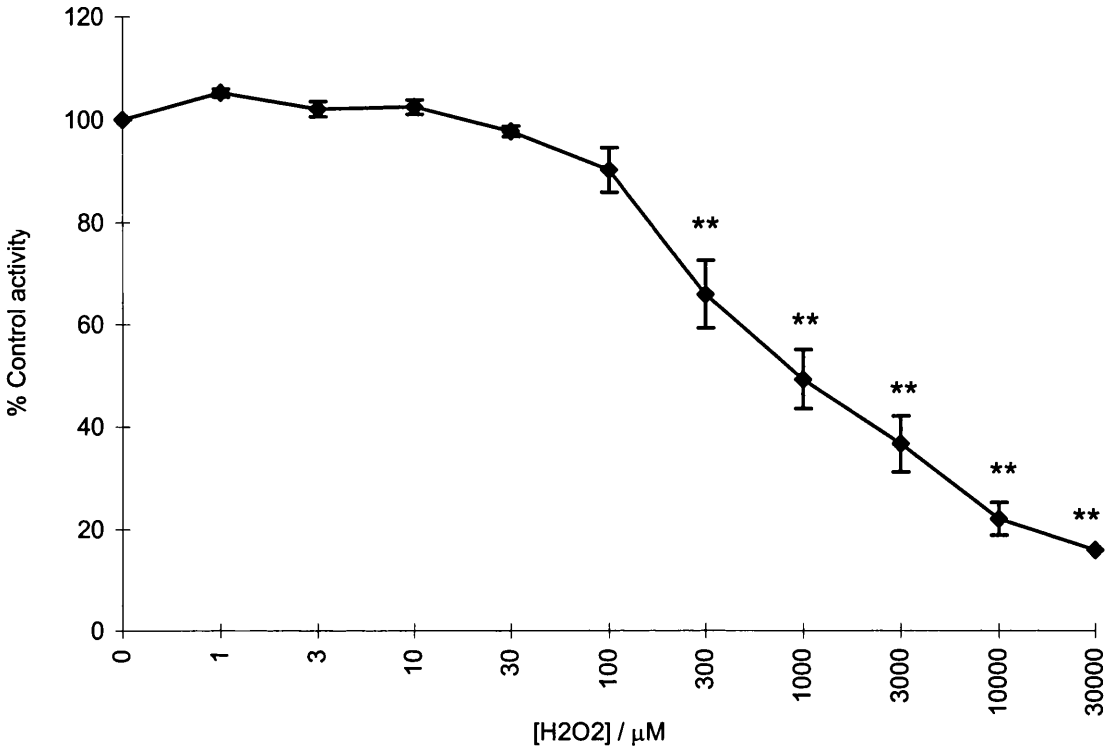


Figure 4.1. Dose-response curve illustrating the effect of H_2O_2 on the cellular activity of DC. DC were derived according to the method of day 4 purification (see Materials and Methods). DC were removed on day 7 and the corresponding concentration of H_2O_2 added. They were then transferred to 96-well plates (100μl/well of $5x10^5$DC/ml) for 24 hours. MTT (final concentration 1mg/ml) was added for the final 4 hours of this culture. Cellular activity was assessed spectrophotometrically based on the reduction of MTT and calculated as detailed in Materials and Methods. Mean ± SD of five independent experiments are shown. Statistical significance is given relative to 0μM H_2O_2. **, $p<0.01$.

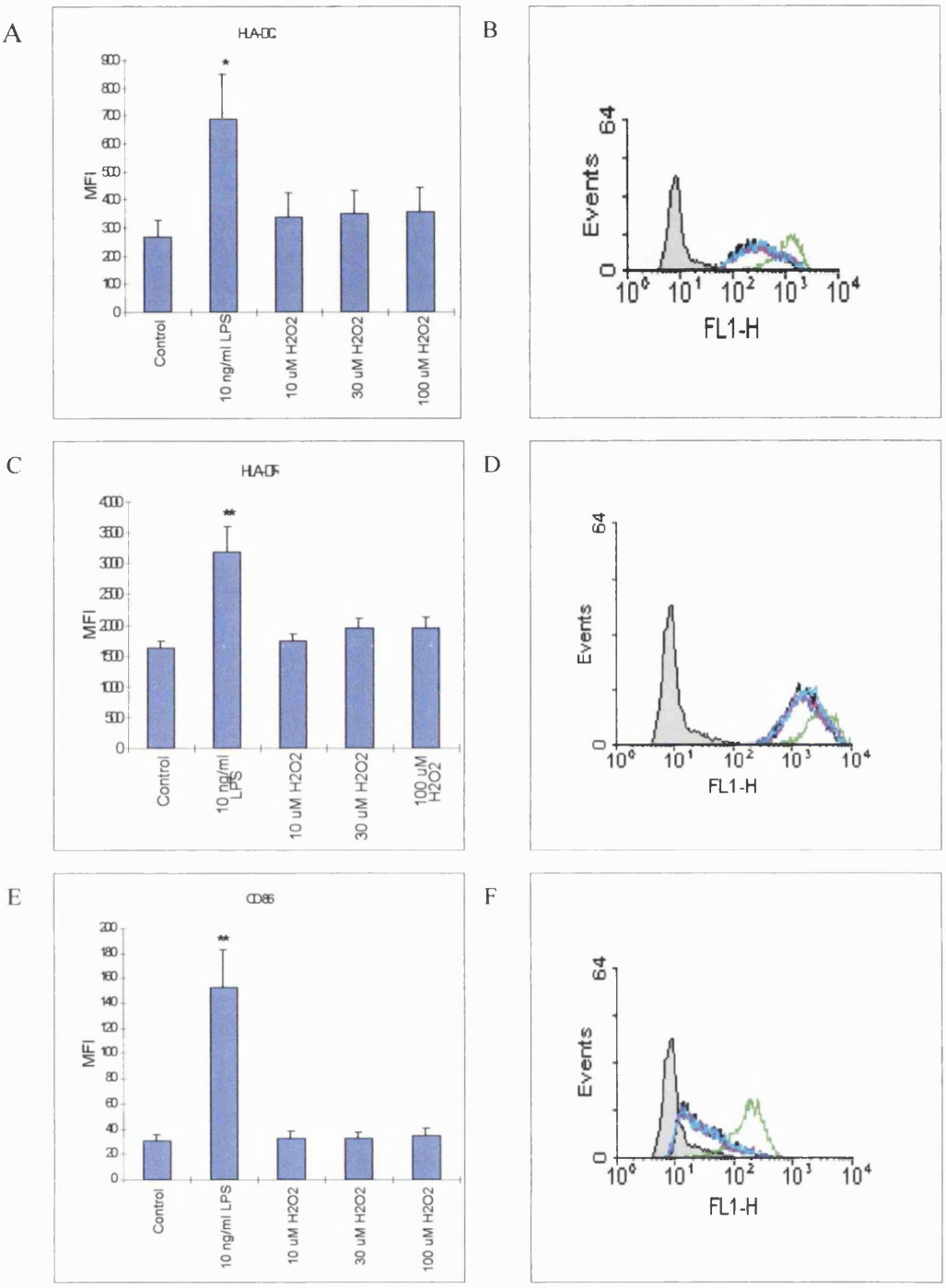


Figure 4.2. Effects of H_2O_2 on the phenotypic maturation of DC. DC were derived according to the method of day 4 purification (see Materials and Methods) and stimulated on day 7 for 24 hours. Surface expression of HLA-DQ and –DR, and CD86 were then assayed (A, C and E, respectively) by FACS analysis. Control values give expression without stimulation. Mean values ± SEM of five independent experiments are shown. The corresponding representative examples are shown in B, D and F. Filled profiles give background IgG2a control; green lines give LPS positive control; all others represent H_2O_2 samples. Statistical significance is given relative to 0μM H_2O_2. *, $p<0.05$; **, $p<0.01$.

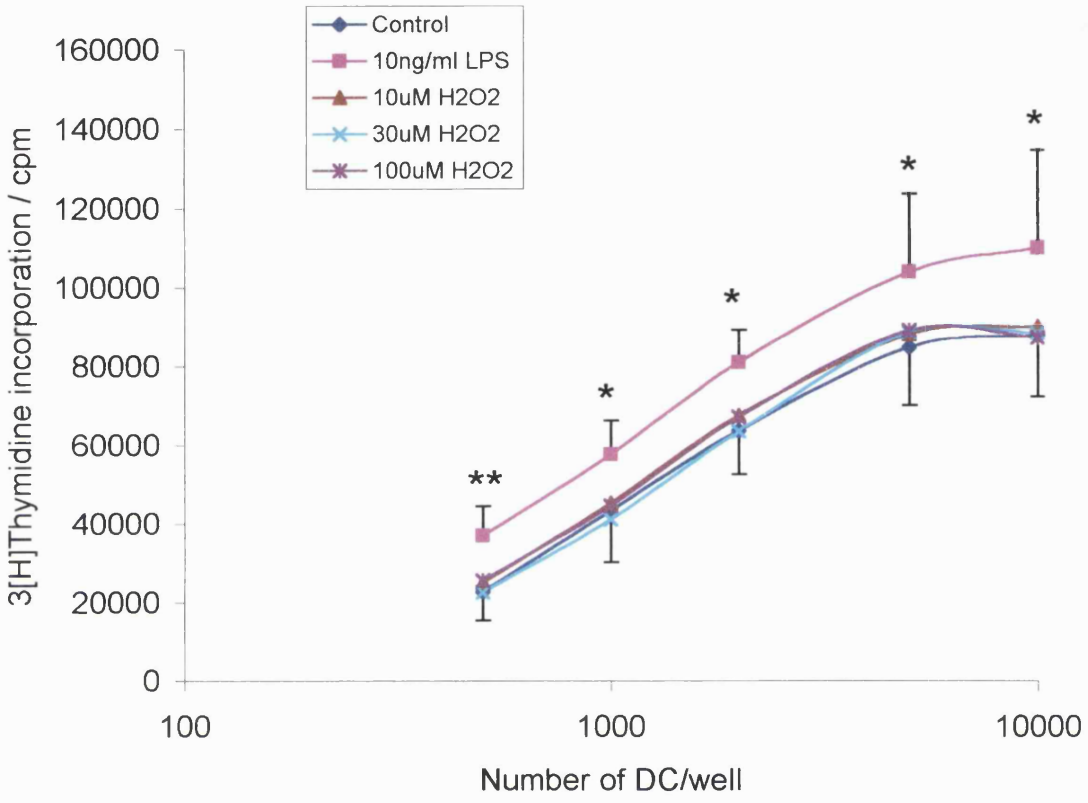


Figure 4.3. Effects of H_2O_2 on the functional maturation of DC. DC were derived according to the method of day 4 purification (see Materials and Methods) and stimulated on day 7 for 24 hours. Functional responses were then assayed by the oxidative mitogenesis assay. Control values give response without stimulation. Mean values ± SEM of four independent experiments are shown. Statistical significance is given relative to 0μM H_2O_2. *, $p<0.05$; **, $p<0.01$.

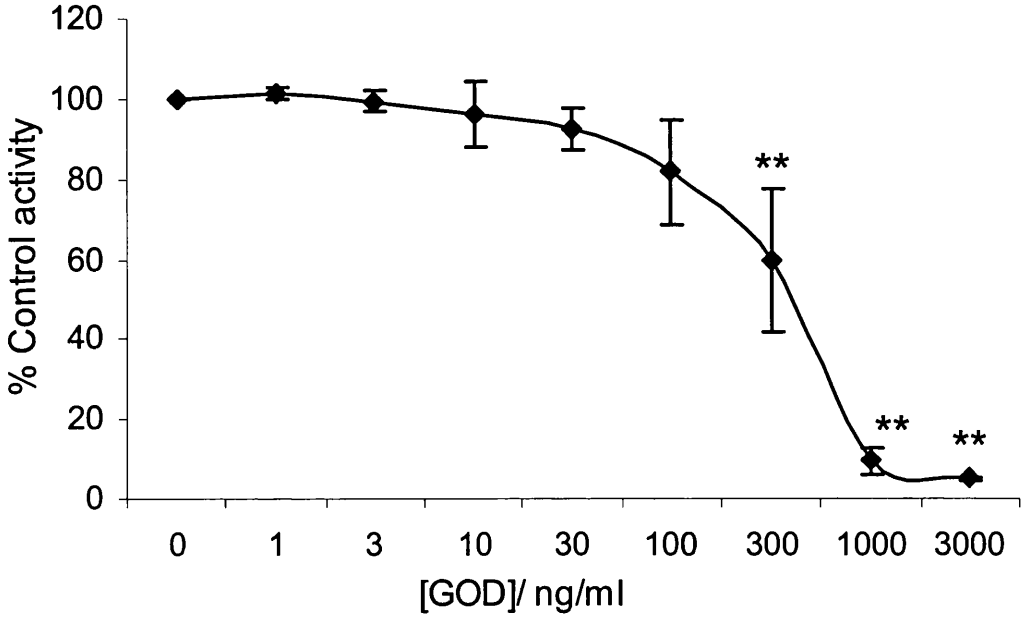


Figure 4.4. Dose-response curve illustrating the effect of GOD on the cellular activity of DC. DC were derived according to the method of day 4 purification (see Materials and Methods). DC were removed on day 7 and the corresponding concentration of GOD added. They were then transferred to 96-well plates (100µl/well of $5x10^5$DC/ml) for 24 hours. MTT (final concentration 1mg/ml) was added for the final 4 hours of this culture. Cellular activity was assessed spectrophotometrically based on the reduction of MTT and calculated as detailed in Materials and Methods. Mean ± SD of five independent experiments are shown. Statistical significance is given relative to 0 ng/ml GOD. **,$p<0.01$.

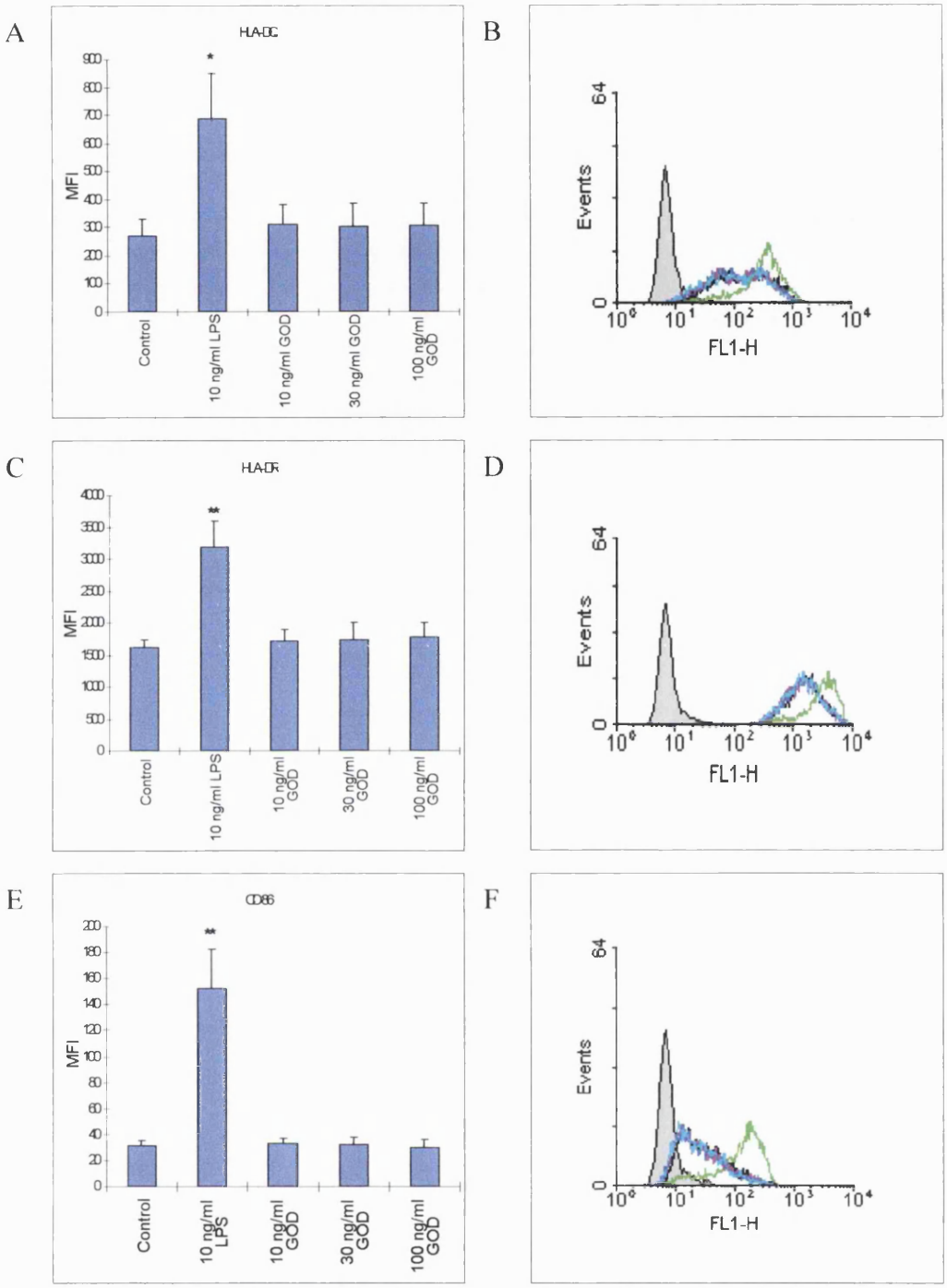


Figure 4.5. Effects of GOD on the phenotypic maturation of DC. DC were derived according to the method of day 4 purification (see Materials and Methods) and stimulated on day 7 for 24 hours. Surface expression of HLA-DQ and –DR, and CD86 were then assayed (A, C and E, respectively) by FACS analysis. Control values give expression without stimulation. Mean values ± SEM of five independent experiments are shown. The corresponding representative examples are shown in B, D and F. Filled profiles give background IgG2a control; green lines give LPS positive control; all others represent GOD samples. Statistical significance is given relative to 0 ng/ml GOD. *, $p<0.05$; **, $p<0.01$.

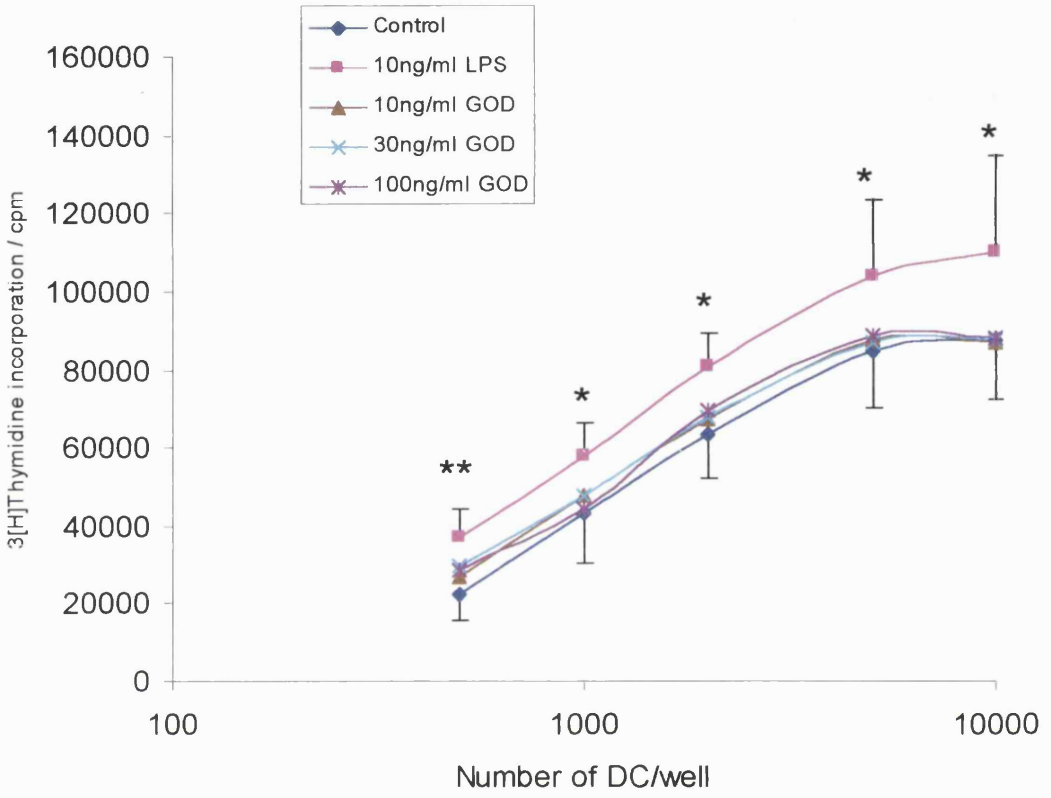


Figure 4.6. Effects of GOD on the functional maturation of DC. DC were derived according to the method of day 4 purification (see Materials and Methods) and stimulated on day 7 for 24 hours. Functional responses were then assayed by the oxidative mitogenesis assay. Control values give response without stimulation. Mean values ± SEM of four independent experiments are shown. Statistical significance is given relative to 0 ng/ml GOD. *, $p<0.05$; **, $p<0.01$.

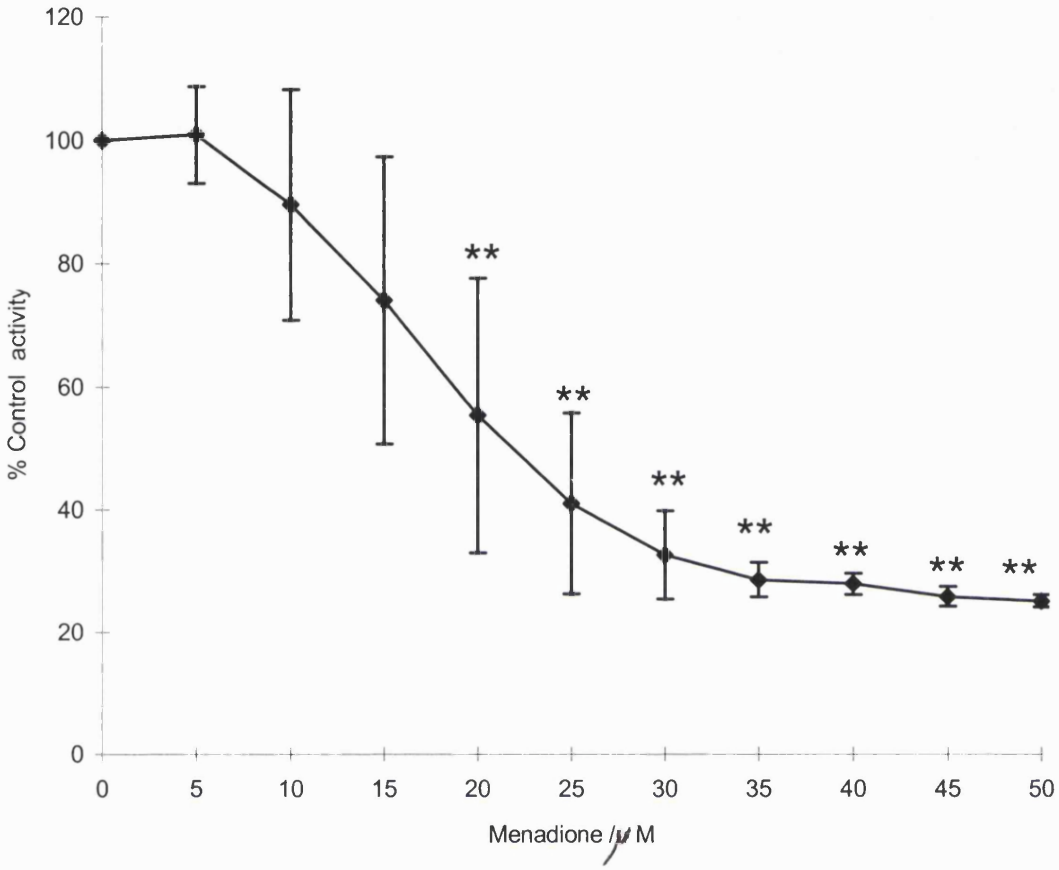


Figure 4.7. Dose-response curve illustrating the effect of menadione on the cellular activity of DC. DC were derived according to the method of day 4 purification (see Materials and Methods). DC were removed on day 7 and the corresponding concentration of menadione added. They were then transferred to 96-well plates (100μl/well of $5x10^5$DC/ml) for 24 hours. MTT (final concentration 1mg/ml) was added for the final 4 hours of this culture. Cellular activity was assessed spectrophotometrically based on the reduction of MTT and calculated as detailed in Materials and Methods. Mean ± SD of five independent experiments are shown. Statistical significance is given relative to 0μM menadione. **,$p<0.01$.

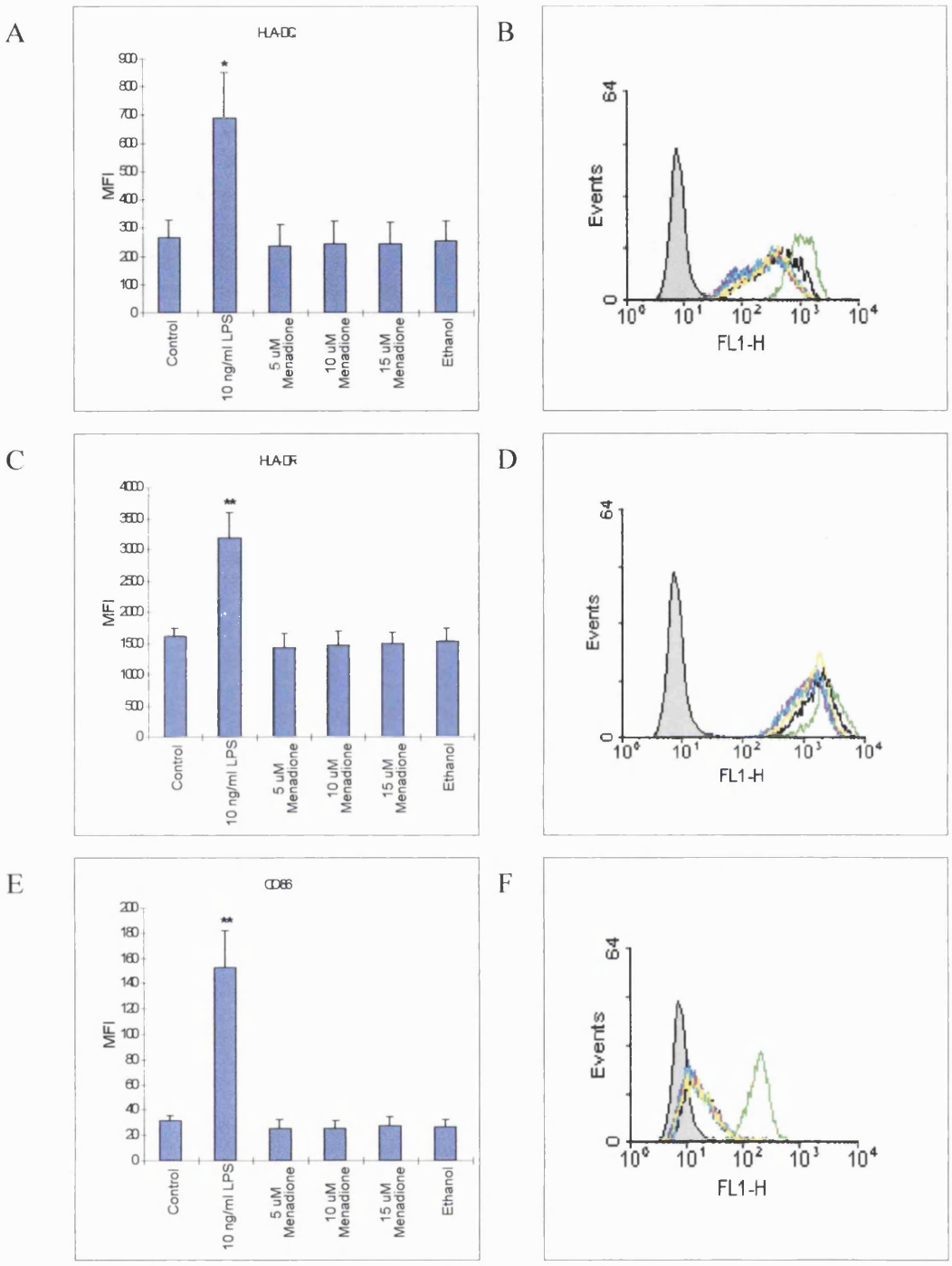


Figure 4.8. Effects of menadione on the phenotypic maturation of DC. DC were derived according to the method of day 4 purification (see Materials and Methods) and stimulated on day 7 for 24 hours. Surface expression of HLA-DQ and –DR, and CD86 were then assayed (A, C and E, respectively) by FACS analysis. Control values give expression without stimulation. Mean values ± SEM of three independent experiments are shown. The corresponding representative examples are shown in B, D and F. Filled profiles give background IgG2a control; green lines give LPS positive control; all others represent menadione samples. Statistical significance is given relative to 0μM menadione. *, $p<0.05$; **, $p<0.01$.

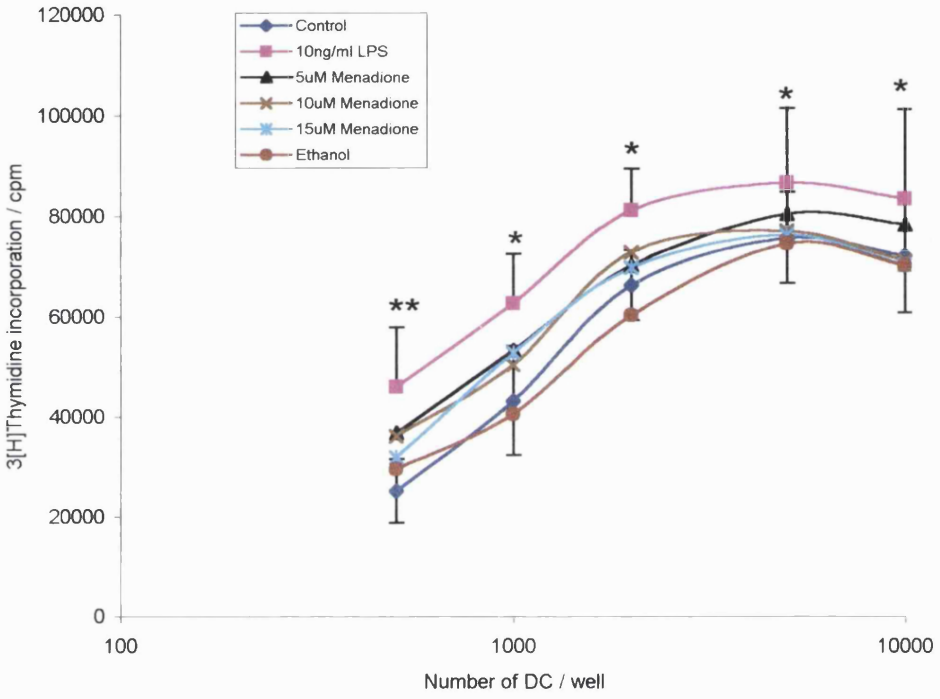


Figure 4.9. Effects of menadione on the functional maturation of DC. DC were derived according to the method of day 4 purification (see Materials and Methods) and stimulated on day 7 for 24 hours. Functional responses were then assayed by the oxidative mitogenesis assay. Control values give response without stimulation. Mean values ± SEM of three independent experiments are shown. Statistical significance is given relative to 0μM menadione. *, $p<0.05$; **, $p<0.01$.

4.2.2 Production of ROS by DC

To test the ability of DC to produce ROS, DC were incubated with 2,7-dichlorofluorescin-diacetate (DCFH-DA), which allows the detection of intracellular ROS at the single cell level by flow cytometry. DCFH-DA diffuses into cells where it is hydrolysed to yield the impermeable non-fluorescent product 2,7- dichlorofluorescin (DCFH). DCFH may then be oxidised by H_2O_2 to the fluorescent compound 2,7- dichlorofluorescein (DCF).

Since the production of ROS by DC has not been reported previously, it was necessary to establish a positive control. Although the addition of H_2O_2 would confirm the responsiveness of intracellular DCFH to ROS, in the event of stimuli not inducing the production of ROS, it would be advantageous if it could be shown that DC contain a pathway for the production of ROS, but which may or may not be activated by various stimuli.

Perhaps the most characterised mechanism for the production of ROS is activation of NADPH oxidase. NADPH oxidase is expressed by a variety of cells, including macrophages, and is primarily responsible for their oxidative burst, whether or not this is present and functional in DC has yet to be investigated. NADPH oxidase is activated by a PKC-dependent signalling pathway, which can, in turn, be stimulated by PMA. Given the similarities between macrophages and DC, it was hypothesised that PMA may activate PKC within DC and, thereby, possibly stimulate the production of ROS *via* the activation of NADPH oxidase. However, it should be noted that activation of NADPH oxidase is not necessarily the mechanism by which PMA stimulates the production of ROS.

Preliminary experiments were conducted to examine the stability of DCFH within DC. Figure 4.10 (A) shows that following a period of 60 minutes culture in phosphate-buffered saline supplemented with DCFH-DA, a bimodal distribution of fluorescence was observed, as would be expected if these cells

were dying and releasing DCFH. Figure 4.10 (B) shows that the cells with the lowest DCF fluorescence were indeed dead or dying, as indicated by their staining with propidium iodide. Future experiments were, therefore, gated to avoid the ectopic population.

A variety of agents were tested for their ability to induce production of ROS by DC. The stimuli tested included bacterial DNA, since this has been shown recently to activate DC and is of particular interest given the suggestion that it may stimulate B cells and monocytes by a mechanism that is dependent upon the intracellular production of ROS (Yi *et al.*, 1996). Figure 4.11 shows an agarose gel electrophoresis demonstrating the isolation of bacterial DNA.

It should be noted that the LPS used to examine the ability of DC to produce ROS was dissolved in phosphate-buffered saline, this was necessary to remove the potential fluorescence associated with phenol red. Stocks of LPS that were dissolved in phosphate-buffered saline were shown to be functional in Table 4.1. It can be seen from Table 4.1 that dissolving LPS in phosphate-buffered saline, as opposed to complete medium, does not prevent LPS from inducing the maturation of DC.

Figure 4.12 demonstrates that 0.3nM PMA induces significant production of ROS within DC, as indicated by the increase in the fluorescence of DCF, in a time-dependent manner (Figure 4.11), thus indicating that this is an enzymatic process and not a result of the interference between PMA and DCF. It can also be seen in Figure 4.12 that a plateau of ROS production is reached after 40 minutes stimulation with 0.3nM PMA, this may indicate that all of the intracellular DCFH had been oxidised to the fluorescent compound DCF, or that the rate of DCF production was equal to the rate that DCF was released from the DC.

Figure 4.12 also illustrates that 10ng/ml LPS was unable to stimulate the production of ROS by DC. However, it is possible that, at low concentrations, the effects of LPS may be dependent upon the presence of LPS binding protein, which was not present because of the potential interference of serum with the

flow cytometer. LPS was further shown not to induce the production of ROS at 10μg/ml, which acts independently of LPS binding protein.

Given the inability of LPS to induce the production of ROS by DC, it was then possible to test the ability of other stimuli to produce ROS. However, human recombinant TNF-α and bacterial DNA were shown not to induce statistically significant increases in the production of ROS by DC (Figure 4.12). Representative examples of the shifts in fluorescence that were associated with the production of ROS are given in Figure 4.13.

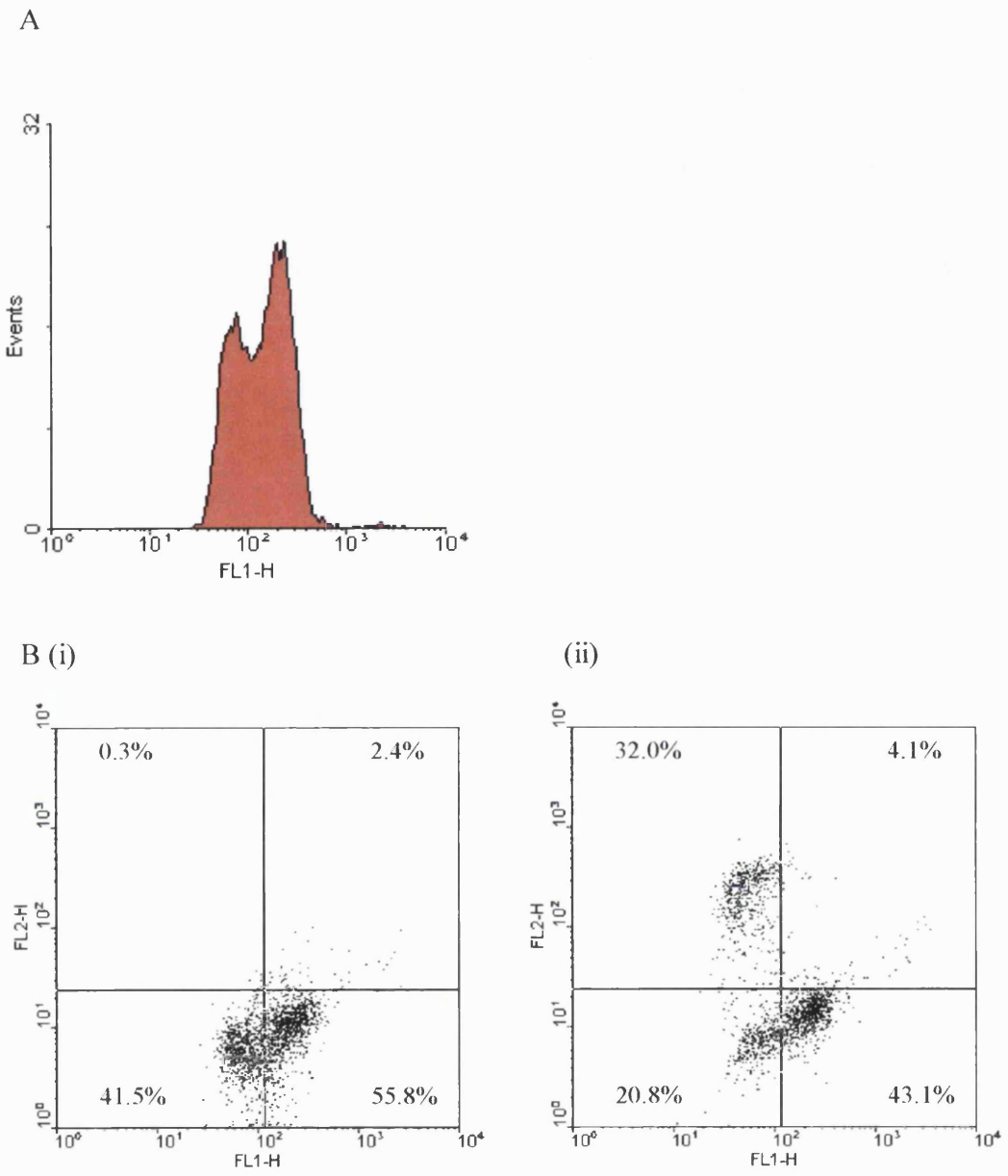


Figure 4.10. DC die after prolonged culture in a solution of phosphate-buffered saline. DC were incubated in PBS at a concentration of $5x10^5$DC/ml. 10μM DCF-DA was added at –15 minutes. (A) Characteristic histogram of DCF fluorescence giving bimodal distribution at 60 minutes. (B) Dot-plots illustrating the death of DC. After 60 minutes, control samples were incubated in the absence (i) or presence (ii) of 2μg/ml propidium iodide. FL1-H represents DCF fluorescence whilst FL2-H represents propidium iodide fluorescence. One representative of two experiments is shown.

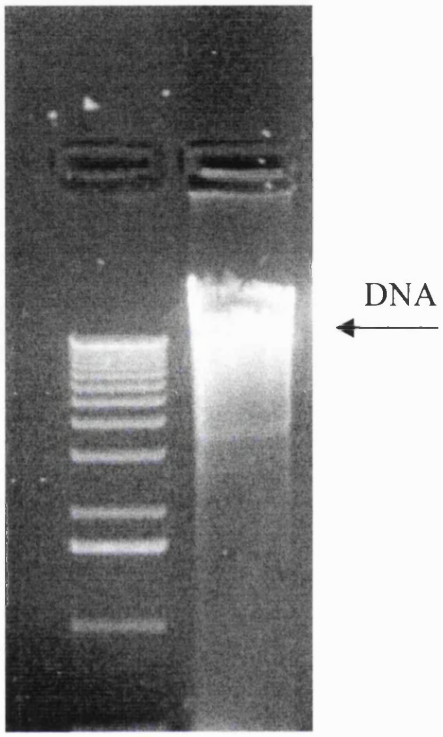


Figure 4.11. Agarose gel electrophoresis illustrating the isolation of DNA from *Escherichia coli*.

Table 4.1. Effects of LPS on DC maturation.

	Control	10ng/ml LPS (Complete medium)	10ng/ml LPS (PBS)	10μg/ml LPS (PBS)
FITC	11.5	9	8	6.5
CD 3	14.5	13	11	12
CD 19	13	10.5	9.5	9.5
CD 86	23	76	51.5	72.5
HLA-DQ	224	630.5	565	676.5
HLA-DR	1147	2333.5	2285	2285.5

LPS was dissolved in either complete medium (standard stock) or phosphate-buffered saline (PBS). Aliquots of these were then added to DC that were derived according to the method of day 4 purification (see Materials and Methods) and stimulated on day 7 for 24 hours. Surface expression of given markers was assayed by FACS analysis. FITC represents background fluorescence in the absence of a primary antibody. Mean fluorescence values are presented from duplicate samples of a single experiment.

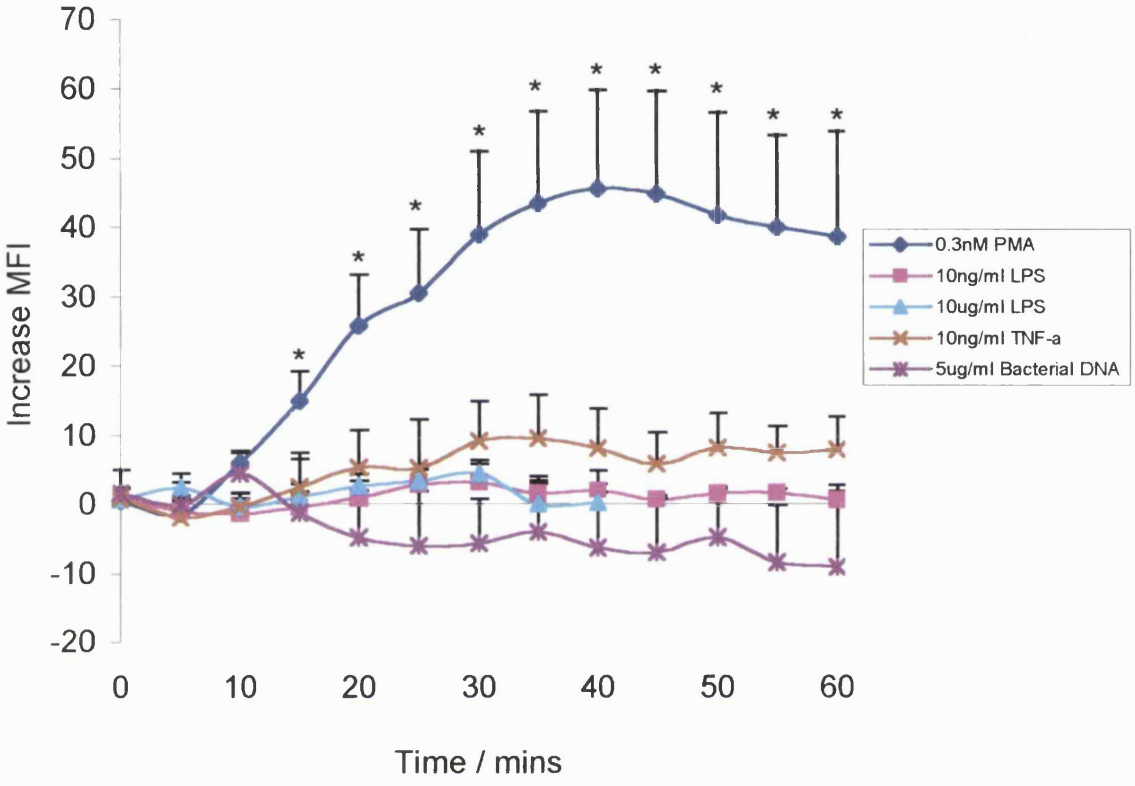
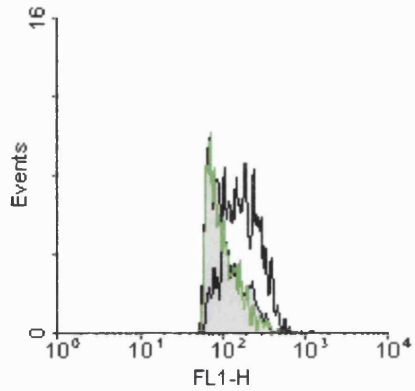
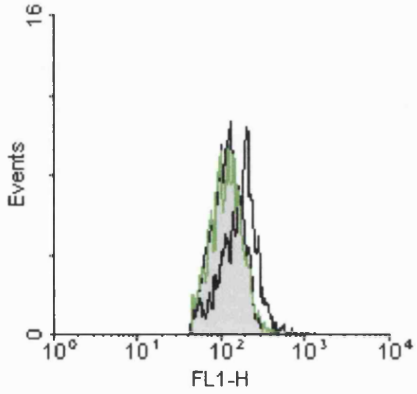


Figure 4.12. Intracellular production of ROS by DC. DC were incubated in phosphate-buffered saline at a concentration of $5x10^5$DC/ml. 10μM DCF-DA was added at –15 minutes. Samples were stimulated at 0 minutes and the fluorescence recorded every 5 minutes by FACS analysis. Values give the increase in fluorescence relative to a phosphate-buffered saline control. Mean ± SEM of 3-6 independent experiments are presented. Statistical analysis is given relative to phosphate-buffered saline control. *, $p<0.05$.

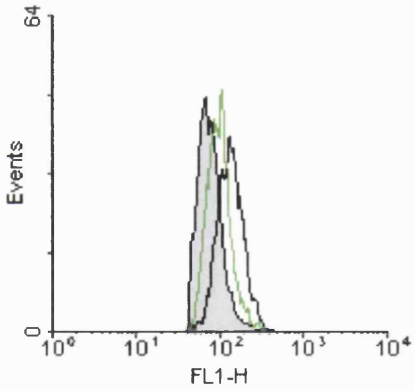
10ng/ml LPS



10μg/ml LPS



10ng/ml TNF-α



5μg/ml Bacterial DNA

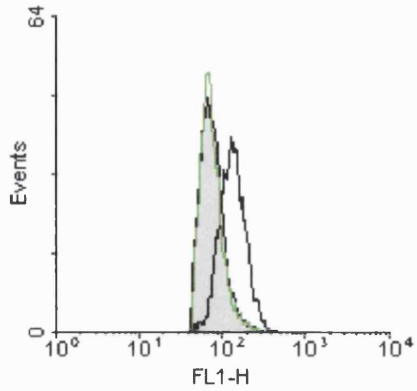


Figure 4.13. Intracellular production of ROS by DC. DC were incubated in phosphate-buffered saline at a concentration of $5x10^5$DC/ml. 10μM DCF-DA was added at –15 minutes. Samples were stimulated at 0 minutes. Histograms give representative shifts in fluorescence after 40 minutes. One representative of two is given for bacterial DNA, all others are representative of three or more independent experiments. Filled profiles give phosphate-buffered saline control; black overlays represent 0.3nM PMA; green overlays represent samples.

4.3 Discussion

4.3.1 Effects of oxidative stress on the phenotypic and functional maturation of DC

Oxidative stress is reputed to induce a number of different cellular effects in a variety of cell populations, and indeed, has been reported influence the maturation of DC (Rutault *et al.*, 1998; Verhasselt *et al.*, 1999). This chapter examines the ability of ROS to induce the maturation of DC directly.

Perhaps the simplest, and indeed the most characterised, way to induce an oxidative stress to a cell population is by the addition of H_2O_2 directly to the culture medium that contains the cells of interest. However, since excessive concentrations of oxidants are associated with toxic side-effects, a complicating factor is that such concentrations of oxidants may prevent or, indeed, inhibit the maturation of DC. In addition, it is possible that DC may be responsive to products or signals derived from apoptotic or necrotic cells. To avoid the problems of excessive toxicity, a range of concentrations of H_2O_2 were applied to purified DC. DC were then cultured for 24 hours, since it is known that other stimuli, including LPS, can induce the phenotypic and functional maturation of DC within this period.

The concentrations of H_2O_2 that were administered to DC were shown not to inhibit significantly the ability of DC to reduce MTT, as an indication of cellular activity (Figure 4.1). The concentrations of H_2O_2 that were added to the DC were, therefore, shown to induce neither inhibitory nor toxic effects on the DC. It can be seen from Figure 4.2 that, following a period of 24 hours culture after the addition of H_2O_2, the DC surface expression of HLA-DQ, HLA-DR and CD86 did not differ significantly from untreated cells. Since the increased expression of HLA-DQ, HLA–DR and CD86 are thought to be fundamental to the maturation process of DC, it can be seen that, under the conditions of these experiments, H_2O_2 did not induce the phenotypic maturation of DC.

Since a relatively limited phenotypic analysis was conducted, the ability of H_2O_2 to influence the functional capacity of DC was also examined. Figure 4.3 shows that the concentrations of H_2O_2 added to DC were unable to induce the functional maturation of DC; this finding supports the earlier observation (Figure 4.2) that H_2O_2 does not induce the maturation of DC.

To investigate whether or not the lack of observable effect of H_2O_2 on DC represented the transitory nature of this stimulus, GOD was subsequently added to DC at concentrations that did not decrease their cellular activity, as assessed by their ability to reduce MTT (Figure 4.4). The enzymatic activity of GOD is thought induce the prolonged release of H_2O_2, which, although not verified, was assumed to account for the inhibitory effects of high concentrations of GOD on the ability of DC to reduce MTT (Figure 4.4).

The effects of GOD, and thus presumably of H_2O_2, were then examined for their ability to induce the phenotypic and functional maturation of DC. The data presented in Figures 4.5 and 4.6, respectively, suggest that a prolonged oxidative stress, induced by the addition of GOD, induces neither the phenotypic nor functional maturation of DC.

Further to investigate the effects of oxidative stress on DC, a third oxidising agent was investigated for its ability to cause the maturation of DC. The agent selected was menadione; this agent is distinct from the previous oxidants tested since it is not only lipid soluble but also releases superoxide through a redox cycling mechanism. Concentration of menadione that were unable to inhibit the ability of DC to reduce MTT (Figure 4.7), as an indication of their cellular activity, were able neither to induce the phenotypic (Figure 4.8) nor functional (Figure 4.9) maturation of DC.

In summary, a range of concentrations of three distinct oxidising reagents were examined for their potential maturational effects on DC. The data discussed above do not provide support for the hypothesis that ROS alone are able to activate DC directly. Following oxidative stress, under all of the conditions

given, no changes were observed in the phenotype or functional properties of DC, whilst LPS (control) was able to mature DC consistently.

4.3.2 Production of ROS by DC

Many cells have been shown to produce ROS both constitutively and upon ligand stimulation. Despite the fact that ROS have been shown not to activate DC, it remained possible that DC could release ROS upon ligand stimulation. ROS released from DC could then, in theory, activate surrounding cells or indeed themselves indirectly, possibly *via* the activation of adjacent T cells.

The results showed that DC can indeed be induced to produce ROS upon stimulation with PMA. It must, therefore, be suggested that DC may stimulate signalling events in surrounding cells *via* their production of ROS.

In contrast, 10ng/ml LPS did not induce ROS production in DC. It remains possible that this was due to the absence of LPS-binding protein in the serum free media. However, since high concentrations of LPS also failed to stimulate ROS, and are known to act independently of LPS binding protein (see Section 3.3.4), it can be concluded that LPS does not activate ROS in DC, and consequently that ROS do not act as second messengers for LPS stimulation.

Furthermore, the inability of LPS to induce the production of ROS by DC has allowed examination of whether or not TNF-α and bacterial DNA, which are frequently contaminated with LPS, may stimulate the production of ROS by DC. It should be noted that TNF-α and bacterial DNA have both been reported to activate DC and are thought to be dependent on the production of ROS to exert their effects in other cellular systems. However, it can be seen from Figure 4.12 that neither TNF-α nor bacterial DNA increase significantly the production of ROS by DC.

4.3.3 Unanswered Questions

Since NF-κB is reputed to be activated by oxidative stress, and is thought to induce the maturation of DC upon activation (Rescigno *et al.*, 1998), it would be interesting to investigate whether or not ROS could induce either the activation or translocation of this transcription factor in DC. However, a positive result would question the role of NF-κB in DC maturation, whilst a negative result would add to the counter evidence that ROS are not a ubiquitous and prerequisite signal for the activation of NF-κB. Thus, neither result would yield any further insight into whether or not oxidative stress could regulate the function of DC directly.

It would be interesting to investigate whether or not ROS could activate DC *via* alternative cell populations and thereby possibly explain the discrepancy between the conclusions of this chapter and the findings of previous reports (Rutault *et al.*, 1998). Although potentially very interesting, this question would be a distinct deviation from the intended direction of this thesis, and would thereby, not further the hypothesis that products derived from tissue damage may act in a specific manner to cause the maturation of DC.

4.4 Conclusions

A range of concentrations of three distinct oxidising agents have been tested for their abilities to cause the maturation of DC directly. It has been shown that purified populations of DC are not induced to mature by the administration of sub-lethal levels of such oxidative stresses. It is, therefore, proposed that the lack of effects of oxidative stress on DC neither represent an artefact of sub-optimal concentrations of ROS nor the specific nature of the stimuli given.

Furthermore, it can be seen that DC are capable of increasing their intracellular production of ROS upon stimulation. However, ROS do not represent a common denominator pathway by which pro-inflammatory stimuli may exert their actions on DC.

It remains distinctly possible that oxidative stress may exert its effects through secondary mechanisms. At this point it is necessary to extend the "danger" hypothesis to include products that are associated with inflammatory conditions, but not actually constituents of tissue damage. Indeed, many of these are known to signal pro-inflammatory responses. Whilst not diverging from the concept that oxidative stress may play a fundamental role in the initiation of adaptive immunity, the potential role of two such products will be examined in the following chapters.

Chapter 5

The Role of Advanced Oxidation Protein Products in the Regulation of Dendritic cell Function

5.1 Introduction

It has been concluded previously that our key to understanding modern immunology may lie in the elucidation of the potential signals that cause maturation of DC, and thus initiate adaptive immune responses. Once these signals are resolved, it may then be possible to either harness or prevent their actions to either promote adaptive immune response or alleviate autoimmunity and chronic inflammation, respectively. Thus, the resolution of such signals may stimulate a new era in immunotherapy.

5.1.1 Hypothesis

The potential signals that may cause maturation of DC would be predicted to have a number of characteristics, as detailed previously (see Section 1.1.2.3.2). However, the hypothesis that necrotic tissues may contain a "danger" signal is questionable given the current data. Perhaps the most noteworthy alternative hypothesis is that an acute oxidative stress may provide the appropriate signal to activate DC. However, Chapter 4 concludes that this is probably not a direct effect of ROS.

It is possible that secondary products of oxidative stress could provide maturation signals to DC. These signals would be distinct from constituents of necrotic tissue damage and pro-inflammatory cytokines, but dependent upon an oxidative stress for their production. Such products must be present at the sites of acute tissue damage and be capable of stimulating intracellular signalling events. One may also expect these alternative signals to be associated with pro-inflammatory effects in other cellular systems. It should also be noted that DC must express a suitable receptor to mediate the recognition of these stress responsive messengers.

Such products may, therefore, not only be derived from micro-environmental constituents but also components of the peripheral circulation that leak into

inflamed sites. The hypothesis that secondary products of oxidative stress may cause maturation of DC must be considered as an elaboration of the modifications proposed by Ibrahim *et al.*, (1995), who suggested that products associated with micro-environmental tissue damage may activate DC.

Since the most significant effects of oxidative stress are thought to originate from protein and DNA damage, perhaps the most likely source of pro-inflammatory derivatives are proteins present in the surrounds of an oxidative stress. One suggestion is that advanced glycation end products (AGE) (see Section 1.3.4.1) could activate DC. This suggestion is supported by the accumulating evidence that AGE act on a number of myeloid cells, have pro-inflammatory effects, and accumulate as a product of physical age and glycaemic levels (Münch *et al.*, 94). Indeed, if this were found to be the case then one would speculate that the actions of AGE on DC might account for the increased predisposition of the elderly to autoimmune diseases. Of additional note is the observation that the biological activity of AGE may be dependent upon their level of oxidation (Baynes, 1991). Therefore, once AGE have accumulated within tissues, they may trigger the activation of DC upon an oxidative stress.

However, if AGE were to represent a universal mechanism for the maturation of DC then ROS, DC and AGE must all be present at the same time, which is unlikely in the young. An alternative candidate that may cause the maturation of DC, which would not require the prior formation of AGE, is the recently defined group of proteins known as advanced oxidation protein products (AOPP) (Wilko-Sarsat *et al.*, 1996). It is, thus, suggested that AOPP may constitute a "danger" signal, this is based on the realisation that AOPP result purely from oxidative stress, may be generated acutely and have been detected *in vivo*.

This chapter examines the hypothesis that AOPP may act as a potential signal to induce the maturation of DC. It should be noted that although AOPP have a number of characteristics, in contrast to other markers of oxidative stress, they do not represent a single molecular structure. Furthermore, it has been suggested that AOPP and AGE may share common biological activities *in vivo*

(Witko-Sarsat *et al.*, 98). If indeed this is the case, then it is likely that AOPP may have pro-inflammatory actions similar to those discussed for AGE (see Section 1.3.4.5).

In summary, DC may not rely on the distinction between apoptosis and necrosis, but on the detection of a suitable "danger messenger", i.e. a molecule or complex that, once oxidised, could activate DC and thereby adaptive immune responses. It is known that fenestration of an endothelium, as a consequence of either a microbial or physical insult, will release into the local milieu a vast diversity of oxidising agents. The local pro-oxidising environment may then lead to the oxidation of surrounding tissue components, in addition to circulating proteins and lipids that gain entry to these sites. It is hypothesised that DC may detect the presence of such oxidised derivatives and, thereby, promote their subsequent activation.

5.2 Results

5.2.1 Formation and characterisation oxidised BSA

5.2.1.1 AOPP and dityrosine formation

Figure 5.1 shows that the oxidation of bovine serum albumin (BSA) can be quantified in terms of the formation of AOPP, relative to a standard curve of chloramine T (see Materials and Methods). It can be seen in Figure 5.1 that the oxidation of BSA by HOCl induced a dose-dependent increase in the formation of AOPP. The first significant increase in the formation of AOPP by HOCl was observed following oxidation of BSA by 30mM HOCl, and maximal AOPP formation occurred following oxidation with 100mM HOCl. Since AOPP are, by definition, known to contain dityrosines, it was possible to confirm the formation of AOPP by analysis of their content of these bonds.

The content of dityrosine residues within samples of oxidised BSA were measured spectrophotometrically and calculated using the known extinction coefficient of dityrosine residues (see Materials and Methods). It can be seen from Figure 5.2 that the first significant increase in the formation of dityrosine residues occurred following oxidation of BSA with 30mM HOCl; this then increased further as higher concentrations of HOCl were applied to the BSA. Indeed, it can be seen that the pattern of dityrosine formation mirrors that of AOPP, although the plateau of dityrosine formation may occur at slightly higher concentrations of HOCl than were required for the formation of AOPP.

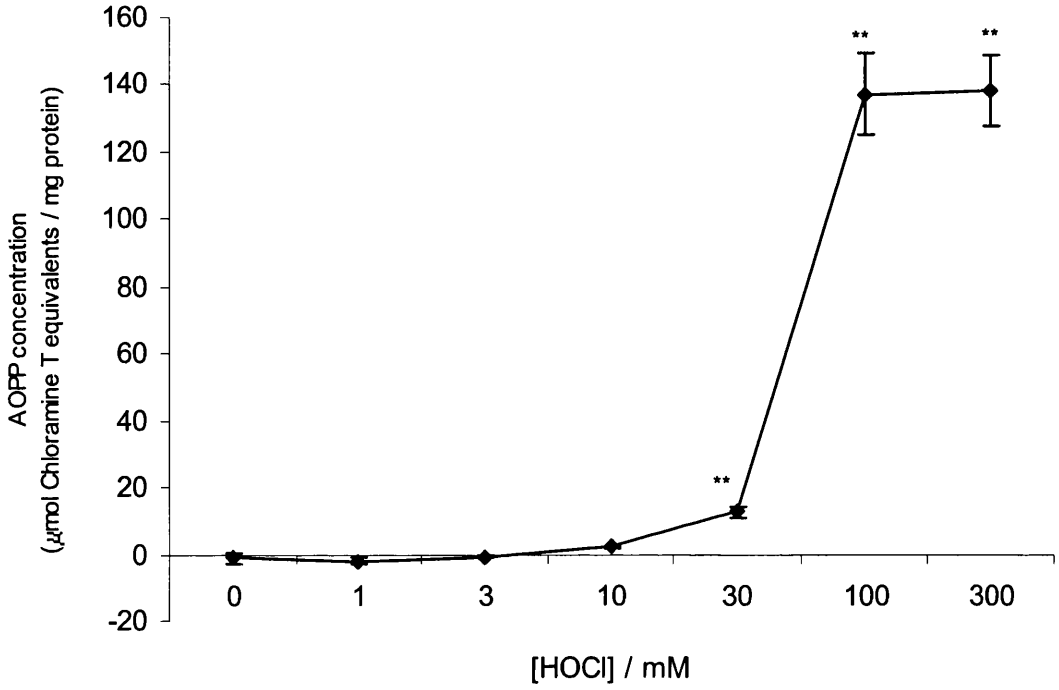


Figure 5.1. Effect of HOCl on the formation of AOPP. Increasing concentrations of HOCl were added to a solution of 40mg/ml BSA in 0.05M phosphate-buffered saline, pH 7.4, and incubated for 30 minutes at room temperature. AOPP concentration was measured spectrophotometrically relative to a standard curve of chloramine T (see Materials and Methods). Results represent mean ± SEM of three independent experiments. Significant increases are given relative to non-oxidised (i.e. 0 mM HOCl) BSA. **, $p<0.01$.

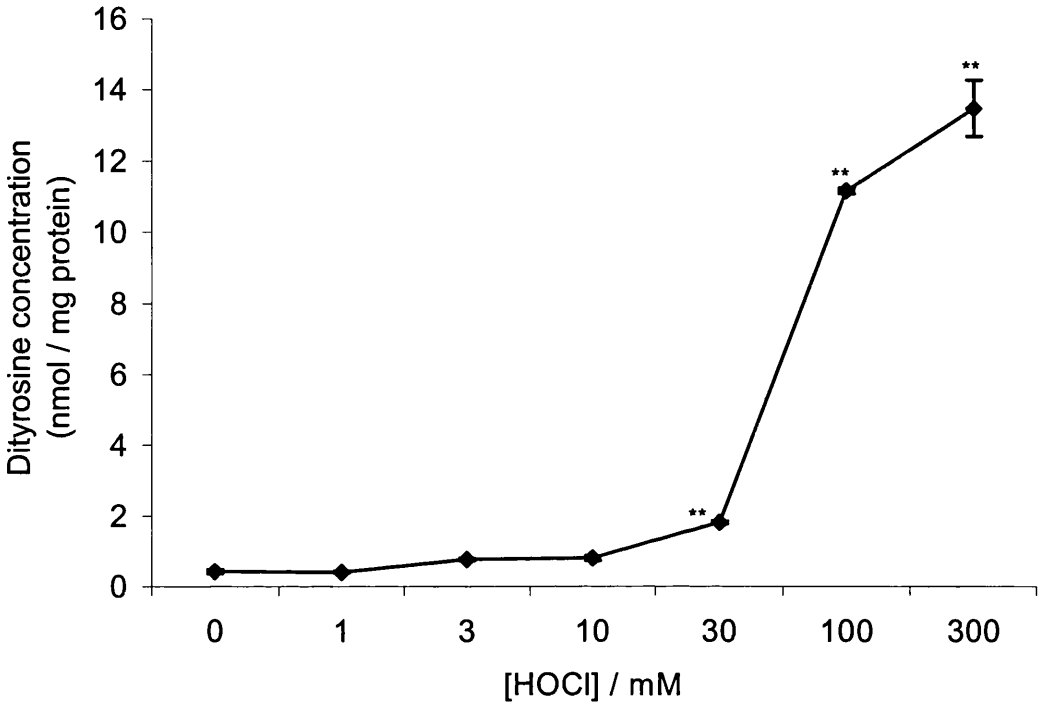


Figure 5.2. Effect of HOCl on the formation of dityrosine. Increasing concentrations of HOCl were added to a solution of 40mg/ml BSA in 0.05M phosphate-buffered saline, pH 7.4, and incubated for 30 minutes at room temperature. Dityrosine concentration was measured spectrophotometrically (see Materials and Methods). Results represent mean ± SEM of three independent experiments. Significant increases are given relative to non-oxidised (i.e. 0 mM HOCl) BSA. **, $p<0.01$.

5.2.1.2 Determination of other amino acid changes

Further to define the biochemical nature of AOPP, various other biochemical properties of HOCl-treated BSA were analysed (Figures 5.3-5.5). The oxidation of proteins is known to alter their content of free amino groups; these were assayed spectrophotometrically, as detailed in Materials and Methods, and quantified on the basis of a known extinction coefficient.

Increasing concentrations of HOCl are shown to decrease the number of free amino groups; this is thought to be the result of chlorination and proteolysis (Olszowska *et al.*, 1989). Low levels of protein oxidation by HOCl induces the formation of N-chloroamine residues, which can be reduced by thiosulphate (which was added to remove excess HOCl) and, therefore, such changes would not be expected to be seen in Figure 5.3. However, at higher doses of HOCl, the modifications are not reversible by thiosulphate and probably result from the formation of N-dichloroamine residues.

It can be seen in Figure 5.3 that the content of free amino groups within BSA decreases as increasing concentrations of HOCl are applied. Following oxidation of BSA with HOCl, the first significant decrease in the concentration of free amino groups occurred after 100mM HOCl had been applied to the BSA. The content of free amino groups was found to decrease further when BSA was oxidised with 300mM HOCl.

An early marker of oxidative stress to proteins is the loss of tryptophanyl residues. Following the oxidation of BSA with HOCl, it can be seen in Figure 5.4 that the content of tryptophanyl residues, as measured by their relative fluorescent intensity (275nm excitation, 334 emission), decreased with increasing concentrations of HOCl. Furthermore, it can be seen in Figure 5.4 that the first significant decrease in the content of tryptophanyl residues, within BSA, occurred following oxidation with 10mM HOCl. However, it is likely that detection of oxidative damage at concentrations of less than 10mM HOCl may

have been masked by the noise of the fluorimeter. At the highest concentrations of HOCl, tryptophanyl residues could not be detected; indicating that extensive proteolysis may have occurred at 100mM and 300mM HOCl.

Another marker of irreversible protein damage is the loss of sulphydryl groups. Low levels of oxidative stress induce the formation of reversible inter- and intramolecular disulphide bonds. Such cross-linking bonds would have been reduced by the addition of thiosulphate to the samples and would, therefore, not be expected to be observed within Figure 5.5. The remaining sulphydryl groups were detected spectrophotometrically and quantified using a known extinction coefficient, as detailed in Materials and Methods.

Figure 5.5 shows that, following the oxidation of BSA with HOCl, the total content of protein sulphydryls and disulphides, which is represented as –SH equivalents, does not differ significantly from non-oxidised BSA over a wide range of HOCl concentrations. Indeed, the first significant decrease in the content of –SH groups occurred following the oxidation of BSA with 300mM HOCl (Figure 5.5).

An important feature of Figure 5.5 is that it shows that at 300mM HOCl any cross-links that are formed are less likely to be mediated by disulphide bonds than dityrosines, since the former of these are cleaved at high concentrations of HOCl.

5.2.1.3 Protein aggregation

To confirm the formation of protein aggregates, and thus AOPP, the oxidised BSA samples were analysed by SDS-PAGE. It can be seen from Figure 5.6 that increasing concentrations of HOCl decreased the intensity of the protein bands that represented non-oxidised BSA. Furthermore, it should be noted that the highest concentrations of HOCl were not associated with precipitation of the protein and did not appear on a 4% gel; indicating that extensive proteolysis may

have occurred. At 100mM HOCl the band associated with non-oxidised BSA was completely absent. However, 100mM HOCl was also associated with the formation of a diffuse band of high molecular weight proteins.

It was necessary to stain the gels with silver stain to detect the low concentrations of proteins within samples of BSA oxidised with 100mM and 300mM HOCl, since inconclusive results were observed with Coomassie blue staining. It should also be noted that these gels were run under reducing conditions, therefore, disulphide bonds are not responsible for the formation of the protein aggregates observed.

5.2.1.4 Extents of protein oxidation to be examined

Since the majority of the significant effects of HOCl on BSA were observed at 30mM HOCl, and maximal formation of AOPP and dityrosine occurred at 100mM HOCl, protein samples oxidised with these two HOCl concentrations were selected to test for their potential effects on the phenotypic and functional properties on DC.

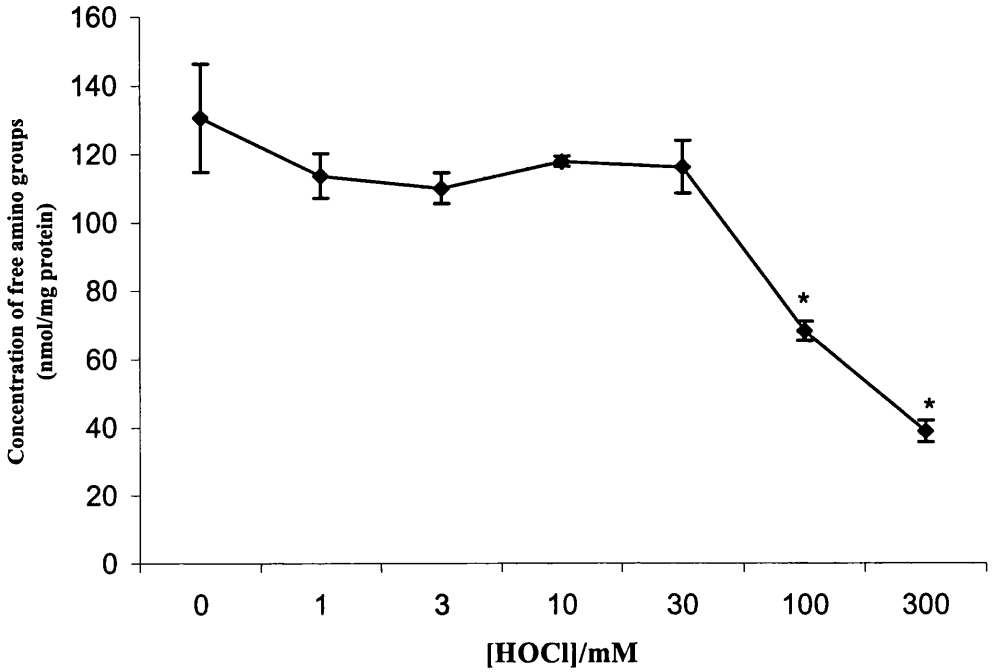


Figure 5.3. Effect of HOCl on the concentration of free amino groups in BSA. Increasing concentrations of HOCl were added to a solution of 40mg/ml BSA in 0.05M phosphate-buffered saline, pH 7.4, and incubated for 30 minutes at room temperature. The concentration of free amino groups was assayed as detailed in Materials and Methods and quantified spectrophotometrically. Results represent mean ± SEM of three independent experiments. Significant differences are given relative to non-oxidised (i.e. 0 mM HOCl) BSA. *, $p<0.05$.

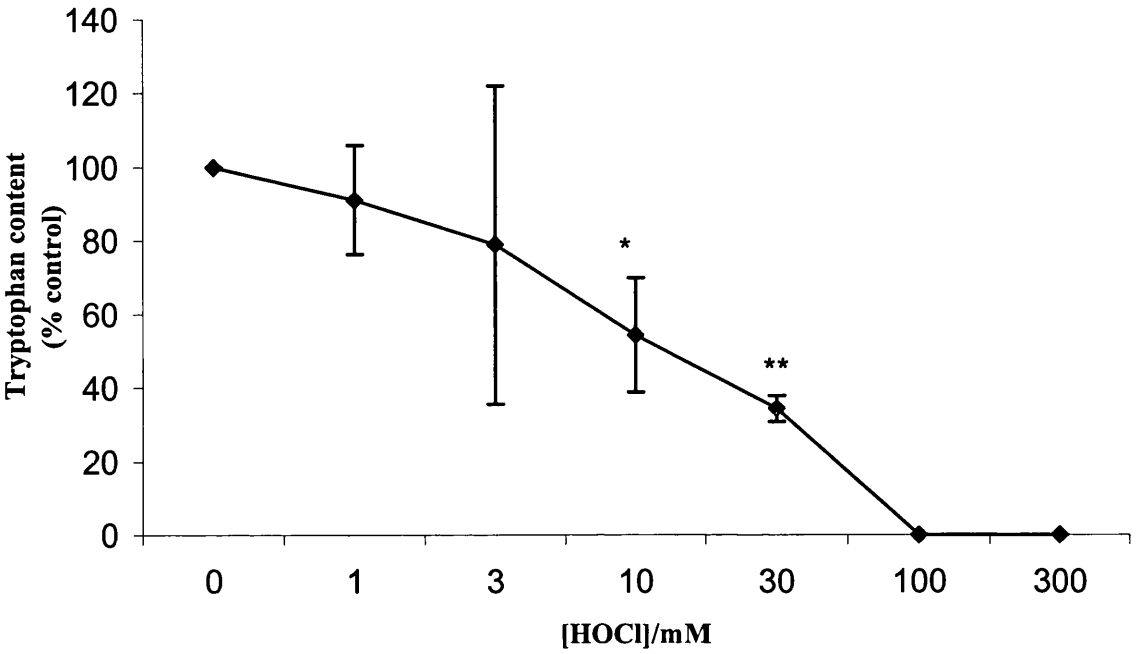


Figure 5.4. Effect of HOCl on the content of tryptophan residues in BSA. Increasing concentrations of HOCl were added to a solution of 40mg/ml BSA in 0.05M phosphate-buffered saline, pH 7.4, and incubated for 30 minutes at room temperature. The content of tryptophan residues was measured fluorometrically. Results represent mean ± SD of three independent experiments. Significant differences are given relative to non-oxidised (i.e. 0 mM HOCl) BSA. *, $p<0.05$; **, $p<0.01$.

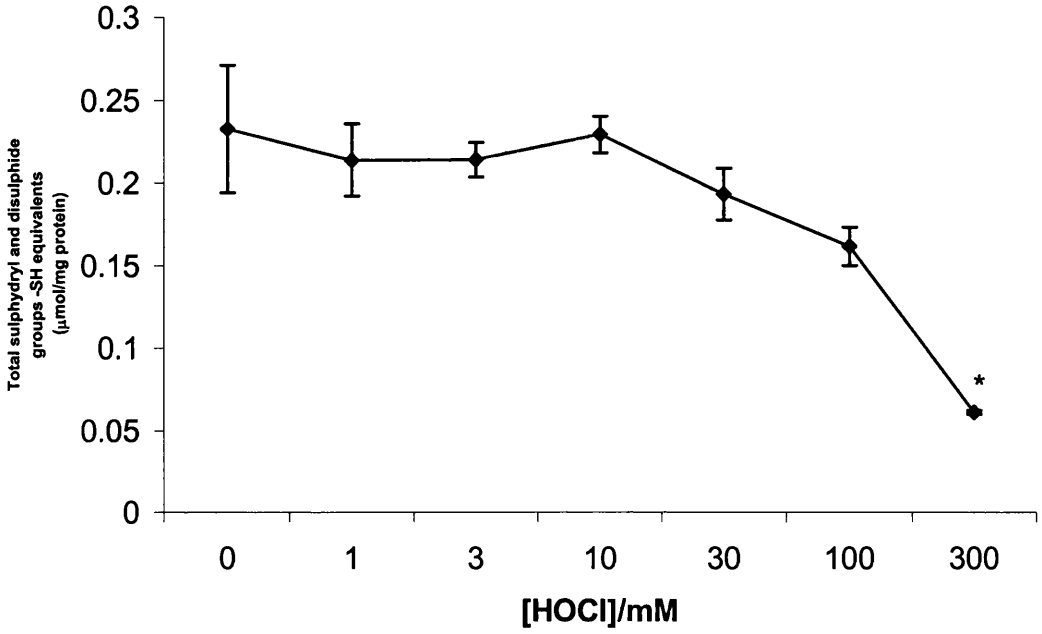


Figure 5.5. Effect of HOCl on the total content of sulphydryl groups in BSA. Increasing concentrations of HOCl were added to a solution of 40mg/ml BSA in 0.05M phosphate-buffered saline, pH 7.4, and incubated for 30 minutes at room temperature. The concentration of sulphydryl groups was assayed as detailed in Materials and Methods and quantified spectrophotometrically. Results represent mean ± SEM of three independent experiments. Significant differences are given relative to non-oxidised (i.e. 0 mM HOCl) BSA. *, $p<0.05$.

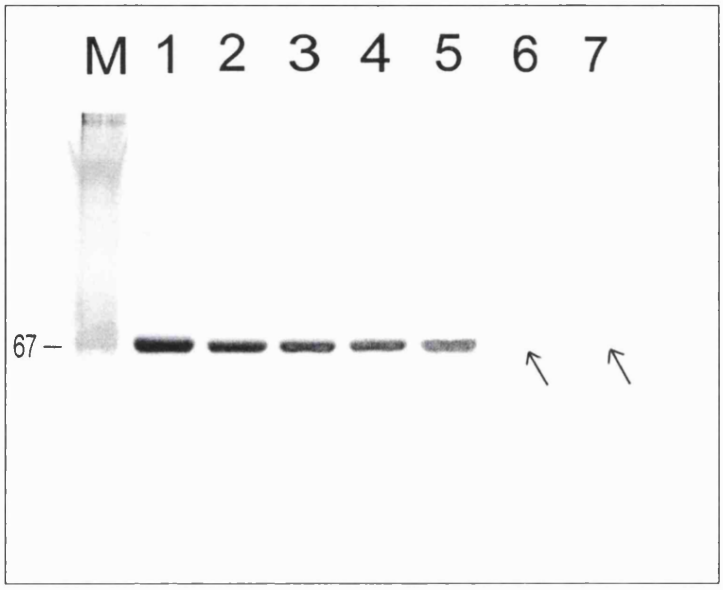


Figure 5.6. SDS-PAGE showing the effects of HOCl on the aggregation of BSA. Increasing concentrations of HOCl were added to a solution of 40mg/ml BSA in 0.05M phosphate-buffered saline, pH 7.4, and incubated for 30 minutes at room temperature. 7.5% Gels were run (10μg/lane) and developed with silver stain. Lane numbers refer to BSA incubated with 0, 1, 3, 10, 30, 100, and 300mM HOCl

5.2.2 Effects of non-oxidised BSA on DC

BSA alone would not be expected to effect either the phenotypic or functional properties of DC. However, standard preparations of BSA are contaminated with numerous proteins and carbohydrates, the actions of which on DC are unknown. It was, therefore, necessary to test whether or not samples of non-oxidised BSA could activate DC, and, if so, at what concentration of BSA these effects could be eliminated.

Figure 5.7 examines the surface expression of HLA-DQ and –DR, and CD86 on DC following 24 hours culture in the presence of non-oxidised BSA and LPS, which is known to induce DC maturation. It can be seen in Figure 5.7 that 10ng/ml LPS induced significant increases in the surface expression of HLA-DQ and –DR, and CD86 on DC, as detected by FACS analysis (see Materials and Methods); this is consistent with the maturational effects of LPS on DC. Furthermore, Figure 5.7 shows that, at concentrations of 1mg/ml and 100μg/ml, samples of non-oxidised BSA also increased significantly the expression of HLA-DQ and –DR, and CD86 on the surface of DC. Such increases in surface molecule expression indicate that DC maturation occurred when they were incubated with equal or greater than 100μg/ml BSA for 24 hours. Moreover, Figure 5.7 also shows that 10μg/ml BSA was the maximum concentration that could be applied to DC without causing their phenotypic maturation.

Further to confirm whether or not samples of non-oxidised BSA were capable of inducing the maturation of DC, the ability of DC that had been incubated in the presence of non-oxidised BSA were tested for their ability to induce the proliferation of resting T cells. Figure 5.8 shows that, in an oxidative mitogenesis assay, DC are capable of inducing the proliferation of T cells, as quantified by ^{3}[H]thymidine incorporation, in a dose-dependent manner. Furthermore, it can be seen from Figure 5.8 that culturing DC for 24 hours in the presence of 10ng/ml LPS increases their capacity to induce T-cell proliferation, as indicated by the observed increase in ^{3}[H]thymidine incorporation.

It can be seen in Figure 5.8 that whilst prior incubation of DC with 10μg/ml BSA did not influence their ability to induce the proliferation of T cells, 100μg/ml BSA induced DC to increase their T-cell stimulatory capacity. The data presented within Figure 5.8 are consistent with the findings observed in Figure 5.7, such that at 10μg/ml non-oxidised BSA was unable to induce the maturation of DC, whilst at 100μg/ml BSA both phenotypic and functional maturation occurred.

In addition to the known contaminants of BSA, it was suspected that these samples may also contain LPS, a known activator of DC. The contamination of BSA with LPS was confirmed by means of the *Limulus amebocyte* lysate assay. Furthermore, in this semi-quantitative assay, the level of LPS within BSA was found to correlate closely with the concentrations that would be expected to activate DC (see Section 3.2.8), such that 1mg of BSA contained between 1ng and 7ng LPS. Hence 10μg BSA will contain a maximum of 70pg LPS, which is well below the threshold of detectable *in vitro* biological activity.

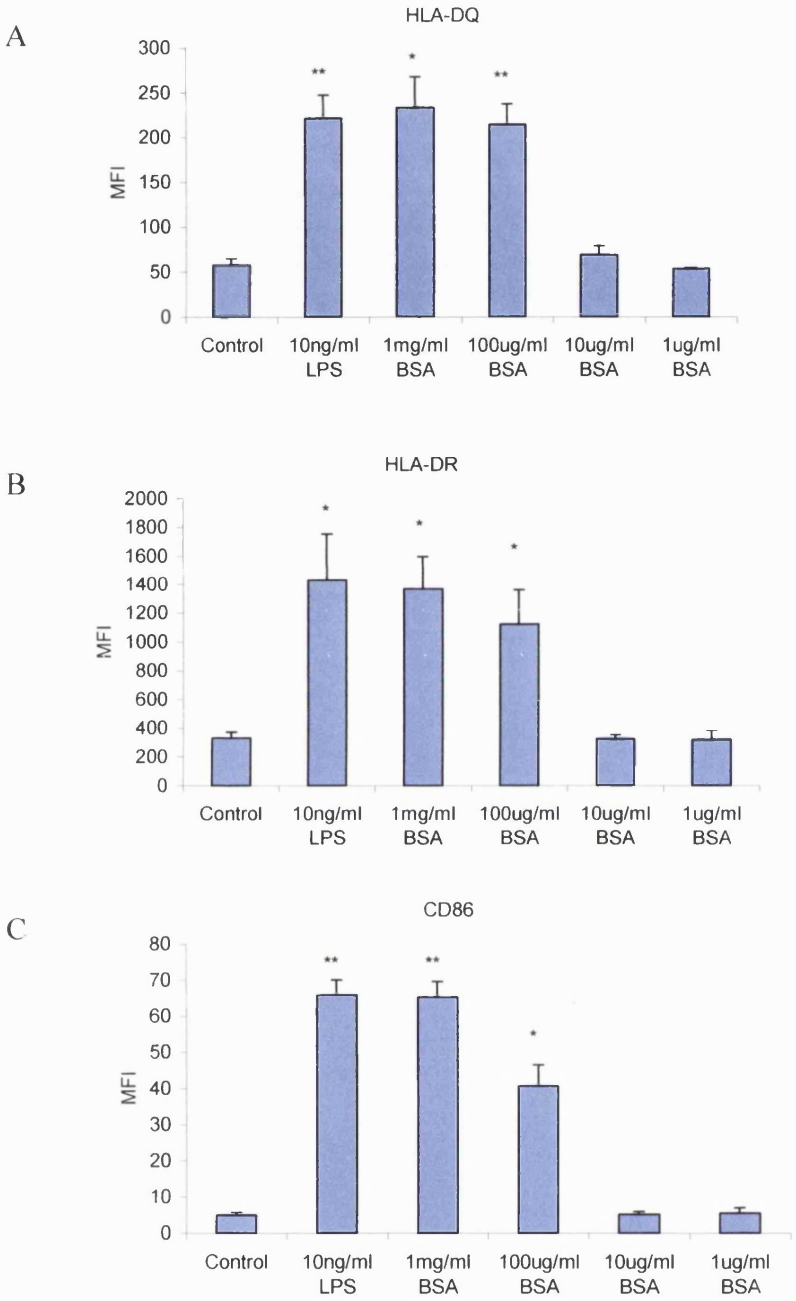


Figure 5.7. Effects of non-oxidised BSA alone on the phenotypic maturation of DC. DC were derived according to the method of day 4 purification (see Materials and Methods) and stimulated on day 7 for 24 hours. Control values represent samples given an equivalent concentration of phosphate-buffered saline to that contained within the BSA samples (final concentration 0.5μM). 10ng/ml LPS is given as a positive control. Surface expression of HLA-DQ and –DR and CD86 were then assayed (A, B and C, respectively) by FACS analysis. Mean values ± SEM of three independent experiments are shown. Significant differences are given relative to non-oxidised (i.e. 0 mM HOCl) BSA. *, $p<0.05$; **, $p<0.01$.

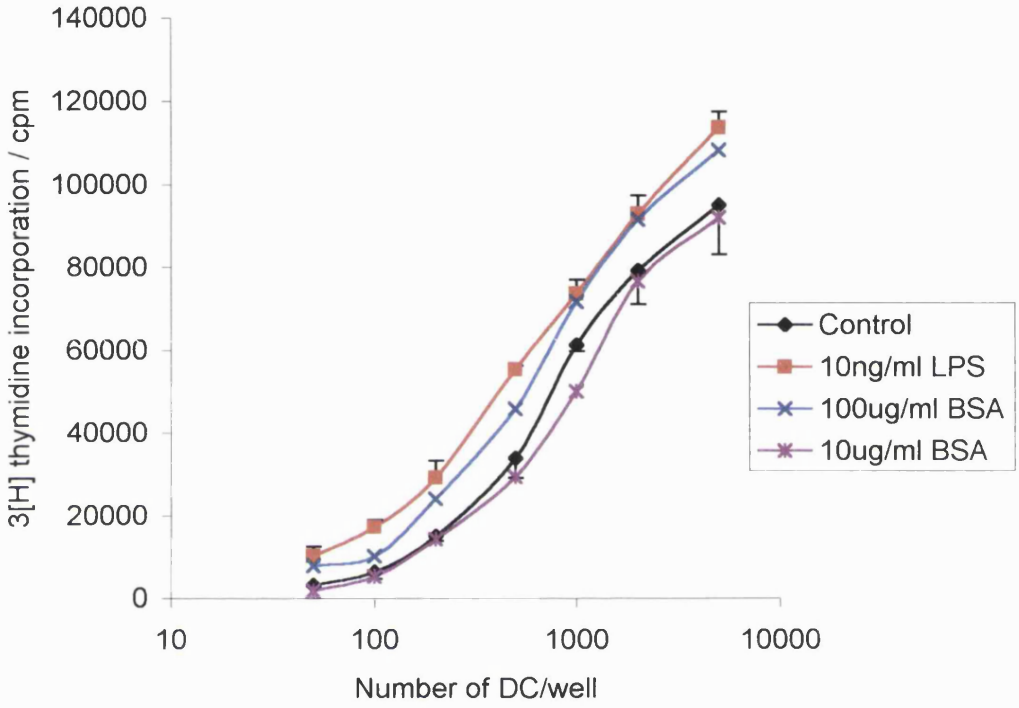


Figure 5.8. Effects of non-oxidised BSA alone on the functional maturation of DC. DC were derived according to the method of day 4 purification (see Materials and Methods) and stimulated on day 7 for 24 hours. Control responses represent samples given an equivalent concentration of phosphate-buffered saline to that contained within the BSA samples (final concentration 0.5μM). 10ng/ml LPS is given as a positive control. The functional capacity of these cells was assayed by the oxidative mitogenesis assay. One representative of three experiments is shown. Mean values ± SD of triplicate samples are presented.

5.2.3 Effects of AOPP on DC

It has been concluded in section 5.2.1.4 that AOPP samples should be derived from BSA that has been oxidised with 30mM and 100mM HOCl. In addition, it has been shown that the maximum concentration of non-oxidised BSA that can be added to DC that induces neither their phenotypic nor functional maturation is 10μg/ml. Based on these initial conclusions, three samples of AOPP were formed and tested for their ability to cause the maturation of DC. The first AOPP sample, representing a control, contained non-oxidised BSA at a final concentration of 10μg/ml (0μM AOPP). The next two samples contained BSA that had additionally been treated with 30mM and 100mM HOCl, respectively. The oxidised samples of BSA were calculated to have final AOPP concentrations of 125μM and 1370μM, respectively (AOPP concentrations were calculated from Figure 5.1).

Figure 5.9 demonstrates the surface expression of HLA-DQ and –DR, and CD86 on DC following 24 hours culture in the presence of AOPP and LPS. It can be seen in Figure 5.9 that 10ng/ml LPS induced significant increases in the surface expression of HLA-DQ and –DR, and CD86 on DC, as detected by FACS analysis (see Materials and Methods); these increases are consistent with the maturational effects of LPS on DC. Since AOPP were dissolved in 0.05M phosphate-buffered saline, control values of surface molecule expression represent the expression following the addition of an equivalent concentration of phosphate-buffered saline (final concentration 0.5μM). Moreover, Figure 5.9 demonstrates that AOPP, at concentrations of 0μM, 125μM and 1370μM, do not increase significantly the surface expression of HLA-DQ, HLA-DR or CD86, on DC. Furthermore, no significant changes were observed in the expression of CD1a nor CD14. It can, therefore, be concluded that, under the conditions of the experiments presented in Figure 5.9, concentrations of AOPP of up to 1370μM do not induce the phenotypic maturation of DC, in a manner akin to the maturational effects of LPS.

Figure 5.10 demonstrates the ability of DC to induce the proliferation of resting T cells, as shown by ^{3}[H]thymidine incorporation, in an oxidative mitogenesis assay. It can be seen in Figure 5.10 that ^{3}[H]thymidine incorporation, and thus the proliferation of T cells, increased with increasing concentrations of DC. Furthermore, following the incubation of DC for 24 hours with 10ng/ml LPS, they were found to increase significantly their ability to stimulate the proliferation of T cells.

Following the addition of 10μg/ml non-oxidised BSA (0μM AOPP) to DC, it can be seen that the DC induced similar levels of T-cell proliferation compared to DC treated with solvent alone (control; phosphate-buffered saline, final concentration 0.5μM). It can, therefore, be seen that, when assessed by an oxidative mitogenesis assay, BSA (final concentration 10μg/ml) that contains 0μM AOPP does not interfere with the ability of DC to induce the proliferation of resting T cells.

Figure 5.10 also shows the effects of incubating DC for 24 hours in the presence of 125μM and 1370μM AOPP on their ability to induce the proliferation of resting T cells. On initial inspection, it appears that DC cultured with the above concentration of AOPP induce slightly higher levels of proliferation of resting T cells. When statistical analysis was conducted, however, it was found that the increase in response observed was only statistically significant for one data point, which represented DC that had been treated with 125μM AOPP for 24 hours and their functional responses examined at 5000DC/well.

It is possible that this single statistically significant increase may represent a chance result and that treating DC with AOPP does not alter their ability to induce the proliferation of T cells. Alternatively, it can be seen from the dose-response curve shown in Figure 5.10 that the responses induced by DC that had been treated with AOPP diverge from control responses at concentration of greater than 200 DC/well. It could be suggested from this divergence that the effects of AOPP on DC may be restricted to events involving large numbers of

DC interactions and that AOPP may, indeed, increase the capacity of DC to induce the proliferation of T cells.

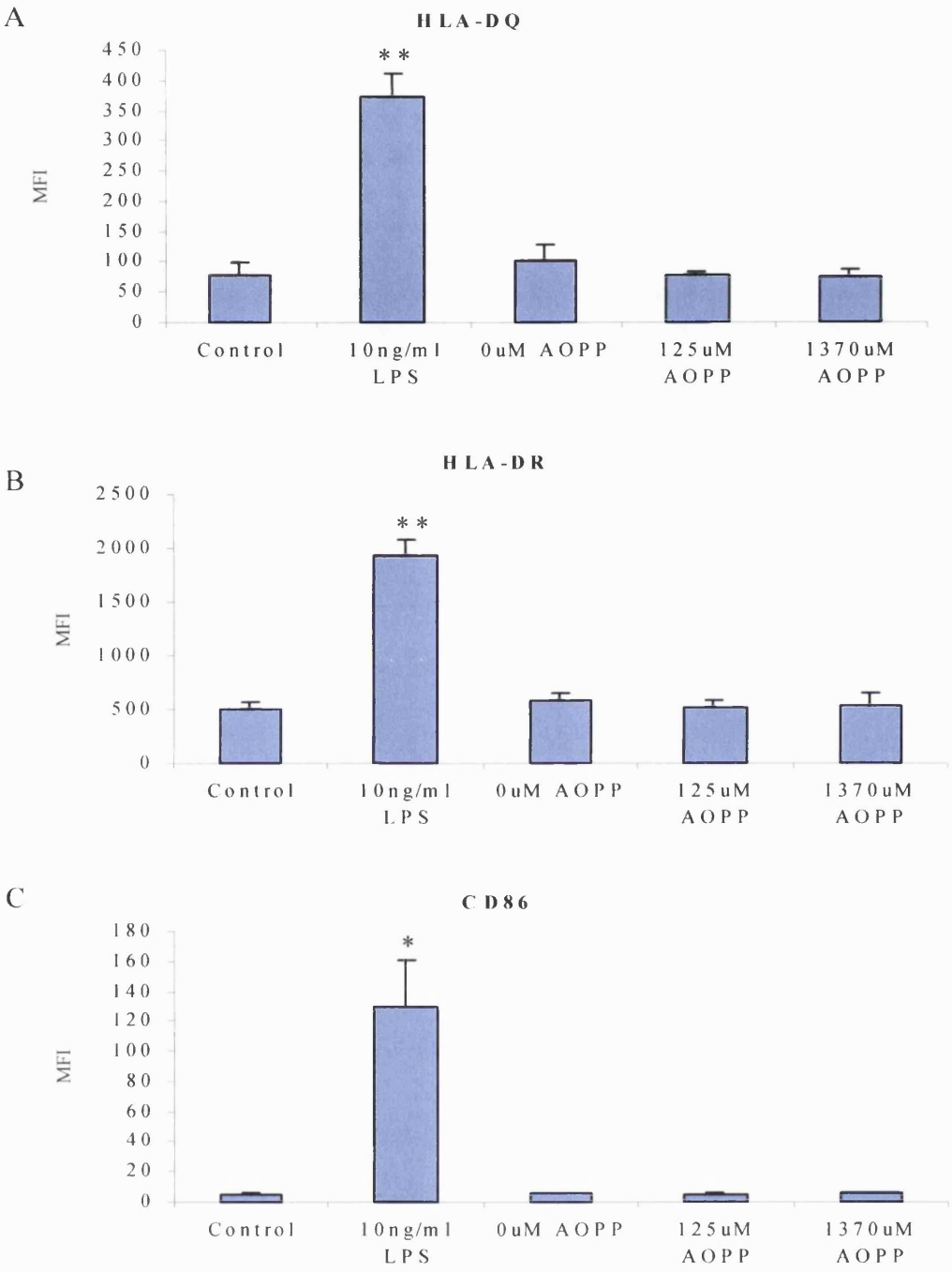


Figure 5.9. Effects of oxidised BSA on the phenotype of DC. DC were derived according to the method of day 4 purification (see Materials and Methods) and stimulated on day 7 for 24 hours. BSA concentrations are all at 10μg/ml. Control values represent samples given an equivalent concentration of phosphate-buffered saline to that contained within the BSA samples (final concentration 0.5μM). 10ng/ml LPS is given as a positive control. Surface expression of HLA-DQ and –DR and CD86 were then assayed (A, B and C, respectively) by FACS analysis. Mean values ± SEM of five independent experiments are shown. Significant differences are given relative to phosphate-buffered saline control. *, $p<0.05$; **, $p<0.01$.

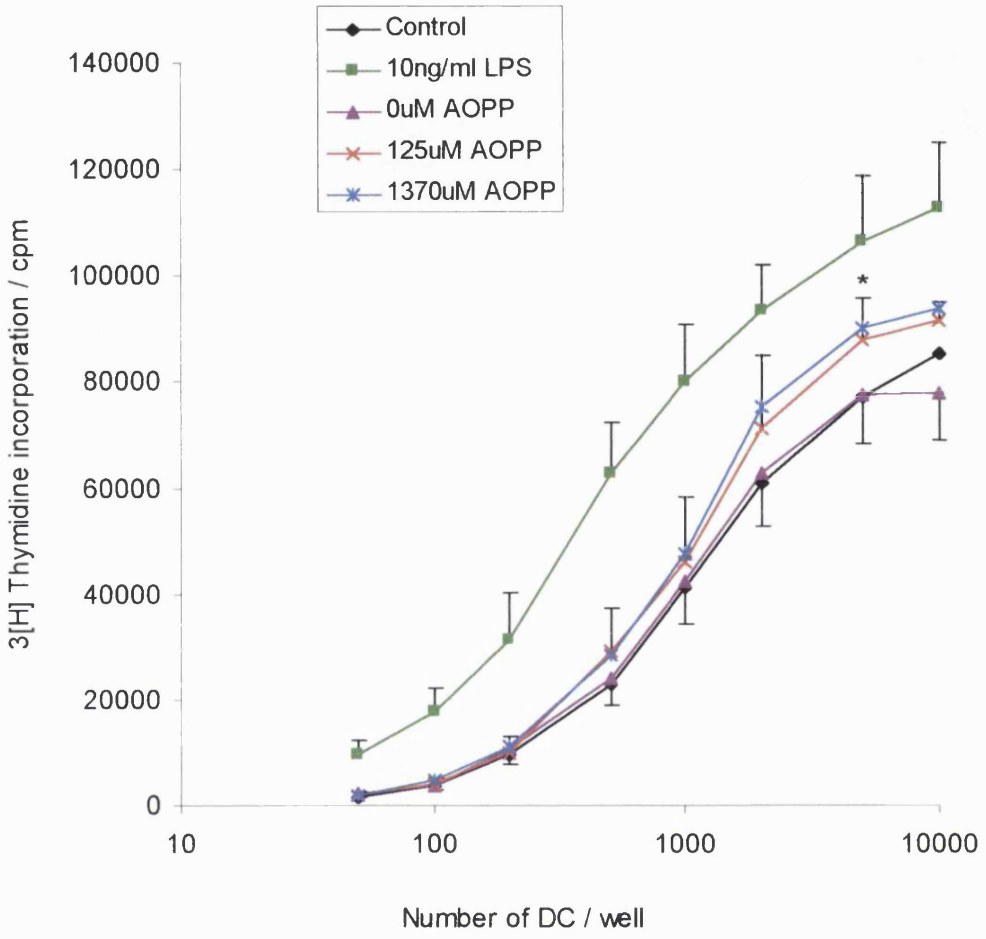


Figure 5.10. Effects of oxidised BSA on the functional responses of DC. DC were derived according to the method of day 4 purification (see Materials and Methods) and stimulated on day 7 for 24 hours. Control responses represent samples given an equivalent concentration of phosphate-buffered saline to that contained within the BSA samples (final concentration 0.5μM). 10ng/ml LPS is given as a positive control. The functional capacity of DC was assayed by the oxidative mitogenesis assay. Mean values ± SEM of three independent experiments are shown. Values given for LPS are significantly ($p<0.05$) above control responses for every concentration of DC given. Significant difference is given for 125μM AOPP relative to phosphate-buffered saline control. *, $p<0.05$.

5.3 Discussion

5.3.1 Formation of AOPP

The hypothesis presented at the start of this chapter is based on the suggestion that an indeterminate group of oxidised proteins may act as a "danger" signal to induce the maturation of DC. The quantification of oxidised protein derivatives in terms of AOPP allows their formation to be determined in a relatively non-specific manner. Thus, it was possible to quantify and test the effects of a cocktail of protein derivatives on DC, as would be expected to occur *in vivo*. If AOPP are found to activate DC, then one could analyse these protein samples to determine their active constituent and, thus, their mode of action.

To investigate the hypothesis that AOPP could activate DC, it was first necessary to oxidise a model protein to form AOPP. Since AOPP are known to be formed from plasma proteins, especially albumin (Witko-Sarsat *et al.*, 1998), the model protein investigated was bovine serum albumin (BSA). Furthermore, given the reported propensity of HOCl to forming AOPP, the effects of this oxidising agent were investigated.

The initial set of experiments was aimed at confirming that HOCl acts on BSA to induce the formation of AOPP. AOPP formation was shown to be dose-dependent (Figure 5.1) and correlate closely with the dimerisation of tyrosine residues (Figure 5.2), providing evidence of cross-linking. Additional evidence of cross-linking was observed when the samples were analysed by protein electrophoresis (Figure 5.6); this demonstrated directly the formation of high molecular proteins. The high molecular weight proteins did not form a discrete band suggesting that they were of various molecular weights and structures. Furthermore, aggregation was likely to be a consequence of intermolecular dityrosine rather than disulphide bond formation, since the gel was run under reducing conditions.

The relative lack of visible protein in the gel, at high concentration of HOCl, may be attributed to fragmentation of BSA. However, one would expect the formation of at least some relatively large fragments, and the gel showed no evidence of this. It is, therefore, suggested that although HOCl may induce the fragmentation of BSA, large protein aggregates are also formed, which are likely to represent AOPP formation.

The formation of oxidised protein derivatives was further supported by the more conventional approaches presented in Figures 5.3-5.5. These data show that oxidation of BSA is associated with the irreversible loss of free amino groups, tryptophanyl residues and total sulphydryl groups. Following the oxidation of BSA with 30mM HOCl, significant changes were observed in all of the above markers of oxidative stress to proteins.

Base upon the biochemical data presented, the test samples of BSA were oxidised by 30mM HOCl and 100mM HOCl. It should be noted that such concentrations of HOCl are significantly higher than those found at sites of inflammation. However, at sites of inflammation numerous other oxidants will also be present which would be expected to have an additive effect on the degree of oxidative damage induced. Indeed we know that sufficient levels of oxidants exist *in vivo* to result in formation of AOPP, so the formation of AOPP in this system is simply an *in vitro* representation of an *in vivo* event. Therefore, the concentrations of HOCl that are required to form AOPP *in vitro* do not necessarily impinge on the validity of using these concentrations of HOCl. Indeed, these concentrations of HOCl are very similar to those reported previously for other *in vitro* studies (Olszowska *et al.*, 1989; Olszowski *et al.*, 1996).

5.3.2 Effects of AOPP on DC

As a result of the contamination of BSA with LPS, the maximum concentration of BSA that could be applied to the DC was 10μg/ml. At this concentration, non-oxidised BSA induced neither the phenotypic nor functional maturation of DC. BSA was then oxidised to the extents defined previously and then added to DC for 24 hours. The effects of AOPP on DC were determined by phenotypic and functional characterisation of these cells. The amount of protein added to DC was quantified prior to oxidation, thereby, controlling for contaminants within BSA rather than the final concentration of protein.

Figure 5.9 illustrates that, at concentrations of 125μM and 1370μM, AOPP do not induce the phenotypic maturation of DC and that any phenotypic effects that may have occurred were not analogous to those induced by LPS. However, AOPP may have direct effects on the ability of DC to induce the proliferation of T cells (Figure 5.10), especially at high concentrations of DC.

The functional effects observed bear a certain resemblance to the actions of superantigens, such that responses can be induced without the maturation of the APC. Superantigens are not processed by the APC but rather directly engage MHC class II molecules on the APC and subsequently bind to the Vβ segments of the TCR. Indeed, it is known that DC are extremely efficient at capturing superantigens and triggering primary T-cell responses (Bhardwaj *et al.*, 1993), which may not be dependent on CD86-CD28 ligation (Damle *et al.*, 1993). It is, therefore, possible that the AOPP formed in this study may act as superantigens, and thus, induce the non-conventional activation of T cells.

Alternatively, it is possible that a small subset of DC may have been activated by AOPP. In this case, at low concentrations of DC the probability of a well containing sufficient, if any, of these highly activated DC would be very low, and therefore, not induce a response. However, at higher concentrations of DC, there would be an increased likelihood that a single well would contain these activated DC, and therefore, elicit a response.

If AOPP activated DC in a manner similar to LPS then a bimodal pattern of activation marker expression would be expected, indicating the presence of a separate population. However, no such pattern was observed, implying either that AOPP do not activate a small subset of DC or that the subset is a very small percentage of the population and not observed within the 5000 events collected.

It is unlikely that the low concentration of LPS within the AOPP samples would induce an effect only at high concentrations of DC/well. Indeed, since we know that the responses of DC to LPS are dose-dependent (Figure 3.18), low concentrations of LPS would be expected to induce a parallel shift in the dose-response curve; however, this was not observed.

Alternatively, it is possible that the relative lack of maturational effects of AOPP may be attributed to the presence of glucocorticoids (see Section 1.1.3.5.2.2), which may have precipitated with albumin during this isolation. However, this is more likely to be relevant for the use of human albumin rather than bovine. Furthermore, since it has been shown that DC can mature in the presence of 100μg/ml BSA, the potential presence of glucocorticoids is unlikely to be a major contributing factor.

The concentrations of AOPP applied to DC were 125μM and 1370μM. These concentrations can be compared to 29.4 ± 4.9μM AOPP found within the plasma of control patients and 76.7 ± 6.8μM in patients with advanced chronic renal failure (Witko-Sarsat *et al.*, 1998). However, little is known about the concentration of AOPP at sites of inflammation, which would be expected to be much greater than within the peripheral circulation.

Since it is apparent that extensive proteolysis had occurred at the highest levels of oxidation, it is likely that AOPP samples contained differing levels of proteins. It would, therefore, be interesting to investigate the effects of aggregated protein alone, which are thought to represent AOPP, on DC. The use of aggregated

proteins would also allow a more accurate determination of the relevant protein concentration added to control sample.

The lack of phenotypic effects of AOPP on DC may also reflect the relatively short timescale of their coculture. The coculture period of 24 hours was based on the fact that, when stimulated by LPS, DC can mature within this period of time. However, LPS is one of the most powerful immune stimuli known (Mims *et al.*, 1993), and may, therefore, act relatively rapidly compared to other stimuli.

Since AOPP would be expected to accumulate as a product of time, AOPP may provide a route for the conversion of a prolonged oxidative stress to a DC activation signal. If this is the case then AOPP may not necessarily activate DC as rapidly as other agents, such as bacterial products, which may signal the presence of an infectious organism, and thus the need for an immediate response.

In this study it has been necessary to use relatively low concentrations of proteins to avoid the effects of LPS contamination on DC. In future investigations it would be interesting to increase the concentration of AOPP applied to the DC, having removed non-protein contaminants, including LPS. It would also be interesting to investigate a variety of other proteins as possible sources of AOPP, since matrix proteins may also represent likely candidates for AOPP-type modification *in vivo*.

5.4 Conclusion

It has been demonstrated that AOPP can play a role in the regulation of T-cell responses by DC, and that they may act as a novel group of immunoregulatory proteins. To clarify the potential physiological and pathological roles of AOPP on DC, the interactions between AOPP and DC would need to be investigated further.

Furthermore, the observed phenomenon that AOPP act solely at high concentrations of DC may be physiologically relevant for a number of chronic inflammatory diseases. In a number of these, for example rheumatoid disease and atherosclerosis (see section 5.1.3), activated DC are found to accumulate in peripheral tissues. If sufficient numbers of DC accumulate over time, then AOPP may activate the surrounding T cells *via* them (the DC), thus perpetuating the chronic inflammation, and hence indicating a potential pathogenic role of AOPP.

Chapter 6

The Role of Oxidised Low-Density Lipoproteins in the Regulation of Dendritic cell Function

6.1 Introduction

The effects of oxidative stress are manifested in a wide variety of ways. Among these effects, peroxidation of membrane lipids is thought to be a secondary event and rarely directly mediated by oxidative stress (Halliwell & Gutteridge 1984). However, some of the effects of oxidative stress are known to be mediated through the oxidation of low-density lipoproteins.

Oxidised LDL (oxLDL) are most frequently discussed in terms of their role as pro-atherogenic agents. However, on further examination of inflammatory sites, it becomes apparent that there is a complex interplay between inflammation and the oxidation of LDL, such that oxLDL are not only pro-inflammatory but that pro-inflammatory sites also promote the oxidation of LDL (Pentikainen *et al.*, 2000). Indeed, the host response to infection and inflammation has been shown to increase the oxidation of LDL in three distinct *in vivo* models (Memon *et al.*, 2000). In addition, it should be noted that oxLDL satisfy all of the aforementioned criteria of likely "danger" signals, and thus constitute potential activating signals for DC.

6.1.1 Hypothesis

Following fenestration of an endothelium, the subsequent inflammatory site provides the ideal conditions that promote the oxidation of LDL. OxLDL may then interact with cells surrounding the site of tissue damage and may promote inflammatory responses *via* their binding to scavenger receptors. Indeed, it has been suggested that the expression of scavenger receptors by a wide variety of cells (including endothelial cells, aortic smooth muscle cells, neurones, and keratinocytes) may aid the regression of an inflammatory response. Furthermore, it has been concluded that DC play an important physiological role in the clearance of oxLDL (Zigg *et al.*, 2000). However, the experimental basis of this has yet to be explored.

Little is presently known about the role of lipid recognition in host defence mechanisms. However, murine peritoneal macrophages bind negatively charged

phospholipid vesicles preferentially, as opposed to neutral and positively charged vesicles. Since phagocytosis is a primitive immunological function, it has been hypothesised that recognition of anionic phospholipids may represent a fundamental mechanism for the identification of non-"self" or damaged-"self" (Ratner *et al.*, 1986).

The hypothesis explored in this chapter is that the potential interaction between oxLDL and DC may lead to the maturation of these cells, and hence to an increase in antigen presentation.

6.1.2 Determination of the oxidation status of LDL

Oxidation of LDL can be induced by a number of different cellular and cell-free systems. However, since the predominant mechanism that occurs *in vivo* has yet to be determined and the extent of the differences between *in vivo* and *in vitro* oxidised LDL is unclear, there is currently no universal protocol for the oxidation of LDL. One of the advantages of this variability among oxidised LDL preparations is that the field is not dependent entirely upon a single protocol, which might be easy to prepare and analyse but which may not yield physiological oxLDL. However, the lack of a universal protocol also increases the variability among oxLDL preparations that are studied *in vitro* and the potential variability in their biological functions.

As a consequence of the complexity of LDL particles, their progressive oxidation can be investigated by assessment of numerous parameters including total lipid peroxides, specific lipid peroxides, oxidised cholesterol, individual aldehydes, thiobarbituric acid-reactive substances (TBARS) and conjugated diene formation, as well as surface charge and fluidity. Oxidation is also associated with loss of reactive amino groups and fragmentation of apoprotein B (apo B).

Perhaps the simplest technique to monitor the early oxidation of LDL is to assess the formation of conjugated dienes, which have an absorbance peak at 234nm, as initially suggested by Esterbauer *et al.*, (1989). From such kinetic data it was

concluded that oxidation of LDL occurs in three consecutive phases. The initial (lag) phase is associated with the depletion of endogenous α-tocopherol followed by carotenes and minimal lipid peroxidation. Following consumption of these anti-oxidants, lipid peroxidation then rapidly ensues during the propagation phase. Subsequently, lipid peroxides decompose to form a variety of breakdown products during the final decomposition phase.

It can be seen that, in addition to native LDL, at least three sub-populations of oxidised LDL can be generated, defined by the endpoints of each consecutive phase. The preparations of oxidised LDL have subsequently been labelled as minimally, moderately and maximally oxidised LDL (Rice-Evans *et al.*, 1996). It should be noted, however, that such definitions are not universal. According to the proposed time course, minimal oxidation can be defined as an increase in A_{234} of 0.2 (100μg LDL protein/ml) and moderate oxidation occurs at the plateau of diene formation. Moderately oxLDL can then undergo further oxidation during the remaining 24 hours to achieve a state of maximal oxidation.

Although the initial stages of oxidation can be monitored readily, based on their formation of dienes, latter stages of oxidation are masked by the production of decomposition products with absorption peaks close to that of dienes. The formation of decomposition products is thought to account for a second increase in A_{234} observed during the oxidation of LDL (Esterbauer *et al.*, 1992). As a result of the second increase in A_{234}, to confirm the occurrence of maximal oxidation of LDL, it is necessary to assess a number of other biochemical changes that occur during extensive oxidation. Indeed, no single biochemical analysis yields sufficient data to follow the entire oxidation process.

The different phases of oxidation can readily be confirmed by assaying levels of thiobarbituric acid-reactive substances (TBARS), lipid peroxides, and surface charge, as indicated by relative electrophoretic mobility. All assays should give similar time profiles up to the stage of maximal lipid peroxide formation, from which point they follow different kinetics (Puhl *et al.*, 1994).

Most researchers characterise the oxidised status of LDL by at least two independent measurements (Puhl *et al.*, 1994). Commonly used determinants are TBARS and electrophoretic mobility. However, in view of the reported variability among TBARS assay protocols, it has been concluded that additional determinants, such as lipid hydroperoxide content, are also beneficial in defining oxidised LDL species (Favier *et al.*, 1995).

Given that different LDL oxidation fractions have been shown to exert distinct cellular effects, it will be necessary to examine the effects of LDL oxidised to each of the predefined stages on DC. For example, minimally modified LDL have attracted a great deal of attention due to the seminal work of Cushing *et al.*, (1990), and have since been shown to act on human endothelial cells and smooth muscle cells to stimulate their production of macrophage chemotactic protein (MCP)-1. In contrast, moderately oxidised LDL is reported to induce apoptosis of human vascular smooth muscle cells (Siow *et al.*, 1999).

An additional distinct (and potentially important feature) of minimally oxidised LDL is that they bind apoprotein receptors and not scavenger receptors; but despite the ability of native LDL and minimally oxidised LDL to bind the same receptors, they are found to elicit completely different cellular effects (Berling *et al.*, 1990).

Currently, there is a paucity of data concerning the cellular effects of maximally oxidised LDL and there is a complete lack of data regarding the interactions of any LDL particles with DC. Therefore, studies of these interactions form the basis of this chapter, with the aim not only of documenting the effects but also of examining the hypothesis that one or more of the forms of oxidised LDL may play a role in regulating or modulating DC, and thus constitute a potential component of the "danger" signal.

6.2 Results

6.2.1 Characterisation of native and oxidised LDL

The purification of LDL is a multi-step process that aims to exclude the majority of protein and non-protein components of serum, whilst leaving the single lipoprotein fraction with a density of between 1.019 and 1.063g/ml, which represents LDL. The removal of the contaminating components of serum allows accurate quantification of the isolated LDL, which is usually measured in terms of grams LDL protein per ml. Figure 6.1 shows that purified LDL samples contained a single protein component, which had a molecular weight similar to the molecular weight of apoprotein B_{100}. The LDL samples were, therefore, assumed to contain apoprotein B_{100}, and no other significant protein contaminants

Figure 6.2 shows a representative example of the increase in A_{234} that occurred during the oxidation of LDL (at a concentration of 100μg LDL protein/ml in low phosphate buffer) by the addition of 5μM Cu^{2+}. It can also be seen from Figure 6.2 that the predefined states of minimal and moderate oxidation can be detected during the time course of LDL oxidation. Maximal oxidation is assumed to have occurred following a total of 24 hours oxidation.

It was subsequently shown that, when the native LDL were oxidised to the predefined states of minimal, moderate and maximal oxidation, the samples of LDL were found to contain reproducible levels of TBARS and lipid hydroperoxides, and to have similar charges (as determined by their migration in agarose gels).

Figure 6.3 shows that native LDL contained low levels of TBARS, which were below the limit of detection. Minimal oxidation was associated with a small but not statistically significant increase in the formation of TBARS. The subsequent oxidation to the stage of moderately oxidised LDL was associated with a significant increase in the formation of TBARS relative to both the native and minimally oxidised samples. The content of TBARS within the LDL samples did

not change significantly during the oxidation of moderately to maximally oxLDL. Furthermore it can be seen in Figure 6.3 that the levels of TBARS within maximally oxLDL remained significantly greater than the levels obtained within corresponding samples of native and minimally oxidised LDL.

The lipid hydroperoxide content of native and oxidised samples of LDL are documented in Figure 6.4. From Figure 6.4 it can be seen that native LDL contained low levels of lipid hydroperoxides. Minimal oxidation of LDL was not associated with a significant increase in the formation of lipid hydroperoxides. The oxidation of LDL from a state of minimal oxidation to one of moderately oxidised resulted in a highly significant increase in the formation of lipid hydroperoxides. The final oxidation of moderately oxidised LDL to a state of maximal oxidation resulted in a highly significant decrease in the content of lipid hydroperoxides within the LDL samples. The decrease is consistent with the reported decomposition of lipid hydroperoxides that is known to occur following moderate oxidation of LDL (Siow *et al.*, 1999). The content of lipid hydroperoxides within the maximally oxidised LDL was neither significantly different from those detected within the native nor minimally oxidised samples.

Figure 6.5 shows a graph of the mean migratory capacity of native and oxidised LDL following agarose gel electrophoresis, as an indication of the relative surface charges of the LDL samples. It can be seen from Figure 6.5 that native and minimally oxidised LDL migrate to similar extents following agarose gel electrophoresis. The relative migration of the LDL particles then increased as the LDL were oxidised from minimally to moderately oxidised states. The relative migration of moderately oxidised LDL was significantly greater than that observed for native and minimally oxidised LDL. The oxidation of moderately oxidised LDL to maximally oxidised LDL was associated with a highly significant increase in the relative migration of the LDL particles. It can also be seen from Figure 6.5 that the migration of maximally oxidised LDL was significantly greater than that observed for both native LDL and minimally oxidised LDL. A representative example of the agarose gel electrophoresis is given in Figure 6.6. From Figure 6.6 the isolation of a single lipoprotein subclass

is also confirmed, since a single lipid band can be observed following agarose gel electrophoresis.

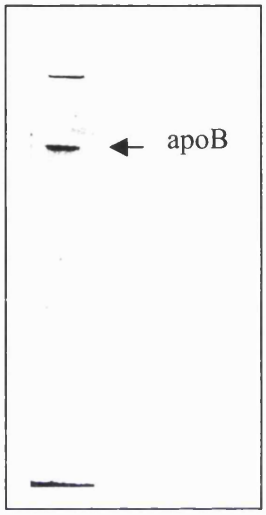


Figure 6.1. SDS-PAGE of native LDL demonstrating a single band corresponding to apoprotein B_{100}. Native LDL were freshly prepared and stored in a low phosphate buffer containing 100μM EDTA. Gel was stained with coomasie blue.

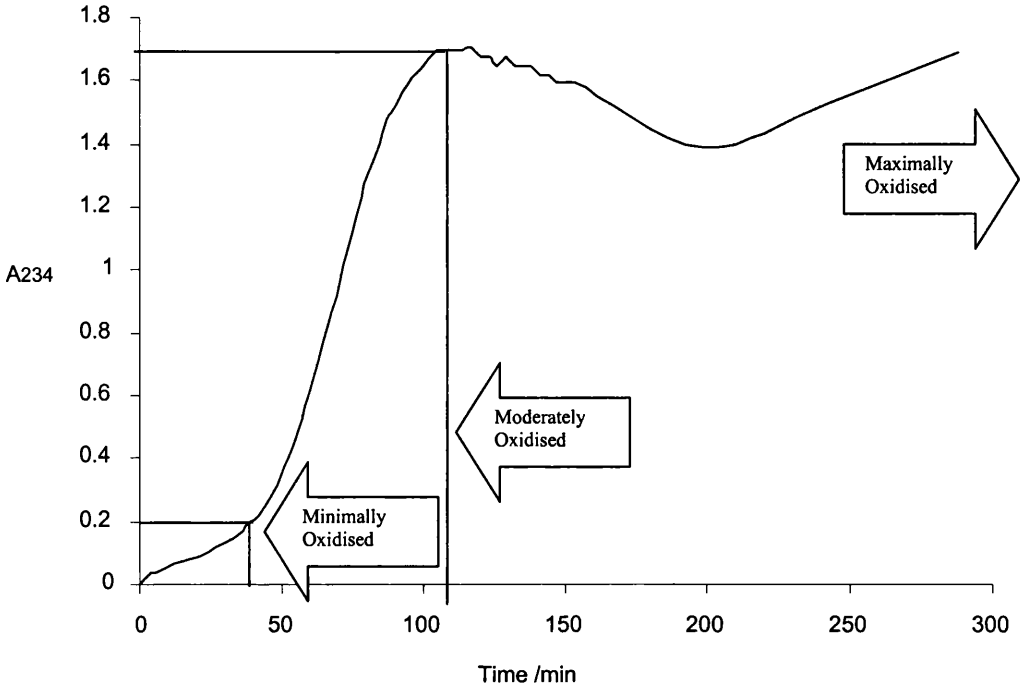


Figure 6.2. Time course of conjugated diene formation during the oxidation of LDL. Native LDL were freshly prepared and stored in a low phosphate buffer. LDL samples were then diluted to 100μg protein/ml and a final concentration of 5μM Cu^{2+} added. The formation of conjugated dienes was monitored spectrophotometrically at 37°C and is given as increase in absorbance at 234nm. One representative example is shown.

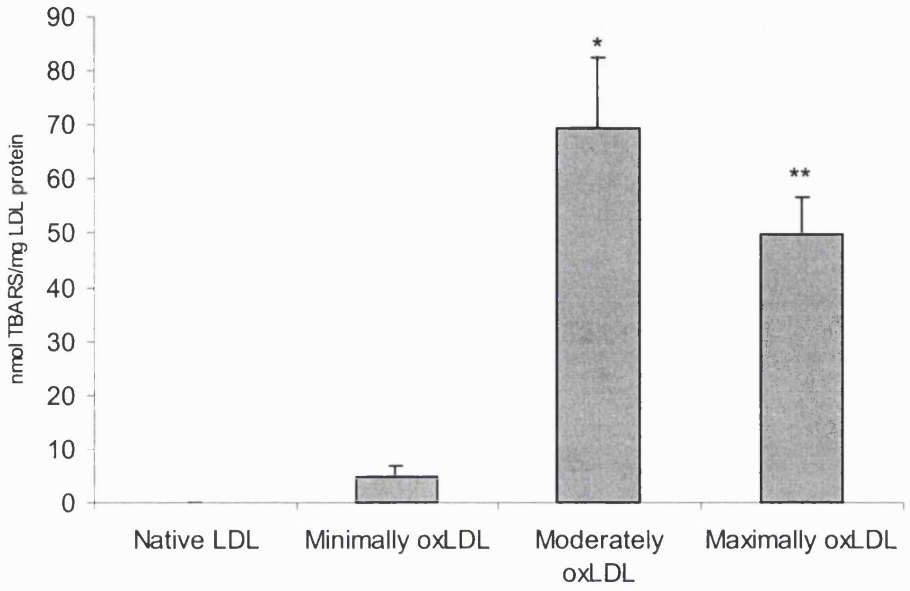


Figure 6.3. Time course of thiobarbituric acid reactive substances (TBARS) formation during the oxidation of LDL. Native LDL were freshly prepared and stored in a low phosphate buffer. LDL samples were then diluted to 100μg protein/ml and a final concentration of 5μM Cu^{2+} added. Samples were prevented from further oxidation, by the addition of 100μM EDTA, at the time points corresponding to the predefined states of oxidation given in Figure 6.2. TBARS were then assayed spectrophotometrically as detailed in Materials and Methods. Mean ± SEM of four independent experiments are shown. Statistical analysis is given relative to both native LDL and minimally oxidised LDL. *, $p<0.05$; **, $p<0.01$.

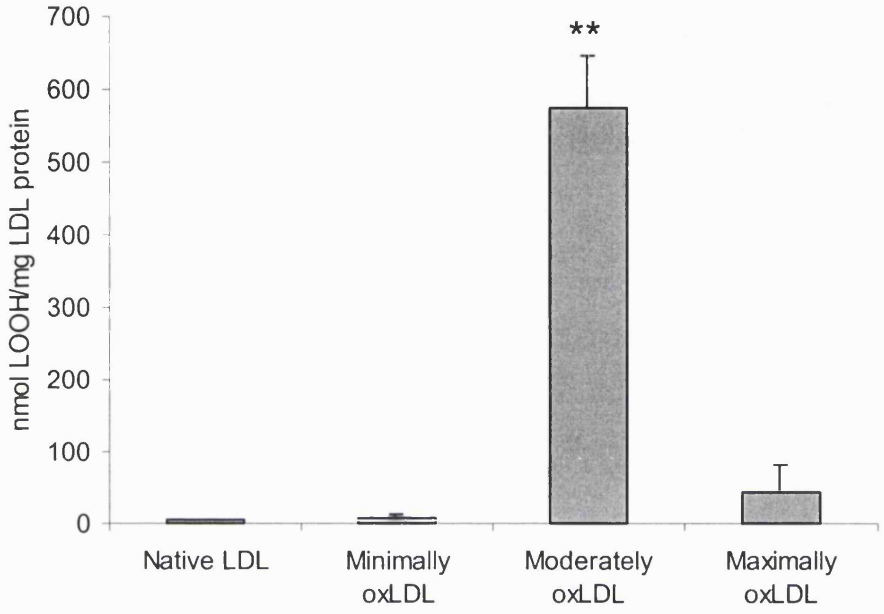


Figure 6.4. Time course of lipid hydroperoxide formation during the oxidation of LDL. Native LDL were freshly prepared and stored in a low phosphate buffer. LDL samples were then diluted to 100μg protein/ml and a final concentration of 5μM Cu^{2+} added. Samples were prevented from further oxidation, by the addition of 100μM EDTA, at the time points corresponding to the predefined states of oxidation given in Figure 6.2. Lipid hydroperoxides were then assayed spectrophotometrically, as detailed in Materials and Methods. Mean ± SEM of four independent experiments are shown. Statistical analysis is given relative to all other samples. **, $p<0.01$.

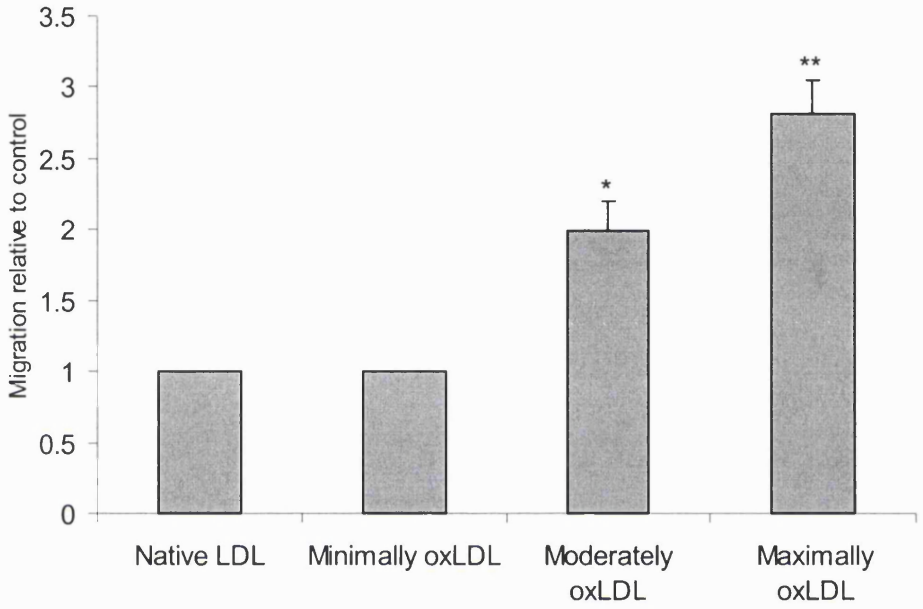


Figure 6.5. Time course of migration of LDL through agarose gels, as an indication of their charge, following oxidation. Native LDL were freshly prepared and stored in a low phosphate buffer. LDL samples were then diluted to 100μg protein/ml and a final concentration of 5μM Cu^{2+} added. Samples were prevented from further oxidation, by the addition of 100μM EDTA, at the time points corresponding to the predefined states of oxidation given in Figure 6.2. Migration is given relative to native LDL. Mean ± SEM of four independent experiments are shown. Statistical analysis is given relative to both native LDL and minimally oxidised LDL. *, $p<0.05$; **, $p<0.01$.

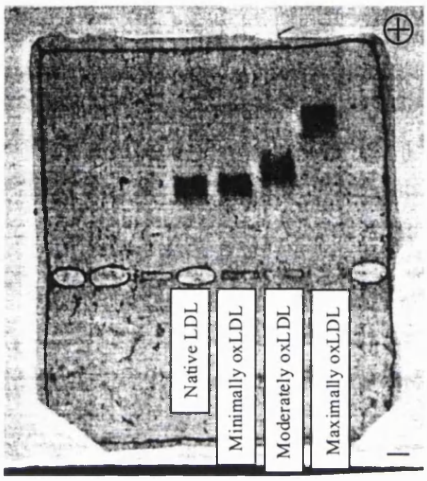


Figure 6.6. Agarose gel electrophoresis illustrating the migration of LDL. Native LDL were freshly prepared and stored in a low phosphate buffer. LDL samples were then diluted to 100μg protein/ml and a final concentration of 5μM Cu^{2+} added. Samples were prevented from further oxidation, by the addition of 100μM EDTA, at the time points corresponding to the predefined states of oxidation given in Figure 6.2. Gel was stained with the lipid dye Sudan Black.

6.2.2 Protocol for functional assays

Chapter 3 details an improved protocol to examine the potential maturational effects of various test stimuli on pure populations of immature DC. However, despite the fact that the suggested protocol was deemed ideal for analysing the effects of various stimuli on DC, the protocol was further modified to investigate the effects of LDL on DC. The reason for the further modification was to eliminate the possibility of any interactions between oxidised LDL and other lipid components of serum, thus ensuring that the direct effects of oxidised LDL were investigated on DC.

The modified protocol used for the investigations within this chapter involved the culturing of monocytes for three days in GM-CSF/IL-4. Following this culture, the non-adherent cells were purified and replated for a further three days in fresh media and cytokines, according to the standard protocol derived in Chapter 3. The resultant immature DC were then washed in RPMI-1640 alone and then re-suspended in LDL samples that had previously been dialysed in RPMI-1640. The starting concentration of LDL for the studies was 10μg LDL protein/ml, since this concentration of LDL has been shown to promote various effects on numerous cell types. However, before the effects of LDL could be examined on DC, it was necessary to verify that DC cultured in the absence of serum (for 24 hours) retained an immature DC phenotype and were viable.

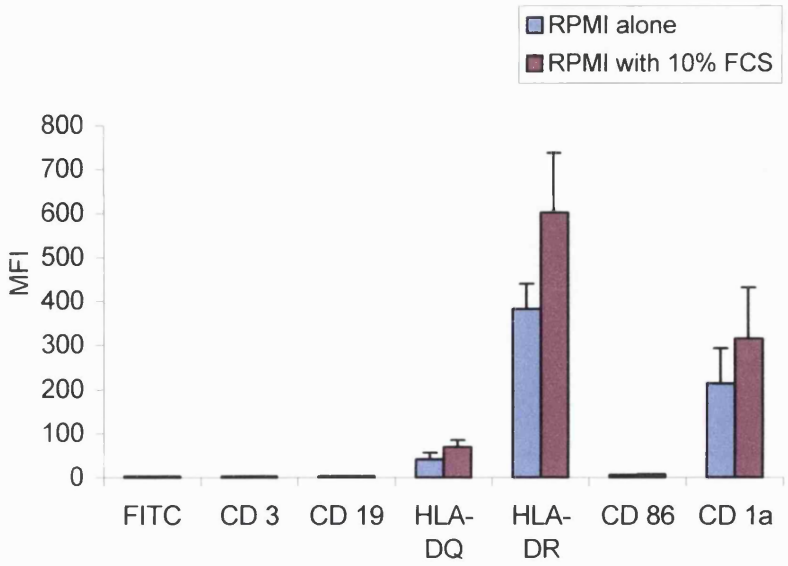
Figure 6.7 shows that culturing immature DC for 24 hours in the absence of serum did not significantly alter the phenotype of the resultant DC, when compared to DC cultured in the presence of serum. It can also be seen from Figure 6.7 that in the absence of serum for 24 hours DC retained a characteristic immature phenotype. A representative example of the phenotypic comparison of DC cultured in the presence and absence of serum is also given in Figure 6.7.

The ability of DC to reduce MTT following a period of 24 hours culture in the absence of serum was then examined to investigate whether or not the absence of serum reduced the cellular activity of immature DC. Figure 6.8 shows that

culturing DC without serum for 24 hours was not associated with a statistically significant reduction in the activity of immature DC, as assessed by their ability to reduce MTT.

Figure 6.8 also illustrates the effects of the native LDL and maximally oxidised LDL on the cellular activity of DC, as a assessed by their ability to reduce MTT. At concentrations of 10μg/ml and 100μg/ml, native LDL was found to increase the cellular activity of DC to similar levels reported for DC in the presence of serum. Since native LDL was alone unable to promote the reduction of MTT, it is possible that the increased cellular activity may reflect activation of the DC by native LDL or restoration of the functional deficit induced by the absence of serum.

From Figure 6.8 it can further be seen that the effects of maximally oxidised LDL on the ability of DC to reduce MTT are highly variably. Indeed, it can be seen from Figure 6.8 that 10μg/ml of maximally oxidised LDL significantly increases the ability of DC to reduce MTT, whilst this increase was not observed at 100μg/ml of maximally oxidised LDL.



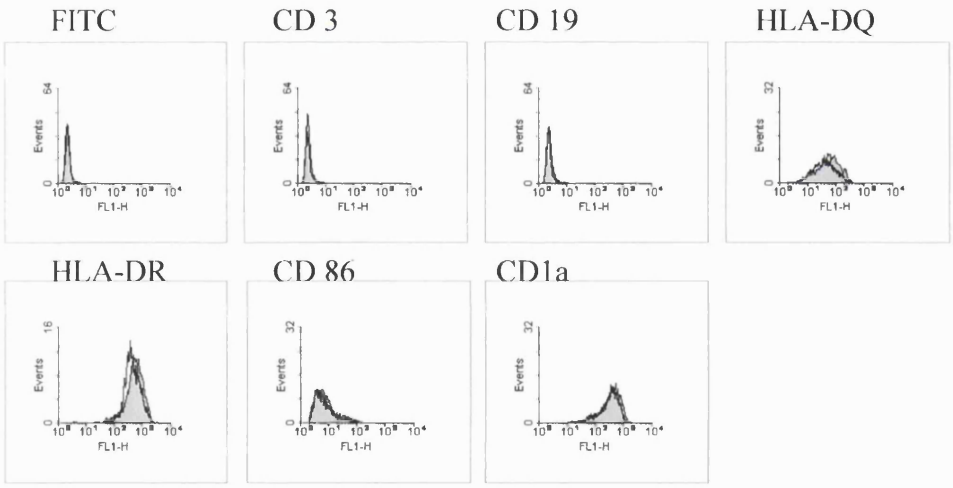


Figure 6.7. Phenotype of DC incubated in the absence of serum for 24 hours. DC were prepared according to the method of day 4 purification. Following 6 days of total culture, DC were washed twice in RPMI-1640 and resuspended at $5x10^5$ DC/ml in the given media for 24 hours. Surface expression of the given markers was then assessed by flow cytometry, as detailed in Materials and Methods. Mean values ± SEM are given from 3 independent experiments. Lower histograms give representative examples. Filled profiles give RPMI-1640 with 10% FCS, overlays give RPMI-1640 alone. Statistical analysis was performed but no statistically significant differences were observed.

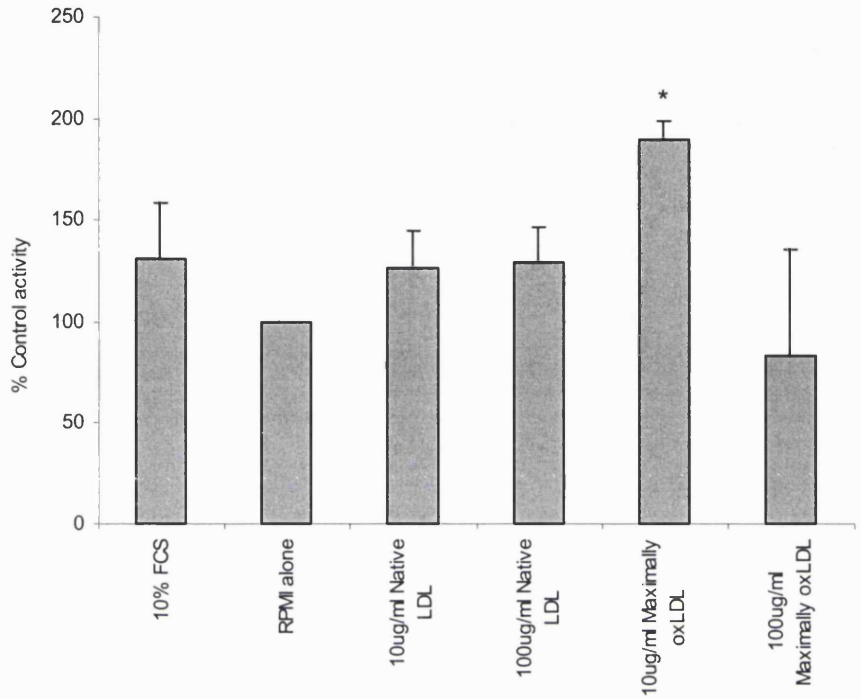


Figure 6.8. Cellular activity of DC following culture for 24 hours in various media. DC were prepared according to the method of day 4 purification. Following 6 days of total culture DC were washed twice in RPMI-1640 and resuspended at $5x10^5$ DC/ml in the given media for 24 hours. MTT was added for the final 4 hours of the culture and cellular activity calculated as detailed in Materials and Methods. Samples were run in triplicate. Mean ± SD of 3-6 independent experiments are shown for each data point. Statistical analysis is given relative to RPMI-1640 alone. *, $p<0.05$.

6.2.3 Effects of LDL on the ability of DC to induce the proliferation of T cells

Following 24 hours of culture in the presence or absence of LDL samples, DC were washed twice in complete medium before their functional responses were assayed by examining their ability to stimulate the proliferation of resting T cells, according to the standard protocol presented in Materials and Methods. The functional experiments presented were, therefore, all conducted in the same media and only differed in the prior treatment of DC for 24 hours in the presence of different LDL samples, as detailed below.

It can be seen from Figure 6.9 that the ability of DC to stimulate the proliferation of resting T cells, as determined by ^{3}[H]thymidine incorporation, increases in a manner that is dependent upon the number of DC / well. It can also be seen from Figure 6.9 that the capacity of DC to induce the proliferation of T cells varies depending on the media in which they are incubated during the 24 hour period prior to conducting the functional assays.

Figure 6.9 shows that in the absence of serum (RPMI-1640 alone), DC have a reduced capacity to induce the proliferation of T cells, when compared to the responses induced by control samples in the presence of serum. The responses induced in the absence of serum were consistently less than those observed in the presence of serum and significantly different at the two data points indicated in Figure 6.9, suggesting that this is not a chance result.

It can also be seen in Figure 6.9 that the addition of 10ng/ml LPS to DC incubated in RPMI-1640 alone can induce a significant increase in the functional responses of DC, as determined by the increased ^{3}[H]thymidine incorporation and thus T-cell proliferation. The dose-dependent nature of the ability of increasing numbers of DC to induce the proliferation of resting T cells and the increased responses observed upon addition of 10ng/ml LPS are also reflected in Figures 6.10-6.12.

Furthermore, it can be seen from Figure 6.9 that the addition of 10ng/ml LPS to DC incubated in the presence of 10% v/v foetal calf serum (FCS) enhanced their capacity to induce the proliferation of resting T cells. The responses observed in the presence of 10% v/v foetal calf serum and 10ng/ml LPS were greater than those found in the presence of 10ng/ml LPS alone. The increase may be attributed to the greater supply of nutrients or the effects of LPS binding protein within the serum.

Upon supplementation with native LDL, the functional responses of DC were found to lie at an intermediate value between those elicited by DC incubated in the presence of RPMI alone and the responses observed in the presence of 10% foetal calf serum. From the intermediate response it can be concluded that native LDL do not inhibit the function of DC. More importantly, Figure 6.9 shows that if the samples of LDL contained LPS, this is at a concentration of less than 10ng/ml, since this concentration of LPS is known to induce significant increases in the functional capacity of DC. Furthermore, it can be seen from Figure 6.9 that any stimulatory effects of oxidised LDL could, indeed, be observed above the responses to native LDL alone.

Figure 6.10 details the effects of minimally oxidised LDL on the ability of DC to induce the proliferation of resting T cells. It can be seen (Figure 6.10) that neither 10μg/ml nor 100μg/ml minimally oxidised LDL induced significant increases in the ability of DC to promote the proliferation of T cells. Following the incubation of DC for 24 hours in the presence of 10μg/ml minimally oxidised LDL, their capacity to stimulate the proliferation of T cells mirrored the responses observed following the incubation of DC in the presence of RPMI-1640 alone. It is, therefore, apparent either that minimally oxidised LDL do not influence the function of DC or that 10μg/ml minimally oxidised LDL is insufficient to observe the potential effects. However, 100μg/ml minimally oxidised LDL induced a slight, but not statistically significant, reduction in the ability of DC to promote the proliferation of T cells, suggesting that concentrations of greater than 100μg/ml minimally oxidised LDL may not increase the stimulatory capacity of DC.

Following 24 hours of culture in the presence of 10μg/ml moderately oxidised LDL (Figure 6.11), the ability of DC to induce the proliferation of T cells was similar to the responses observed in the presence of an equivalent concentration of native LDL. At concentrations of 100μg/ml, moderately oxidised LDL was found not to significantly alter the functional capacity of the DC. However, as seen with 100μg/ml minimally oxidised LDL, 100μg/ml moderately oxidised LDL may have a slight inhibitory effect on the ability of DC to induce the proliferation of T cells, as indicated by the reduced ^{3}[H]thymidine incorporation.

Figure 6.12 shows that 10μg/ml maximally oxidised LDL was able to increase the functional capacity of DC. The increased responses observed were significantly greater, at two concentrations of DC/well, than those induced by an equivalent concentration of native LDL. It is, therefore, suggest that the increases do not represent a chance result and that maximally oxidised LDL may induce the functional maturation of DC. The fact that the increases in the ability of DC to induce the proliferation of T cells were less for 10μg/ml maximally oxidised LDL than 10ng/ml LPS may simply reflect the fact that sub-maximal responses were elicited under the different conditions.

Following the incubation of DC with 100μg/ml maximally oxLDL the functional capacity of DC was found to decrease relative to DC incubated in RPMI alone. The reduction in functional response was statistically significant at two concentration of DC/well, suggesting 100μg/ml maximally oxidised LDL may induce an inhibitory effect on the capacity of DC to stimulate the proliferation of T cells.

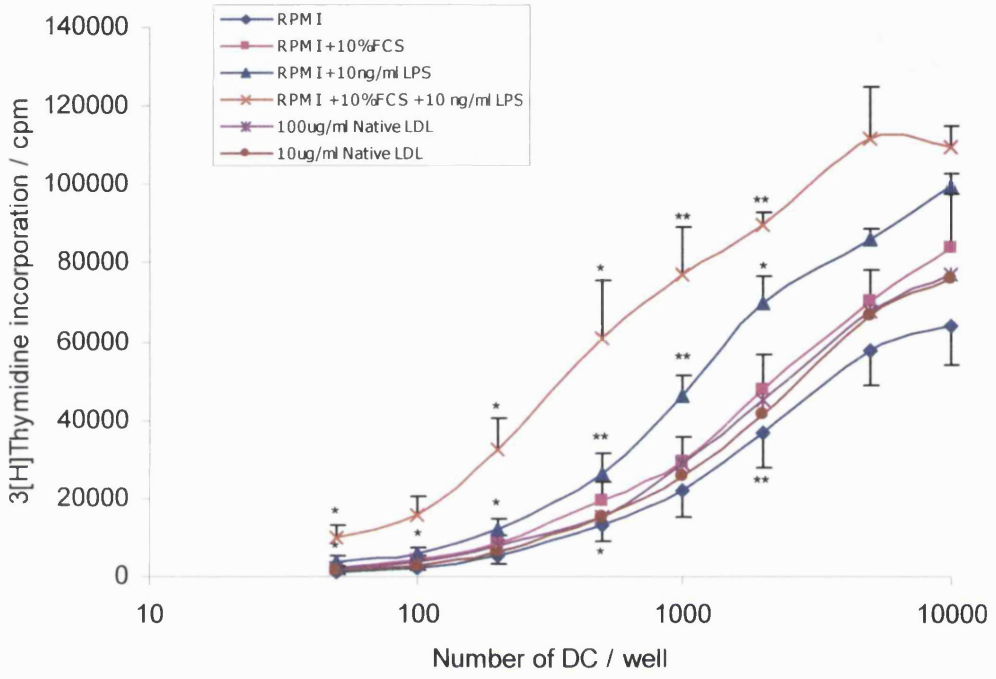


Figure 6.9. Effects of native LDL on the ability of DC to induce the proliferation of T cells. DC were prepared according to the method of day 4 purification. Following 6 days of total culture DC were washed twice in RPMI-1640 and resuspended at $5x10^5$ DC/ml in the given media for 24 hours. DC were subsequently harvested, washed twice in complete medium and added to 10^5 purified autologous T cells for a further 48 hours, according to the oxidative mitogenesis assay. Functional responses were assessed by ^{3}[H]Thymidine incorporation during a further 16 hours of incubation. Samples were run in triplicate. Mean ± SEM of 4-5 independent experiments are shown. Statistical analysis is given relative to RPMI-1640 alone. *, $p<0.05$; **, $p<0.01$.

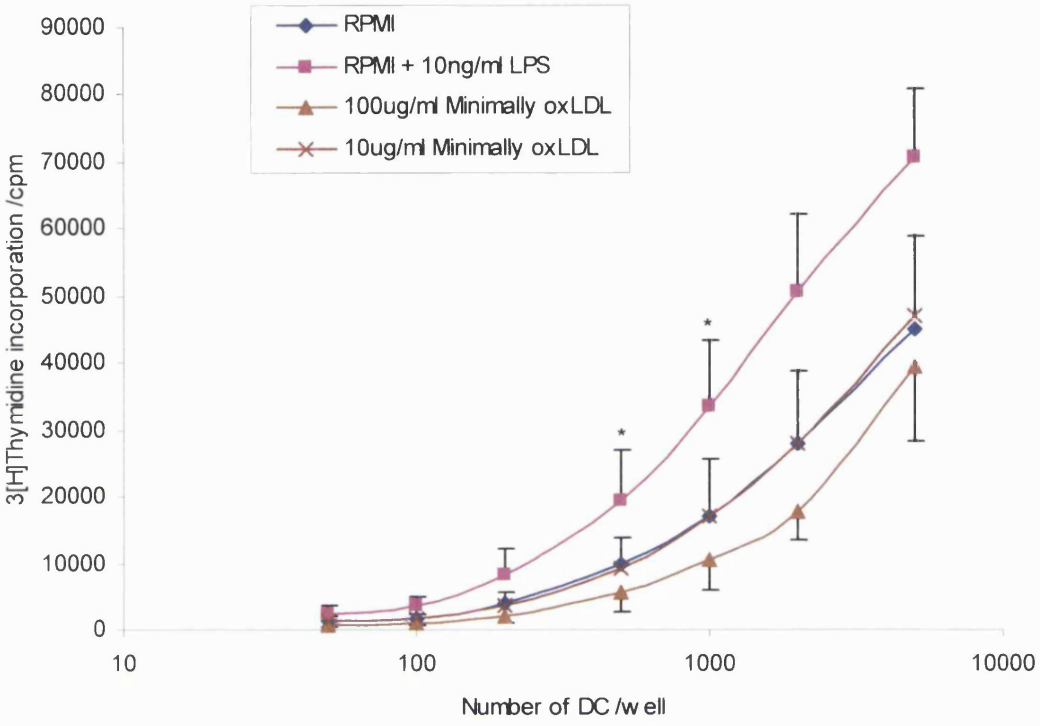


Figure 6.10. Effects of minimally oxidised LDL on the ability of DC to induce the proliferation of T cells. DC were prepared according to the method of day 4 purification. Following 6 days of total culture DC were washed twice in RPMI-1640 and resuspended at $5x10^5$ DC/ml in the given media for 24 hours. DC were subsequently harvested, washed twice in complete medium and added to 10^5 purified autologous T cells for a further 48 hours, according to the oxidative mitogenesis assay. Functional responses were assessed by ^{3}[H]Thymidine incorporation during a further 16 hours of incubation. Samples were run in triplicate. Mean ± SEM of 3 independent experiments are shown. Statistical analysis is given relative to RPMI-1640 alone. *, $p<0.05$.

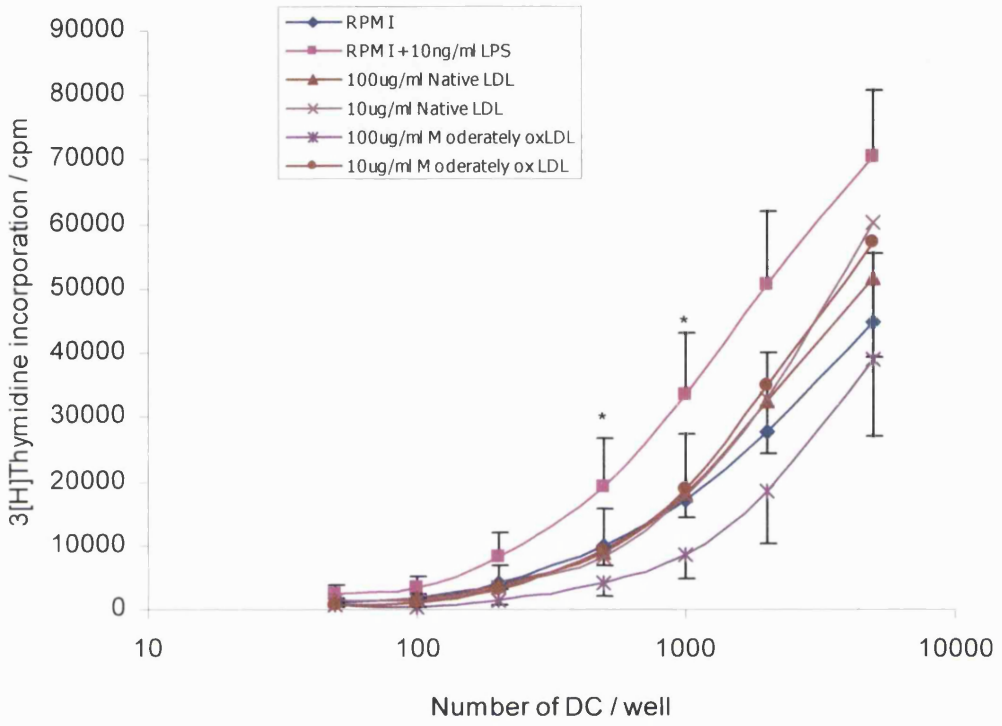
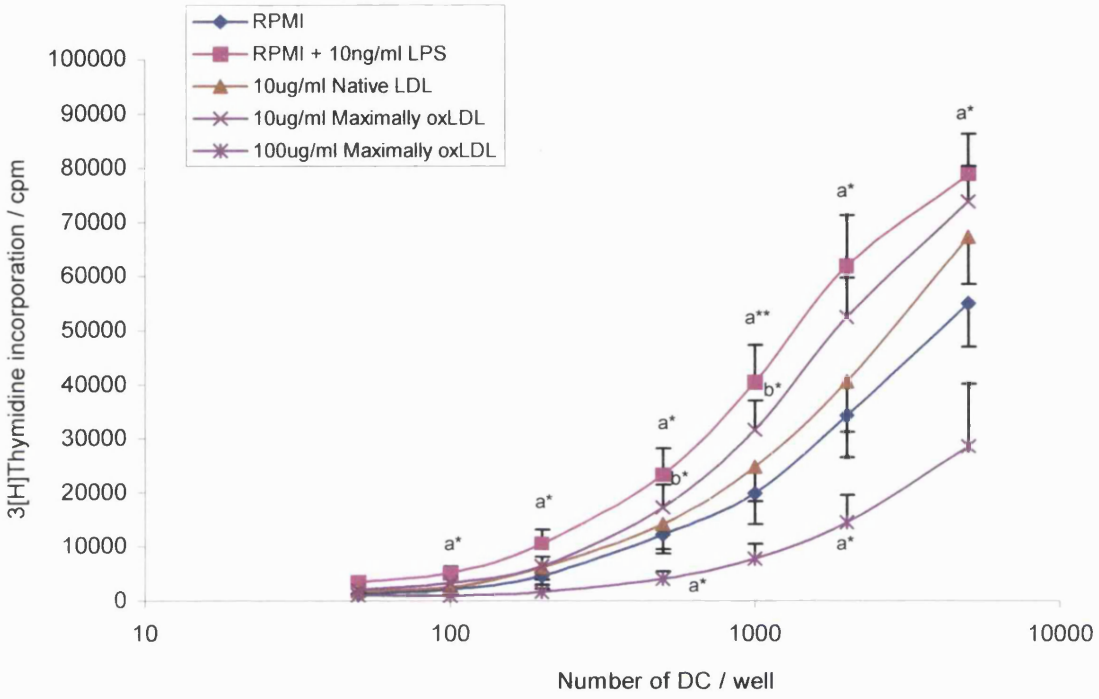


Figure 6.11. Effects of moderately oxidised LDL on the ability of DC to induce the proliferation of T cells. DC were prepared according to the method of day 4 purification. Following 6 days of total culture DC were washed twice in RPMI-1640 and resuspended at $5x10^5$ DC/ml in the given media for 24 hours. DC were subsequently harvested, washed twice in complete medium and added to 10^5 purified autologous T cells for a further 48 hours, according to the oxidative mitogenesis assay. Functional responses were assessed by ^{3}[H]Thymidine incorporation during a further 16 hours of incubation. Samples were run in triplicate. Mean ± SEM of 3 independent experiments are shown. Statistical analysis is given relative to RPMI-1640 alone. *, $p<0.05$.



a, relative to RPMI alone
b, relative to 10μg/ml native LDL
*, $p<0.05$
**, $p<0.01$

Figure 6.12. Effects of maximally oxidised LDL on the ability of DC to induce the proliferation of T cells. DC were prepared according to the method of day 4 purification. Following 6 days of total culture DC were washed twice in RPMI-1640 and resuspended at $5x10^5$ DC/ml in the given media for 24 hours. DC were subsequently harvested, washed twice in complete medium and added to 10^5 purified autologous T cells for a further 48 hours, according to the oxidative mitogenesis assay. Functional responses were assessed by ^{3}[H]Thymidine incorporation during a further 16 hours of incubation. Samples were run in triplicate. Mean ± SEM of 5-6 independent experiments are shown.

6.2.4 Phenotypic effects of maximally oxidised LDL on DC

It can be seen from section 6.2.3 that the most significant functional effects of oxidised LDL on DC are likely to be mediated through the action of maximally oxidised LDL. Furthermore, it can be seen that at a concentration of 10μg/ml, maximally oxidised LDL induce functional changes in DC that are consistent with the known effects of DC maturation. To investigate further the potential maturational effects of 10μg/ml maximally oxidised LDL on DC, phenotypic analysis of DC was conducted.

From Figure 6.13 it can be seen that maximally oxidised LDL induce significant increases in the surface expression of HLA-DR, and the co-stimulatory molecules CD40, CD86 on DC, when compared to DC incubated in an equivalent concentration of native LDL and medium containing RPMI alone. The surface expression of HLA-DQ was unaltered by the incubation of DC with 10μg/ml maximally oxidised LDL. In addition, no significant changes were observed in the expression of CD1a nor CD14, indicating that the cells did not dedifferentiate in the presence of maximally oxidised LDL.

Despite the clear observation that the phenotypic maturational effects of 10μg/ml maximally oxidised LDL were relatively small when compared to those induced by 10ng/ml LPS, Figure 6.13 indicates that, within a period of 24hours, 10μg/ml maximally oxidised LDL can induce a phenotypic maturation effect on DC.

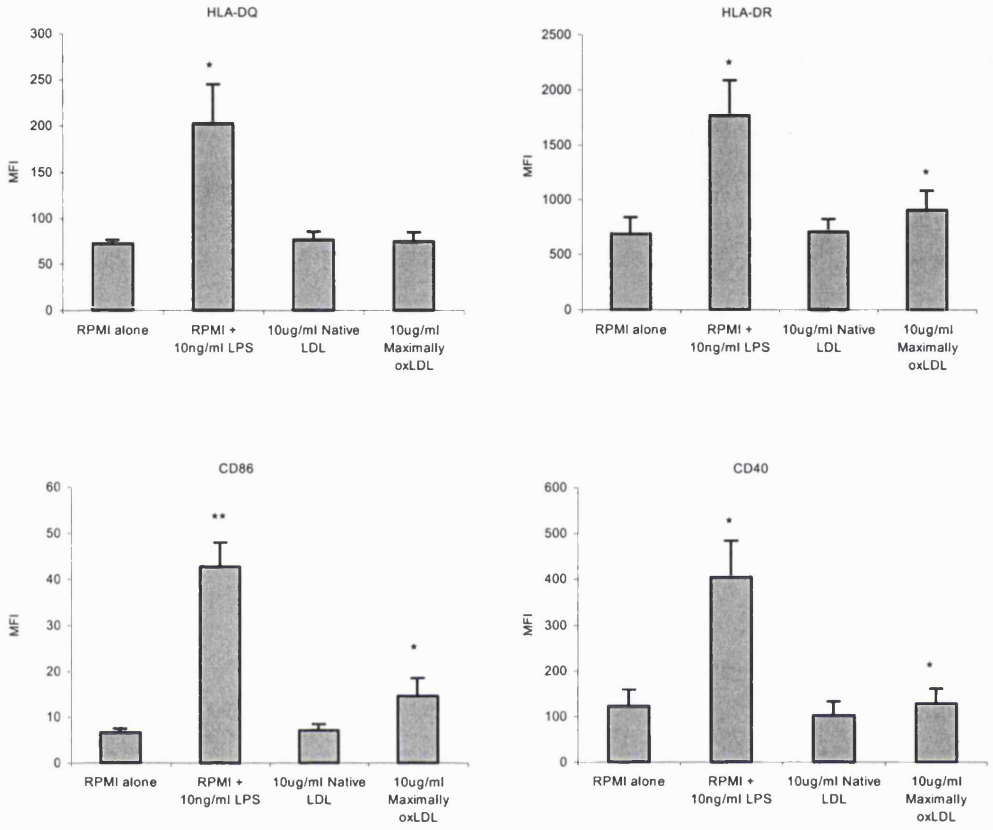


Figure 6.13. Effects of maximally oxidised LDL on the phenotype of DC. DC were prepared according to the method of day 4 purification. Following 6 days of total culture DC were washed twice in RPMI-1640 and resuspended at $5x10^5$ DC/ml in the given media for 24 hours. DC were subsequently harvested and the surface expression of the given markers assessed by flow cytometry, as detailed in Materials and Methods. Mean values + SEM are given from 3-4 independent experiments. Statistical analysis is given relative to both RPMI-1640 alone and 10μg/ml native LDL. *, $p<0.05$; **, $p<0.01$.

6.2.5 Effect of oxidised LDL on the apoptosis of DC

The functional data presented in Figure 6.13 suggests that high concentrations of maximally oxLDL may inhibit the ability of DC to induce the proliferation of resting T cells. However, an alternative hypothesis was that maximally oxidised LDL may have reduced the number of viable DC present within these proliferation assays. If the increased rate of cell death were a consequence of necrosis, then the DC would not exclude trypan blue and would not have been considered as viable DC before being plated with the T cells for the functional assay, so this would not account for the decrease in functional response.

However, if DC had been induced to undergoing apoptosis, as has been reported for numerous other cells types following incubation with oxidised LDL (see section 1.4.5), then some of the cells may still have been able to exclude trypan blue and would have been included within the functional assays. The apoptotic cells would then finally die during the functional assays, resulting in a reduced level of T-cell proliferation. To investigate the hypothesis that high concentrations of maximally oxidised LDL can induce apoptosis of DC, following 24 hours culture in the presence of the LDL samples previously investigated, DC were stained with annexin V and propidium iodide and analysed for their percentage expression by flow cytometry, as detailed in Materials and Methods. Figure 6.14 shows a representative examples of the data obtained, whilst the differences between culture conditions can, perhaps, be interpreted most readily by analysis of Figure 6.15, which shows the mean of three such experiments.

Figures 6.14 and 6.15 illustrate that, within the gated DC population, a very low percentage of the cells died by necrosis (annexin$^-$, P.I.$^+$), following incubation for 24 hours in any of the conditions given. It could also be suggested that the removal of serum from the culture medium increased the percentage of DC that underwent apoptosis (annexin$^+$). However, the increase was not statistically significant. Supplementation of RPMI-1640 with native LDL results in similar

cell survival profiles as those found when DC were incubated for 24 hours in RPMI-1640 alone.

Minimally oxidised LDL and moderately oxidised LDL did not induce any significant changes in the proportion of DC that undergoing apoptosis. It can be seen (Figures 6.14 and 6.15) that at 10μg/ml and 100μg/ml minimally oxidised LDL induced similar levels of apoptosis in DC, this was also observed for moderately oxidised LDL. Furthermore, it could be suggested that with increasing levels of oxidation, LDL induced a greater proportion of DC to enter apoptosis. However, the only statistically significant increase in the percentage of cells entering late apoptosis (annexin$^+$, P.I.$^+$) occurred when DC were incubated with 10μg/ml and 100μg/ml maximally oxidised LDL $p<0.05$ and $p<0.01$, respectively.

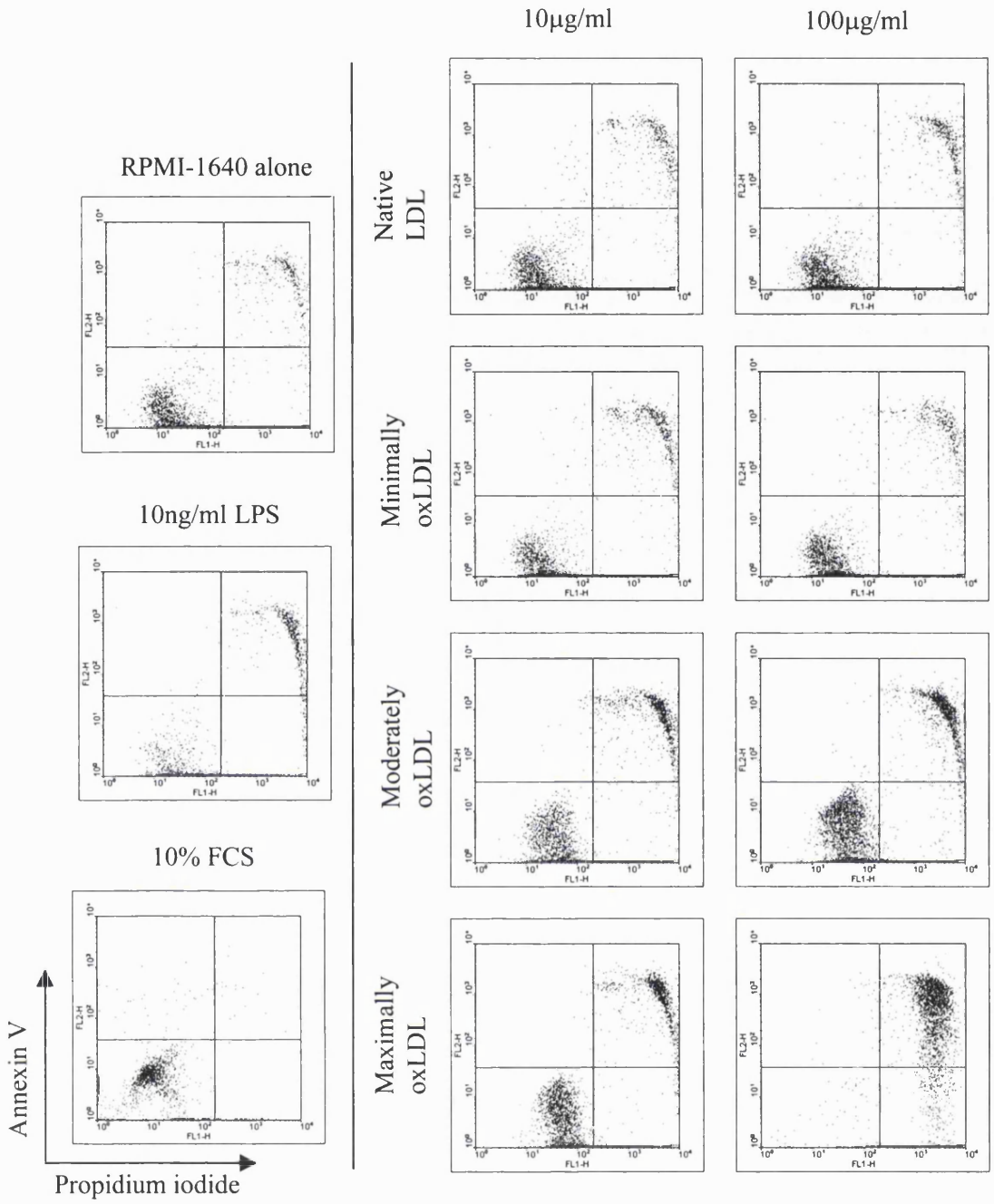


Figure 6.14. Effects of LDL on the viability of DC. DC were prepared according to the method of day 4 purification. Following 6 days of total culture DC were washed twice in RPMI-1640 and resuspended at $5x10^5$ DC/ml in the given media for 24 hours. DC were subsequently harvested and stained with Annexin V and propidium iodide, as detailed in Materials and Methods. One representative of three experiments is shown, except for the 10% FCS sample, which is representative of two assays.

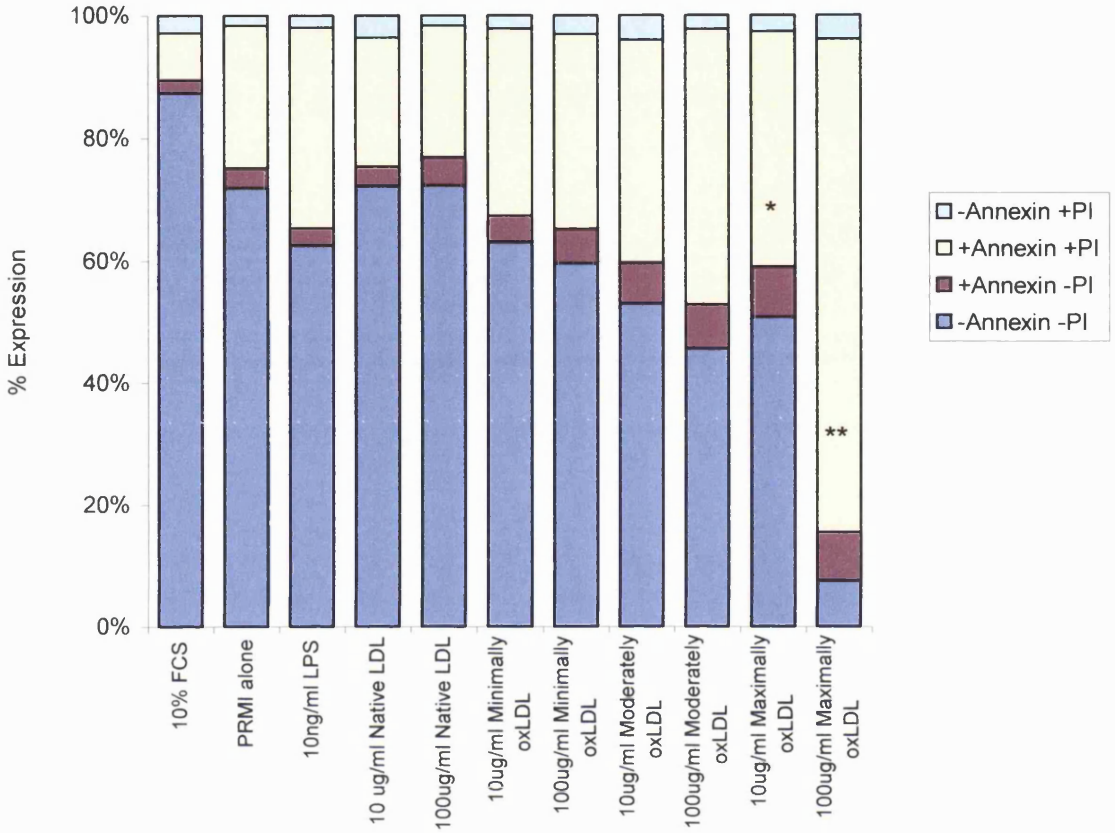


Figure 6.15. Effects of LDL on the viability of DC. DC were prepared according to the method of day 4 purification. Following 6 days of total culture DC were washed twice in RPMI-1640 and resuspended at $5x10^5$ DC/ml in the given media for 24 hours. DC were subsequently harvested and stained with Annexin V and propidium iodide, as detailed in Materials and Methods. Each data point represents the mean percentage expression of three independent experiments, except for 10% FCS which is the mean of two values. Statistical analysis is given relative to RPMI-1640 alone. *, $p<0.05$; **, $p<0.01$.

6.2.6 Cluster formation induced by native LDL

Throughout all of the above experiments, an additional consistent finding was that in the absence of serum (RPMI-1640 alone) DC did not form the characteristic clusters, which were observed for all other experiments (10% v/v foetal calf serum). However, upon addition of native LDL, homotypic aggregation was observed (Figure 6.16). Although the degree of DC clustering was not quantified, it can be seen from Figure 6.16 that 100μg/ml native LDL induced greater aggregation of DC than 10μg/ml native LDL, suggesting that the formation of DC clusters is dependent upon the concentration of LDL. Furthermore, Figure 6.16 shows that as the LDL were increasingly oxidised they induced decreasing amounts of aggregation. It is probable that the lack of observable DC aggregation in the presence of maximally oxidised LDL may partially reflect the increased rate of apoptosis within these samples.

The ability of native LDL to induce the aggregation of a homotypic cell population has not been reported previously for any other cell type. It is possible that the adherence of macrophages to plastic *in vitro* may inhibit their formation of homotypic clusters. However, purified populations of T cells were shown neither to aggregate in the presence of native nor oxidised LDL.

Figure 6.16 also shows that, in the absence of serum, 10ng/ml LPS can induce the homotypic aggregation of DC. It can, therefore, be seen that mature DC readily form homotypic aggregates in the absence of native LDL, suggesting that the effects of native LDL on the formation of DC clusters may be restricted to immature DC.

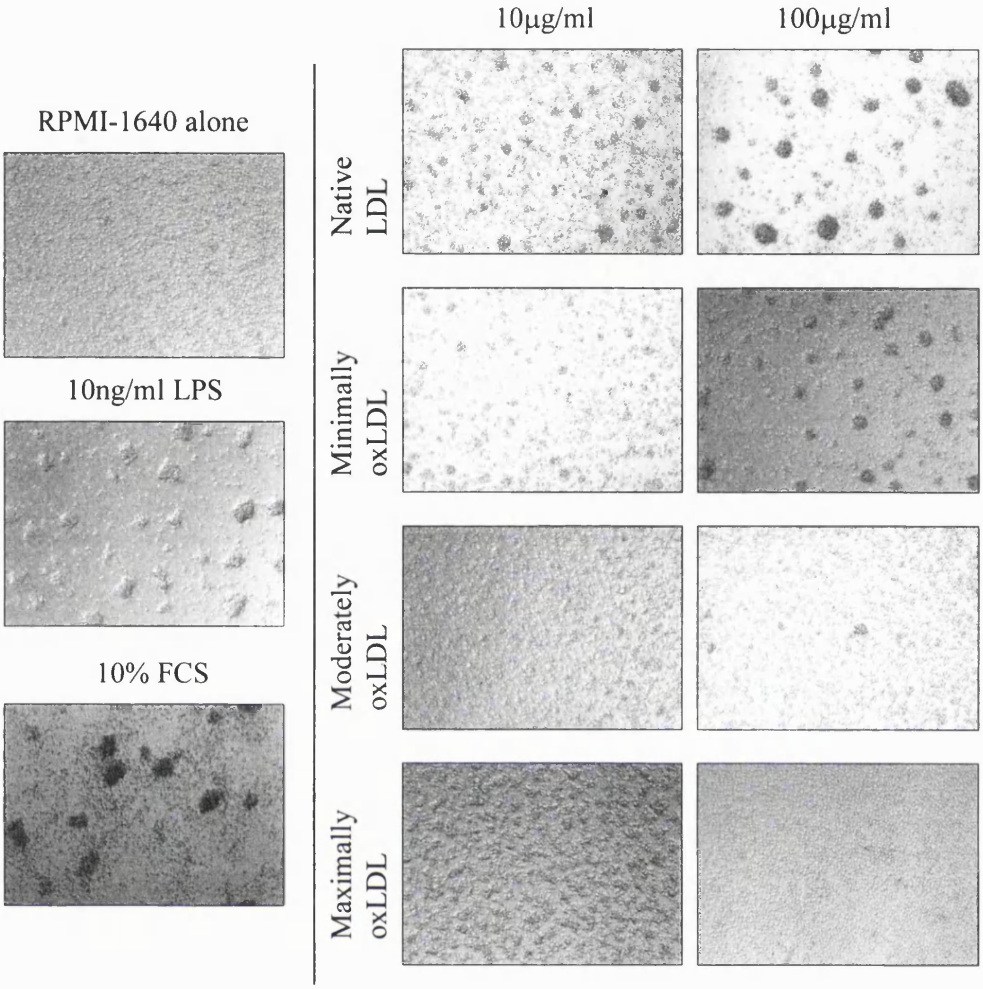


Figure 6.16. Effects of LDL on the formation of homotypic DC clusters. DC were prepared according to the method of day 4 purification. Following 6 days of total culture DC were washed twice in RPMI-1640 and resuspended at $5x10^5$ DC/ml in the given media for 24 hours.

6.3 Discussion

The initial set of experiments described in this chapter was aimed at verifying the isolation and oxidation of LDL. From Figures 6.1 and 6.6 it can clearly be seen that the LDL samples contain negligible protein and lipid contaminants, and that 5μM Cu^{2+} could catalyse the oxidation of native LDL to the predefined states of minimal, moderate and maximal oxidation (Figure 6.2), as determined by the formation of conjugated dienes. Figures 6.3-6.5 show that native and oxidised LDL contained reproducible levels of TBARS and lipid hyderoperoxides and had similar surface charges, as indicated by their relative migration through an agarose gel. The above characteristics of the LDL used within these studies are consistent with the expected values reported by other groups (Jialal *et al.*, 1990; Siow *et al.*, 1999; Rice-Evans *et al.*, 1996).

It was then shown that the suggested protocol for investigating the effects of LDL on DC resulted in the formation of immature DC (Figure 6.7) that were viable (Figures 6.8 and 6.15) and could be induced to mature, when given a suitable stimulus (Figures 6.9 and 6.13).

Following the incubation of DC in the presence of RPMI-1640 alone, compared to 10% v/v foetal calf serum for 24 hours, DC were found to have a decreased ability to reduce MTT (Figure 6.8) and stimulate the proliferation of T cells (Figure 6.9). The decreases in the responses of DC could be a result of an increase in the proportion of cell that had been induced to undergo apoptosis ($Annexin^+$, $P.I.^+$) (Figure 6.15) or a generalised decrease in cellular function as a consequence of serum depletion.

Given the known sensitivity of immature DC to the effects of LPS contamination, it was imperative to ensure that the LDL preparations contained minimal, if any, LPS. This was achieved by examining the effects of native LDL on the functional capacity of immature DC. From Figure 6.9 it was also shown that the potential effects of oxidised LDL samples could, in theory at least, be observed above the responses induced following culture of DC with native LDL,

since such responses are shown not to represent the maximum stimulatory capacity of DC. It could also be suggested from Figure 6.9 that supplementation of at least part of the lipid component of serum may help to restore the functional deficit induced by serum depletion. Phenotypic analysis of DC also showed that, 10μg/ml of LDL must contain less than 1ng/ml LPS since this concentration of LPS induces significant increases in the surface expression of CD86 (Figure 3.18), yet no such phenotypic changes were observed (Figure 6.13).

When examining the effects of LDL on the cellular activity of DC, as assessed by the ability of DC to reduce MTT, it was found that 10μg/ml maximally oxidised LDL significantly increased the reduction of MTT. The increase in the reduction of MTT by DC may be explained by the recent finding that the ability of cells to reduce MTT is dependent upon vesicular trafficking (Liu *et al.*, 1997), and that oxidised LDL are known to interfere with these pathways (see section 1.4.6). Native and the other forms of oxidised LDL alone were unable to induce the reduction of MTT. It was, therefore, concluded that the ability of DC to reduce MTT does not give a reliable assessment of the cellular activity, and thus, the viability of DC in the presence of oxidised LDL.

It can be seen that, among oxidised LDL, maximally oxidised samples exert the most significant effects on DC, as shown for the ability of DC to stimulate the proliferation of T cells (Figure 6.12) and on the induction of apoptosis (Figure 6.15). Indeed, the only statistically significant effects of oxidised LDL on DC were observed for maximally oxidised LDL.

6.3.1 Maturation of DC by maximally oxidised LDL

This chapter reports the novel observation that oxidised LDL are capable of maturing DC. Despite the fact that relatively small increases were observed in both the functional and phenotypic properties of DC, these data are mutually supportive since both of these characteristics change in a manner akin to the maturation that can be induced by other factors.

To investigate further the effects of oxidised LDL on DC, the effects of increasing the culture period in the presence of varying concentrations of maximally oxidised LDL need to be investigated. However, a clear concern would be the viability of DC in the absence of serum. It would, therefore, be interesting to investigate the effects of maximally oxidised LDL on DC incubated in the presence of serum, artificial serum and lipid depleted serum, over increased incubation periods. Such experiments would be aimed at amplifying the stimulatory effects of oxidised LDL on DC. However, the data within this chapter are sufficient to conclude that maximally oxLDL induce the phenotypic (Figure 6.13) and functional (Figure 6.12) maturation of DC, albeit to a lesser extent than 10ng/ml LPS.

The finding that oxidised LDL increase the expression of CD40 on DC may prove to be of particular significance. CD40 mediates its actions through ligation with CD40L (also known as CD154 and gp39) and is known to play an important role in both cellular and humoral immune responses. It has been shown that interruption of CD40-CD40L signalling may reduce the immune response associated with numerous autoimmune diseases, including acute and chronic graft-versus-host disease (Stuber *et al.*, 1999), multiple sclerosis (Howard *et al.*, 1999), and lupus nephritis (Kalled *et al.*, 1998).

The potential importance of CD40 signalling in atherosclerosis is suggested not only by the abundant expression of this co-stimulatory molecule and its ligand within atherosclerotic plaques, but also the recent evidence that blocking CD40 can alleviate many of the inflammatory responses associated with this disease (Mach *et al.*, 1998). The ability of oxidised LDL to increase the expression of CD40 on DC may, therefore, represent a novel pro-atherogenic mechanism through the increased signalling of these pathways. However, the *in vivo* responses induced by CD40-mediated signalling in atherosclerosis have yet to be established.

6.3.2 Maximally oxidised LDL induce apoptosis of DC

Oxidised LDL are known to induce the apoptosis of numerous cell types and these actions are thought to be of pathological significance. Indeed, the apoptosis of vascular smooth muscle cell, possibly induced by oxidised LDL, is thought to be a major contributing factor to the rupturing of atherosclerotic plaques. The novel finding that maximally oxidised LDL induce apoptosis of DC (Figures 6.14 and 6.15) may also be of pathological significance.

Given that DC are known to play an important role in the maintenance of tolerance, it is possible that one of the pathophysiological effects of oxidised LDL may be the breakdown of tolerance, leading to an increased predisposition to an auto-immune disease. It is of interest to note that many diseases that are associated with increased concentrations of oxidised LDL are found to have an auto-immune component, including not only widely recognised auto-immune diseases (e.g. rheumatoid disease) but also atherosclerosis.

It is interesting to note that, as with oxidised LDL, it appears that LPS too may induce both maturation and apoptosis of DC. It remains to be established whether the dying cells represents a distinct cell population or the dichotomy between maturation and death. A similar pattern has been reported for many cells in response to TNF-α, and indeed, in this system in response to maximally oxidised LDL. It appears that there may be a very fine balance between activation of DC and their self-limiting (apoptotic) responses. It is interesting to speculate that the molecular basis of activation versus apoptosis may be activation of competing intracellular signalling cascades, possibly involving stress kinases (see section 1.1.3.6.2).

The increased rate of apoptosis upon incubation of DC with LPS, shown in Figure 6.15, contrasts with the findings of Recigno *et al.*, (1998) who reported a decrease in apoptosis upon maturation. However, the degree of apoptosis induced by LPS (Figure 6.15) was found not to be statistically significant, therefore no firm conclusions can be drawn. An important caveat is that the experiments

within this chapter were conducted in the absence of serum. It would be interesting to investigate whether or not the additional stress induced by the absence of serum alters the responses of DC to maturation signals, such that in the presence of a pre-existing stress DC are more susceptible to apoptosis, and, therefore, less likely to initiate immune responses.

6.3.3 Cluster formation of DC

The ability of native LDL to induce the clustering of homotypic cell populations is not only a novel phenomenon but also potentially an important one.

The current dogma is that DC do not form homotypic aggregates, but that these cells do form aggregates that comprise DC interacting with T cells (Inaba & Steinman, 1986) and B cells (Kushnir *et al.*, 1998). Heterotypic clusters involving DC are characteristic of adaptive immune responses and are known to be formed *in vivo* (Kushnir *et al.*, 1998).

In addition, mouse spleen DC (Inaba & Steinman. 1986) and human tonsillar DC (King & Katz, 1989) form heterotypic cluster with surrounding lymphocytes *in vitro*. Moreover, DC differentiated from monocytes in the presence of GM-CSF/IL-4 are reported to be dependent upon lymphocytes for their cluster formation (Pickl *et al.*, 1996). However, with surprising consistency, all of the above groups have not addressed the possibility that DC may form homotypic aggregates. The reason for the lack of such experiments may reflect the dogma that homotypic DC aggregates do not exist, or do not play a role *in vivo*.

It has been reported, recently, that the *in vitro* differentiation of Langerhans' cells from $CD34^+$ hemopoietic progenitor cells may be dependent upon cluster formation for their generation (Riedl *et al.*, 2000). Given the potential role of clusters in the differentiation of DC, an as yet unasked question must be raised regarding the potential role of aggregation, and more specifically of T cells, in the differentiation of monocytes to DC in the presence of GM-CSF/IL-4.

Since IL-4 receptors have been described on monocytes (Fisher *et al.*, 1998) but not monocyte-derived DC, it is possible that the latter stages of the differentiation of monocytes to DC may be dependent upon the local presence of T cells that express IL-4 receptors. However, since it has been shown that purified monocyte-derived DC can differentiate further (from day 4) in the absence of T cells (see Chapter 3), it is unlikely that the latter part of the differentiation of monocytes to DC is dependent upon the local presence of T cells. Indeed, in the absence of IL-4, monocyte-derived DC are reported to differentiate into a cell population that does not express CD1a (Palucka *et al.*, 1998). However, it was shown in Chapter 3 that, in the absence of T cells, DC differentiate from day 4 to day 8 into a population of cells that express increased levels of CD1a. It is, therefore, suggested that T cells may not play an important role in the differentiation of monocytes to DC.

It has been known for many years that DC accumulate in the target organs of a number of endocrine autoimmune diseases (such as Graves' disease, Hashimoto goitre and type I diabetes) (Kabel *et al.*, 1988; Kabel *et al.*, 1989; Voobij *et al.*, 1989) and diseases with an autoimmune component (such as atherosclerosis and rheumatoid disease) (Lord & Bobryshev, 1999; Thomas *et al.*, 1994a). Furthermore, it has been suggested that the accumulation of DC may be the earliest hallmark of an impending autoimmune reaction (Kamperdijk *et al.*, 1993).

It has been reported, recently, that DC accumulate, even in children, at sites of major hemodynamic stress prior to atherosclerotic plaque formation. Furthermore, since it is thought that haemodynamic stress increases the risk of plaque formation, it could be suggested that early accumulation of DC could contribute to the disease process. The potential involvement of DC in aterosclerosis has been suggested previously (Bobryshev *et al.*, 1996). However, since atherosclerosis is generally considered to be a chronic inflammatory disease rather than an autoimmune disease, the role of DC accumulation in the initiation of atherosclerosis needs to be defined. One hypothesis might be that

atherosclerosis may be initiated by an autoimmune response, possibly to oxidised LDL.

In many diseases associated with an auto-immune response, it is unclear whether or not DC simply accumulate in number or form homotypic aggregates. The formation of homotypic DC aggregates *in vivo* is controversial, but, such aggregates have been identified in atherosclerotic plaques (Lord & Bobryshev, 1999) and in the thyroid of iodine-deficient Wistar rats (Kamperdijk *et al.*, 1993). In this latter condition the aggregation of DC precedes the development of autoantibodies and the infiltration of T cells, and may parallel similar consequences of iodine-deficiency in humans (Kamperdijk *et al.*, 1993). However, the putative homotypic clustering of DC, and the potential implications of such interactions, have yet to be firmly established or investigated.

It is interesting to note that both rheumatoid disease and atherosclerosis are associated with increased localised concentration of oxLDL and DC. However the data reported here suggests that it is the native and not oxLDL that induce DC clustering. Therefore, native and oxidised LDL may act in seriatim to promote an auto-immune reaction. It is known that vascular lesions are associated with an increased subendothelial concentration of native LDL (see section 1.4.4.1) and this may lead to clustering of DC (Figure 6.14). As the site of DC clustering is also the site of oxidation of LDL, the next step in the sequence would be that low levels of oxidised LDL mature DC, leading to the presentation of auto-antigens in the presence of costimulation, and, therefore, increasing the predisposition to a local auto-immune focus. However, when the higher concentrations of oxLDL accumulate, then apoptosis may ensue.

6.4 Conclusions

It has been documented that LDL are able to influence the ability of DC to induce the proliferation of resting T cells. Moreover, the effects of oxidised LDL are distinct from those of native LDL and, thus, the effects of oxidative stress may be mediated on DC *via* the oxidation of LDL. The effects of oxidised LDL on DC may contribute significantly to the pathogenesis of a number of diseases and, therefore, may represent a pertinent therapeutic target.

The potential importance of the findings presented within this chapter can be demonstrated readily in relation to atherosclerosis (Figure 6.17), although they may also help to explain features of other disease processes. Figure 6.17 illustrates that sites of physical stress within blood vessels are associated with an accumulation of DC within the tunica intima (Bobryshev & Lord, 1998). Subsequently, endothelial dysfunction may ensue as a consequence of smoking, which induces a generalised oxidative stress and increases the susceptibility of LDL to oxidation. Endothelial dysfunction may then promote the accumulation of leukocytes and native LDL into the tunica intima. Native and oxidised LDL are then ideally located to induce DC clustering and maturation, respectively. Both the clustering and maturation of DC may be important factors in the promotion of an autoimmune reaction and may play an important contribution to the accumulation of T cells that are known to occur throughout the formation of atherosclerotic lesions (Ross, 1999). Indeed, T cells specific to oxidised LDL have been isolated from atherosclerotic lesions in humans, indicating that oxidised LDL itself may also represent an important immunogen *in vivo*.

Furthermore, it could be suggested that LDL may play an important role in the migratory patterns of DC during atherosclerosis. In healthy arteries, DC are generally considered to exist at low numbers within the tunica adventitia. Figure 6.18 illustrates that at sites of atherosclerotic lesions DC may be found within the tunica intima as well as the tunica adventitia. It is known that oxidised LDL may act as chemoattractants for circulating monocytes, which may then differentiate

into DC. The clustering of DC within the tunica intima, possible mediated by the presence of native LDL, is also illustrated on Figure 6.18.

As an atherosclerotic lesion progresses into an advanced plaque, the concomitant prolonged oxidative stress may lead to the formation of maximally oxidised LDL. Maximally oxidised LDL may influence the accumulating DC by several different mechanisms, as illustrated in Figure 6.18. Firstly, low concentrations of maximally oxidised LDL may stimulate the maturation of DC, which would increase the predisposition to an autoimmune response and possibly stimulate the proliferation of T cells, thus helping to perpetuate a chronic inflammatory state (see section 1.1.4). As maximally oxidised LDL accumulate in number, they may induce the apoptosis of DC (along with other cells) and thereby contribute to the formation of the necrotic core within advanced atherosclerotic plaques.

Finally, since it is known that oxidised LDL stimulate the migration of monocytes from the peripheral circulation but inhibit the motility of macrophages, it is also interesting to speculate that oxidised LDL may inhibit the emigration of mature DC. If oxidised LDL are indeed found to inhibit the emigration of maturing DC, then it is possible that they may contribute to both the accumulation and activation of DC found at sites associated with oxidised LDL, such as the synovial joint in patients with rheumatiod disease, and atherosclerotic lesions.

It appears, therefore, that the most significant effects of LDL on DC may be mediated through the actions of native LDL, which induce cluster formation, and maximally oxidised LDL. The effects of maximally oxidised LDL on DC are unlikely to play a significant contribution to the development of early atherosclerotic lesions, since they are unlikely to be formed at this stage. However, it is distinctly possible that maximally oxidised LDL may act to perpetuate inflammation and precipitate autoimmunity *via* their action on DC.

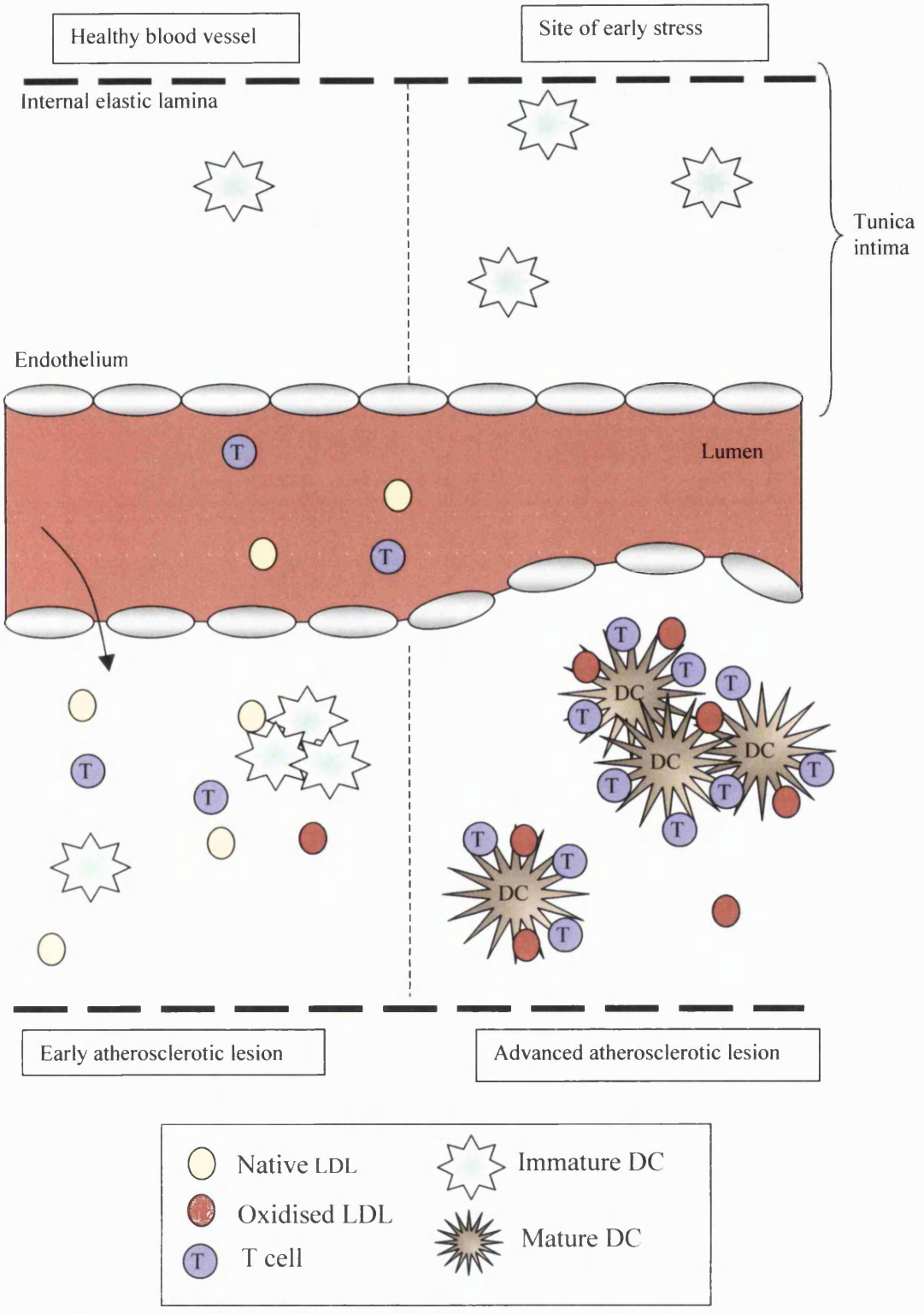


Figure 6.17. Schematic diagram illustrating the potential role of DC in atherosclerosis. See section 6.4 for further details.

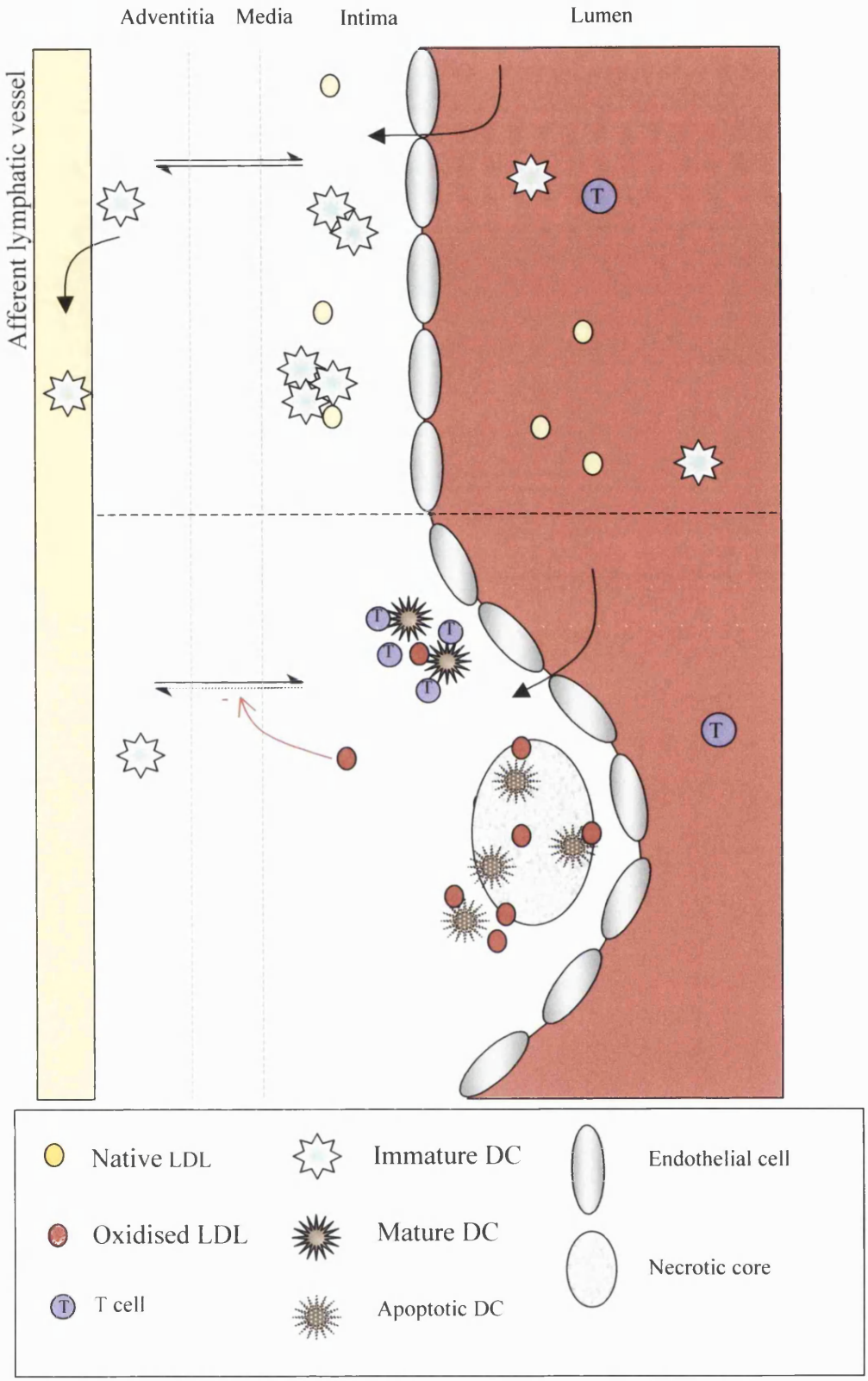


Figure 6.18. Schematic diagram illustrating the potential effects of native LDL and oxidised LDL on the migration of DC in atherosclerosis. See section 6.4 for further details.

Chapter 7

General Discussion

7.1 Introduction

In this thesis, the "danger" hypothesis has been explored and examined to investigate whether or not DC can indeed be activated by tissue injury. After characterisation of DC differentiation, improved experimental methods were developed to monitor DC activation. These methods were used to investigate the effects of oxidative stress, a fundamental component of tissue injury, on DC activation. The "danger" hypothesis was then expanded to include the suggestion that products derived from oxidative stress could constitute components of an elusive "danger" signal, and two such products were then examined in relation to their effects on DC maturation.

7.2 Danger Hypothesis

The initial aim of this thesis was to examine the paradigm that the immune system recognises the need to initiate adaptive immune responses through the detection of a "danger" signal. A presupposition of the hypothesis investigated was that, since DC are the most potent activators of adaptive immune responses known, they are likely to be activated by the putative danger signal(s). However, since the formulation of the "danger" hypothesis it has received little critical evaluation. Perhaps the main reason for the lack of evaluation is that, despite the fact that the hypothesis has been discussed for almost a decade, there is little experimental evidence either to support or to reject the hypothesis. Moreover, in the few instances when supporting evidence has been found, these conclusions have not been substantiated in later publications (see section 1.1.2.3.2). Indeed, it appears equally likely that products derived directly from tissue damage are either insufficient alone to activate DC, or that that these do not represent the predominant signal that induces the maturation of DC, at least *in vitro*.

Given the association between tissue damage and oxidative stress, and the suggestion that oxidants may represent a novel group of pro-inflammatory

signalling molecules, perhaps the most noteworthy alternative hypothesis is that oxidative stress may activate DC. Thus, it was suggested that oxidative stress may constitute the elusive "danger" signal.

However, Chapter 4 shows that a key biochemical product of oxidative stress did not activate DC directly, and further supports the notion that oxidative signalling mechanisms may not represent prerequisite signals for the activation of DC. Rather than pursuing the earlier observations further, these findings prompted a re-evaluation and an extension of the "danger" hypothesis to incorporate the suggestion that it is the derivatives of oxidative stress which act as likely candidate signals that are capable of maturing DC. Furthermore, two particular products were defined and used for these investigations. These two signals were selected deliberately from entirely different precursor pools, namely proteins and lipids, and are, perhaps the two most probable candidates from each of these groups that might have a relationship to the maturation of DC *in vivo*.

The results obtained in Chapter 5 suggest that AOPP may indeed interact with DC. However, the hypothesis that AOPP may constitute a "danger" signal was not supported by the data, since phenotypic maturation of DC was not observed. Despite the lack of DC maturation, the AOPP study illustrated how the production and potential function of superantigens at a site of tissue damage may represent a fundamental component of an inflammatory response. Indeed, it seems possible that, beyond a critical threshold level of DC accumulation, AOPP may act to enhance the ability of DC to induce the activation of resting T cells. Perhaps equally important is the hypothesis that AGE- and AOPP-modified "self" proteins could play a significant role in a number of disease states that are associated with oxidative stress. One examples of how this might arise is that it has been noted that several of the auto-antigens targeted in scleroderma may result from the unique susceptibility of such proteins to oxidative modifications and, thus, to fragmentation. The episodic ischaemic reperfusion that occurs in scleroderma may be the factor that precipitates oxidative stress and, subsequently, the generation of new immunogenic epitopes. It is not yet established whether or not the auto-antigens in scleroderma, many of which are

oxidised protein derivatives, represent AOPP, and their interactions with DC have yet to be investigated specifically. However, recent findings suggest such experiments may yield interesting results, and also prompt the questions as to whether the auto-antigens in scleroderma represent immunocryptic epitopes or epitopes rendered immunogenic by oxidative stress or whether the presentation of oxidised proteins may promote an auto-immune response – by expressing modified "self" in the context of a relatively non-specific activation of surrounding T cells and DC.

The ability of oxidised LDL to act upon and indeed mature DC was a novel finding with potentially important implications. An interesting feature of the data was that it was the oxidised LDL with the greatest toxicity (maximally oxidised LDL) that also caused the maturation of DC. Since it has been shown that maximally oxidised LDL induce apoptosis of DC and apoptotic cells are thought not to activate DC, increased toxicity is unlikely to explain the maturation of DC by maximally oxidised LDL. Indeed, given that apoptosis is a normal physiological process, products derived from this would not be expected to activate DC (Ibrahim et al., 1995). Moreover, the ability of maximally oxidised LDL to induce apoptosis of DC might increase the rate of cross-presentation of antigens, from such apoptotic cells by viable DC in the local milieu, to $CD8^+$ lymphocytes (see section 1.1.3.4.2).

The notion that activation of DC by 10μg/ml maximally oxidised LDL is not a side effect of the toxic nature of these species is supported further by the finding that 10μg/ml moderately oxidised LDL induced a similar degree of apoptosis, yet did not induce DC maturation. Therefore, the maturation of DC is unlikely to be induced by products derived from apoptotic cells. Moreover, it is suggested that maximal oxidation of LDL may occur *in vivo* and could promote chronicity of chronic inflammatory diseases and diseases with an auto-immune component, *via* the activation of DC. Of particular interest is the potential that oxidised LDL could act on DC to promote atherosclerosis and rheumatoid disease, since these diseases are examples where there is a known association with increased levels

of oxidised LDL and increased numbers of activated DC at the sites of inflammation.

In summary, therefore, it has been shown in this thesis that products derived from oxidative modifications may have potential immunostimulatory effects. Furthermore, the effects of these oxidative modifications are not likely to be confined to acute inflammation, and/or the early stages of disease processes, so that products such as maximally oxidised LDL and AOPP may act to perpetuate rather than to initiate disease. It is possible that biochemical products of oxidative stress such as oxidised LDL and AOPP may act in concert and promote the chronicity of numerous diseases associated with aberrant DC functions. This concept requires further careful analysis in the future.

7.3 Functional responses

The induction of T-cell proliferation by DC is dependent upon the culmination of numerous factors. Therefore, such assays are infamous for producing inconsistent results. However, examination of the ability of DC to induce the proliferation of T cells reveals information regarding the functional endpoint of DC responses and is, therefore, an invaluable final "gold standard" for these cells.

This thesis has concentrated on examination of DC effects on T cells under different conditions wherever feasible, thus negating the need to question whether or not the observed effects may result in downstream changes in biological activity. In addition, examining the phenotype of DC has also clearly been important in the initial verification of DC isolation and in characterising the responses of DC to various stimuli.

The phenotypic data also provides an accurate index of the state of DC maturation that is independent of their viability. Although maturation is thought to be associated with longevity of DC, immature DC are thought not to apoptose

significantly within the time-frame of these studies (Recigno *et al.*, 1998), suggesting that the reported increases in the ability of DC to induce the proliferation of T cells reflects the maturation of DC rather than an artefact of increased DC survival.

A recurrent observation throughout this thesis has been that the stimulatory capacity of DC appears only to increase marginally upon maturation. There are a number of potential reasons for this marginal increase. Firstly, most of the proliferation data are presented as the mean of three or more experiments. Therefore, although the apparent increases in stimulatory capacity appear relatively small, the differences are masked by the variability of functional assays, which arises from analysing pooled data. Moreover, since the DC investigated within this study were functionally "more active" than DC differentiated in the conventional manner, the differences upon maturation may be less pronounced than they would otherwise be, as there must be an absolute maximum stimulatory capacity.

Finally, it should be noted that the oxidative mitogenesis assay provides the primary signal to the T cells. Therefore, the differences in activity are mainly dependent upon the degree of costimulatory signals provided by the DC. It is important to recognise that *in vivo* the primary signals are provided by the presence of specific cognate antigens. Therefore, the baseline proliferation would be much lower than that obtained within these assays. Indeed, *in vivo* immature DC would be expected not to induce significant T-cell proliferation at all. The situation is slightly complicated by the fact that *in vivo* a much lower percentage of the T cells would be responsive to a specific mature DC, thereby reducing the responses induced by each DC, but nonetheless it is possible that *in vitro* one is forced to shift the signal to noise ratio in order to demonstrate an effect that would be observed readily *in vivo*.

Therefore, it is suggested that the detailed quantitative increases in stimulatory capacity of DC are not as important as the fact that their overall stimulatory capacity has increased. Changes in the functional and phenotypic properties of

DC can be taken to indicate whether or not the potential signals could act to cause the maturation of DC *in vivo*, and partial functional and phenotypic maturation may simply reflect the dose-dependent nature of DC maturation signals (as shown for LPS) (Figure 3.18), a subject which has rarely been investigated previously.

7.4 Rate of DC Maturation

In Chapters 5 and 6, the abilities of AOPP and oxidised LDL to induce the maturation of DC were less pronounced than the responses induced by LPS. This difference may be a result of the fact that AOPP and oxidised LDL do not induce conventional maturation of DC, as previously suggested for AOPP. Alternative explanations include the possibility that sub-optimal concentrations of stimuli had been applied to the DC, and that AOPP and oxidised LDL may induce the maturation of DC at a slower rate than LPS, as discussed in previous chapters.

If products of oxidative stress are found to act on DC over prolonged periods of time, then it is possible that the activation of adaptive immunity could be segregated into two main categories depending upon the rate that responses are elicited.

Firstly, a rapid response is required following the presence of infectious agents, since these may be severely detrimental to the host. In many cases, the presence of pattern recognition receptors (as proposed by Janeway and colleagues) could account for activation of DC by infectious agents. It should be noted that the fact that infectious agents can activate DC *via* pattern recognition receptors is not disputed. However, the relative importance of pattern recognition receptors and responses that may be initiated *via* the detection of putative "danger" signals have yet to be determined.

It is then possible that a distinct set of responses may be elicited following physical tissue damage. Such responses may not be dependent on pathogenic

agents for their initiation and may, thus, act in addition to responses that result from the detection of an infectious agent. The advantage of a "danger" signal may be that it would not necessarily signal the presence of an infectious agent and could require a prolonged signal to induce DC maturation. Indeed, a slower response may limit the secondary tissue damage that is known to occur during acute inflammation.

Since LPS was known to induce the maturation of DC within 24 hours, this was taken to imply that other stimuli may also act within a similar timeframe. In retrospect, however, stimuli may activate DC at different rates, especially if they require internalisation or further processing. Indeed, when investigating the effects of oxidised LDL on DC it is also possible that the rate of internalisation may have been reduced as a result of the absence of serum, and more specifically of cholesterol. It would be interesting to investigate the hypothesis that "danger" signals may act on DC less rapidly than signals induced by microbial constituents.

7.5 Clarifying the "danger" hypothesis further

On reflection about the "danger" hypothesis, it may be that one of the virtues of the hypothesis is that the "danger" signal may not be as elusive as has been suggested previously.

In essence, the "danger" hypothesis aims to explain the initiating factors that induce the expression of costimulatory molecules on antigen presenting cells. The presence of these costimulatory signals, are, in most cases, thought to be essential in the initiation of adaptive responses. Indeed, the few exceptions to the general rule that costimulatory signals are required to mount adaptive responses occur in the presence of excessive antigenic stimulation (Bingaman *et al.*, 2000) and represent experimental rather than physiological responses.

One should consider that, *in vivo*, "danger" signals would not act on DC in isolation. The "danger" signals may activate DC indirectly *via* surrounding cells and molecules. It must, therefore, be suggested that the specific targets of a "danger" signal are not overly important, since, once an inflammatory response is initiated, the subsequent inflammatory cascade would be expected to initiate the maturation of DC. Thus, the simple fact that a mediator that is capable of activating DC is released at a site of tissue damage may be the important feature of a response initiated by "danger". Therefore, investigation of the direct effects of potential "danger" stimuli on the responses of DC may not represent the *in vivo* mechanism by which these stimuli act.

It follows that the next question to resolve is: what is the initial feature of an innate immune response that occurs in the absence of pathogen recognition? Although superficially there may seem to be a number of potential answers, such as "neutrophil or monocyte recruitment," this does not really answer: "What signal is it that proceeds, and activates, the cascade of reactions that initiate innate and then adaptive responses, with the concomitant maturation of DC?"

If one accepts that the destruction of tissues could initiate immune responses, then the quest for an elusive ubiquitous "danger" signal is futile, since there are many plausible answers already known. Perhaps the most noteworthy answer could be the direct release of mediators from intracellular granules, which would occur following the rupturing of cells that contain pre-formed mediators. The mixture of cell populations present, including neutrophils, monocytes and macrophages, which contain pre-formed mediators, may explain some of the spurious results (within the literature) suggesting that necrotic cellular products may activate DC.

A candidate cell that is present in most tissues, and particularly rich in pre-formed mediators, is the mast cell. The pre-formed mediators known to be present within mast cells include the chemotactic factors for eosinophils and neutrophils (ECF and NCF, respectively), interleukins (IL-3, 4, 5 and 6), GM-CSF, TNF and histamine. The mediators released could act in concert to promote

an acute inflammatory response in the absence of an infectious agent, leading to the recruitment and activation of numerous cell populations, including DC. In addition, another factor that may contribute to the maturation of DC, in the absence of the recognition of an infectious agent by pattern recognition receptors, are neurogenic components of inflammation. Several neuropeptides are known to act on Langerhans' cells and promote an inflammatory response (Lambert *et al.*, 98).

Thus, it is evident that, in the absence of pathogen recognition, DC will be activated by a variety of different mechanisms following tissue damage and the associated inflammatory response. Furthermore, the dogma that there may be a universal "danger" signal that is currently elusive is not necessarily true. Indeed, it is more likely that, following a physical or microbial insult, several mechanisms may "kick in" and/or synergise to initiate innate and adaptive responses. It must, therefore, be suggested that the key "danger" signal is not the signal that initially induces an immune response, but is in fact the signal that prevents the resolution of an inflammatory response, which may act *via* the accumulation of mature DC.

7.6 Implications of the data

If the above discussion is an accurate reflection of current knowledge, then the quest of modern immunologists should be to identify the elusive signals that promote the inappropriate perpetuation of DC (and inflammatory) responses. If such inappropriate mechanisms could be defined, then the results and hypotheses presented within this thesis may be of particular significance.

In general terms, this thesis demonstrates that products derived from oxidative damage constitute signals that are likely to influence the function of DC *in vivo*. Furthermore, since the products are highly oxidised, they would be expected to act cumulatively, with maximum effect at the later stages of disease processes. Thus, AOPP and maximally oxidised LDL may represent fundamental signals

for the perpetuation of inflammatory responses *via* their actions on DC. Furthermore, they may amplify the immune response by themselves acting as antigens, and this may be of particular significance for oxidised LDL.

Although the bulk of the study (and of this discussion) has focused on the potential role of oxidative stress in the regulation of DC maturation, this has to be seen in the context that the most immediately relevant practical finding was that the early purification of DC increases their functional potential, and avoids some of the pitfalls and inconsistencies of previous work. The use of DC that have an amplified and well-defined capacity to stimulate the proliferation of T cells may prove particularly beneficial for use in DC-based immunotherapy.

7.7 Future Directions

This project has yielded many fascinating results and even more potential directions that it would be interesting to pursue. Perhaps the most novel concept to investigate would be whether or not atherosclerosis could be initiated by an auto-immune reaction. The potential role of oxidised LDL in DC accumulation, aggregation, activation and apoptosis, in this process, is of particular interest (as discussed in Chapter 6).

Many of the potential future directions that relate to other specific hypotheses presented within this thesis have been raised within their respective chapters. A recurrent theme was that it would be interesting to determine whether or not the effects of AOPP and oxidised LDL could be amplified by increasing their incubation time with DC. If derivatives of oxidative stress are found to activate DC at a slower rate than microbial products then this would support the hypothesis that DC may also be able to determine the urgency at which responses are required, as discussed previously.

Specifically in relation to the potential role of AOPP in the regulation of DC function, it would be interesting to examine the effects of higher concentrations

of AOPP on the maturation of DC. It would also be interesting to examine whether or not AOPP are capable of binding receptors on the surface of DC and then, if this was found to be the case, investigate whether or not DC express the putative receptors for AOPP on their surface.

The release of cytokines by DC represents an important mechanism by which they induce cellular responses. It would, therefore, be of particular interest to investigate the effects of oxidative stress and oxidation derivatives on the cytokine profiles of immature and mature DC. In particular, it would be interesting to investigate the production of IL-12 by DC following their culture in the presence of AOPP and maximally oxidised LDL. IL-12 is thought to be released from DC in a manner that is dependent upon their state of maturation and is known to play an important role in the development of Th1-type responses and in the activation of NK cells during host defence. Assessment of IL-12 production by DC would help to confirm whether or not AOPP and maximally oxidised LDL are capable of activating signalling pathways that are involved in the maturation of DC, which may be reflected at the T-cell level.

It would be worth while seeking the optimal conditions for the maturation of DC by maximally oxidised LDL in more detail. Once these conditions are established it would then be possible to explore the mechanism by which oxidised LDL induce the maturation of DC, and then, possibly, find a means to prevent the maturation of DC by maximally oxidised LDL.

Since it has been suggested that the "danger" signals of interest may be most important in relation to the chronic inflammatory milieu, it would be interesting to examine the effects of non-chlorinated oxidants and free radicals on the ability of proteins to induce the maturation of DC. The potential advantage of using non-chlorinated oxidants is that the chronic inflammatory site contains very few neutrophils, and rather more macrophages (Stephenson, 1992). The switch in the predominant phagocyte at the site of inflammation is associated with a reduction in the relative expression of myeloperoxidase and thus in the formation of HOCl.

In addition, it is likely that in the inflammatory milieu, there may be several "danger" signals. *In vivo*, therefore, these signals may act synergistically to cause DC activation. Thus, it may be that free radicals, AOPP and oxidised LDL can cause the maturation of DC when they act in synergy with other "danger" signals, such as reduced pH or anoxia. Thus, it would be interesting to investigate whether or not a variety of potential "danger" signals could potentiate the effects of derivatives of oxidative stress on DC.

An alternative further direction could involve investigating the role of NF-κB in the responses of DC to oxidative stress. NF-κB has been suggested frequently to mediate the effects of oxidative stress in numerous cell types and has been implicated in the maturation of DC. However, oxidative stress alone is insufficient to induce the maturation of DC. It would be interesting to investigate this apparent paradox further and also to examine whether or not maximally oxidised LDL may exert their effects on DC *via* the activation of NF-κB. An understanding of the signalling mechanism by which maximally oxidised LDL exert their actions on DC may aid the elucidation of potential mechanisms by which such interactions could be promoted or inhibited.

Finally, it would be interesting to investigate the apparent dichotomy between activation and apoptosis of DC, with the aim of identifying a novel means by which the functions of DC could be modulated to potentiate or inhibit immune responses.

Chapter 8

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