

A ROLE FOR POLYUNSATURATED FATTY ACIDS IN Th1-MEDIATED DISEASE

**A thesis submitted for the degree of Ph.D.
by
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DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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PUBLICATIONS

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ABSTRACT

The anti-inflammatory effects of polyunsaturated fatty acids (PUFA) have been well documented, however their exact method of action remains elusive.

Dendritic cells (DC) are the most potent antigen presenting cell (APC) and we found that n-3 and n-6 PUFA suppressed IL-12p70 and enhanced IL-10 production rendering the DC less inflammatory. Furthermore, PUFA inhibited DC maturation by impeding the upregulation of co-stimulatory markers and MHCII expression. Given the critical role of IL-12 and IL-10 in T helper cell differentiation we looked at the effects of PUFA-modified DC on subsequent T cell development and found them capable of inhibiting IFN- γ , IL-17, IL-2, and IL-4 production from CD4⁺ T cells.

PUFA are reported to be natural ligands for PPAR γ , therefore we investigated whether they exerted their effects on DC maturation by activation of this nuclear receptor. We found PPAR γ expression was enhanced in PUFA-treated DC, and confocal microscopy revealed an increased association of PPAR γ with NF κ B. However, the PUFA-induced changes in DC cytokine production and cell surface marker expression were not reversed in the presence of the specific PPAR γ antagonist (GW9662) and were therefore deemed PPAR γ -independent.

Finally we carried out a number of feeding studies in mice where a CLA-incorporated diet was used to ascertain whether results we recorded *in vitro* were mirrored in an *in vivo* situation. Mice fed a CLA-rich diet had less circulating inflammatory cytokines (IL-12p70 and IFN- γ) following endotoxin challenge in an LPS-shock model. Furthermore, feeding animals a CLA-supplemented diet significantly protected against the development of colitis in a DSS-induced model of inflammatory bowel disease, thus indicating that PUFA can exert beneficial and protective effects in inflammatory disease.

ABBREVIATIONS

AA	Arachidonic acid
AG	Antigen
AIA	Antigen-induced arthritis
AICD	Activation-induced cell death
ALA	Alpha-linolenic acid
AP-1	Activator protein-1
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
BBB	Blood brain barrier
BD	Becton Dickinson
BSA	Bovine serum albumin
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Crohn's disease
CD(number)	Cluster of differentiation
CIA	Collagen induced arthritis
CIF	Complex intestinal microflora
CLA	Conjugated linoleic acid
CNS	Central nervous system
COX	Cyclo-oxygenase
CSF	Cerebro-spinal fluid
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
DC	Dendritic cell
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulphoxide
DNCB	Dinitrochlorobenzene
DSS	Dextran sodium sulphate
DTH	Delayed type hypersensitivity
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-related kinase
FCS	Foetal calf serum
Foxp3	Fork-head box P3
GALT	Gut-associated lymphoid tissue
GATA-3	GATA-binding protein 3
GIT	Gastrointestinal tract

GMCSF	Granulocyte-macrophage colony stimulating factor
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
IFN	Interferon
IKK	I κ B kinase
IL	Interleukin
IL-1Ra	IL-1 receptor antagonist
IP	Intraperitoneal
IRF	Interferon regulatory family
IS	Immunological synapse
IV	Intravenous
JNK	Jun amino-terminal kinase
KLH	Keyhole limpet haemocyanin
KO	Knockout
LA	Lauric acid
LAT	Linker for activation of T cells
LFA-1	Leukocyte function-associated molecule-1
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LTB	Leukotriene
MAPK	Mitogen-activated protein kinase
MCP	Macrophage chemoattractant protein
MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MLN	Mesenteric lymph node
MS	Multiple sclerosis
MUFA	Monounsaturated fatty acid
NEMO	NF κ B essential modulator
NFAT	Nuclear factor of activated T cells
NF κ BD	NF κ B decoy
NF κ B	Nuclear factor- κ B
NK	Natural killer
NKT	Natural killer like T cell
NOD	Nucleotide oligomerization domain
OVA	Ovalbumin
PAF	Platelet activating factor
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PG	Prostaglandin

PMA	Phorbol 12-Myristate 13-Acetate
PP	Peyer's patches
PPAR	Peroxisome proliferator-activated receptor
PPRE	PPAR response element
PUFA	Polyunsaturated fatty acid
PVDF	Polyvinylidene Fluoride
RA	Rheumatoid arthritis
RBC	Red blood cell
RSG	Rosiglitazone
RT	Room temperature
RXR	Retinoic X receptors
SCF	Stem cell factor
SDS	Sodium dodecylsulphate
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGF	Transforming growth factor
Th	T-helper
TLR	Toll-like receptor
TMB	3,3',5,5'-tetramethyl-benzidine
TNBS	Trinitrobenzenesulfonic acid
TNF	Tumour necrosis factor
Treg	T regulatory
TXB	Thromboxane
UC	Ulcerative colitis
VCAM-1	Vascular cell adhesion molecule

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CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The immune system is an interactive network of lymphoid organs, cells, humoral factors, and cytokines, which can be divided into two parts; innate and adaptive immunity. Although considered separate, there is a great deal of interaction between the two.

1.1.1 OVERVIEW OF INNATE IMMUNITY

The term innate immunity is sometimes used to include physical, chemical, and microbiological barriers, but more usually encompasses the elements of the immune system (neutrophils, monocytes, macrophage, dendritic cells, complement, cytokines and acute phase proteins) which provide the first line of defence against invading pathogens [reviewed by Beutler (2004)]. The innate response is largely mediated by phagocytes (e.g. macrophage and neutrophils), which engulf and kill invading microbes, and produce a range of inflammatory mediators, including cytokines and chemokines, which concurrently coordinate additional host responses. Macrophage reside in tissues at sites of pathogen entry, including the lungs and gut, and are among the first cells to encounter invading microbes. Neutrophils are short-lived and abundant in the blood but are recruited to sites of infection by activated macrophage and inflammatory products (Aderem & Ulevitch, 2000). Macrophage and neutrophils have a large number of surface receptors which recognise pathogens and instigate phagocytosis. These include pattern recognition receptors (e.g. Toll-like receptors), and cell surface receptors for the Fc portion of antibodies. Ligand binding to these receptors induces the plasma membrane to surround and engulf the microbe in a membrane-bound vesicle known as a phagosome (Beutler, 2004). Phagosome maturation in macrophage and neutrophils results in the killing of internalised microorganisms, and complete degradation of phagocytic cargo, whereas maturation of phagosomes in dendritic cells (DC) serves to prevent complete degradation of antigens so they can be presented with major

histocompatibility complex (MHC) II molecules to initiate adaptive immune responses (Blander & Medzhitov, 2006).

1.1.2 TOLL-LIKE RECEPTORS (TLRS)

The innate system is not antigen specific. Our understanding of how immune cells discriminate self from non-self was transformed by the discovery of Toll-like receptors (TLRs). TLRs are so termed as they share homology with the *Drosophila* protein, Toll. One of the first mammalian receptors to be identified was TLR4 which was shown to bind lipopolysaccharide (LPS), an endotoxin from gram-negative bacteria. Since then, at least 11 TLRs sharing homology with TLR4 have been identified in mammals, each able to recognize unique molecular patterns associated with different classes of pathogens. These conserved motifs are termed pathogen associated molecular patterns or PAMPs. TLRs 1, 2, 4, 5, 6 are involved in the recognition of mainly bacterial products, where non-self distinction is a fairly straightforward process. TLRs 3, 7, 8, 9, are involved in detecting viruses and nucleic acids which are not unique to the microbial world. In this case, self/non-self discrimination is mediated by receptor accessibility, as host nucleic acids do not normally enter the intracellular compartments that contain these TLRs (Aderem & Ulevitch, 2000; Iwasaki & Medzhitov, 2004).

Consistent with their role in pathogen recognition, TLRs are mainly expressed in cells of the innate system, such as macrophage, DC, neutrophils, mast cells, mucosal epithelial/endothelial cells and fibroblasts. Distinct subsets of these cells differ in expression patterns of TLRs enabling them to induce specific types of immune responses to specific pathogens (Andreaskos *et al.*, 2004). TLR ligation can trigger various mitogen-activated protein kinase (MAPK) signalling cascades and activate nuclear factor- (NF)- κ B, both of which regulate the transcription of many genes involved in immunity and inflammation, resulting in the production of lipid signalling molecules such as prostaglandins, and protein signalling molecules such as cytokines (O'Neill, 2006). Although NF κ B is activated by all TLRs, recent studies have highlighted specific members of the interferon-regulatory family (IRF) as other crucial factors in the induction of

TLR-responsive genes, which share many signalling pathway molecules with NF κ B. IRFs were discovered when Myd88-deficient mice were still able to produce IFNs following TLR3 and TLR4 ligation. It is now well documented that TLR3 and TLR4 signalling can induce type I IFNs by mechanisms independent of Myd88 using IRF3 and IRF7 (Moynagh, 2005). The coordinated activation of IRF3, IRF7, and NF κ B provide maximal induction of type I IFNs, whereas activation of IRF5 and NF κ B ensures functional promotion of proinflammatory genes (Sankar *et al.*, 2006).

1.2 OVERVIEW OF ADAPTIVE IMMUNITY

Adaptive or acquired immunity utilises antigen-specific receptors on T and B cells to drive targeted effector responses. T and B lymphocytes originate in the bone marrow from progenitor cells. B cells remain in the bone marrow and undergo further development, whereas T cells migrate and mature in the thymus. The antigen-specificity of B cells is due to membrane-bound antibody whereas T cell specificity is attributed to the T cell receptor (TCR). Specificity in both cell types is due to random rearrangements of the genes encoding them resulting in the manifestation of a huge repertoire of receptors (Nemazee, 2006). Naïve lymphocytes enter peripheral lymphoid organs including lymph nodes, spleen, mucosal lymphoid tissue and gut-associated lymphoid tissue (GALT) and it is in these highly organised tissues that the majority of immune responses occur. Lymphocyte development ensures that each cell bears surface receptors for a single antigen, but the high degree of specificity combined with the huge lymphocyte repertoire means only a relatively small number of lymphocytes are able to recognise any given antigen. To increase the chances of antigen encounter, lymphocytes continually circulate between the blood and peripheral lymph nodes. It is only after they encounter their specific antigen (and co-stimulatory molecules) on an antigen-presenting cell that they are retained in the lymph node where they proliferate and differentiate into effector T cells (Ebert *et al.*, 2005; Bono *et al.*, 2007). The acquired response results in clonal expansion of specific lymphocytes over several days after initial activation. A number of these antigen-specific cells persist for some time after removal of initiating

antigen giving rise to memory cells and a more effective immune response to antigen re-exposure.

1.3 ANTIGEN PRESENTING CELLS

Antigen presenting cells (APC) are a heterogeneous family of cells capable of internalising, processing and presenting exogenous antigen to antigen-specific T cells as peptide-MHC complexes. They can be classified as “professional” APC, i.e. myeloid DC, which are uniquely capable of activating naïve and memory T cells, and the “non-professional” B cells, monocytes, macrophage and endothelial cells, which are more involved in the activation of memory T cells (Rutella & Lemoli, 2004).

Cell-mediated and humoral immune responses require cytokines from T helper (Th) cells. The fact that CD4⁺ T helper cells only recognise antigen when it is coupled to an MHCII molecule on the surface of an APC, ensures the regulated activation of Th cells, as does the fact that DC are the only APC that express MHCII and are simultaneously capable of delivering the co-stimulatory signals necessary to activate naïve T cells (Trombetta & Mellman, 2005).

1.3.1 DENDRITIC CELLS

Dendritic cells are bone marrow derived antigen presenting cells present in small numbers in most tissues encompassing less than 1% of total mononuclear cells. They have a disproportionately large influence on the immune response as they link innate and adaptive immunity by being the only APC capable of activating naïve T cells (Stagg *et al.*, 2003). They have two main functions; acquisition of antigen and stimulation of lymphocytes. Immature DC reside at sites of potential pathogen entry, constantly patrolling and sampling the environment for foreign antigen. Recognition of invading pathogens is mediated by a set of pattern recognition receptors that recognise conserved PAMPs (Sousa, 2004). One of the major families of receptors is the TLR family [*see section 1.1.2*] and TLR ligation leads to DC activation and immediately results in the production of proinflammatory cytokines, such as IL-2, IL-12, IL-15, and IL-18 (Andreaskos *et al.*, 2004) which contribute to local innate immune responses. TLR signalling

also initiates a program of DC maturation, which transforms the immature DC into a potent effector DC capable of driving naïve T cells to differentiate into effector T helper cells. During DC maturation there is an up-regulation of MHCII-peptide complex expression which determines the specificity of the T cell response (signal 1), as well as an increase in costimulatory molecule expression, such as CD80, CD86 and CD40, that determines the ability of naïve T cells to expand (signal 2). DC also express a selective set of T cell-polarizing molecules (signal 3); either soluble or membrane-bound that determines the balance between T helper cell subset development (Th1, Th2, Th17 or regulatory T cells). The expression profile of these T cell-polarizing molecules is determined when PAMPs bind to pattern recognition receptors (e.g., TLR) on the DC in their immature state [Reviewed by Kapsenberg (2003) and de Jong *et al.* (2005)].

1.3.2 DENDRITIC CELL ACTIVATION AND MATURATION

DC maturation is triggered by exogenous and/or endogenous mediators, including pro-inflammatory cytokines (IL-1 β , granulocyte-macrophage colony stimulating factor (GM-CSF), tumour necrosis factor- (TNF)- α), bacterial or viral components (lipopolysaccharide (LPS), double stranded DNA), and ligation of the TNF receptor family during interactions with T cells (e.g. CD40) (Rutella & Lemoli, 2004).

DC can capture exogenous antigen through various routes including macropinocytosis and receptor-mediated endocytosis (e.g. C-type lectin receptors (DEC205)). They can acquire immune complexes and opsonised particles via Fc γ -receptors, and are able to phagocytose bacteria and virus particles, all of which ultimately leads to cell activation (Banchereau *et al.*, 2000). Activation triggers a maturation process, and the DC gain the ability to stimulate naïve T cells whilst migrating to draining lymph nodes.

The maturation process is associated with several events including the loss of phagocytic/endocytic receptors, upregulation of MHCII and co-stimulatory molecules (CD80, CD86, CD40), and increased motility (Banchereau *et al.*, 2000). The maturation process also sees a rearrangement of chemokine receptors and adhesion molecules on the DC surface. CCR5, a receptor for CCL3 (also

known as macrophage inflammatory protein, or MIP-1 α) is highly expressed on immature DC and directs cells to sites of inflammation. Upon activation, CCR5 is down-regulated and receptors for lymphoid chemokines are increased, e.g. CCR7, whose ligand is CCL21 or MIP-3 α , which is found on lymphatic endothelial cells during inflammation and aids DC migration to T cell areas of lymphoid tissue (Imhof & Aurrand-Lions, 2004).

When DC reach the T cell rich areas of the lymph node they produce stimulatory cytokines (e.g. IL-12, IL-6). Once their sentinel function is complete, DC commit suicide by apoptosis, and in doing so, help restore homeostasis (Rescigno *et al.*, 1999).

1.3.3 ANTIGEN PROCESSING AND PRESENTATION

The major histocompatibility complex or MHC is a large genetic complex with multiple loci that encodes two MHC classes: MHCI is a complex of glycoproteins found on the membrane of nearly all nucleated cells. There are three class I loci in humans; A, B, and C, and two in mice; K and D. MHCII is a complex of heterodimeric glycoproteins consisting of an alpha and beta chain expressed by APC. There are three class II loci in humans (DR, DP, and DQ) and two in mice (IA and IE). Both MHCI and II genes are highly polymorphic, and therefore multiple MHCI and II molecules are expressed on immune cells and bind to a spectrum of antigenic peptides. Antigen is degraded and complexed with either MHCI or MHCII molecules depending on the route by which the antigen enters the cell. If the antigen is produced endogenously, i.e. within the cell, it is complexed to MHCI through an intracellular processing pathway. Alternatively, professional antigen presenting cells (dendritic cells, macrophage, and B cells) take up antigen by endocytosis or phagocytosis, and the exogenous antigen is processed and complexed to MHCII molecules before being expressed on the APC surface (Guermontprez *et al.*, 2002; Trombetta & Mellman, 2005).

The expression of MHCII genes is enhanced by inflammatory and immune stimuli, particularly cytokines such as interferon- (IFN)- γ , which stimulates the transcription of MHCII genes (Lee *et al.*, 2006). In immature DC, MHCII molecules are rapidly internalised and have a short half-life. The DC degrade

antigens within a MHCII-rich endosomal compartment, or MIIC, and are able to conserve sufficient peptide structure to present on their cell surface coupled to MHCII molecules (Trombetta & Mellman, 2005). After DC maturation, there is a burst of MHCII synthesis and translocation to the surface where it remains stably complexed to peptide for days and available for CD4⁺ T cells (Rescigno *et al.*, 1999). MHC-peptide complexes are 10-100 times higher on DC than macrophage or B cells (Banchereau & Steinman, 1998) making them the superlative APC.

1.3.4 ACTIVATION OF T CELLS

During its interaction with a DC, the T cell organises signalling molecules in a specialised junction known as the immunological synapse (IS). The IS facilitates the integration of multiple signals delivered by TCR and costimulatory receptor engagement, ensuring sustained signalling, gene transcription, and cell-cycle progression (Viola *et al.*, 2006).

The most common form of the TCR is composed of an α - and β -chain. These α - and β -chains are non-covalently associated with a complex of low-molecular weight transmembrane proteins, known as the CD3 complex (Alegre *et al.*, 2001) [see figure 1.1]. TCR ligation by MHC coupled to an antigen induces signal transduction through the CD3 complex into the nucleus, activating genes involved in T cell proliferation. Accessory molecules on the T cell must be simultaneously engaged by corresponding ligands on the APC to allow the T cell to become fully activated. Without these costimulatory signals, the T cells will either become anergic or die by apoptosis (Parkin & Cohen, 2001). Anergy occurs under two circumstances; one is if the TCR is engaged by a non-professional APC that lacks costimulatory molecules on its surface; the second is if TCR ligation is followed by the binding of cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) with CD28 on the T cell (Romagnani, 2006) [see section 1.5.1].

It is thought that MHC-peptide molecules can serially engage several TCRs on a single T cell, progressively amplifying the magnitude of intracellular signals, eventually crossing the threshold for T cell activation (Alegre *et al.*, 2001). It is only with prolonged TCR stimulation and co-stimulatory molecule ligation, in

the presence of polarising cytokines that naïve T cells become effector cells [section 1.4.2 describes the different T helper cell subsets and **Table 1.1** shows the cytokines involved in T helper cell differentiation]. This is because TCRs in naïve T cells are inefficiently coupled. The ligation of CD28 by B7 molecules recruits membrane rafts, containing kinases and adaptors, to the synapse, which amplifies the signalling process up to 100-fold (Sallusto & Lanzavecchia, 2002). It has been shown that naïve T cells require 6-30 hours of stimulation before committing to cell division, whereas memory/effector cells are able to respond within 0.5-2 hours. After engagement with antigen, the TCR is internalised and degraded and the differentiated cells lose their lymph-node homing receptors and acquire receptors that control their migration to inflamed tissues where they execute their effector functions (Sallusto & Lanzavecchia, 2002). Division and clonal expansion of each T cell results in up to 1000 progeny, most of which are armed effector cells (Parkin & Cohen, 2001).

Studies suggest that tissue-resident DC migrate to draining lymph nodes in the absence of stimulation as part of the homeostatic process, where they can tolerise any self-reactive T cells they come into contact with or generate regulatory T cells (Pasare & Medzhitov, 2004). Under inflammatory conditions lymph nodes receive large numbers of highly stimulatory DC for sustained periods leading to rapid proliferation and differentiation of antigen-specific T cells (Sallusto & Lanzavecchia, 2002).

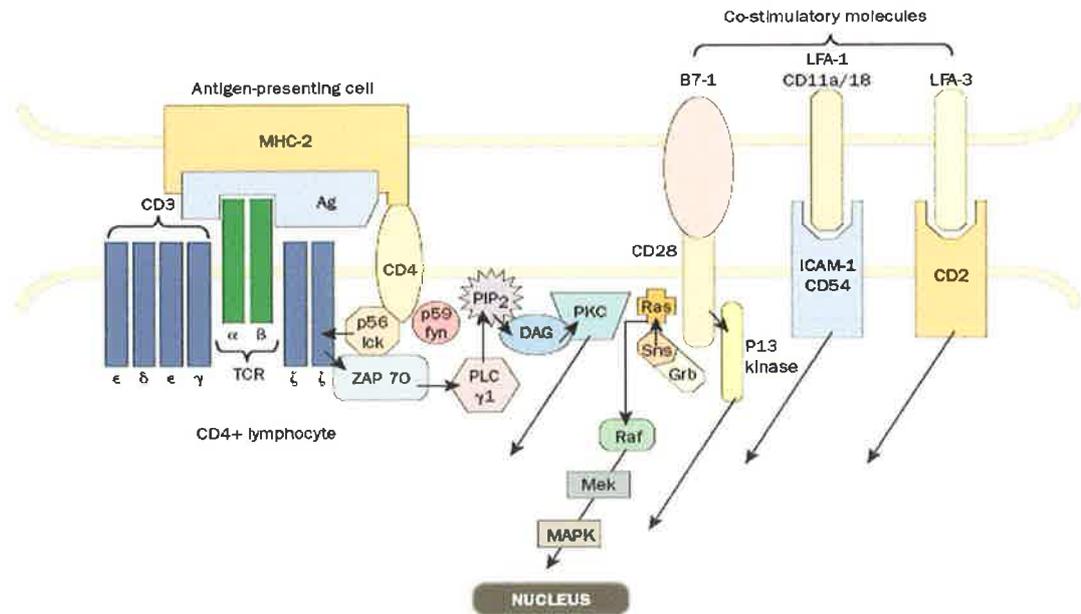


FIGURE 1.1 [Taken from Parkin & Cohen (2001)]

Diagrammatic representation of TCR ligation and the accessory molecules involved in during T cell activation. In T lymphocytes the receptor has two forms, the most common consists of an α and β chain. Cross-linking of the TCR with antigen-MHCII causes aggregation with the CD3 complex that then transmits signals into the cell eventually leading to T cell proliferation. Concurrent ligation of co-receptors on the surface of the T cell (co-stimulatory molecules such as CD80, CD86 and CD40) signal the cell to become activated. Without these cosignals the cell will become either anergic or apoptotic.

1.4 T CELLS

The precursors of T lymphocytes arise in the bone marrow and mature in the thymus. They are divided into functionally distinct populations; T helper cells and cytotoxic T cells (CTLs) characterised by the expression of CD4 and CD8 on their cell surface, respectively. T cells are able to recognise antigen complexed to MHC proteins; CD8⁺ T cells recognise MHCI-peptide complexes, whereas CD4⁺ T cells recognise MHCII-peptide complexes, but they are only activated if accessory molecules on the T cell are ligated (e.g. CD28 with B7-1 or B7-2 on APC) (Parkin & Cohen, 2001).

TABLE 1.1: Summary of the CD4⁺ T-helper subsets, the cytokines they secrete, the cytokines that influence their differentiation, and their main immunological functions.

T-HELPER SUBSET	Polarised by	Cytokines produced	Inhibited by	Protective activity
Th1	IL-12 ICAM-1 IL-27	IFN- γ IL-2 TNF- α/β	IL-4 IL-17	Cell-mediated immunity against intracellular bacteria & some viruses
Th2	IL-10 OX40L IL-4	IL-4 IL-5 IL-6 IL-13	IFN- γ IL-12	Humoral responses against helminth infections & multicellular organisms
Th17	IL-23 IL-1 β , IL-6 TNF- α , TGF- β (in mouse only)	IL-17A IL-17F IL-22 IL-6	IFN- γ IL-4	Extracellular bacteria & some fungi (<i>Klebsiella pneumoniae</i> ; <i>Candida albicans</i>)
Treg	IL-10 TGF- β	IL-10 TGF- β	IL-6	Homeostasis – self-tolerance & control of excessive responses against non-self

1.4.1 CD8⁺ T CELLS

Cytotoxic T cells (CD8⁺) recognise MHC I-peptide complexes. They become activated and proliferate but do not generally secrete many cytokines. Instead they exhibit cytotoxic activity, eliminating tumour cells, cells that display viral antigens and any cells displaying foreign antigen coupled to MHC I (Parkin & Cohen, 2001).

1.4.2 CD4⁺ T CELLS

Many cell types are involved in an immune response, but CD4⁺ T cells are critical in determining the outcome of any infection. The direction of the ensuing immune response is resultant on the cytokines CD4⁺ T cells secrete, which act as

growth and differentiation factors for themselves and other cell types (Szabo *et al.*, 2003).

CD4⁺ T lymphocytes differentiate into distinct effector cell subsets characterised by their function and cytokine production profile. The fate of naïve T cells is determined by three signals: Firstly ligation of TCRs by MHCII-peptide complexes presented by DC. Then co-stimulation (discussed below). The third signal depends on the conditions under which the DC are primed and is imprinted by the binding of pathogens to DC via pattern recognition receptors (e.g., TLR) [see section 1.1.2] (Kapsenberg, 2003). Until fairly recently the general consensus was that there were only 2 types of T helper lineages; Th1 and Th2. **Figure 1.2** below depicts the processes involved in the polarisation of Th1 and Th2 cells.

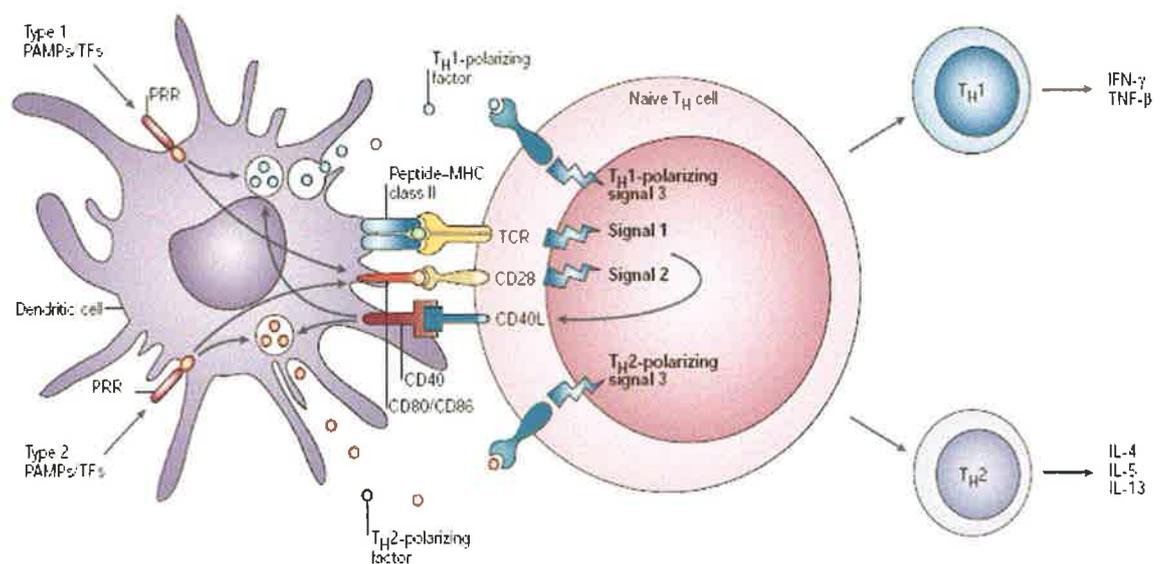


FIGURE 1.2 [Taken from Kapsenberg (2003)]

T-cell stimulation and Th1/Th2-cell polarisation require three DC-derived signals; Signal 1 is the antigen-specific signal via TCR ligation with MHC class-II-peptide complexes: Signal 2 is the co-stimulatory signal, mainly mediated by triggering of CD28 by CD80 and CD86 on DC: And signal 3 is the polarizing signal that is mediated by various soluble or membrane-bound factors, such as IL-12, and depends on the activation of particular PRRs by PAMPs

1.4.2.1 TH1 AND TH2 CELL DEVELOPMENT

The development of naïve T cells into a Th1 or Th2 phenotype is greatly influenced by the surrounding cytokine milieu; Th1 differentiation is regulated by IL-12, IL-27, type I IFNs, and IFN- γ ; whereas Th2 differentiation is largely influenced by IL-4 (Agnello *et al.*, 2003). Other Th1-polarising factors include transcription factors, such as T-bet, signal transducer and activator of transcription- (STAT)-1, and STAT-4, and the adhesion molecule, ICAM-1 (intracellular adhesion molecule-1), while transcription factors, including STAT6 and GATA-3, and the costimulatory molecule, OX40L promote Th2 differentiation (Agnello *et al.*, 2003).

The Th1 and Th2 subsets have a relatively restricted cytokine production profile; Th1 cells produce IL-2, TNF- β , and IFN- γ , which activate macrophage, natural killer (NK) cells, and CTL, and are the principal effectors of cell-mediated immunity and delayed type hypersensitivity (DTH), primarily targeting intracellular pathogens (Trinchieri, 2003). Th2 cells produce IL-4, (which stimulates IgE production), IL-5, (an eosinophil activating factor), and IL-10 and IL-13, which together with IL-4 suppress cell-mediated immunity and inflammation (Gouwy *et al.*, 2005). Extracellular pathogens, such as helminthic worms, tend to induce differentiation along the Th2 pathway. One important aspect of the Th1/Th2 paradigm is that Th1-type cytokines, especially IFN- γ , inhibit the differentiation of Th2 cells, while Th2-type cytokines, especially IL-4, inhibit the differentiation of Th1 cells (Calder, 2002). This inhibitory feedback mechanism aids to further polarise the T helper response.

Other than classification via cytokine profiles, several surface markers have been recognized that distinguish Th1 and Th2 cell populations. For example, the IL-12 receptor- β 2 (IL-12R β 2) and the chemokine receptors CXCR3 and CCR5 are preferentially expressed on Th1 cells. In contrast CCR3, CCR4, and CCR8 are more common on Th2 cells (Langenkamp *et al.*, 2003).

1.4.2.2 TH17 CELLS

The recently discovered Th17 cells were found to develop via a lineage distinct from Th1 and Th2 cells by (Harrington *et al.*, 2005; Mangan *et al.*, 2006). Th17 cells are characterised by the production of a distinct cytokine profile, including IL-17 and IL-6, and are thought to have evolved to combat pathogens distinct

from those targeted by Th1 and Th2 cells (Weaver *et al.*, 2006). IL-17 has emerged as a key pro-inflammatory cytokine and is known to induce the production of IL-1, TNF- α , and GM-CSF. Differentiation of this subset in mice is controlled by transforming growth factor- (TGF)- β , IL-6, and IL-23, and inhibited by IL-4 and IFN- γ as naïve murine T cells cultured with TGF- β and IL-6, in the absence of IFN- γ and IL-4 were found to differentiate into Th17 cells and up-regulate the IL-23R (Kikly *et al.*, 2006). IL-23, a member of the IL-12 family, appears to be required for the expansion and survival of the Th17 subset but not for their initial differentiation (Tato & O'Shea, 2006). A recent paper by Sutton and colleagues (2006) addresses the possibility that IL-1 is also involved in promoting pathogenic Th17 cells in a murine model of EAE (experimental autoimmune encephalomyelitis - animal model equivalent to multiple sclerosis). Sutton and colleagues also reported that TNF- α synergizes with IL-23 to enhance IL-17 production in an IL-1-dependant manner in the same model. It is important to note here that although TGF- β has been shown to be involved in Th17 cell differentiation in mice, it is not essential for Th17 cell development in humans (Acosta-Rodriguez *et al.*, 2007; McGeachy & Cua, 2008).

It is unknown what Th17 cells do under steady-state conditions, but they have been shown to be key in the clearance of some extracellular bacteria and fungi (e.g. *Klebsiella pneumoniae* and *Candida albicans*), and have been implicated as being responsible for many inflammatory disorders previously attributed to Th1 cells, including rheumatoid arthritis and EAE (Romagnani, 2006). Th17 cells are thought to illicit inflammatory responses by the actions of their cytokine production on fibroblasts, endothelial cells, epithelial cells, and macrophage, and through the recruitment of polymorphonuclear leukocytes (Romagnani, 2006)

1.4.2.3 T REGULATORY CELLS

T helper phenotypes are regulated by a heterogenous family of cells known as T regulatory cells or Tregs characterised by their ability to suppress adaptive T cell responses and help prevent autoimmunity [reviewed by Mills (2004)].

In general, Tregs can directly target effector T cells, compete with pathogenic T cells for access to APC, or directly target APC. For example, DC co-cultured with CD4⁺CD25⁺ regulatory T cells down-regulate the expression of costimulatory molecules, release large amounts of IL-10 and become unable to

effectively trigger T cell activation (Rutella & Lemoli, 2004). There are at least two types of Tregs; CD4⁺CD25⁺ Tregs that are developmentally programmed and maintain tolerance to self antigen, and those that are inducible and thought to control excessive inflammation by producing large amounts of IL-10 and TGF- β (Iwasaki & Medzhitov, 2004).

Naturally occurring CD4⁺CD25⁺ Tregs constitutively express the IL-2 receptor α -chain (CD25). In contrast to recently activated T cells that express CD25 at a low and transient level, CD4⁺CD25⁺ Tregs have a high and sustained level of CD25 expression (Rutella & Lemoli, 2004). CD4⁺CD25⁺ Tregs reside in secondary lymphoid organs and their development in the thymus is controlled by the transcription factor fork-head box P3 (Foxp3). Evidence suggests that these cells have evolved to prevent activation of auto-reactive T cells that have escaped thymic deletion and they are therefore primarily involved in central and peripheral tolerance to autoantigens (Pasare & Medzhitov, 2004) and exert potent immunosuppressive functions, suppressing IFN- γ production and the proliferation of both CD4⁺ and CD8⁺ T cells via cell contact and IL-2 inhibition. Approximately half of CD4⁺CD25⁺ Tregs constitutively express CTLA-4 and/or membrane-bound TGF- β , both of which are thought to be involved in their regulatory function (Rutella & Lemoli, 2004; Bacchetta *et al.*, 2005).

A second set of suppressor cells known as inducible or adaptive regulatory T cells include Tr1 and Th3 cells. They originate, like Th1 and Th2 cells, from uncommitted peripheral naive or central memory Th cells following activation by antigen-presenting DC under certain immunological situations. For example, the absence of costimulatory molecules on immature DC induces T cell tolerance following repetitive TCR stimulation (de Jong *et al.*, 2005). It is interesting to note that the immature state of DC can be maintained even after activation in the presence of certain anti-inflammatory cytokines, such as IL-10 or TGF- β , suggesting an inhibitory feedback loop between adaptive Tregs and tolerogenic DC (de Jong *et al.*, 2005). Adaptive Tregs appear to have evolved to prevent destruction of host tissues caused by chronic inflammation. They respond to innocuous foreign antigens by secreting immunosuppressive cytokines; Tr1 cells secrete high levels of IL-10; and Th3 cells secrete high levels of TGF- β , and they are associated with mucosal tolerance to ubiquitous antigens and non-pathogenic microflora (Pasare & Medzhitov, 2004). IL-10 inhibits the production of TNF- α

and IL-12 by DC and macrophage, and down-regulate the expression of MHCII and costimulatory molecules on DC, whereas TGF- β impedes Th1 responses through its inhibitory effects on the transcription factor T-bet and IL-12R expression (Mills, 2004).

1.5 COSTIMULATION/COINHIBITION

Costimulatory signals are defined as those that act in conjunction with antigen-receptor signals, leading to growth-factor production, cell activation, and cell proliferation and survival. In contrast, co-inhibitory signals attenuate antigen-receptor signals, resulting in decreased cell activation, limited growth-factor production, inhibition of cell cycle progression, and sometimes increased cell death (Collins *et al.*, 2005).

Engagement of costimulatory molecules in the course of DC-T cell interactions is critical for generating efficient T helper responses, as absence of co-stimulation can result in cell anergy or apoptosis (Alegre *et al.*, 2001). On the other hand, inappropriate or poorly controlled effector T cells can cause host pathology and are particularly harmful if directed against a self-antigen or commensal flora, therefore effector T cell responses are under stringent regulatory control (Weaver *et al.*, 2006)

1.5.1 CD80, CD86, CD28, AND CTLA-4 INTERACTIONS

The B7 ligands, B7.1 (CD80) and B7.2 (CD86), and their receptors CD28 and CTLA-4 (CD152), exemplify a costimulatory-coinhibitory system that acts to regulate immune responses.

CD80 and CD86 are type I transmembrane proteins primarily expressed on DC that share 25% sequence homology and interact with the same receptors on T cells, namely CD28 and CTLA-4 (Bhatia *et al.*, 2006). Engagement of CD28 with B7 ligands delivers a positive signal, resulting in T cell proliferation, cytokine production and prevention of Treg induction, whereas the binding of CTLA-4 with B7 results in negative signalling leading to attenuation of T cell activation and induction of T cell anergy (Bhatia *et al.*, 2006).

CD86 is constitutively expressed on the cell surface of APC and is rapidly upregulated upon interaction with CD28 with maximal expression after 48 hours. The expression of CD80 is more slowly induced and not detectable until 24 hours post-stimulation, with maximal expression 48-72 hours later. It is also stable for longer (up to 4-5 days) (Maerten *et al.*, 2003). It has been suggested that CD80 favours Th1 development, while CD86 augments the Th2 response (Maerten *et al.*, 2003), and that CD80 favours binding to CTLA-4 whereas CD86 shows a preference for CD28 (Bhatia *et al.*, 2006). The preferential binding of CD86 to CD28 (and CD80 to CTLA-4) may partly be explained by expression kinetics; CD28 and CD86 are both constitutively expressed on T cells and APC respectively, and are available at the time of T cell activation. CD86 appears to be a weaker ligand than CD80, and the relatively weak interaction between CD28/CD86 may be enough to initiate T cell activation. As the T cell becomes activated, the expression of CTLA-4 is induced on T cells along with the expression of CD80 on APC. The stronger interaction between CD80 and CTLA-4 may override the weak interaction between CD86 and CD28 thereby delivering a negative signal to the T cell (Bhatia *et al.*, 2006). Evidence also suggests that CD80 has a superior affinity for CTLA-4 due to its dimeric structure, whereas CD86 has a relatively weak affinity and appears monomeric in structure (Manzotti *et al.*, 2006). CD86 knockout mice have a more severe immune deficiency compared to CD80 knockouts, suggesting CD86 is a more important costimulatory molecule. However, this may be because CD86 expression is more abundant, which makes it difficult to differentiate between functional and expressional differences (Manzotti *et al.*, 2006).

1.5.1.1 CTLA-4 (CYTOTOXIC T LYMPHOCYTE-ASSOCIATED ANTIGEN 4)

CTLA-4 binds B7 molecules with a 20-fold higher affinity than CD28. CD28 is constitutively expressed on resting CD4⁺ cells whereas CTLA-4 is only upregulated following T cell activation and has a more complex expression pattern (Manzotti *et al.*, 2006). In resting T cells, CTLA-4 is predominantly found in intracellular vesicles. Upon activation, it is transported to the T cell surface where it is rapidly internalised and undergoes a pattern of recycling. CD28 has a slow turnover rate, whereas CTLA-4 has a half life of 2 hours, which still seems to be sufficient to terminate T cell activation (Bhatia *et al.*, 2006).

CTLA-4 regulates T cell responses by facilitating apoptosis, and suppressing cytokine production. Binding inhibits the production of IL-2 from T cells and reduces the expression of the IL-2R, and can also arrest cells in the G1 phase of the cell cycle (Ly *et al.*, 2006).

The immunosuppressive importance of CTLA-4 is shown in knock-out mice, which develop a lethal lymphoproliferative disease with progressive accumulation of T cells at peripheral lymphoid organs, as well as in solid organs (heart, lungs, liver) (Alegre *et al.*, 2001). Engagement of CTLA-4 seems to reduce the proportion of cells that proliferate or secrete cytokines, rather than reduce the magnitude of the response, meaning more APC are required to elicit a response in the presence of CTLA-4 ligation. This suggests that CTLA-4 raises the threshold for T cell activation. It is unknown whether CTLA-4 antagonises CD28 by binding B7s, or directly/indirectly reduces TCR signals, but CTLA-4 cross-linking has been shown to inhibit activation of the MAPKs, Jun amino-terminal kinase (JNK) and extracellular signal-related kinase (ERK), and to reduce the activation of several transcription factors (NF κ B, NFAT and AP-1) (Alegre *et al.*, 2001). CTLA-4/TCR co-ligation has also been shown to induce TGF- β production by naïve CD4⁺ T cells, suggesting a potential immunosuppressive mechanism (Maerten *et al.*, 2003). Another possible mechanism of action has been proposed by Schneider and colleagues (2006): It is known that TCR ligation reduces or arrests T cell motility (the stop signal), an event that is required for stable T cell-APC interaction and efficient activation. Schneider and colleagues found that the ligation of anti-CD3 reduced the movement of mouse and human primary T cells, whereas the co-ligation of CTLA-4 reversed the arrest such that treated and untreated cells moved at similar speeds. Their findings suggest that CTLA-4 overrides the stop signal induced by the antigen receptor complex. By limiting T cell-APC contact times, CTLA-4 would reduce the efficiency of antigen presentation, and the number of TCR ligation events, resulting in reduced T cell signalling and activation.

1.5.2 CD40-CD40L

This is the most widely studied co-stimulatory pathway. The interaction between CD40 and CD40L is bidirectional, since it not only activates APC but also

enhances T cell activation. CD40 is a member of the TNF receptor superfamily and expressed most notably on APC (DC, macrophage, B cells) (van Kooten & Banchereau, 2000). Its ligand, CD40L (CD154), is principally expressed on activated CD4⁺ T cells, but also found on NK cells, B cells, CD8⁺ T cells and basophils (Danese *et al.*, 2004). CD28 engagement enhances and maintains CD40L expression on activated T cells, and cytokines, including IL-2, IL-12, and IL-15, have also been shown to upregulate its expression (Maerten *et al.*, 2003). CD40-CD40L interaction on monocytes and DC results in enhanced cell survival, secretion of cytokines: IL-1, IL-6, IL-8, IL-10, IL-12, TNF- α , MIP-1 α , and the upregulation of costimulatory molecules: CD54/ICAM-1, CD58/LFA-1, CD80, and CD86 (van Kooten & Banchereau, 2000). Importantly, CD40 stimulation induces DC to produce IL-12, and therefore has a role in Th1 polarisation (Danese *et al.*, 2004). Binding initiates various MAPK signalling pathways (including ERK, JNK, p38) ultimately resulting in the activation of transcription factors including NF κ B (Danese *et al.*, 2004). In DC, CD40 expression seems to be a critical step in final maturation into a competent antigen-presenting cell as it has been shown that CD40/CD40L knockout mice have abnormalities in their ability to prime naïve CD4⁺ T cells (Watts, 2005). Increased expression of CD40 and CD40L have been found in the ileum and colon of patients with Crohn's disease (CD) and ulcerative colitis (UC) respectively and the CD40-CD40L pathway participates in the pathogenesis of inflammatory bowel disease (IBD) especially as a result of macrophage activation and induction of IL-12 production (Maerten *et al.*, 2003).

1.5.3 OX40-OX40L

OX40 ligand (OX40L) is a type II transmembrane protein expressed on active B cells, DC and macrophage, and on activated T cells. It can also be found on endothelial cells at the site of inflammation possibly aiding extravasation of OX40-positive T cells (Maerten *et al.*, 2003). OX40L on APC has been shown to be required for the sustained activation of T cells (Grunig *et al.*, 2005), and OX40L signalling is important in human DC differentiation leading to increased production of IL-12, TNF- α , and IL-6 (Maerten *et al.*, 2003). Engagement of CD40 upregulates the expression of OX40L, and OX40-OX40L interactions

enhances CD40 and B7 expression on APC (Maerten *et al.*, 2003), exemplifying a positive feedback mechanism for costimulatory molecule expression.

OX40 (CD134) is a transmembrane protein whose expression is restricted to CD4⁺ T cells *in vivo* (also found on CD8⁺ *in vitro*) (Barr *et al.*, 2006). Ligation of OX40 enhances activation, proliferation and survival of T cells, and has been shown to promote both Th1 and Th2 cytokine production (Maerten *et al.*, 2003). OX40 is preferentially expressed on Th2 cells in some models, but it can also be expressed on Th1 cells and is therefore capable of affecting both Th1 and Th2 responses [reviewed by Watts (2005)]. Watts suggests that OX40 does not determine whether a response becomes Th1 or Th2, but rather contributes to ongoing Th1 or Th2 development. It may have a greater effect on Th2 cells owing to their higher level of OX40 expression. Ito and colleagues (2006b) suggest that OX40L may control the magnitude of the Th response, as OX40L inhibited the generation of IL-10 producing Tr1 cells during IL-12-induced Th1 responses or IL-4-induced Th2 responses. Indeed dysregulated OX40 expression may have a role in chronic infection as it has been detected for prolonged periods in the central nervous system of mice with EAE, as well as in inflamed tissues in a number of autoimmune and inflammatory diseases in mouse and human (Watts, 2005).

1.5.4 ICAM-1 (INTERCELLULAR ADHESION MOLECULE-1)

Adhesion molecules are surface-bound molecules involved in cell contact interactions, directing cell migration, phagocytosis and cellular cytotoxicity. Signal transduction after ligation of the adhesion molecule, also leads to cell activation, alteration in receptor expression, cytokine production, and effects on cell survival. Cells can express adhesion molecules constitutively, or upregulate them following exposure to cytokines, chemokines, or other proinflammatory molecules. Some adhesion molecules are expressed mainly on leukocytes, and others on endothelial cells, enabling interaction between the two (Weaver *et al.*, 2006). One such adhesion molecule, ICAM-1, is found on DC and binds leukocyte function-associated molecule-1 (LFA-1) on T helper cells (de Jong *et al.*, 2005). DC acquire ICAM-1 expression during their maturation and the ICAM-1/LFA-1 interaction supports cell adhesion during DC-T cell contact.

ICAM-1 has also been implicated in the induction of Th1 responses, particularly in the absence of T cell-polarizing cytokines, such as IL-4 or IL-12. The molecular mechanisms underlying this Th1 polarization are thought to involve the MAP-kinases ERK and JNK (de Jong *et al.*, 2005).

1.5.5 CCR5

Chemokines are small cytokines with chemoattractant properties that are able to coordinate tissue homeostasis and inflammation. Consequently, the dysregulated expression of chemokines and their receptors are involved in the development of several pathologies, including autoimmunity and chronic inflammation.

CCR5 is a chemokine receptor expressed on resting and memory T cells (primarily of the Th1 phenotype), monocytes, macrophage, and immature DC (Oppermann, 2004), and its expression is upregulated by various proinflammatory mediators (Blanpain *et al.*, 2002). Expression of 'inflammatory' chemokine receptors, such as CCR5, allows leukocytes to respond to inflammatory chemokines, (including MIP-1 α and MIP-1 β) which are secreted by monocytes/macrophage, neutrophils, DC, and endothelium, directing immune cells to sites of inflammation (Luster *et al.*, 2005).

During maturation in inflamed tissues, DC down-regulate the expression of CCR5, and upregulate CCR7 expression. CCR7 binds to chemokines expressed on endothelial cells lining the lymphatic vessels, facilitating the migration of activated DC to the T-cell-rich areas of draining lymph nodes, where they await interaction with antigen-specific T cells (Cravens & Lipsky, 2002). Viola and colleagues (2006) recently demonstrated that, among others, the T-cell chemokine receptor CCR5 is recruited into the IS during T-cell-APC interactions, resulting in the formation of a stable IS and reinforcing the attraction between T cells and their partners.

1.6 CYTOKINES

Complex interactions between cells of the immune system are partly mediated by a group of low molecular weight proteins called cytokines. Cytokines are produced by virtually all immune cells and have a wide variety of functions: Their biological effect depends on the cytokine and the cell involved, but typically they affect cell activation, division, apoptosis, or movement, and are able to mediate biological effects at only picomolar concentrations due the high affinity between cytokine and receptor (Parkin & Cohen, 2001). Cytokines produced by leukocytes and having effects mainly on other white cells are termed interleukins (ILs). Cytokines that have chemoattractant activity are called chemokines. Those that cause differentiation and proliferation of stem cells are called colony-stimulating factors, and those that interfere with viral replication are called interferons (Parkin & Cohen, 2001). Cytokines can also be segregated according to their biological function; those that promote inflammation (i.e. Th1/Th17 responses) are termed pro-inflammatory cytokines, and include IFN- γ , TNF- α , IL-17, IL-12, and IL-1 β ; while those generated during a Th2 or regulatory response are generally considered anti-inflammatory, for example, IL-4, IL-10, and TGF- β (Hill & Sarvetnick, 2002). However, this concept is debatable as some pro-inflammatory cytokines can activate homeostatic mechanisms to suppress inflammation, whilst some “anti-inflammatory” cytokines have a role in autoimmunity, and therefore have dual activity (Hill & Sarvetnick, 2002). Examples of the regulatory mechanisms controlling the production and various actions of some key cytokines are listed in **Table 1.2** and will be considered in more detail in the following sections:

TABLE 1.2 Summary of some key cytokines highlighting their source and mode of action

CYTOKINE	SOURCE	MODE OF ACTION	PRO/ANTI-INFLAMMATORY
IL-1 β	Monocytes, macrophage, B cells, DC	Induces inflammatory response; co-stimulates T cells, enhances NK cell activity, chemoattractant	Pro-inflammatory
IL-2	Active T cells (CD4 ⁺ & CD8 ⁺)	T cell growth factor, but has regulatory roles & can promote AICD in T cells	Both
IL-4	Active Th2 cells	Th2 polarising cytokine, but can synergise with IL-12 to induce IFN- γ production from T cells	Anti-inflammatory (Th2)
IL-5	Active Th2 cells	Stimulates proliferation & differentiation of B cells, promotes eosinophil activation	Anti-inflammatory (Th2)
IL-6	Monocytes, macrophage, DC, Th17 cells	Promotes inflammation, activates T & B cells, & involved in Th17 cell differentiation	Pro-inflammatory [but also drives B cell activation]
IL-10	Lymphocytes, DC, macrophage	Immunosuppressive & regulatory functions; inhibits Th1 response & cytokine production	Anti-inflammatory (Treg)
IL-12	Macrophage, monocytes, DC, neutrophils	Directs Th1 cell development, & stimulates APC, NK cells, & CD8 ⁺ CTLs	Pro-inflammatory
IL-13	Active Th2 cells	Activates B cells & induces isotype switching, generally immunosuppressive	Anti-inflammatory (Th2)
IL-17	Th17 CD4 ⁺ T cells	Induces cytokine & chemokine production, DC maturation, & neutrophil chemotaxis	Pro-inflammatory (Th17)
IL-18	Monocytes, macrophage & DC	Induces low levels of IFN- γ alone, but serves as a cofactor for IL-12-induced Th1 development	Pro-inflammatory
IL-23	Monocytes, macrophage & DC	Expansion & survival of Th17 cells, induces proinflammatory cytokine production	Pro-inflammatory
IL-27	Monocytes, macrophage, & DC	Member of IL-12 family & aids IL-12 & IL-18 in Th1 cell development	Pro-inflammatory
IFN- γ	Th1 & CD8 ⁺ T cells, NK cells	Th1 expansion, induction of cytokine & chemokine secretion, increases NK cell function	Pro-inflammatory (Th1)
TNF- α	Macrophage, DC, T cells, NK cells	Amplifies inflammation & induces cytokine release	Pro-inflammatory
TGF- β	T cells (especially Th3) & many non-lymphoid cells	Immunosuppressive but involved in Th17 cell differentiation	Anti-inflammatory but multi-faceted (mainly Treg)

1.6.1 INTERLEUKIN-12 (IL-12)

IL-12 is a heterodimeric cytokine composed of a 35-kDa light chain (p35) and a 40-kDa heavy chain (p40). The genes encoding the two sub-units are located on different chromosomes, and both genes need to be expressed co-ordinately in the same cell to produce the biologically active heterodimer, IL-12p70 (Trinchieri, 2003). Besides forming IL-12p70, p40 can dimerise with a p19 sub-unit to form IL-23, which has functions similar to, yet distinct from, IL-12 (Watford *et al.*, 2003) [see section 1.6.2]. There is an almost ubiquitous cellular expression of p35 mRNA (albeit at low levels); whereas the expression of p40 is restricted to cells that produce the biologically active IL-12p70 heterodimer. The less abundant p35 sub-unit is considered the rate-limiting factor for IL-12p70 production, since the production of p40 exceeds that of p35 by 40-500 fold (Aste-Amezaga *et al.*, 1998). Studies in mice suggest that excess p40 is secreted as a homodimer (referred to as p80) and as a monomer: Murine p80 binds to the IL-12R with a similar affinity to the heterodimer and is therefore a natural inhibitor (Trinchieri, 2003). However, much less is known about the availability of this homodimer in humans. Its existence is questionable, and its immunological role uncertain [for a review see Cooper & Khader (2007)]. Naturally occurring IL-12p80 has been detected in the bronchoalveolar lavage (BAL) of human asthma sufferers (Walter *et al.*, 2001) suggesting that this molecule may occur under specific conditions, but more research is required to fully ascertain its immunological existence and relevance.

Similar to other pro-inflammatory cytokines, the production of IL-12 is strictly controlled by positive and negative regulatory mechanisms. IL-12p40 and p70 are produced by monocytes, macrophage, dendritic cells, and neutrophils, and induced by a variety of pathogenic organisms, including gram positive and negative bacteria, viruses, and fungi (Watford *et al.*, 2003). Various cytokines, most importantly IFN- γ , but also TNF- α , and GMSCF, can increase the ability of cells to produce IL-12 (Hasko & Szabo, 1999). Interactions with antigen, or ligation of CD40 by CD40L during T cell-APC interactions also augment IL-12 production by macrophage and DC (Hasko & Szabo, 1999), and stimulation through CD28 upregulates the expression of the IL-12R in T cells (Trinchieri, 2003). Surprisingly, the two Th2 cytokines, IL-4 and IL-13, are also potent

enhancers of IL-12 production. At early time points (<24 hours), they inhibit p40 production, whereas at later times, they strongly enhance it (Trinchieri, 2003). IL-10 is an efficient inhibitor of IL-12 production blocking the transcription of both genes. TGF- β inhibits IL-12 production by reducing the stability of IL-12p40 mRNA, and IFN- α and IFN- β have also been shown to suppress IL-12 production (Trinchieri, 2003).

The high affinity IL-12 receptor requires the co-expression of 2 sub-units; IL-12R- β 1 and - β 2, and is expressed on macrophage, DC, NK cells and active T cells (Hasko & Szabo, 1999). It is the β 2 sub-unit that permits signal transduction after receptor ligation by providing a cytoplasmic STAT-4 binding site allowing STAT-4 mediated responses to IL-12 to occur (Brombacher *et al.*, 2003). Both IL-12R subunits are upregulated on macrophage and DC following their activation (Bastos *et al.*, 2004). TCR ligation, IL-12 itself, IFN- γ , TNF- α , and co-stimulation through CD28 all upregulate the transcription and expression of the IL-12R on T cells. In contrast, IL-4, IL-10, and TGF- β generally decrease receptor expression and IL-12 responsiveness (Trinchieri, 2003).

IL-12 is an important link between innate and adaptive immunity as its targets include T cells, DC, and NK cells, and one of its main functions is to direct newly activate T helper cells to a Th1 phenotype (Hasko & Szabo, 1999) [See **Figure 1.4** for a diagrammatic illustration of the main roles of IL-12]. IL-12 can upregulate MHCII, CD80 and CD86 expression on APC (Bastos *et al.*, 2004), and in T cells it synergises with IL-2, TCR-CD3 signalling, and CD28 ligation, to rapidly induce IFN- γ production. IL-12 can also enhance the generation of CTLs by augmenting their cytotoxic activity (Trinchieri, 2003).

IL-12 has been shown to be directly and predominantly involved in autoimmune diseases including multiple sclerosis (MS), IBD, and rheumatoid arthritis (RA), and overproduction is important in inflammatory states such as septic shock (Hasko & Szabo, 1999) consequently it represents a potential therapeutic target for the treatment of these inflammatory disorders. As it shares the common p40 subunit and the IL-12R β 1 receptor subunit with IL-12, IL-23 was predicted to have a function similar to that of IL-12. However studies using p19 and p40 knock-out mice showed an abrogation of some inflammatory diseases suggesting that IL-23 is in fact responsible for much of the autoimmune pathology previously attributed to IL-12 (Zhang *et al.*, 2007).

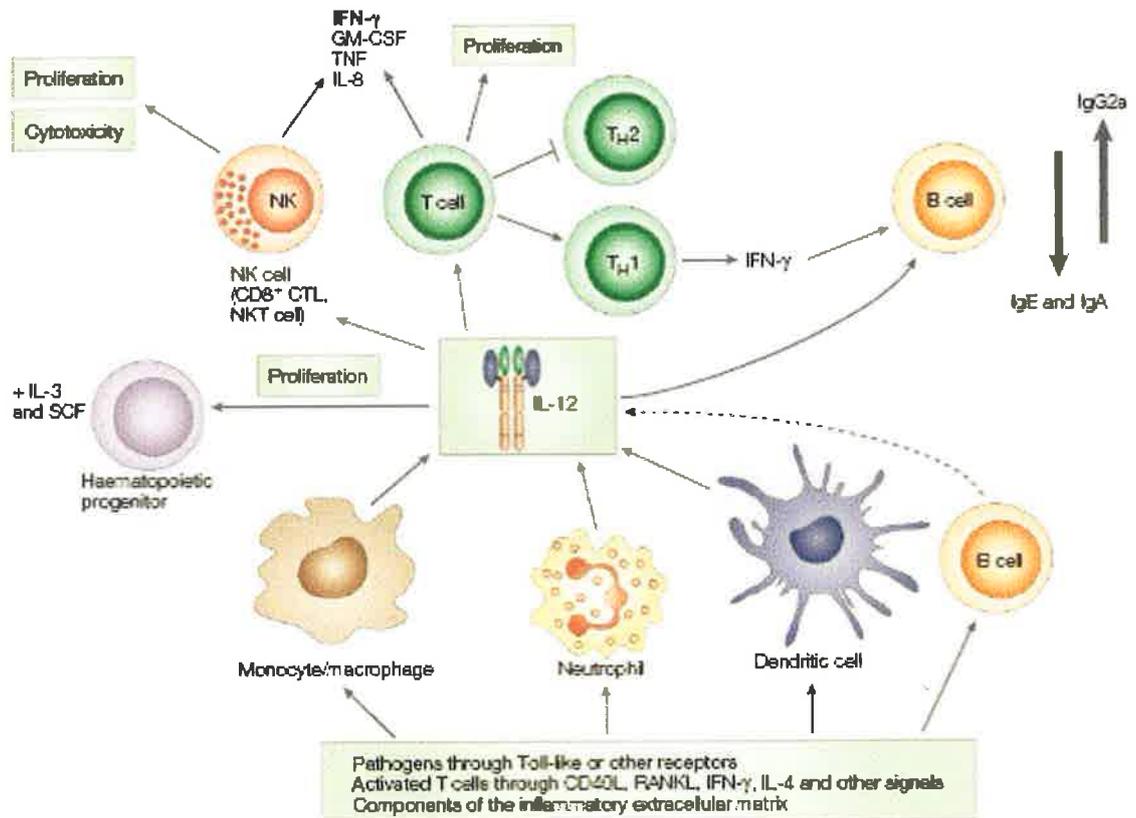


FIGURE 1.4 [Taken from Trinchieri (2003)]

Diagrammatic representation of the major biological effects of the cytokine IL-12p70.

1.6.2 IL-23

IL-23 is a heterodimeric cytokine belonging to the IL-12 family. It consists of a p40 sub-unit (shared with IL-12) and a novel p19 sub-unit. Similar to IL-12, the formation of biologically active IL-23 requires the synthesis of both subunits (p19 and p40) within the same cell (Langrish *et al.*, 2004). IL-23 is mainly produced by activated myeloid cells such as DC and macrophage (Zhang *et al.*, 2007). The IL-23 receptor consists of two sub-units; IL-23R which binds p19, and IL-12R β 1 which binds p40 (de Jong *et al.*, 2005). The IL-23R is not expressed on naïve T cells, but is predominantly found on activated/memory T cells, T cell clones, and NK cells, and found at low levels on monocytes, macrophage, and DC (Langrish *et al.*, 2004). IL-23 is associated with the expansion and survival of already polarised IL-17-producing T helper cells (Th17), but does not appear to be necessary for their initial differentiation (Bettelli *et al.*, 2007) [see section 1.4.2.2]. It was recently shown that TGF- β and

IL-6 upregulate IL-23R expression, demonstrating that they too are involved in the generation of Th17 cells (Zhang *et al.*, 2007).

The role of IL-23 rather than IL-12 in autoimmune diseases was resolved using knock out mice and antibodies to p40 and p19. Mice lacking p19 but not p40 were resistant to disease induction in EAE and IBD models. And administration of anti-p19 or anti-p40 antibodies (but not anti-p35) inhibited the production of a range of proinflammatory cytokines including, IL-17, IL-6, IFN- γ , IL-1 β and TNF- α (Langrish *et al.*, 2005; Kikly *et al.*, 2006) implicating IL-23 as a major causative agent of inflammatory pathology, and not IL-12 as originally thought.

1.6.3 IL-17

IL-17 is mainly secreted by CD4⁺ Th17 cells. It acts *in vitro* and *in vivo* as a potent inflammatory cytokine, coordinating tissue inflammation by inducing the expression of proinflammatory cytokines, (such as IL-1, IL-6, IL-8, and TNF- α), and chemokines (MCP-1 and MIP-2), which mediate immune cell infiltration and tissue destruction (Langrish *et al.*, 2004). IL-17 is involved in the proliferation, maturation and chemotaxis of neutrophils, co-stimulates T cells, and enhances the maturation of dendritic cells (Kolls & Linden, 2004). The IL-17R has an extensive distribution and is found in various tissues and cells including, lungs and liver, epithelial cells, lymphocytes, and various myeloid cell types (Kolls & Linden, 2004). Signalling through the IL-17R has been reported to activate all three classes of MAP kinases (ERK, JNK, and p38), and to activate NF κ B (Zhang *et al.*, 2007), leading to T cell proliferation and the production of inflammatory mediators (Langrish *et al.*, 2004). Experimental evidence implicates IL-17 as being responsible for some of the pathology seen in autoimmune diseases, such as RA, where elevated levels of IL-17 have been found in the synovial fluid and sera of RA sufferers, and in Crohn's disease, where the number of IL-17 positive T cells in patients with the active disease was more than 20-fold higher compared to control patients (Kikly *et al.*, 2006).

1.6.4 INTERFERON (IFN)- γ

IFN- γ is a pleiotropic cytokine that has a role in both innate and adaptive immunity. The pro-inflammatory effects of IFN- γ at the site of tissue inflammation are well established; one of its major roles is to activate macrophage and DC, leading to increased phagocytosis, elevated MHC I and II expression, and the induction of IL-12 production (Szabo *et al.*, 2003). It also induces the release of TNF- α and IL-1 from APC, and upregulates the production of pro-inflammatory chemokines to augment the inflammatory process (Hill & Sarvetnick, 2002). It enhances natural killer cell function, and aids macrophage and neutrophil intracellular killing by increasing nitric oxide and superoxide production (Parkin & Cohen, 2001).

The exact role of IFN- γ in Th1 cell development is unclear as experiments have yielded inconclusive results [reviewed by Szabo *et al.*, (2003)]. Data indicates that IFN- γ promotes Th1 differentiation by upregulating the transcription factor, T-bet (O'Shea *et al.*, 2002). Alternatively, its role in Th1 promotion may be due to the inhibition of Th2 differentiation, as it has been shown that if IFN- γ is present, IL-12R expression and the ability to produce IFN- γ is maintained during Th2 development, however its presence does not induce IFN- γ production nor does it alter IL-4 levels (Szabo *et al.*, 2003).

CD4⁺ T cells (predominantly Th1 cells), CD8⁺ T cells, and natural killer cells are the major sources of this cytokine, although other cell types have been found to secrete IFN- γ including DC, macrophage, and B cells (Szabo *et al.*, 2003). All three members of the IL-12 family induce IFN- γ production in T cells and NK cells; IL-12 and IL-27 rapidly induce IFN- γ production in naive Th cells, and are important for maintaining Th1 responses. IL-27 seems to be dependent on IL-12 or IL-18 in this respect, whereas IL-23 is less efficient compared to IL-12 in inducing IFN- γ production and has more involvement in the expansion of the Th17 cell subset (de Jong *et al.*, 2005). IFN- γ in turn promotes IL-12 production from APC creating a positive feedback loop between the Th1 cytokines and enhancing the Th1 response (Hasko & Szabo, 1999). Since IFN- γ defines a Th1 or inflammatory response, the use of antibodies against this cytokine to treat inflammatory disorders have been evaluated with some positive results, for example anti-IFN- γ therapy has proved efficacious in patients with moderate to

severe active CD (Nakamura *et al.*, 2006), however in a number of other autoimmune diseases, the genetic deletion of IFN- γ and/or the IFN γ R actually increased disease severity (for example in EAE and collagen-induced arthritis (CIA)) (Rosloniec *et al.*, 2002).

1.6.5 IL-1, IL-6 AND TNF- α

Phagocytosis of microbial cells and stimulation by bacterial products, cytokines and immune complexes results the production of TNF- α , IL-1 β , and IL-6 from innate cells and triggers an inflammatory response (Blander & Medzhitov, 2006). However when inflammation occurs in an uncontrolled manner, disease ensues and high levels of TNF- α , IL-1 β , and IL-6 are particularly destructive and implicated in chronic inflammatory diseases like IBD and RA (Calder, 2006).

1.6.5.1 TNF- α

TNF- α is considered a prototypical proinflammatory cytokine and is secreted by activated macrophage, monocytes, dendritic cells and chronically activated T lymphocytes (Nakamura *et al.*, 2006). It activates a range of cells including macrophage and endothelial cells, and induces the production of chemokines and pro-inflammatory cytokines (including IL-1 and IL-6). TNF- α upregulates adhesion molecules on endothelial cells, stimulates fibroblast proliferation, and recruits leukocytes from the circulation into inflamed tissue (Wong *et al.*, 2008). TNF has also been shown to influence the function of APC, but its effects are complicated. In some circumstances, TNF can activate APC, augment antigen-presentation capability and upregulate the expression of co-stimulatory molecules. However, it can also inhibit the function of mature DC by impairing antigen presentation and inducing their apoptosis (O'Shea *et al.*, 2002).

Increased levels of circulating TNF have been measured soon after the onset of illness in almost every severe systemic infectious state in which it has been sought (Clark, 2007). Its over-production is documented in rheumatoid arthritis, Crohn's disease, multiple sclerosis and in many other autoimmune diseases and consequently its inhibition or blockade has proved efficacious in treating many of these disorders (Wong *et al.*, 2008).

1.6.5.2 IL-1

IL-1 is another cytokine that plays a major role in the inflammatory process:

IL-1 refers to two different cytokines, termed IL-1 α and IL-1 β , which are produced by many different cell types following stimulation by bacterial products, cytokines and immune complexes (Jacques *et al.*, 2006). IL-1 α is a cell-associated cytokine believed to function as an autocrine messenger, whereas IL-1 β is solely active in its secreted form and mainly produced by monocytes and macrophage (Apte *et al.*, 2006). Several signalling pathways lead to the transcriptional upregulation of IL-1 β including IL-1 β itself, TNF- α and TLR ligands, such as LPS (Braddock & Quinn, 2004).

There is a third ligand in the IL-1 family: The naturally occurring receptor antagonist of IL-1, termed IL-1Ra, is a structural variant of IL-1 that binds to both IL-1 receptors (IL-1 has two receptors; biologically active IL-1RI and inert IL-1RII (Arend, 2002)) with an avidity nearly equal that of IL-1 but it fails to activate cells. IL-1Ra is also secreted from monocytes, macrophage, neutrophils, and other cells and the balance between IL-1 and IL-1Ra in local tissues plays an important role in the susceptibility to and severity of many diseases (Arend, 2002). As with TNF- α , IL-1 has been implicated as having a pro-inflammatory, tissue-destructive role in many human diseases: An increased production of IL-1 β has been demonstrated in the circulation of patients with RA and IBD and the levels of measured IL-1 β have been shown to correlate with disease severity (Braddock & Quinn, 2004)

1.6.5.3 IL-6

The same triggers mentioned before instigate IL-6 production from innate cells, i.e. phagocytosis, TLR signalling and cytokine binding. And again, inflammation-associated cytokines including IL-6, (and IL-1 β and TNF- α) are produced by a variety of cell types at sites of inflammation, the most important of which are macrophage and monocytes (Gabay, 2006). The binding of CD28 to B7 on DC also induces the production of IL-6 (Bhatia *et al.*, 2006).

IL-6 plays an important role in leukocyte trafficking to sites of inflammation. It has been shown to activate endothelial cells to produce chemokines and express adhesion molecules facilitating leukocyte migration. And IL-6 is also involved in promoting B cell differentiation and T cell activation (Gabay, 2006). Another

one of its functions is to block apoptosis in cells during the inflammatory process, keeping them alive in very toxic environments and pro-longing the immune response (Hodge *et al.*, 2005). IL-6 was recently reported to render effector T cells insensitive to the actions of Tregs. Therefore the high levels of IL-6 produced during inflammatory disease may lead to an ineffective Treg response (Stagg *et al.*, 2004).

Dysregulated overproduction of IL-6 has been found to play pathological roles in chronic inflammatory diseases such as RA and CD. Anti-IL-6 receptor antibodies have been developed as a therapeutic agent for these diseases, with some efficacy revealed in clinical trials (Ito, 2004; Nishimoto & Kishimoto, 2004).

1.6.5.4 THE ROLE OF TNF- α , IL-1 AND IL-6 IN TH17 DEVELOPMENT

Considering their simultaneous production and overlapping functions, it is unsurprising that IL-1, TNF- α , and IL-6 have been implicated in promoting a specific set of T helper cells: Recent studies have found a third effector CD4⁺ Th pathway whose differentiation and growth is controlled by TGF- β , IL-6, and IL-23. These T cells have been termed Th17 based on their production of IL-17, but they also produce a range of other factors known to drive inflammatory responses, including TNF- α , IL-6 and GM-CSF (Langrish *et al.*, 2005; Kikly *et al.*, 2006). More recently, it was shown that IL-1 α and IL-1 β are involved in IL-23-mediated IL-17 production, and that TNF- α also has a synergistic effect on IL-17 production (Sutton *et al.*, 2006).

1.6.6 IL-2

IL-2 was one of the first cytokines identified and is an important T cell growth factor, supporting the proliferation of Th1 and Th2 cells, as well as CD8⁺ T cells (Stockinger, 2007). IL-2 is mainly produced by activated T lymphocytes and exerts its biological activity by binding the high affinity IL-2 receptor (IL-2R). The IL-2R consists of three subunits; the α -chain (IL-2R α ; also known as CD25); the β -chain (IL-2R β ; also known as CD122) and the common cytokine-receptor γ -chain (γ c; also known as CD132), however only the IL-2R β and γ c sub-units are required for signal transduction. The genes that encode IL-2 and the subunits

of the IL-2R are among the first genes activated in T cells following TCR ligation, and cytokines such as IL-1, IL-2, IL-7, IL-12, IL-15, and TNF- α , play a critical role in regulating the expression of both the IL-2 and IL-2R α genes after T cell activation (Kim *et al.*, 2006).

Following CD4⁺ or CD8⁺ T cell stimulation, exogenous IL-2 levels are sufficient to induce a more than 1000-fold clonal expansion of these cells (Malek & Bayer, 2004), although it is interesting to note that IL-2/IL-2R-deficient mice develop a lethal lymphoproliferative autoimmune disease, making it clear that IL-2 is also involved in regulatory mechanisms controlling T-cell proliferation and/or tolerance (Malek & Bayer, 2004). Another regulatory activity of IL-2 is its ability to trigger activated T cells to undergo apoptosis or activation-induced cell death (AICD). AICD occurs when the TCR is engaged by antigen after substantial IL-2-induced clonal expansion *in vitro*. It is thought that AICD is an important mechanism that greatly reduces the number of antigen-specific T cells and limits the immune response (Malek & Bayer, 2004). It was recently found that Th17 cell development is also inhibited by IL-2, supposedly because Th17 subset development is supported by TGF- β which in turn is able to block T cell proliferation via its inhibitory effect on IL-2 (Stockinger, 2007).

1.6.7 IL-10

Interleukin-10 is a cytokine produced by a variety of cells, including T and B lymphocytes, dendritic cells, and macrophage. It has strong anti-inflammatory activities, and was previously described as macrophage deactivating factor. IL-10 suppresses the production of inflammatory cytokines such as IL-1, IL-6, IL-12, and TNF- α from APC (Papadakis & Targan, 2000), and reduces the secretion of IL-2 and IFN- γ from Th1 cells, as well as inhibiting their proliferation and chemotaxis (Mocellin *et al.*, 2004).

T cell anergy can be induced when CD4⁺ or CD8⁺ T cells are activated in the presence of IL-10, or with DC previously treated with IL-10 (Mocellin *et al.*, 2004). The induction of this anergy is partially dependent on the ability of IL-10 to limit IL-12 production, and down-regulate costimulatory molecules on dendritic cells, as it has been shown to inhibit the full maturation of DC by decreasing MHCII, ICAM-1, CD80, and CD86 expression, all of which

culminate in impaired T cell responses (de Jong *et al.*, 2005). IL-10 deficiency in mice results in an overproduction of inflammatory cytokines and leads to the development of chronic inflammatory diseases (Conti *et al.*, 2003), and the fact that regulatory CD4⁺ T cells contribute to the induction of peripheral tolerance via the production of IL-10 and TGF- β , reiterates the importance of IL-10 as an immunosuppressive cytokine. The production and action of the two regulatory cytokines, IL-10 and TGF- β , are interrelated and probably involve a positive feed-back loop, in which IL-10 enhances TGF- β expression and vice versa (Mocellin *et al.*, 2004).

1.6.8 TRANSFORMING GROWTH FACTOR (TGF)- β

Similar to IL-10, TGF- β is generally considered an anti-inflammatory or regulatory cytokine. It is secreted from certain classes of T cells (in particular regulatory Th3 cells) and many other non-lymphoid cells (Hanada & Yoshimura, 2002). Although largely immunosuppressive and involved in regulating the proliferation, apoptosis, and differentiation of multiple cell types (Chen & Wahl, 2003), TGF- β has also been implicated in promoting the differentiation of the recently discovered proinflammatory Th17 T helper subset (Kikly *et al.*, 2006). TGF- β appears to be multi-faceted; it has an important role in restraining essentially all immune cells (particularly self-reactive T cells), is crucial for oral tolerance, and has the capacity to restore immune homeostasis (Wahl, 2007). Conversely, it is implicated in directing naïve T cells to develop into pro-inflammatory Th17 effector cells, which are thought to contribute to the pathology associated with certain autoimmune diseases [*see section 1.4.2.2*] and perhaps because of this, was found to be up-regulated in some patients suffering from IBD (Hanada & Yoshimura, 2002). Evidence suggests that Th1 cells suppress the expansion of TGF- β secreting cells and TGF- β signalling, whereas TGF- β interferes with IL-12 synthesis and signalling (Strober *et al.*, 2002). Furthermore, TGF- β has been shown to impede IFN- γ production from T cells (Bastos *et al.*, 2004). Therefore, Th1 and TGF- β -secreting cells appear to be mutually exclusive, whereas Th2 cells and TGF- β -producing cells can co-exist (Strober *et al.*, 2002).

It is of interest to note that as well as being involved in T helper cell development, TGF- β has many other functions. These include, wound healing, mast cell recruitment and fibrosis, and effects on IgE-mediated release of effector molecules, such as histamine, proteases and TNF- α . Furthermore, early work connected TGF- β with the generation of antibody-secreting B cells and isotype switching to IgA (Ashcroft *et al.*, 1999; Wang *et al.*, 2006; Wahl, 2007; Leask, 2008).

1.6.9 IL-4

In common with TGF- β , IL-4 displays immunoregulatory functions, such as inhibiting LPS-induced IL-1 β and TNF- α production in APC. IL-4 is a potent inducer of the Th2 response, and plays a central role in polarizing naïve CD4⁺ T cells toward a Th2 phenotype, both *in vitro* and *in vivo* (Agnello *et al.*, 2003). It is widely accepted that the main source of IL-4 is the Th cell population itself, indicating that IL-4 acts as an autocrine factor amplifying IL-4 production from developing Th2 cells (de Jong *et al.*, 2005). Indeed, T cells stimulated through the TCR in the presence of IL-4 develop into Th2 effector cells capable of producing IL-4, IL-5, and the related cytokine, IL-13 (Agnello *et al.*, 2003).

Although generally considered a Th2 cytokine, under certain circumstances IL-4 synergises with IL-12 to induce production of IFN- γ by DC. The effect of IL-4 seems to be dependant on the maturational stage of the APC target; the presence of IL-4 at early stages of macrophage or DC activation can inhibit STAT4 and suppress IFN- γ production, whereas IL-4 fails to inhibit STAT4 and can actually increase IFN- γ production in mature cells (Bastos *et al.*, 2004).

1.7 NUCLEAR FACTOR- (NF) - κ B

Activation of NF κ B plays a central role in inflammation and is involved in regulating genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes, such as COX-2 (cyclooxygenase 2) (Hanada & Yoshimura, 2002). The NF κ B family consists of five related transcription factors; RelA (p65), NF κ B1 (p50), NF κ B2 (p52), c-Rel and RelB. The functional NF κ B protein is made up of combinations of either

homodimers or heterodimers of these proteins but the most common transcriptionally active form is a heterodimer of the p65 subunit associated with p50 (Hatada *et al.*, 2000). The p50 and p65 sub-units are widely expressed in various cell types, whereas the expression of RelB is restricted to regions of the thymus, lymph nodes and Peyer's patches, and c-Rel is confined to haematopoietic cells and lymphocytes (Li & Verma, 2002).

1.7.1 NF κ B ACTIVATION

Inactive NF κ B is sequestered in the cytoplasm by a family of inhibitory proteins known as inhibitors of κ B, or I κ B. Cytokines and pathogens act through distinct signalling pathways resulting in the activation of I κ B kinase (IKK), which is a complex composed of two catalytic subunits, IKK α and IKK β , and a scaffold protein called NEMO (NF κ B essential modulator - also known as IKK γ). Activated IKK initiates I κ B phosphorylation, and once I κ B proteins are degraded NF κ B is able to translocate to the nucleus where it can modulate gene expression (Hanada & Yoshimura, 2002). The most common I κ B proteins are I κ B α , I κ B β and I κ B ϵ . It has been well documented that I κ B α regulates transient NF κ B activation and that I κ B β maintains persistent NF κ B activation. Accordingly, I κ B α is degraded rapidly in response to stimuli and quickly resynthesized (Li & Verma, 2002). NF κ B signalling can occur through either the classical (canonical) or alternative (non-canonical) pathway. The classical pathway utilises IKK β and IKK γ , and is activated by members of IL-1R/TLR superfamily via cytokines or PAMPs. The alternative pathway is IKK α dependant and initiated by members of the TNF superfamily (e.g. CD40L) (Bonizzi & Karin, 2004).

1.7.2 ACTIONS OF NF κ B

NF κ B is a key player in controlling both innate and adaptive immunity as it is associated with the transcription of a large number of genes, including cytokines and chemokines, as well as being essential for lymphocyte survival and activation (Li & Verma, 2002). For example, NF κ B activation increases the expression of vascular cell adhesion molecule- (VCAM)-1 and ICAM-1, while

NFκB inhibition reduces leukocyte adhesion and transmigration (Hanada & Yoshimura, 2002). NFκB is involved in the production of IL-18 and IFN-γ, which are highly important in orchestrating a Th1 response (Li & Verma, 2002). DC maturation seems to be NFκB-dependant; following *in vitro* stimulation, the ERK kinase signalling pathway is triggered to sustain cell survival, and NFκB is activated to facilitate DC maturation, characterised by an upregulation of costimulatory molecules and MHCII expression (Banchereau *et al.*, 2000). With regard to T cells, TCR ligation only weakly activates NFκB. It is only after co-stimulation through CD28 that full T cell and NFκB activation occurs (Schulze-Luehrmann & Ghosh, 2006). It is interesting to note that NFκB activation is also involved in the resolution stage of inflammation and is associated with the expression of anti-inflammatory genes, since inhibiting NFκB during the resolution stage prolongs the inflammatory response and prevents apoptosis (Hanada & Yoshimura, 2002). Nonetheless, the constitutive activation of NFκB is often seen in inflammatory diseases, such as RA, IBD and MS. Nuclear NFκB activity is consistently detected in biopsies from these patients, accompanied by the enhanced recruitment of inflammatory cells and production of pro-inflammatory mediators, such as IL-1, IL-6, IL-8 and TNF (Li & Verma, 2002).

1.8 PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR (PPAR)-γ

It is well established that lipids and their metabolites (e.g. prostaglandins and leukotrienes) have the ability to regulate immune responses. Lipid mediators exert their effects via specific cell surface receptors, and PPARs – peroxisome proliferator activated receptors – are one such group of lipid-activated transcription factors (Szatmari *et al.*, 2006). PPARs are present in a variety of cell types and are capable of influencing lipid and lipoprotein metabolism, glucose homeostasis, cellular differentiation, and the inflammatory response (Faveeuw *et al.*, 2000). There are three isoforms; PPARα, PPARδ, and PPARγ, which can either dimerise with retinoic X receptors (RXR) and bind to a prescribed DNA sequence, termed the PPAR response element (PPRE), or influence gene expression by interacting with other transcription factors to

enhance or inhibit their binding to DNA (Yaqoob, 2003; Sampath & Ntambi, 2005).

PPAR γ is the isoform predominantly expressed in cells of the myeloid line (e.g., monocytes/macrophage and DC) and is selectively activated by polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA). PPAR α on the other hand is mostly expressed by lymphocytes and activated by a large variety of fatty acids (Stulnig, 2003). Fatty acids, and various fatty acid-derived metabolites, can affect gene expression by binding to and activating PPARs, but it is difficult to know whether these nuclear receptors play a significant role in fatty acid-mediated immunomodulation *in vivo* or *in vitro* as studies have yielded conflicting results (Fritsche, 2006). The fact that PPAR γ deletion is embryonically lethal, also makes it difficult to ascertain the exact involvement of PPAR γ in immune response modifications (Klotz *et al.*, 2007).

1.8.1 EFFECTS OF PPAR BINDING

PPAR γ ligand binding has an anti-inflammatory effect on monocytes/macrophage and DC but the exact mechanism of action is yet to be fully elucidated. PPAR γ ligation has been shown to inhibit the secretion of IL-12 (Faveeuw *et al.*, 2000), decrease IL-15, TNF- α , and IL-6 production (Nencioni *et al.*, 2002), and alter the expression of costimulatory markers on DC leading to an impaired lymphocyte-stimulating capacity (Klotz *et al.*, 2007). Faveeuw and colleagues (2000) used the PPAR γ activator, rosiglitazone (RSG) and found that it negatively interfered with NF κ B activation. However, RSG did not affect DC maturation or the ability of DC to activate naïve T cells. Klotz and colleagues (2007) showed that PPAR γ activation interferes with LPS-induced NF κ B-DNA binding, as did Bassaganya-Riera *et al.*, (2002) who reported that the activation of PPAR γ decreased pro-inflammatory cytokines via its antagonistic activity on NF κ B, JNK, and p38 in macrophage. Resting T lymphocytes predominantly express PPAR α , but PPAR γ is also expressed and upregulated during T cell activation (Stulnig, 2003). PPAR γ ligands attenuated proliferative responses (Clark *et al.*, 2000) and inhibited IL-2 and IFN- γ secretion (Cunard *et al.*, 2002) in murine T cells.

PUFAs from both n-3 and n-6 families reportedly bind equally well to PPARs. Chain length and number of double bonds seems irrelevant, as there appears to be no distinction in the binding affinity and/or activating capacity between PUFAs. Such data might mean that differential immune modulation by various PUFAs is unlikely to be mediated through PPAR. Yet, it is possible that differences in routing and cellular metabolism of various PUFAs *in vivo* could result in unique patterns of PPAR activation for different n-3 and n-6 PUFAs (Yaqoob, 2003).

The potential involvement of PPARs in PUFA-mediated modifications of the immune response will be discussed more fully in *section 1.10.4.1*.

1.9 INFLAMMATION AND INFLAMMATORY DISEASE

Under most circumstances, inflammation is the body's natural response to infection or injury, but when dysregulated it can lead to extensive tissue damage and chronic disease, as seen in RA, MS and CD. Although these diseases affect different tissues and are associated with different genetic, hormonal, and environmental risk factors, at their tissue-damaging stage, they all seem to share common pathogenic processes: There is an abnormal accumulation of inflammatory cells (such as T lymphocytes, macrophage, neutrophils and plasma cells) that along with tissue endothelial cells, epithelial cells, and fibroblasts release a complex array of lipids, growth factors, cytokines, and destructive enzymes that cause local tissue damage and sometimes hyperproliferation and fibrosis (Andreaskos *et al.*, 2004).

Granulocytes, which include neutrophils, eosinophils and basophils, are cells of the innate system that provide the first line of defence against invading pathogens. During inflammation, mediators released from damaged cells and sentinels of the innate system (e.g. chemokines and eicosanoids) upregulate the expression of adhesion molecules (ICAM-1, VCAM-1) and act as chemoattractants triggering leukocyte migration (Luster *et al.*, 2005). Neutrophils are abundant in the blood but extravasate to accumulate rapidly at the site of infection. Under steady-state conditions tissue-resident DC and macrophage constantly patrol and sample the milieu for foreign organisms, but

during an inflammatory response, monocytes are recruited from the circulation and once in the tissues can differentiate into either macrophage or DC (Luster *et al.*, 2005). Upon activation, cells of the innate system secrete several proinflammatory cytokines such as IL-1, IL-6, IL-12, and TNF- α , which further attract and stimulate innate immune cells (Hanada & Yoshimura, 2002). The adaptive immune system becomes involved when dendritic cells are activated after taking up antigen in infected/inflamed tissue. DC mature into efficient APC as they migrate to T cell zones of lymphoid tissues, up-regulating the expression of adhesion (ICAM-1) and co-stimulatory molecules (CD80, CD86 and CD40) (Guermonprez *et al.*, 2002). In the lymph node, antigen-specific T cells are activated and proliferate to produce hundreds of effector T cell clones, which then migrate to inflamed sites where they carry out their effector functions. Cytokines are normally produced only transiently upon by an inducing stimulus and are down-regulated rapidly. However, at chronic inflammatory sites, cytokine expression is upregulated for extended periods of time (Andreaskos *et al.*, 2004).

Understanding the nature of inflammation with regard to disease chronicity has become an area of major research and great therapeutic interest. The events that trigger autoimmunity or that determine the disease type, manifestation and progression remain elusive. Three of the most common inflammatory diseases, MS, RA and IBD, will now be considered in more detail:

1.9.1 MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is the most common inflammatory disease of the central nervous system (CNS), affecting approximately 2.5 million people worldwide (Holmoy & Vartdal, 2007). Although MS is not usually life-threatening, those with the disease suffer substantial disability through deficits in sensation and in motor, autonomic, and cognitive function (Sospedra & Martin, 2005).

Multiple sclerosis is considered a classical T cell-mediated autoimmune disease that develops in genetically susceptible individuals but it is thought that environmental factors are also involved in disease manifestation, and the search for an antigenic trigger is on-going (McFarland & Martin, 2007).

The theory of CD4⁺ T cell involvement in disease progression is based on the composition of infiltrating cells entering the brain and cerebrospinal fluid (CSF) and based on data from the animal model of MS, EAE (Sospedra & Martin, 2005). In the EAE model, the injection of myelin components into susceptible animals leads to a CD4⁺-mediated autoimmune disease, furthermore, EAE cannot be transferred by antibodies, emphasizing the importance of CD4⁺ T cells (Sospedra & Martin, 2005).

It was initially thought that the CD4⁺ T cells mediating disease in MS had a Th1 phenotype characterized by the production of IFN- γ . Evidence now indicates that the T cells critical for inflammation and disease, at least in the EAE model, are characterized by the production of IL-17 (McFarland & Martin, 2007). For example, neutralization of IL-17 but not IFN- γ in IL-25^(-/-) mice prevented EAE, suggesting that IL-17 is a major disease-promoting factor (Kleinschek *et al.*, 2007); and it has been shown that IFN- γ -producing cells are present in EAE-resistant, IL-23-deficient mice, suggesting that the IL-23-driven immune response is independent of the IFN- γ -Th1 pathway (Langrish *et al.*, 2005). In addition to evidence from animal models, a link between disease and IL-17 is emerging in studies of patients with MS: IL-17 mRNA is more abundant in the blood and the mononuclear cells of cerebrospinal fluid in MS sufferers, and the expression of IL-23 is higher in myeloid DC from patients with MS than in healthy controls (McFarland & Martin, 2007).

Other proinflammatory cytokines from both T cells and APC (e.g., IL-12, IFN- γ , IL-23, TNF- α) are thought to play a role in the pathogenesis of MS via immune system activation in the periphery and/or by directly damaging tissue. Elevated numbers of blood cells secreting TNF- α and higher numbers of peripheral blood mononuclear cells (PBMC) expressing IFN- γ mRNA have been found in MS, but therapeutic trials involving neutralising antibodies for these cytokines resulted in disease exacerbation (Holmoy & Vartdal, 2007). Data concerning anti-inflammatory cytokines in MS are similarly contradictory. Decreased numbers of PBMC secreting IL-10 and unexpectedly high numbers of cells expressing IL-4 mRNA have been observed in MS CSF lesions (Sospedra & Martin, 2005). A defective regulatory response has also been suggested as a possible additive source in disease pathology as Hafler and colleagues (2005) found that the

regulatory function of the CD4⁺CD25^{hi} T-cell subpopulation was defective in patients with MS.

Other factors that contribute to a proinflammatory environment in MS include adhesion molecules, such as LFA-1, which facilitate the migration of auto-reactive T cells through the blood brain barrier (BBB). And chemokines (RANTES/CCL5, IP-10/CXCL10, IL-8) that recruit other immune cells, including monocytes, CD8⁺ T cells, B cells, and mast cells, from the peripheral blood, all of which orchestrate the formation of the inflammatory lesion (Sospedra & Martin, 2005). Therefore, it is clear to see that many components of the innate and adaptive immune systems all contribute to aspects of the disease process in multiple sclerosis.

1.9.2 RHEUMATOID ARTHRITIS

Rheumatoid arthritis is a chronic inflammatory disease that mainly targets the synovial membrane, cartilage and bone. It affects 1% of the population and is associated with significant morbidity and increased mortality (McInnes & Schett, 2007).

T cells are implicated in the pathogenesis of rheumatoid arthritis by virtue of the detection of high numbers of T cells in the inflamed synovium and the demonstrated requirement of T cells in various animal models of arthritis. Furthermore, T cell-derived cytokines are directly implicated in many of the immune processes that are associated with the pathogenesis of rheumatoid arthritis (Brand *et al.*, 2003). RA has largely been considered a Th1-mediated disorder, thought to be driven by a population of T cells producing inflammatory cytokines and chemokines, such as IFN γ and TNF- α (McInnes & Schett, 2007). However, more recently, studies in animal models favour a new model that implicates Th17 cells as crucial effectors. For example, Irmeler *et al.*, (2007) demonstrated that IFN- γ is not essential for a potent inflammatory response and that its absence actually increased inflammation in a murine model of AIA (antigen-induced arthritis). The researchers found that disease severity in IFN- γ knockout mice was characterized by a prominent elevation of IL-17 and the inhibition of IL-17 decreased disease pathology, thus implicating IL-17 as the

destructive cytokine in this disease setting. Whether these mouse models faithfully represent human disease is currently unclear. Nevertheless, consistent with the Th17-cell model, IFN- γ is lacking or present at low levels, in the synovial membrane of patients with rheumatoid arthritis and it is rarely detectable in the synovial fluid (Brand *et al.*, 2003).

As in MS, other cytokines and cell types are implicated in disease pathology: Sites of inflammation contain macrophage, mast cells, CD4+ and CD8+ T cells, NK cells, B cells and plasma cells. And the synovial milieu contains various cytokines, such as IL-1 β , IL-6, IL-7, IL-12, IL-15, IL-18, IL-23p19 and TGF- β (Andreakos *et al.*, 2004), that in turn can support the expansion and differentiation of T-helper cells. Myeloid and plasmacytoid DC also contribute to the presence of cytokines and subsequent T-cell differentiation (Brand *et al.*, 2003). Again, there appears to be a defective regulatory mechanism in RA: Although regulatory cytokines such as IL-10 and IL-1ra are expressed by synovial mononuclear cells, they are not present in sufficient local concentrations to counteract the actions of the dominant proinflammatory cytokine milieu. And the naturally occurring Tregs (Foxp3+CD4+CD25+) detected in the synovium of patients with active disease and particularly in synovial fluid, seem to have an impaired regulatory function (McInnes & Schett, 2007).

1.9.3 INFLAMMATORY BOWEL DISEASE

Ulcerative colitis (UC) and Crohn's disease (CD), collectively termed inflammatory bowel disease (IBD), affect millions of people worldwide and are characterized by a chronic uncontrolled inflammation of the intestinal mucosa (Uhlig *et al.*, 2006). It is a chronic intermittent disease with patients suffering from a variety of symptoms including, abdominal pain, diarrhoea, malnutrition, and anaemia (Cobrin & Abreu, 2005). CD is characterized by transmural, patchy inflammation of any part of the gastrointestinal tract but it most commonly involves the ileum and the colon. UC on the other hand, is confined to the large bowel beginning in the rectum and progressing proximally, mainly affecting the superficial layers of the bowel (Papadakis & Targan, 2000).

There are two main hypotheses as to the cause of IBD: The first is the presence of an abnormal microflora that provokes a pathological response from a normal

mucosal immune system. The second, more widely accepted theory, is that of a dysregulated mucosal immune response to normal constituents of the gut microflora leading to sustained inflammation (Strober *et al.*, 2007). The latter theory has been reinforced by the identification of Crohn's disease related mutations in the nucleotide oligomerization domain-2 (NOD2) gene (Stokkers & Hommes, 2004). NOD proteins are a distinct subset of PRRs expressed by enterocytes, macrophage, dendritic cells and T cells, that are involved in the regulation of inflammation and apoptosis (Inohara & Nunez, 2003; Stokkers & Hommes, 2004). Muramyl dipeptide (MDP), a derivative of bacterial peptidoglycan, is the NOD2 ligand and binding induces NF κ B activation and the production of pro-inflammatory mediators (van Beelen *et al.*, 2007). Polymorphisms in NOD2 can lead to hypo-responsiveness or hyper-responsiveness to bacteria; in either case, resulting in susceptibility to chronic inflammation (Cobrin & Abreu, 2005).

1.9.3.1 THE IMMUNE RESPONSE IN CD

Mucosal permeability refers to the degree to which the mucosal epithelium is permissive to the passage of luminal substances. In animal models, it has been shown that defects in permeability of the intestinal epithelium can cause IBD and indeed it has been documented that patients with active CD also have an increased intestinal permeability (Cobrin & Abreu, 2005). Innate immune cells, particularly DC and macrophage, are significantly involved in both homeostasis in the normal gut and in a dysregulated immune response during intestinal inflammation (Wirtz & Neurath, 2007). An increase in intestinal permeability would allow the passage of bacteria which could lead to inflammation possibly through the activation of DC. Studies have implicated a role for DC in disease manifestation: The transfer of CD4⁺CD45Rb^{HI} T cells into SCID mice induces colitis, and researchers have found that the transplanted T cells form aggregates with CD11c⁺ DC in the lamina propria where they undergo proliferation which leads to intestinal pathology (Bilsborough & Viney, 2004).

A number of studies have proposed a defective regulatory response as a possible mechanism in the inflammatory process of IBD: Adoptively transferring CD4⁺CD25⁺ Treg cells reversed established pathology in a model of bacterial-induced colitis (Maloy *et al.*, 2005), and Mottet and co-workers (2003) also

showed that transferring CD4⁺CD25⁺ T cells cured colitis using the CD4⁺CD45Rb^{HI} T cell transfer model of IBD.

Again, cytokines are the main mediators of tissue destruction in IBD, acting locally to upregulate adhesion molecules and enhance the recruitment of effector cells, including neutrophils and phagocytes, which in turn amplifies the inflammatory response and leads to tissue damage (Ardizzone & Porro, 2005). Cytokines, chemokines, and growth factors such as TNF- α , IL-1, IL-2, IL-6, IL-10, IL-12, IL-18, IFN- γ , MIP-1, MCP-1, and TGF- β were all found to be upregulated at both the mRNA and protein level in CD patients (Andreacos *et al.*, 2004). Like MS and RA, CD was previously considered a Th1-type disease, but recent evidence suggests that IL-23 and IL-17 play a central role in disease pathology (Yen *et al.*, 2006; Caruso *et al.*, 2008). IL-17 expression is not detectable in the normal colon of humans, but is readily detectable in the colon of CD patients (Mizoguchi & Mizoguchi, 2008). And blockade of IL-23 attenuated intestinal inflammation, which was accompanied by decreased production of many proinflammatory cytokines, including TNF- α , IFN- γ , IL-6 and IL-1 β , several of which have been implicated in the pathogenesis of IBD (Sheibanie *et al.*, 2007). However, in a DSS model of colitis, treatment with anti-IL-17 monoclonal antibody increased the mucosal expression of TNF- α , IFN- γ , IL-6, and actually enhanced the development of DSS-colitis in mice, suggesting an inhibitory role for IL-17 in this disease model (Ogawa *et al.*, 2004). Furthermore, Fuss and colleagues (2006) found both IL-23 and IL-12p70 were upregulated in active CD sufferers, as well as the downstream effector cytokines, IL-17 and IFN- γ . It is, therefore, probably an over-simplification to consider the IL-23/IL-17 and IL-12/IFN- γ pathways as independent and mutually exclusive in such a complex disease setting as CD (Sheibanie *et al.*, 2007). Owing to the impact of genetic variability and environmental influence, patients experience altered gut immunity, resulting from a variety of aberrant innate and/or adaptive immune responses, leading to the clinical heterogeneity seen amongst CD sufferers.

1.9.3.2 DSS MODEL OF COLITIS

Feeding dextran sodium sulphate (DSS) polymers in the drinking water of mice causes a reproducible acute colitis. Symptoms include weight loss, shortening of

the colon, neutrophil infiltration and epithelial changes including fibrosis and ulceration. The initial acute phase of inflammation is followed by a slow regeneration of the colonic epithelium and a concurrent chronic inflammatory process associated with high mucosal levels of IFN- γ and IL-4 (Elson *et al.*, 2005; Wirtz *et al.*, 2007). It is thought that DSS is directly toxic to gut epithelial cells which leads to a disrupted epithelial barrier thereby increasing the hosts exposure to normal intestinal flora (Melgar *et al.*, 2005).

1.9.3.3 TREATMENT OF CD

Conventional therapy for IBD relies on corticosteroids combined with immunosuppressive agents for maintenance. These drugs are not always effective and can inflict serious side effects (Stokkers & Hommes, 2004).

Other agents being investigated for the treatment of Crohn's disease include inhibitors of T-cell activation, peroxisome proliferator-activated receptors, proinflammatory cytokine receptors and Th1 polarisation factors (Ardizzone & Porro, 2005). Some examples are listed below:

- Infliximab, a monoclonal antibody against the pro-inflammatory cytokine TNF- α has been successfully applied as a treatment for Crohn's disease (Wong *et al.*, 2008)
- A humanised monoclonal antibody to CD40L, toralizumab, entered a phase II clinical trial for CD but the trial was halted due to hazardous side-effects (Ardizzone & Porro, 2005).
- *Lactococcus lactis* has been used for thousands of years in the fermentation of milk products and has been engineered to secrete soluble, fully active cytokines. The treatment of colitogenic mice with IL-10-producing *L. lactis* was shown to prevent disease pathology (Steidler, 2002)
- De Vry and co-workers (2007) investigated the therapeutic potential of a locally administered, non-viral NF κ B decoy (NF κ BD) in an experimental model of IBD and saw a dose-dependent reduction in disease severity.

While traditional medical treatment has focused on non-specific suppression of the inflammatory response, an improved understanding of the immune defects in IBD will improve the spectrum of therapeutic agents available and permit individualized therapy. Given the vast increase in prevalence of many autoimmune/inflammatory conditions (e.g. MS, CD, and RA) and the severe side effects observed with conventional treatments, the search for adequate non-pharmaceutical treatments and preventative interventions has led to huge advances in nutrient-based research (Roche, 2004). One of those being explored is polyunsaturated fatty acids.

1.10 POLYUNSATURATED FATTY ACIDS (PUFA)

1.10.1 STRUCTURE AND CLASSIFICATION

Fatty acids are the main components of dietary lipids. The presence or absence of double bonds denotes whether a fatty acid is unsaturated or saturated, respectively. Polyunsaturated fatty acids contain more than one double bond and are generally named based on the number of carbon atoms in their chain, the number of double bonds present, and the position of the first double bond from the methyl- (ω) terminus of the molecule. In an n-6 PUFA the first double bond is located on carbon number 6, whereas it is found on carbon number 3 in an n-3 PUFA. Mammals cannot synthesize linoleic (n-6) or linolenic (n-3) acids, so they are essential in the diet, nor can they interconvert n-6 and n-3 fatty acids. Desaturase and elongase enzymes generate families of n-3 and n-6 PUFAs by extending dietary PUFAs and inserting double bonds (Wan *et al.*, 1989). The n-3 fatty acids, EPA or eicosapentaenoic acid (20:5) and DHA or docosahexaenoic acid (22:6) can be obtained from high-fat fish and marine mammals, and α -linolenic acid (ALA [18:3n-3]) is contained in certain nuts and plants (Sampath & Ntambi, 2005). Conjugated linoleic acid, (CLA) refers to a group of n-6 PUFAs that exist as positional and stereo-isomers of conjugated dienoic octadecadienoate (18:2). The c9,t11-CLA isomer (also called "rumenic acid") is the predominant geometric isomer found in foods derived from ruminants, such as meat and dairy products (Belury, 2002). Cell-specific metabolism as well as the expression of fatty acid-regulated transcription factors (e.g. PPARs) are likely

to play an important role in determining how cells respond to changes in PUFA composition (Jump, 2002).

1.10.2 GENERAL EFFECTS OF PUFA IN DIET

Ingestion of n-3 and n-6 PUFAs results in their incorporation into virtually every cell in the body, (e.g., erythrocytes, platelets, endothelial cells, monocytes, lymphocytes, granulocytes) (Simopoulos, 2002; Kew *et al.*, 2004; Zeyda & Stulnig, 2006) and nutritional studies show PUFAs confer various health benefits, including protection against the onset of diabetes (Suresh & Das, 2003; Das, 2005), efficacious immune response modifications (Hwang, 2000; Bhattacharya *et al.*, 2006), decreased incidence of coronary heart disease (Simopoulos, 1999; Hamer & Steptoe, 2006), and therapeutic potential in certain cancers (Song *et al.*, 2006; Hwang *et al.*, 2007; Schley *et al.*, 2007).

Omega-3 and -6 fatty acids have long been recognized as having anti-inflammatory activity, and their use in inflammatory diseases, such as rheumatoid arthritis and Crohn's disease, is expanding (Giugliano & Esposito, 2006). Many nutritional intervention studies have concentrated on evaluating the immunosuppressive and anti-inflammatory properties of specific PUFA but their exact mechanism of action is yet to be fully understood. For reviews see Fritsche (2006) and Shaikh & Edidin (2006). There appear to be four broad targets for the anti-inflammatory effects of PUFA, none of which are mutually exclusive (see **Figure 1.5** for a diagrammatic chart of the proposed immunomodulatory effects of PUFA), these are; modified eicosanoid production, changes in cytokine biosynthesis, altered membrane composition and function, and modifications in gene expression, which will be discussed in detail:

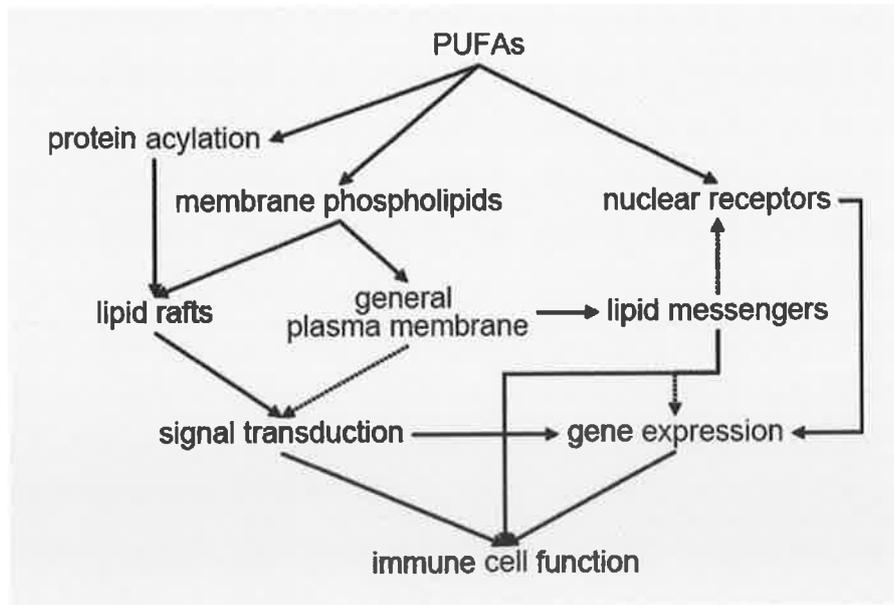


FIGURE 1.5 [Taken from Stulnig (2003)]

Possible proposed mechanisms of immune cell modifications by PUFA. Research suggests that there are four broad targets for the anti-inflammatory effects of PUFA, none of which are mutually exclusive, these are; modified production of lipid messengers, changes in cytokine biosynthesis (via changes in signal transduction, gene expression etc.), altered membrane composition and function, and modifications in gene expression. The diagram above demonstrates how inter-linked all of these mechanisms are, and how each PUFA-induced modification ultimately leads to altered immune cell function.

1.10.3 EICOSANOIDS

Changes in eicosanoid production through dietary intervention is relatively well understood [reviewed by Calder (2006)]. During inflammation, arachidonic acid (AA [20:4n-6]) is released from the membrane phospholipids of immune cells and metabolised to produce eicosanoids, which have diverse inflammatory properties, including neutrophil chemotaxis, increased vascular permeability, and vasodilation. The cyclooxygenase pathway gives rise to prostaglandins (PG) and thromboxanes (TXB), and the lipoxygenase pathway (LOX) produces leukotrienes (LTB) (Luster *et al.*, 2005). Increased proportions of dietary PUFA in cell membranes is partly at the expense of arachidonic acid resulting in decreased production of downstream eicosanoid products (Mantzioris *et al.*, 2000; Albers *et al.*, 2002). EPA can also act as substrates for COX and LOX

enzymes giving rise to a less potent family of eicosanoids; i.e., mediators formed from EPA are less inflammatory than those produced from AA (Belury, 2002; Calder, 2006). Serhan (2007) recently described a novel group of anti-inflammatory and pro-resolving mediators termed resolvins, lipoxins, and protectins formed from EPA and DHA. Sneddon and co-workers (2006) found that CLA inhibited the binding of monocytes to endothelial cells by suppressing the production of platelet-activating factor (PAF) formed from AA metabolism.

1.10.4 GENE EXPRESSION

Fatty acid-mediated alterations in nuclear receptor activation and gene expression is reviewed by Khan & Heuvel (2003).

1.10.4.1 PUFA AND PPARS

Fatty acids can affect gene expression through binding and activating a family of nuclear receptors known as peroxisomal proliferator-activated receptors (Sampath & Ntambi, 2005) [see section 1.8]. Studies have shown that PPAR ligation suppresses the production of proinflammatory cytokines and can affect cellular differentiation and functional properties (Clark *et al.*, 2000; Szatmari *et al.*, 2004). However there is conflicting data as to whether immune modulation by PUFA is mediated through PPAR. Bassaganya-Riera and colleagues (2004) found that CLA increased PPAR γ expression and decreased tissue damage (through an NF κ B-dependent mechanism) in a pig model of colitis. This was also seen in a study by Hontecillas and co-workers (2002) where feeding pigs CLA before perturbation of their gastrointestinal system with enteric bacteria markedly upregulated PPAR γ resulting in decreased mucosal inflammation. In a more recent paper Bassaganya-Riera and Hontecillas (2006) found that CLA-supplementation delayed the onset of colitis, whereas n-3 PUFA failed to protect from IBD but did augment disease resolution. It is thought that the n-3 PUFA accelerated colonic regeneration and clinical remission by activating PPAR δ . Li and associates (2005), on the other hand, demonstrated that both EPA and DHA activate PPAR γ in HK-2 cells. They used the high-affinity antagonist, BADGE, which abolished PPAR γ activation by the n-3 PUFAs and attenuated their inhibitory action on LPS-induced NF κ B activation, pointing towards a PPAR-

dependent mechanism for the immunomodulatory action in HK-2 cells. It is important to note however, that the researchers could not exclude the possibility that PUFA metabolites were acting as PPAR ligands and mediating the recorded anti-inflammatory effects. Reports by Zhang and Fritsche (2004) and Zeyda and co-workers (2005) found that PPAR γ antagonists were unable to reverse the inhibitory actions of PUFAs, suggesting a PPAR-independent mechanism for their immunomodulatory effects. Therefore, evidence to date is inconclusive, and more research is required to determine whether PPAR are involved in PUFA-generated immunological modifications.

1.10.4.2 PUFA AND NF κ B

There is evidence to suggest that both the n-3 and n-6 PUFAs may exert their regulatory effects through alterations in NF κ B activation:

An *in vivo* study by Bassaganya-Riera and colleagues (2004) found that DSS challenge increased NF κ Bp65 activation in control mice, whereas NF κ Bp65 activation resembled non-DSS challenged mice in animals fed a CLA-supplemented diet. Researchers have reported similar findings when using n-3 PUFA. EPA was able to impede TNF- α -induced NF κ B activation in pancreatic cells, and similar to the findings with CLA, it was proposed that this effect involved a decrease in the degradation of the inhibitory subunit of I κ B. Weldon and colleagues (2007) demonstrated that EPA and DHA down-regulate nuclear NF κ Bp65 levels, and increase cytosolic I κ B α levels in THP-1 macrophage. However, conflicting data was recorded in a study by Zeyda *et al.*, (2005), who treated human monocyte-derived DC with AA and EPA. They found that I κ B degradation and NF κ B activation were not affected by either PUFA even though the membrane composition of the cells were markedly altered, and the maturation process of PUFA-treated DC was impaired (DC did not upregulate CD40 or CD80, or increase IL-12 and TNF- α production). LPS stimulation increased NF κ B activity approximately two-fold independent of fatty acid treatment, suggesting that PUFA can modify immune cell responses without interfering with NF κ B activation.

1.10.5 CYTOKINE SYNTHESIS AND CELL PROLIFERATION

There is contrasting evidence surrounding the effects of fatty acids on cell proliferation and cytokine production. Many studies have reported a PUFA-induced decrease in lymphocyte proliferation (Pompos & Fritsche, 2002; Tricon *et al.*, 2004; Ly *et al.*, 2005). Zhang and co-workers (2006a) found that a diet rich in n-3 PUFA did not alter the ability for cells to differentiate into a Th1 phenotype, but halved the number of cells proliferating in response to IL-2. A previous paper by the same research group (Zhang *et al.*, 2005) reported that fish oil significantly increased the percentage of Th2-polarised cells and suppressed Th1-cell expansion. Tricon *et al.*, (2004) found that CLA decreased mitogen-induced cell activation in a dose-dependent manner in human T lymphocytes. In complete opposition to these reports, a recent human trial detected enhanced lymphocyte proliferation in response to n-3 PUFA supplementation and increased phagocytic capacity in macrophage and neutrophils (Gorjao *et al.*, 2006). The same study saw an upregulation of the proinflammatory cytokines TNF- α and IFN- γ , as did Vaisman and colleagues (2005) who gave n-3 PUFA supplements to children for 12 weeks and assayed cytokine production in LPS-stimulated PBMCs. The levels of the pro-inflammatory cytokines IL-1 β , TNF- α and IL-6 were all higher in LPS-stimulated cultures from the n-3 supplemented subjects. There appears to be a larger body of evidence supporting a PUFA-induced decrease in proinflammatory cytokine production; studies by (Fritsche *et al.*, 1999; Fritsche *et al.*, 2000; Weatherill *et al.*, 2005; Zeyda *et al.*, 2005; Bhattacharya *et al.*, 2007) collectively saw reductions in IL-12, IFN- γ , TNF- α , IL-2, IL-6, and IL-1 β . Moller and Lauridsen (2006) examined the *ex vivo* synthesis of eicosanoids and cytokines from porcine alveolar macrophage: LPS-stimulated macrophage from the fish oil-fed group produced less TNF- α , IL-8, LTB₄, and PGE₂ relative to the other diet groups. PUFA not only decrease pro-inflammatory cytokines as discussed above, but also increase anti-inflammatory cytokines, for example, CLA supplementation increased the levels IL-10 in a study involving healthy human volunteers (Song *et al.*, 2005). Interestingly Zeyda and associates (2005) suggested that since the concentration of PUFA needed to inhibit cytokine production in DC is very low (EC₅₀ = 2 μ M) and the extent of inhibition (>90%) is more pronounced compared to the effects seen on

other cell types (e.g. T cells EC50 = 5-25µM, and inhibition = 50% maximum), the *in vivo* action of PUFA may primarily involve DC.

1.10.6 CHANGES IN CELL SURFACE MARKER EXPRESSION

With regard to cell surface marker expression, cells cultured with PUFAs frequently show a reduction in MHCII and adhesion molecule expression (Hughes *et al.*, 1996; Chen *et al.*, 2005). Studies have also shown PUFA to have inhibitory effects on costimulatory molecule expression and the ability of DC to present antigen (Sanderson *et al.*, 1997; Hughes & Pinder, 2000; Zeyda *et al.*, 2005). For instance: Hughes *et al.*, (1996) showed that EPA and DHA inhibited the expression of MHCII and ICAM-1 on IFN γ -stimulated human monocytes *in vitro*: DC isolated from rats fed n-3 PUFA had a reduced MHCII, CD2, CD18 and CD11a on their surface and consequently could not present antigen to KLH-sensitised responder spleen cells in a study carried out by Sanderson's research group (1997). Conversely Kew and colleagues (2004) found that dietary supplements of EPA or DHA had no significant effect on cytokine production or adhesion molecule expression in peripheral blood mononuclear cells from healthy volunteers.

Again, rather than dampening the inflammatory response, Ly and associates (2006) showed that dietary EPA and DHA may promote anti-inflammatory or regulatory mechanisms. They saw an upregulation of CTLA-4 expression in murine CD4⁺ T cells in n-3 PUFA-fed mice. CTLA-4 inhibits T cell activation by reducing IL-2 production and IL-2R expression, and by arresting cells in the G1 phase of cell cycle, and therefore its upregulation was suggested as a mechanism by which PUFA down-regulate T cell responses

1.10.7 CELL MEMBRANE ALTERATIONS

Cell membrane fluidity is determined by its lipid composition; increased saturated fatty acids and cholesterol increase rigidity, whereas increasing unsaturated fatty acids makes it more fluid. Membrane fluidity influences the behaviour of membrane-bound enzymes, proteins, and receptors (Das, 2006); for

example, fluidity has been shown to increase the number of insulin receptors on the membrane and therefore decrease insulin resistance (Das, 2005).

Much research is focused on how PUFAs may modulate immune function through alteration or disruption of membrane micro-domain organisation (Ma *et al.*, 2004; Stulnig & Zeyda, 2004; Shaikh & Edidin, 2006). Evidence suggests that the translocation of immune receptors into specialized membrane regions called lipid rafts regulates immune cell activation. PUFA treatment and their subsequent membrane incorporation can alter lipid raft conformation leading to the displacement of signalling molecules which directly correlates with impaired signalling (Zeyda & Stulnig, 2006). The Src kinase family of protein tyrosine kinases, and the transmembrane adaptor protein, LAT (linker for activation of T cells), play an important role in T cell activation (Yaqoob, 2003). Lck and Fyn are two Src kinases concentrated on the cytoplasmic side of lipid rafts, and LAT is another signalling molecule constitutively present in rafts. All are activated following TCR stimulation to facilitate other downstream signalling events. The treatment of Jurkat T cells with EPA displaced Lck, Fyn, and LAT from lipid rafts (Stulnig *et al.*, 1998; Stulnig *et al.*, 2001) and Zeyda and co-workers (2005) propose that lipid raft modifications underlie the inhibitory effects of PUFA on T cell signalling. Ly and co-workers (2006) reported that n-3 PUFAs can alter the ability of CD28 to trigger synapse formation and/or modulate signal-transducing proteins that associate with the rafts after T cell activation, which may also relate to the dislodgement of the Src kinases and LAT.

Despite the many decades of research with PUFAs, the cellular and molecular basis underlying their regulatory and immunoprotective actions remain relatively unknown.

1.11 AIMS AND OBJECTIVES

The current study was undertaken to try and elucidate the mechanisms used by PUFA to elicit their anti-inflammatory effects. Considering the pivotal role of dendritic cells in the initiation of an immune response, and their possible role as instigators of T cell-mediated autoimmune disease, the effects of PUFA on DC and the subsequent effects of these PUFA-altered DC on adaptive immunity was examined using *in vitro* and *in vivo* methods. As proof of principle, two inflammatory models were used to assess the protective effects of increased PUFA in the diet.

The main aims of the study were-

- To examine the effects of PUFA on DC maturation, migration and cytokine production *in vitro*
- To investigate whether CLA-modulated DC have effects on subsequent T helper cell responses both *in vitro* and *in vivo*
- To ascertain a possible mechanism used by PUFA to elicit their anti-inflammatory effects in DC by looking at alterations in transcription factor expression and activation (namely PPAR γ and NF κ B)
- To determine the modulatory effects of a CLA-rich diet on inflammatory responses *in vivo* using a murine model of septic shock
- To examine the effect of dietary CLA in an inflammatory disease setting using a murine model of colitis (IBD)

CHAPTER 2

MATERIALS & METHODS

CHAPTER 2 MATERIALS & METHODS

2.1 MATERIALS

MATERIAL	SOURCE
TISSUE CULTURE MATERIALS/REAGENTS	
Foetal Calf Serum (FCS)	Gibco
Penicillin Streptomycin	Gibco
Sterile Petri Dishes	Nunc
6, 24, 96-well tissue culture plates	Nunc
RPMI-1640	Gibco
LPS (<i>E.coli</i> serotype R515)	Alexis Biochemicals
Dimethyl sulphoxide (DMSO)	Sigma
Recombinant IL-2	BD Pharmingen
OVA Peptide 323-339	GenScript Corp
GMCSF	J558 GMCSF-producing cell-line
Trypan blue (0.4% v/v)	Sigma
Xvivo15® medium	Lonza
G418 Geneticin	Gibco
Anti-mouse CD3e monoclonal antibody	BD Pharmingen
Anti-mouse CD28 monoclonal antibody	BD Pharmingen
CellTiter 96® AQueous One Solution	Promega
Keyhole Limpet Hemocyanin (KLH) (<i>Megathura crenulata</i>)	Calbiochem
Rosiglitazone (RSG)	Sigma
Phorbol 12-Myristate 13-Acetate (PMA)	Sigma
6.5mm Transwell® plate (8.0µm pore)	Corning
Recombinant MIP-3β/CCL19	R&D Systems
FATTY ACIDS	
Eicosapentaenoic Acid (EPA)	Sigma
Docosahexaenoic Acid (DHA)	Sigma

Conjugated linoleic acid cis-9, trans-11 (CLA)	Cayman
Alpha-linolenic acid (ALA)	Sigma
Lauric Acid (LA) – Dodecanoic acid	Sigma

FATTY ACID-INCORPORATED DIETS

Control diet – incorporated with 1% (w/w) purified, synthetic linoleic acid

CLA diet - incorporated with 1% (w/w) purified, synthetic conjugated linoleic acid

Purified oils were purchased from Loders Croklaan, Netherlands, and incorporated into animal feed by Special Diets Services, Essex, UK. Prior to purchase feed was irradiated and checked for purity, and subsequently stored in sealed bags at 4°C until use.

INHIBITORS

PPAR γ antagonist (GW9662)	Sigma
ERK/MEK inhibitor (U0126)	Sigma

ELISA REAGENTS

96-well microtitre plate	Nunc
3,3',5,5'-tetramethyl-benzidine (TMB)	Sigma
Tween 20	Sigma
Bovine serum albumin (BSA)	Sigma
ELISA DuoSet kits	R&D Systems
IL-23 (p19/p40) "Ready-SET-Go!" ELISA kit (5x assay diluent and TMB also provided)	eBioscience

PROTEIN EXTRACTION REAGENTS

IGEPAL	Sigma
Bio-Rad protein assay	Bio-Rad Laboratories
HEPES	Sigma
MgCl ₂	Sigma
KCl	Sigma
PMSF	Sigma
EDTA	Sigma
Glycerol	Sigma

DTT	Sigma
WESTERN BLOT REAGENTS	
Anti-IκB-α (C-20)	Santa Cruz Biotechnology
Anti-NFκBp65 (C-21)	Santa Cruz Biotechnology
Anti-mouse IgG peroxidase	Sigma
Anti-goat IgG peroxidise	Sigma
Supersignal Chemiluminescent substrate	Pierce
Sodium dodecylsulphate (SDS)	Sigma
Trizma base	Sigma
β-mercaptoethanol	Sigma
Protein molecular weight marker	Bio-Rad Laboratories
PVDF membrane	Pall Life Sciences
Whatman filter paper	Whatman
TEMED	Sigma
Ammonium persulphate	Sigma
Hyperfilm ECL	Amersham, GE Healthcare

TABLE 2.1 Antibodies used for FACs analysis of cell surface markers; suppliers and concentrations used.

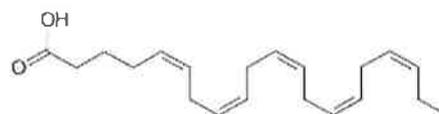
ANTIBODY	FLUORO-CHROME	SUPPLIER	ISOTYPE CONTROL	AMOUNT USED (per 10 ⁶ cells)
CD3	FITC	BD	Rat IgGb	0.5μg
CD4	PE-Cy5	BD	Rat IgG2a	0.5μg
CD11c	PE-Cy5.5	Caltag	Ham IgG	0.25μg
CD25	PE	BD	Rat IgG2b	0.5μg
CD28	PE	BD	Ham IgG2	0.5μg
CD40	FITC	BD	Ham IgM	0.5μg
CD48	FITC	Acris	Rat IgG2a	10μl (1 test)
CD80	PE	BD	Ham IgG	0.5μg
CD86	FITC	BD	Rat IgG2a	0.5μg
CTLA-4 (CD152)	PE	BD	Ham IgG1	0.5μg
CCR5 (CD195)	PE	BD	Rat IgG2c	0.5μg
IL-10R	PE	BD	Rat IgG1	0.5μg
MHCII	FITC	BD	Rat IgG2a	0.5μg

2.2 METHODS

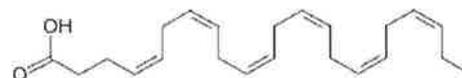
2.2.1 PREPARATION OF FATTY ACIDS

Fatty acids were dissolved in sterile DMSO to a stock concentration of 100mM, and sterile filtered again. 10µl aliquots were kept at -20°C and were thawed once and discarded after use to prevent oxidation. Fatty acids were purchased from suppliers and were tested for purity by HPLC prior to purchase. To further confirm batch reliability, new stocks of PUFA were tested for their consistent inhibitory effect on pro-inflammatory cytokine production (IL-12p70).

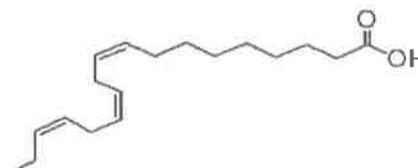
1. **Eicosapentaenoic acid (EPA) 20:5n-3**
 $C_{20}H_{30}O_2$
MW: 302.45
cis-5,8,11,14,17-eicosapentaenoic acid



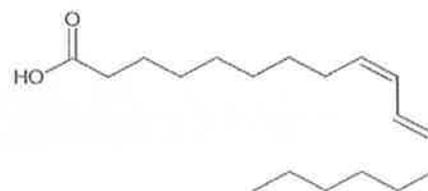
2. **Docosahexaenoic acid (DHA) 22:6n-3**
 $C_{22}H_{32}O_2$
MW: 328.49
cis-4,7,10,13,16,19-docosahexaenoic acid



3. **α -Linolenic acid (ALA) 18:3n-3**
 $C_{18}H_{30}O_2$
MW: 278.45
*cis*9, *cis*12, *cis*15-octadecatrienoic acid



4. ***cis*-9, *trans*-11-conjugated Linoleic Acid (c9, t11-CLA)**
 $C_{18}H_{32}O_2$
MW: 280.5
9Z,11E-octadecadienoic acid



5. **Lauric acid (LA) - SATURATED**
12:0 $CH_3(CH_2)_{10}COOH$
MW: 200.32
Dodecanoic acid

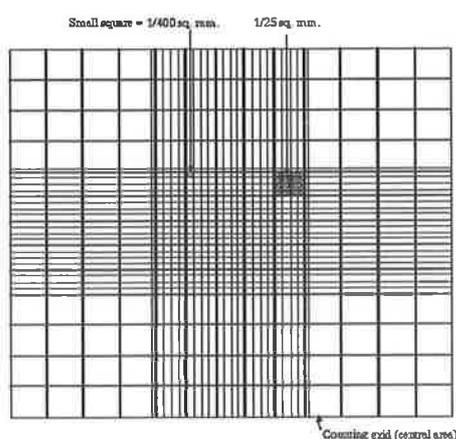


2.3 CELL CULTURE

All tissue culture was carried out using aseptic techniques in a class II laminar airflow unit (Holten 2010 – ThermoElectron Corporation, OH, USA) and cells were maintained in a 37°C incubator with 5% CO₂ and 95% humidified air (Model 381- Thermo Electron Corporation OH USA). Cells were grown in complete RPMI-1640 medium [Appendix A]. FCS was heat inactivated (56°C for 30 minutes) to inactivate complement and then aliquoted for storage at -20°C. Each batch was tested for LPS contamination using an endotoxin testing kit (Sigma Aldrich). Supplemented medium was stored at 4°C.

2.3.1 CELL ENUMERATION AND VIABILITY ASSESSMENT

Cell viability was assessed using the trypan blue dye exclusion test which is based on the ability of viable cells to actively exclude dye, owing to an intact cell membrane. Dead cells are unable to exclude the dye and appear blue when viewed under a microscope. 100µl of cell suspension was mixed with 150µl PBS and 250µl of trypan blue solution (0.4% (v/v)). After ~2 minutes, this solution was applied to a Brightline haemocytometer (Sigma MO USA) and the cells were counted under high-power magnification (×40). Cells inside the central grid of 25 squares were counted [Figure. 2.1].



A viable cell count was determined using the following formula:

$$\text{Cells/ml} = N \times 5 \times 10^4$$

Where, N = cell number counted, 5 = dilution factor, and 10⁴ = constant.

FIGURE 2.1 Diagrammatic Representation of Haemocytometer used to count cells

2.3.2 ISOLATION OF BONE MARROW-DERIVED DC

2.3.2.1 DAY 1 – BONE MARROW HARVEST

Bone marrow from the tibiae and femurs of BALB/c mice was extracted by flushing cRPMI through the bones using a syringe and a 27.5g needle into a sterile Petri dish. The bone marrow was broken up whilst being transferred to a falcon tube using a syringe and 19.5g needle. Cells were centrifuged for 5 minutes at 1200rpm, supernatant removed and the bone marrow cells resuspended in cRPMI to allow for 1ml of cells per petri dish required depending on experimental design. A cell count was performed using the trypan blue exclusion method to ensure an adequate number of viable cells had been obtained.

9ml of cRPMI supplemented with GMCSF [*see section 2.3.3*] and 1ml of cells were added to each petri dish and the appropriate amount of fatty acid or vehicle control added. The cells were incubated at 37°C.

2.3.2.2 DAY 4 – FEEDING CELLS

Petri dishes were tipped slightly so the cell monolayer was easily visible, and using a transfer pipette approximately 6-7mls of media was removed and discarded. Care was taken so as not to disturb the cell monolayer. 10mls of pre-warmed cRPMI supplemented with GMCSF was gently added back into each petri dish and the appropriate amount of fatty acid or vehicle control re-added. Cells were incubated at 37°C.

2.3.2.3 DAY 7 – COUNTING & PLATING CELLS

To remove semi-adherent and ,therefore, immature dendritic cells, the existing media was repeatedly pipetted onto the petri dish surface using a transfer pipette before being collected in a falcon tube. Each petri dish was examined under a microscope to ensure a majority of the cells were recovered (~90% recovery). Tubes were centrifuged at 1200rpm for 5 minutes and cells were resuspended and counted using the trypan blue exclusion method. The cell concentration was adjusted with cRPMI – generally cells were plated at 1×10^6 /ml, (or at 2×10^5 /ml for co-culture experiments). The respective fatty acids or vehicle control was re-added before cells were plated.

2.3.3 CULTURE OF THE J558 GMCSF-SECRETING CELL LINE

The mouse gene for GMCSF (granulocyte macrophage colony stimulating factor) was cloned into a mammalian expression vector (Karasuyama *et al.*, 1990) and transfected into the plasmacytoma line X63-AgS. Cell stocks were kindly donated by Professor Kingston Mills (Trinity College, Dublin).

After removal from liquid nitrogen and rapid thawing, cells were washed in 30mls cRPMI, then resuspended in 5mls of selection medium consisting of 1mg/ml G418 Geneticin (GibcoBRL) in complete RPMI for 2 passages. Cells were seeded at 1×10^6 cells/ml each time and culture flasks stood upright in the incubator. After the second passage cells were washed twice in cRPMI, counted and seeded in cRPMI at 1×10^6 cells/ml. When cells reached a medium density they were subsequently seeded at 2.5×10^5 cells/ml at each passage. Up to and including passage 9, supernatant was collected from the J558 cells and the amount of secreted GMCSF quantified by ELISA (R&D). For bone marrow culture, GMCSF was used at a concentration of 40ng/ml.

2.3.4 ADDITION OF FATTY ACIDS & ROSIGLITAZONE (RSG)

The vehicle control (DMSO), and the fatty acids, CLA, and LA, were added to cells at a concentration of $50 \mu\text{M}$. The n-3 PUFA, EPA, DHA, and ALA, were added at $25 \mu\text{M}$ unless otherwise stated. RSG was dissolved to a stock concentration of 100mM in DMSO and stored at 4°C . It was added to cells at a final concentration of $5 \mu\text{M}$. PUFA and RSG were added on day 1 of DC culture.

2.3.5 ADDITION OF INHIBITORS

The PPAR γ antagonist (GW9662) was dissolved in sterile DMSO to give a 10mM stock solution and stored at 4°C . It was added to cells ($10 \mu\text{M}$) either 1 hour prior to LPS activation or on day 1 of DC culture as indicated.

The U0126 ERK or MEK1/2 inhibitor was dissolved in sterile DMSO to give a 1mM stock, and stored at -20°C . It was added to cells ($5 \mu\text{M}$) 1 hour prior to LPS- or OVA-stimulation.

2.3.6 LPS ACTIVATION

Cells were activated with 100ng/ml LPS unless otherwise stated, and incubated for 24 hours before being used in the relevant assays.

2.3.7 ADDITION OF OVA PEPTIDE (323-339)

The OVA peptide sequence is given below and represents a T and B cell epitope of OVA: **ISQAVHAAHAEINEAGR** [C₇₄H₁₂₀N₂₆O₂₅: MW 1773.9]

1g vials of lyophilized OVA peptide were kept at -20°C and dissolved in 1ml of sterile water to give a 1mg/ml stock which was also kept at -20°C. OVA was added to DC (whilst in their petri dishes) at 5µg/ml for 24 hours before use in the co-culture experiments. The amount of OVA added was based on there being 14mls of media per petri dish. Therefore, 70µl of OVA from the 1mg/ml stock was added to each petri dish.

2.3.8 MTS ASSAY FOR PUFA AND LPS DOSE RESPONSE

The CellTiter 96® AQueous One Solution (Promega) is a colorimetric method for determining the number of viable cells in a sample. It contains an MTS tetrazolium compound (Owen's reagent) which is bioreduced by cells into a soluble coloured formazan product. The quantity of formazan product is measured at an absorbance reading of 490nm and is directly proportional to the number of living cells in the culture medium.

2.3.8.1 PUFA DOSE RESPONSE

Bone marrow-generated DC were cultured for 7 days (*see section 2.3.2*). Fatty acids were added at different concentrations (25, 50, 100µM) on day 1 of the cell culture. Cells were collected, counted and plated (100µl per well at 1x10⁶ cells/ml) in a 96-well plate on day 7 before being stimulated with LPS (100ng/ml) for 24 hours.

2.3.8.2 LPS DOSE RESPONSE

DC were grown for 7 days in the presence of fatty acids: DMSO, CLA, LA and SA were added at 50µM, and EPA, DHA, and ALA were added at 25µM. Cells

were collected, counted, and plated in a 96-well plate (100µl per well at 1×10^6 cells/ml). LPS was added at three different concentrations; 10, 100, and 1000ng/ml.

2.3.8.3 MTS ASSAY

24 hours after the addition of LPS, 20µl of the CellTiter 96® AQueous One solution was added to each well of the 96-well plate. The plates were incubated for 2 hours at 37°C in 5% CO₂ and absorbance read at 490nm. The cell viability of each sample was calculated by treating the absorbance of the vehicle control (DMSO) as 100% and comparing the remaining samples to this and expressing results as percentage viability.

2.3.9 CHEMOTAXIS ASSAY

Bone marrow-derived DC were cultured with fatty acids, rosiglitazone (5µM), and/or the specific PPAR_γ inhibitor GW9662 (10µM) for 7 days, as described in *section 2.3.2*. Cells were then plated at a concentration of 1×10^6 cells/ml in a 6-well plate (3mls/well) and stimulated with LPS (100ng/ml) for 24 hours. Following incubation, cells were removed from wells using a transfer pipette and counted. Transwell® plates were used in accordance with the manufacturer's instructions; 3×10^5 cells were added to the insert well in 100µl of media, and 600µl of media containing the chemokine CCL19 (100ng/ml) was added to the bottom chamber. Plates were incubated at 37°C for 5 hours. Cells that had moved to the bottom chamber were collected in eppendorf tubes, centrifuged and resuspended in 4% (v/v) formaldehyde/PBS before being transferred to FACS tubes. Migrated cells were counted for 60 seconds on a FACSCalibur.

2.3.10 CD4⁺ T CELL ISOLATION

2.3.10.1 ERYTHROCYTE LYSIS

(R&D Systems - mouse erythrocyte lysing kit WL2000)

Spleens were removed aseptically and collected in HBSS/10% (v/v) FCS on ice. A single cell suspension was achieved by pushing each spleen through a cell

strainer (40µm, BD falcon). Cells were then washed in HBSS/10% (v/v) FCS and erythrocytes lysed by adding a 1x solution of M-lyse for approximately 10 minutes at room temperature (2ml of 1x solution per spleen). Following incubation, cells were vortexed for 2 seconds. 40ml of wash buffer was then added and the cells centrifuged and resuspended in 2ml of 1x column wash before being counted and adjusted to $\leq 2 \times 10^8$ cells/ml.

2.3.10.2 CD4⁺ T CELL ISOLATION

(R&D Systems - Mouse T cell CD4 Subset Column Kit MCD4C-1000)

Less than or equal to 2×10^8 of cells (in 2ml of column wash) was mixed with 1 vial of monoclonal antibody cocktail (1ml CD4⁺ enrichment cocktail as supplied in R&D column kit) and incubated at room temperature for 15 minutes. During this incubation period the column was washed with 10ml of column buffer. After incubation, cells were washed twice with 10mls of 1x column wash and finally resuspended in 1ml column wash. The cells were added to the column and any liquid displaced from the column collected in a sterile falcon tube. Once the cells had moved onto the column, the bottom cap was replaced and the cells left in the column at room temperature for 10 minutes. This allowed B cells, non-selected T cells and monocytes to bind to the glass beads coated with anti-immunoglobulin via both F(ab) and Fc interactions. The column was eluted with 10mls of column buffer. Eluted and therefore CD4 positive T cells were centrifuged (250g for 5 minutes), resuspended in cRPMI, and counted using the trypan blue exclusion method. Cells were adjusted to the required concentration with cRPMI.

2.3.11 DC-T CELL CO-CULTURE

Dendritic cells were grown as previously described in the presence of DMSO (50µM), CLA (50µM), LA (50µM), RSG (5µM) and where indicated, the PPAR γ inhibitor, GW9662 (10µM) until day 6 of culture. At this time (i.e., 24 hours prior to commencement of the co-culture experiment) cells were activated with OVA peptide (5µg/ml) and/or LPS (100ng/ml) if required. The ERK inhibitor U0126 was added 1 hour before activation where indicated. The cells were

incubated for a further 24 hours at 37°C in 5% CO₂. After 24 hours, DC were collected and washed twice in sterile PBS/2%FCS to remove any traces of RPMI before being irradiated. DC were irradiated (at Trinity College, Dublin under supervision) with 40Gy (4000rads) using a gamma irradiator with a Caesium-137 source. Cells were then counted and resuspended in cRPMI at a final concentration of 2x10⁵cells/ml.

CD4⁺ T cells were isolated from the spleens of OVA transgenic D011.10 mice as described in *section 2.3.9*. A large portion of the T lymphocytes from these mice express a TCR specific for a peptide within the ovalbumin (OVA) molecule (OVA (323-339)). When this antigen is presented by DC complexed to MHCII (together with costimulation), these naive CD4⁺ T cells become activated and proliferate (Pompos & Fritsche, 2002).

Following purification, OVA transgenic CD4⁺ T cells were adjusted to 2x10⁶cells/ml. Equal volumes of T cells and DC were added to a sterile 96-well plate to give a final volume of 200µl/well. Fatty acids were added as required (co-culture DAY1). Plates were incubated at 37°C in 5% CO₂ for 5 days. On day 5 of the co-culture, plates were centrifugally pulsed to move cells to the bottom of the wells. 200µl of media was carefully removed from each well and frozen for future cytokine analysis. 200µl of fresh media was added to each well along with fatty acids, and plates were incubated at 37°C in 5% CO₂ until day 7. On day 7 of the co-culture, plates were again pulsed before 100µl of media was removed. 100µl of newly harvested OVA-activated DC were added at a concentration of 2x10⁵cells/ml for the second round of T cell stimulation. Recombinant murine IL-2 (Becton Dickinson) was also added at this time (10U/ml). At the end-point of the co-culture experiment (day 10), 200µl of media was removed after plates were pulsed and the supernatant frozen for future cytokine analysis.

2.3.12 EX VIVO T CELL CULTURE FROM FED ANIMALS

CD4⁺ T cells were isolated from the spleens of BALB/c mice as previously described (*section 2.3.9*) and adjusted to 1x10⁶/cells per ml.

For *ex vivo* receptor-mediated activation, cells were stimulated with 5µg/ml (in PBS) plate bound anti-CD3 (BD Pharmingen). Plates were incubated for 1hour

at 37°C to allow binding of anti-CD3 before adding cells. Following the addition of cells, 10µg/ml soluble anti-CD28 was also added (BD Pharmingen). 10U/ml of recombinant IL-2 (BD Pharmingen) was added 24 hours after initial activation to promote clonal expansion. Cells were then incubated for a further 48 hours at 37°C before supernatant was removed for cytokine analysis or cells processed for FACS analysis.

2.4 CYTOKINE ELISAS

2.4.1 BASIC PRINCIPLES OF ELISA

ELISAs can be used to quantify the amount of cytokine produced in solution. In a sandwich ELISA, a fixed quantity of capture antibody specific for the cytokine being detected is bound to a 96-well plate (the capture antibody is usually diluted in buffer, such as PBS and incubated overnight at RT). The plate is then washed to remove excess or unbound antibody and a blocking buffer, usually containing BSA, is added to prevent non-specific binding of subsequently added reagents. Samples of unknown antigen concentration and a series of recombinant cytokine standards of known concentration are added to the plate and incubated overnight at 4°C. The plate is washed again to remove any unbound antigen or cytokine and a biotinylated detection antibody for the cytokine is added and incubated (usually for 2 hours at RT). After incubation, the plate is washed and then streptavidin-horseradish-peroxidase (HRP) is added to the plate. Streptavidin binds biotin with high affinity and is conjugated to HRP, which is an enzyme that catalyses the oxidation of its substrate tetramethylbenzidine (TMB) by hydrogen peroxide, forming a blue compound. Following streptavidin-HRP incubation the plate is washed again and TMB is added, which forms a blue colour that increases in intensity depending on how much cytokine is present. The reaction is stopped by adding sulphuric acid to the plate, which turns the solution a yellow colour and allows the absorbance to be read at 450nm. Thus, the rate of colour formation is proportional to the amount of cytokine present.

Figure 2.2 summarises the principles of a sandwich ELISA.

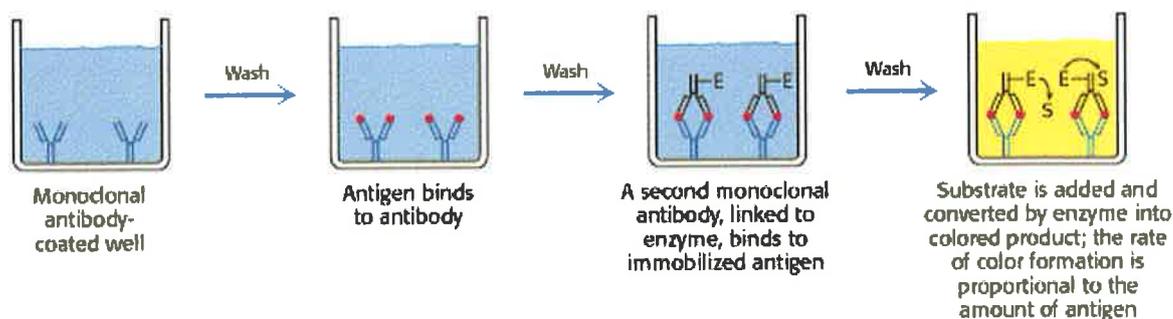


FIGURE 2.2 Schematic illustrating the principles of a sandwich ELISA

(Taken from: <http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=stryer.figgrp.515>)

Throughout this study, the concentration of IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-17, TNF- α and IFN- γ in cell supernatants was determined using ELISA Duoset kits from R&D Systems in accordance with the manufacturers' instructions. The concentration of IL-23p19 in supernatants was quantified using a "Ready-SET-Go!" ELISA kit (eBioscience).

2.4.2 IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-17, TNF- α ELISA

96-well Nunc microtitre plates were coated with 100 μ l of the relevant capture antibody diluted to working concentration in PBS (1:180 dilution of stock antibody - see **Table 2.2**) and incubated overnight at room temperature. After washing plates x4 with wash buffer (PBS/0.05% (v/v) Tween 20®), wells were blocked with 300 μ l of reagent diluent (PBS/1% (w/v) BSA) for at least 1 hour at room temperature. After repeating the washing step, 50 μ l of reagent diluent, and 50 μ l of supernatant or serially diluted standards (top standard serially diluted x6 in reagent diluent - see **Table 2.2**) were added to wells in duplicate, and plates were incubated overnight at 4°C. The following day plates were washed x4 with wash buffer. 100 μ l of the relevant biotinylated detection antibody, diluted in reagent diluent (1:180 dilution), was added to each well and plates were incubated for 2 hours at room temperature. Plates were washed x4 with wash buffer and 100 μ l of streptavidin-HRP (1:200 dilution in reagent diluent) was added to each well. Plates were incubated for 20 minutes at room temperature. Finally, wells were washed x4 with wash buffer. 100 μ l of TMB (Sigma Aldrich) was added to each well and plates incubated in the dark. The reaction was

stopped by adding 50µl 2N H₂SO₄ per well once the colour had developed. Optical densities were read immediately at 450 nm microplate-reader (VERSA Amax microplate reader, Molecular devices, CA, USA). The cytokine concentrations in the supernatants were determined from standard curves.

2.4.3 IL-1β, IL-2, AND IFN-γ

The method above was followed with two deviations:

Blocking buffer used was 1% (w/v) BSA/PBS + 0.05% (w/v) NaN₃ and the reagent diluent was 0.1% (w/v) BSA/TBS + 0.05% (v/v) Tween.

2.4.4 IL-12p40

Samples were diluted 1:100 in reagent diluent and 100µl of diluted samples and undiluted standards were added to plates in duplicate. Concentrations of samples were multiplied by the dilution factor once calculated from the standard curve.

2.4.5 IL-23p19

The IL-23 “Ready-SET-Go!” ELISA kit was purchased from eBioscience.

96-well plates were coated with 100µl of IL-23p19 capture antibody diluted 1/250 in PBS and plates were incubated overnight at 4°C. After washing x4 with wash buffer (PBS/0.05% (v/v) Tween 20®), wells were blocked with 300µl of assay diluent for 1 hour at room temperature. The assay diluent was provided in the kit at a 5x stock and diluted in distilled water before use. After repeating the washing step, 50µl of assay diluent, and 50µl of supernatant or serially diluted standards (top standard serially diluted x6 in assay diluent – see **Table 2.2**) were added to wells in duplicate, and plates were incubated overnight at 4°C. The following day plates were washed x4 with wash buffer. 100µl of the biotinylated p40 detection antibody, diluted in assay diluent (1:500 dilution), was added to each well and plates were incubated for 1 hour at room temperature. Plates were washed x4 with wash buffer and 100µl of streptavidin-HRP (1:250 dilution in assay diluent) was added to each well. Plates were incubated for 20 minutes at room temperature. Finally, wells were washed x4 with wash buffer. 100µl of TMB (eBioscience – supplied with kit) was added to each well and plates

incubated in the dark. The reaction was stopped by adding 50µl 2N H₂SO₄ per well once the colour had developed. Optical densities were read immediately at 450 nm microplate-reader (VERSA Amax microplate reader, Molecular devices, CA, USA). The cytokine concentrations in the supernatants were determined from standard curves.

TABLE 2.2 Concentrations of standards, and capture and detection antibodies used in sandwich ELISA assays

CYTOKINE ASSAYED	COATING ANTIBODY CONC (µg/ml)	STANDARD TOP CONC (pg/ml)	DETECTION ANTIBODY CONC (ng/ml)
IL-1β	4.0	1000	400
IL-2	1.0	1000	400
IL-4	4.0	1000	400
IL-6	2.0	1000	200
IL-10	4.0	2000	500
IL-12p40	4.0	2000	400
IL-12p70	4.0	2500	400
IL-23	(p19) 1/250 dilution	4000	(p40) 1/500 dilution
IL-17	2.0	1000	200
TNF-α	0.8	2000	75
IFN-γ	4.0	2000	400

2.5 CELL SURFACE MARKER STAINING

2.5.1 BASIC PRINCIPLES OF FLOW CYTOMETRY

Flow cytometry is a process used to characterise the properties of individual cells as they pass by laser beams of light. It can provide information about the size and granularity of a cell, and the expression of protein markers on, or in a particular cell.

To analyse protein expression, cells are incubated with specific monoclonal antibodies which bind to the protein of interest. These antibodies are usually conjugated to fluorochromes that emit light at various wavelengths after excitation by the laser beam, enabling the proteins to be detected. After cells have been labelled with fluorochrome-conjugated antibodies, the cell suspension is forced, along with sheath fluid, through a nozzle enabling the cells to be individually spaced in a stream of liquid. As each cell passes the laser beam, (usually an argon light with an excitation wavelength of 488nm), the light is scattered and the fluorochrome-conjugated antibodies fluoresce. The scattered light is detected by photomultiplier tubes that measure both the size of a cell, detected as forward scatter (FSC), and cell granularity, detected as side scatter (SSC). This enables the distinction of different cell types based on their size and granularity. For example, macrophage are large and granular compared to lymphocytes, which are a much smaller and less granular.

The fluorescence emitted from the fluorochrome-conjugated antibodies are also detected by photomultiplier tubes. There are four main fluorochromes used in flow cytometry:

1. FITC (fluorescein isothiocyanate) - emits light at a maximum intensity of 530nm and is detected by the fluorescent detector FL1.
2. PE (phycoerythrin) - emits light at 578nm and is detected by FL2
3. PerCP (peridin chlorophyll protein) - emits light at 675nm and is detected by FL3
4. APC (allophycocyanin) – emits light at 660nm and is detected by FL4.

The information from the flow cytometer (FACS [fluorescence activated cell sorter] caliber; Becton Dickinson) is then feed to a computer where the data can be analysed.

(<http://www.petermac.org/pdf/Intro%20to%20Flow%20Cytometry%20.pdf>).

Isotype controls should be used at identical concentrations and staining conditions as the target primary antibodies during sample preparation for flow cytometry. An isotype control is an antibody of the same immunoglobulin subclass and from the same species as the primary antibody. However, this antibody is not raised against anything. They are used to estimate the non-specific binding of target primary antibodies to cell surface antigens. Non-

specific binding is due to Fc receptor binding or other protein-protein interactions, and isotype controls normally show negligible cross-reactivity with cell surface antigens on tissue sections or in cellular preparations

2.5.2 CELL SURFACE MARKER STAINING METHOD

Cells were removed from tissue culture plates and collected in falcon tubes. An equal volume of FCS was added for 15 minutes to prevent non-specific binding. Tubes were centrifuged at 1200rpm for 5 minutes and cells resuspended in FACS buffer [see **Appendix**]. 200µl of cells were added to a 96-well round bottom plate to give approximately 400,000 cells per well. 1 well per treatment group (i.e., each PUFA treatment +/- LPS) was allocated for each antibody group plus 1 well for each corresponding isotype control group.

Plates were centrifuged at 2000rpm for 10 minutes and supernatant carefully removed from the wells. 100µl of the correct antibody or isotype mixture was added to the appropriate wells. Plates were incubated in the dark at 4°C for 30 minutes. Following incubation, plates were centrifuged at 2000rpm for 10 minutes at 4°C. Cells were washed twice in 200µl FACS buffer, and then fixed in 200µl 4% formaldehyde/PBS before being transferred to labelled FACS tubes. Samples were acquired immediately or left overnight in the dark at 4°C. 30,000 events were acquired per sample using a 4-colour FACS (fluorescence activated cell sorter) caliber (Becton Dickinson). Data was analysed using CellQuest software and samples gated on CD11c⁺ cells for DC samples, or CD3⁺/CD4⁺ cells for T cell samples to ensure histograms and dot plots represented pure cell populations.

TABLE 2.3 Antibody groups generally used for DC flow cytometry

Fluorochrome	Antibody Grp1	Antibody Grp2	Antibody Grp3	Antibody Grp4
FITC	CD40	CD86	MHCII	CD48
PE	IL-10R	CD80	CCR5	
PerCy5.5	CD11c	CD11c	CD11c	CD11c

TABLE 2.4 Antibody groups used for T cell flow cytometry

Fluorochrome	Antibody Grp1	Antibody Grp2	Antibody Grp3	Antibody Grp4
FITC	CD3	CD3	CD3	CD3
PE	CD25	CD28	CTLA-4	CCR5
PeCy5	CD4	CD4	CD4	CD4

2.6 NUCLEAR AND CYTOSOLIC PROTEIN

2.6.1 EXTRACTION PROTOCOL

Stock buffers were made up in dH₂O and kept at 4°C (1M HEPES pH 7.9, 1M MgCl₂, 1M KCl, 0.5m PMSF, 1M NaCl, 0.5M EDTA, 20% (v/v) & 25% (v/v) glycerol). 1M DTT was kept at -20°C.

To make a 0.5M solution of PMSF, 0.3484g PMSF was added to 4ml acetone and the solution was protected from light. PMSF and DTT were added to the buffers just before use and all steps were carried out on ice.

2.6.2 BUFFERS

The buffers below were used on 4x10⁶ cells and were scaled up/down where necessary. On day of extraction buffers A-D were made up without PMSF/DTT and stored on ice. PMSF/DTT were added just before use (*)

BUFFER A	STOCK	TO MAKE 10mls
10mM Hepes	1M	100µl
1.5mM MgCl ₂	1M	15µl
10mM KCl	1M	100µl
0.5Mm PMSF*	0.5M	10µl
0.5Mm DTT*	1M	5µl
dH ₂ O	1M	9.77ml
BUFFER B		
1µl IGEPAL added per ml of BUFFER A		

BUFFER C	STOCK	TO MAKE 10mls
20mM Hepes	1M	200 μ l
50mM MgCl ₂	1M	15 μ l
120mM NaCl	1M	4.2ml
0.2mM EDTA	0.5M	4 μ l
25% glycerol		2.5ml
0.5mM PMSF*	0.5M	10 μ l
dH ₂ O		3.07ml

BUFFER D	STOCK	TO MAKE 10mls
10mM Hepes	1M	100 μ l
50mM KCl	1M	500 μ l
0.2mM EDTA	0.5M	4 μ l
20% glycerol		2ml
0.5mM PMSF*	0.5M	10 μ l
0.5Mm DTT*	1M	5 μ l
dH ₂ O	1M	7.38ml

2.6.3 PROTEIN EXTRACTION

Media was discarded and cells were washed x2 in ice-cold sterile PBS (see Appendix). 1ml of buffer A was added to each well and cells were removed from the surface of the well by scraping with a transfer pipette. Samples were then transferred to sterile eppendorfs and centrifuged at 10,000rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 20 μ l buffer B. Tubes were incubated on ice for 10-20 minutes and vortexed every 3-5 minutes before being centrifuged at 10,000rpm for 10 minutes at 4°C. The subsequent supernatant was transferred to labelled tubes as the **cytoplasmic extract** and stored at -20°C

The remaining pellet was resuspended in 15 μ l buffer C and mixed well. Tubes were incubated on ice for 15 minutes and vortexed every 3-5 minutes. Tubes were again centrifuged at 10,000rpm for 10 minutes at 4°C, and the subsequent supernatant was transferred to new labelled tubes. This was the **nuclear extract**. 75 μ l of buffer D was added to each tube and samples were stored at -80°C.

2.6.4 BRADFORD ASSAY

Determination of protein concentration was performed according to the method originally described by (Bradford, 1976). A 1mg/ml stock of BSA in dH₂O was prepared. Standards were diluted from this stock to a concentration to 25, 20, 15, 10, 5, 2.5, 1.25 and 0µg/ml. Standards for cytosolic samples were diluted in PBS whereas standards for nuclear extracts were diluted in buffer D. Cytosolic samples were diluted 1:75 with PBS, and nuclear samples diluted 1:20 with buffer D before 160µl of samples and standards were added to a 96-well plate in duplicate. 40µl of BIORAD protein assay was added to all samples and standards and mixed well. The plate was left at room temperature for 10 minutes to allow the reaction to take place before plates were read at 595nm. The protein concentrations of the samples were calculated from the relevant standard curve.

2.7 WESTERN BLOTS

2.7.1 PRINCIPLES WESTERN BLOTTING

Protein blotting is an analytical method that involves the immobilisation of proteins onto membranes before detection using antibodies. In western blotting, prior to protein immobilisation, sample proteins are separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE). During SDS-PAGE proteins are electrophoretically separated based on weight as they migrate through a polyacrylamide gel matrix. In order to make the proteins accessible to antibody detection, an electric current is used to pull proteins from the gel onto a membrane made of nitrocellulose or polyvinylidene fluoride (PVDF). Before incubating with antibody, the membrane is blocked to prevent non-specific binding and then incubated with primary antibody. After rinsing, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody. This is known as a secondary antibody, and it is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase (HRP). Most commonly, a HRP-linked secondary is used in conjunction with a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein present. A sensitive sheet of

photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot. The image on the film can be analysed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density.

2.7.2 PREPARATION OF SAMPLES FOR GEL ELECTROPHORESIS

Nuclear and cytosolic fractions were equalized for protein by performing an acetone precipitation step. 5x volume of acetone was added to a calculated and equal concentration of protein for each sample and tubes were incubated at -20°C for 2 hours. Tubes were then centrifuged at 12000rpm for 5 minutes, the acetone pipetted off and any remaining liquid allowed to evaporate at RT. 15µl of sample buffer [see Appendix] was added to each tube. Tubes were vortexed vigorously to ensure full resuspension of the protein pellet, and boiled at 100°C for 5 minutes.

2.7.2 GEL ELECTROPHORESIS

Proteins were fractionated by SDS denaturing polyacrylamide gel electrophoresis (SDS PAGE). Acrylamide gels (10%) [see Appendix] were cast between two glass plates and affixed to the electrophoresis unit using spring clamps. Electrode running buffer [see Appendix] was added to the upper and lower reservoirs. 12µl of prepared samples were loaded into the wells and run at 30mA per gel for approximately 30 minutes.

Pre-stained protein molecular weight markers (Bio-Rad laboratories) ranging from 10–250 kDa was added to one well in each gel.

2.7.3 WESTERN BLOTTING

Western blotting was carried out using the semi-dry method for the transfer of electrophoresed proteins to immobilising membranes. During gel electrophoresis, polyvinylidene fluoride (PVDF) transfer membrane (0.45µm, Pall Life Sciences) was immersed in methanol for 60 seconds and then equilibrated in transfer buffer [see Appendix] for approximately 20-30 minutes

prior to semi-dry transfer. Whatmann 3mm filter paper of the same dimensions was also saturated in transfer buffer prior to semi-dry blot sandwich assembly, which was assembled in the order cathode, 2x filter paper, acrylamide gel, PVDF membrane, 2x filter paper and anode. Electrophoretic transfer was performed at 200mA for 75 minutes. Following semi-dry transfer, the PVDF membrane was removed and processed for immunoblotting.

2.7.4 IMMUNODETECTION AND DEVELOPMENT

Following transfer, non-specific sites on the membrane were blocked with freshly prepared PBS/10% (w/v) Marvel/0.05% (v/v) Tween 20® (blocking buffer) for 1 hour with gentle agitation at room temperature. Membranes were then washed x3 with PBS/0.05% (v/v) Tween 20® (washing buffer) and incubated with appropriate primary antibodies, diluted in PBS/5% (w/v) Marvel/0.05% (v/v) Tween 20® (reagent diluent). Membranes were gently agitated with the primary antibodies at room temperature for 1 hour then left overnight at 4°C. Following overnight incubation, membranes were washed x8-10 times over 1 hour with washing buffer. Membranes were then incubated with the relevant horseradish peroxidase-conjugated secondary antibody, diluted as required in reagent diluent, for 1 hour with gentle shaking. Following incubation with secondary antibody, membranes were washed x8-10 with washing buffer over the course of 1 hour.

HRP-labelled antibody complexes were visualised using the enhanced chemiluminescence (ECL) method. Membranes were incubated for 5 minutes in 3ml of Supersignal West Pico Chemiluminescent substrate (Pierce ILL USA). Excess substrate was decanted and the membrane placed between acetate sheets and immediately exposed X-ray film (Amersham, GE Healthcare) in a dark room under red light. The film was developed using a film Hyperprocessor (Amersham Pharmacia Biotech). Exposure times varied depending on the concentration of protein used and the intensity of signals obtained, so varied from 1 minute to overnight exposure. The density of the resultant bands were calculated using the densitometry program on the Syngene gel analysis and documentation system (Syngene NJ USA).

2.7.5 STRIPPING AND RE-PROBING MEMBRANES

To reprobe membranes, antibody complexes were removed by incubating membranes in 10ml stripping buffer [see **Appendix**] at 56°C for 30 minutes followed by x6 5 minute washes in PBS/0.05% (v/v) Tween 20®. At this point the membrane was either re-probed with antibodies or stored at 4°C.

2.8 ADOPTIVE TRANSFER EXPERIMENT

Bone marrow derived dendritic cells were grown as described in *section 2.3.2* in the presence of GMSCF along with DMSO, CLA or LA (50µM). On the sixth day of culture, DC were stimulated with KLH (10µg/ml) or media alone. After 24 hours cells were harvested, washed in PBS, and injected into the footpads of recipient BALB/c mice (10^5 cells per footpad in 25µl PBS). Popliteal lymph nodes and spleens were isolated from recipient mice 7 days-post cell transfer and placed on ice in Xvivo15 media until processed. To obtain a single cell suspension, lymph nodes were pushed through a 40µM cell strainer, washed in Xvivo15 media and resuspended at a concentration of 1×10^6 cells/ml. A single cell suspension of splenocytes was achieved by following the RBC lysis step detailed in *section 2.3.9*, i.e. spleens were pushed through a cell strainer (40µM), erythrocytes lysed, cells washed and finally resuspended at a concentration of 2×10^6 cells/ml.

200µl of cells at the above concentrations were added in triplicate to 96-well round-bottom plates pre-coated with media, KLH (2, 10 and 50µg/ml), or 1µg/ml anti-mouse CD3 (BD Pharmingen) and 20ng/ml PMA (Sigma Aldrich). Supernatant was removed from the plates after 72 hours and assessed for levels of IL-2, IL-4, IL-10, IL-17, and IFN-γ using specific immunoassays (R&D DuoSet ELISA kits).

2.9 PROLIFERATION ASSAY USING [³H]-THYMIDINE INCORPORATION

2.9.1 PRINCIPLES OF THYMIDINE INCORPORATION AS A METHOD OF MEASURING PROLIFERATION

Proliferation techniques employ the principle that cells will incorporate radiolabelled precursors of DNA and protein into newly synthesized macromolecules. One of the most frequently used radiolabelled precursors is thymidine, which is generally labelled with tritium (³H). Quantitative measurement of the incorporation of tritiated thymidine into cultures of stimulated lymphocytes is routinely used as an indication of the immunocompetence of the cells and of their proliferation.

In general, [³H] labelled thymidine is added to cells for a controlled length of time; cells are harvested onto glass fibre filters, and radioactivity measured by scintillation counting. Cell growth is correlated to the amount of labelled thymidine which was incorporated during DNA synthesis. A scintillation counter measures radiation from beta-emitting nuclides. Samples are covered in a scintillation fluid containing an aromatic solvent and small amounts of other additives known as fluors. Beta particles emitted from the labelled thymidine in the sample transfer energy to the solvent molecules, which in turn transfer their energy to the fluors; the excited fluor molecules dissipate the energy by emitting light. In this way, each beta emission results in a pulse of light and the scintillation counter reports the number of photons that it detects as counts per minute (cpm).

2.9.2 PROLIFERATION ASSAY METHODOLOGY

The proliferation of T cells was assessed using the thymidine incorporation method: Following supernatant removal from the fed T cells and in the adoptive transfer experiment (*section 2.8*), 1 μ Ci/well of [³H]-thymidine (Amersham, GE Healthcare) diluted in 200 μ l Xvivo15 medium was added to each well and cells were cultured for a further 6 hours at 37°C. After incubation plates were frozen at -20°C. To assess thymidine incorporation, plates were thawed and cells harvested onto glass fibre filters (Wallac), and allowed to dry overnight. The

filters were then sealed inside plastic sample bags with 5mls of non-aqueous scintillation fluid (BetaScint, Wallac) and read using a Beta-plate scintillation counter (Wallac) [this was carried out at Trinity College, Dublin under supervision]. Results are expressed as mean counts per minute (cpm) of [³H]-thymidine incorporation for triplicate cultures of lymphocytes.

2.10 FEEDING STUDIES

Animals were purchased at 4-6 weeks of age from Harlan UK Ltd and immediately fed a control diet (1% (w/w) α -linoleic acid) or a CLA-incorporated diet (1% (w/w) CLA) for 5 weeks. The feed was kept at 4°C and changed on a daily basis to prevent PUFA peroxidation.

2.10.1 LPS SHOCK MODEL

Animals were fed the CLA-supplemented diet or control diet for 5 weeks. There were 10 animals in each diet group, half of which served as a control and did not receive the endotoxin. The remaining five animals in each diet group received an intravenous injection of 3 μ g LPS (Alexis Biochemicals) into the tail vein in 100 μ l sterile PBS. Animals were left for 6 hours after which time they were sacrificed and blood collected in sterile eppendorf tubes. Blood was allowed to clot overnight at 4°C and plasma removed after centrifugation at 12000rpm for 5 minutes. Plasma samples were stored at -20°C and analysed for the presence of various cytokines using specific immunoassays.

2.10.2 DSS COLITIS MODEL

3 groups of mice (6 per group) were fed either the CLA- or LA-incorporated diet for 5 weeks. One group of mice on each diet were then administered with 3% (w/v) DSS in their drinking water *ad libitum* for 5 days. Normal water was replaced after this for a subsequent 5 days. The third group of mice was fed the control diet and had normal drinking water for the duration of the experiment to serve as a control. All mice were weighed daily and assessed for outward signs of colitis i.e. rectal bleeding, loose stools and diarrhoea (see **Table 2.5** below for

details of scoring). Mice were sacrificed and the colon removed, weighed, measured and preserved in formalin for histological examination. Histological examination of tissue was carried out by Professor Brian Sheehan at the veterinary college, UCD.

TABLE 2.5 *Scoring system for the comparative analysis of intestinal bleeding*

SCORE	% WEIGHT LOSS	STOOL CONSISTENCY
0	0	Solid, no blood
1	1-5	Semi-solid, blood tinged
2	5-10	Semi-solid – Fluid, blood present
3	10-20	Blood Fluid
4	>20	Mouse sacrificed

2.11 STATISTICS

One-way analysis of variance (ANOVA) was used to determine significant differences between conditions. When this indicated significance ($p < 0.05$), post-hoc Student-Newmann-Keul test was used to determine which conditions were significantly different from each other. There was no significant difference between cells alone and DMSO (vehicle control) treated cells, therefore DMSO was used as the reference treatment.

An unpaired t-test or Mann-Whitney U test was performed to assess statistical differences between two treatment groups where indicated.

The Western blot and confocal microscopy experiments in chapter 5 represented by figures 5.5, 5.9 and 5.11 were carried out in conjunction with Clare Reynolds in the Nutrigenomics Research Group, UCD.

CHAPTER 3

***IN VITRO* EFFECTS OF PUFA ON BONE MARROW-DERIVED DENDRITIC CELLS**

3.1 CHAPTER 3 INTRODUCTION

Inflammation is the causative process in many chronic human diseases, including rheumatoid arthritis and Crohn's Disease (Andreacos *et al.*, 2004). Since many of the treatments available to sufferers of such diseases often have severe side-effects (e.g. steroidal drugs), there is a constant quest for more natural therapeutic agents. Polyunsaturated acids (PUFA) are found naturally in the diet; the n-3 PUFA, EPA and DHA, are found in fish oils, and CLA, an n-6 PUFA derivative, is present in meat and dairy products. These fatty acids have received much attention recently and dietary intervention studies have shown that PUFA have beneficial effects in a range of disorders, for example, they have been reported to offer some protection against coronary heart disease (Hamer & Steptoe, 2006), and the development of autoimmune diseases (Simopoulos, 2002). However, after much research the exact mechanism(s) PUFA use to manipulate the immune response still remains unclear.

The innate immune system can initiate adaptive immune responses to both foreign and self-antigen ultimately resulting in an effective immune response or a possible autoimmune reaction, respectively. Dendritic cells are the most potent antigen presenting cell being the only cell capable of activating naïve T cells (Kapsenberg, 2003). Thus, they are uniquely able to link the innate and adaptive arms of the immune system. Given their central role in controlling immunity, and the fact that several reports suggest that DC contribute to the onset of autoimmune disease (Lipscomb & Masten, 2002; Turley, 2002; Bayry *et al.*, 2004), DC are logical targets for treating inflammatory disorders, and because of this we hypothesized that dendritic cells (DC), may be one of the targets of PUFA, and alterations in such an influential cell type may change the ensuing adaptive immune response and give rise to the many beneficial effects seen after PUFA supplementation both *in vitro* and *in vivo* (Belury, 2002; Simopoulos, 2002).

Some autoimmune diseases are typified by an ongoing chronic inflammatory process involving excessive production of pro-inflammatory cytokines and a dysregulated Th1 and/or Th17 lymphocyte response, whereas many other autoimmune diseases, in particular those mediated by autoantibodies, involve a dysregulated Th2 response (Cravens & Lipsky, 2002; Kikly *et al.*, 2006).

Cytokines are normally produced only transiently upon the action of an inducing stimulus and are subsequently down-regulated. However, at chronic inflammatory sites, cytokine expression is upregulated for extended periods of time (Strober *et al.*, 2002; Andreakos *et al.*, 2004). DC have a pivotal role in generating T helper cell responses. Following activation, DC begin a maturation process which sees a down-regulation of inflammatory chemokine receptors (e.g. CCR5), and the upregulation of receptors for chemokines that direct them to T cell-rich areas of lymph nodes, for example, CCR7 is upregulated conferring responsiveness to MIP-3 β (CCL19) which is secreted by endothelial cells of lymphatic vessels and by cells within the T cell-rich areas of draining lymph nodes, which draws DC and naïve T cells together (Banchereau *et al.*, 2000; Langenkamp *et al.*, 2000). Once in the lymph node, the activation of naïve antigen-specific T cells is solely attributable to DC. The differentiation of T cells into various subsets is partly determined by the cytokines DC secrete; i.e. IL-12 drives the differentiation of T cells to a Th1 phenotype; IL-23, IL-1 and IL-6 are involved in generating Th17 cells; IL-4 induces Th2 cell differentiation; and IL-10 promotes the induction of type I T regulatory cells (Langrish *et al.*, 2004; de Jong *et al.*, 2005; Kikly *et al.*, 2006). The pivotal role of these cytokines in directing the development of T helper cell subsets suggests that modulating their production by DC may modulate the subsequent adaptive immune response. Therefore the effect of PUFA on LPS-induced cytokine release from dendritic cells was assessed to evaluate any beneficial changes that may occur.

In order to become fully activated by a dendritic cell, a naïve T cell must have its TCR engaged by a specific antigen coupled to an MHCII molecule. In addition to this, the T cell requires co-stimulation via CD40, CD80, and/or CD86 on the dendritic cell in order to reach and cross an activation threshold facilitating T cell differentiation and proliferation (de Jong *et al.*, 2005). Modulations in co-stimulatory and/or co-inhibitory surface marker expression could alter the ability of a DC to activate naïve T cells, whilst altered responsiveness to chemokines would hamper the bridging of innate and adaptive immune cells. Therefore, changes in surface marker expression on DC and migratory capacity to the lymph node homing chemokine, CCL19, induced by PUFA were also examined in this study.

3.2 RESULTS

3.2.1 THE CONCENTRATIONS OF PUFA USED HAVE NO SIGNIFICANT EFFECT ON CELL VIABILITY

Cells from the bone marrow of BALB/c mice were differentiated into dendritic cells with GMSCF and concurrently cultured with increasing concentrations (25 μ M, 50 μ M and 100 μ M) of PUFA (EPA, DHA, ALA or CLA) or LA (saturated fatty control) for 7 days. The molarity of DMSO was not calculated; instead the same volume of DMSO as fatty acid was added to cells (i.e. 2.5 μ l DMSO for 25 μ M, 5 μ l for 5 μ M, and 10 μ l for 100 μ M). The resultant fatty acid-treated immature DC were harvested and plated at a concentration of 1x10⁶ cells/ml. The viability of DC following incubation with different concentrations DMSO or fatty acid was determined using Cell Titer 96 Aqueous One Solution (Promega, WI, USA) according to the manufacturer's instructions. The concentrations of fatty acids selected for use in future experiments did not have significant cytotoxic effects on DC *in vitro*, i.e. 50 μ M DMSO, 25 μ M EPA, 25 μ M DHA, 25 μ M ALA, 50 μ M CLA, 50 μ M LA, and 50 μ M SA. 100 μ M of EPA and DHA was found to be cytotoxic so no data is available for cell viability or cytokine production at this concentration [Figure 3.1].

3.2.2 PUFA DOSE-DEPENDENTLY MODULATE CYTOKINE PRODUCTION BY DC

Fatty acids are able to inhibit LPS-induced IL-12p70 and IL-12p40 production in a dose dependent manner [Figure 3.2]. Both the biologically active IL-12p70 and the IL-12p40 sub-unit are dose-dependently produced by DC following stimulation with increasing amounts of LPS. Increasing concentrations of the polyunsaturated fatty acids; EPA, DHA, ALA, and CLA, were able to inhibit this LPS-induced IL-12 production in a dose dependent manner. Unexpectedly the saturated fatty acid, LA, also had an inhibitory effect on IL-12p40 and IL-12p70 production, although its effect on the latter was not as acute as the effects seen with the unsaturated fatty acids and the inhibition was not dose dependent.

Figure 3.3 shows that activating DC with increasing doses of LPS (10, 100 and 1000ng/ml) induces DC to respond by producing larger amounts of both IL-

IL-12p40 and IL-12p70. PUFA were able to inhibit this LPS-induced IL-12 production regardless of LPS dose. Again, LA was able to inhibit IL-12p40 production at all doses of LPS assayed, however it caused a significant increase in IL-12p70 production following DC activation with each concentration of LPS. Data from the MTS, and PUFA and LPS dose response assays were used to decide which concentration of PUFA would be used in future experiments. These were 50 μ M DMSO, 25 μ M EPA, 25 μ M DHA, 25 μ M ALA, 50 μ M CLA and 50 μ M LA. A dose of 100ng/ml LPS was used during *in vitro* cell culture also based on the dose response data and on studies conducted by other researchers who found this to be the optimal dose of endotoxin.

3.2.3 PUFA MODULATE *IN VITRO* LPS-INDUCED CYTOKINE PRODUCTION BY DC

DC were differentiated with GMSCF and concurrently cultured with either DMSO (vehicle control – 50 μ M), EPA (25 μ M), DHA (25 μ M), ALA (25 μ M), CLA (50 μ M), or LA (50 μ M) for a total of 7 days. DC were then stimulated with 100ng/ml LPS (*E.coli* serotype R515) and after 24 hours, supernatant was removed and assessed for levels of IL-12p70 and IL-12p40 [Figure 3.4], IL-10 [Figure 3.5], IL-23p19 and IL-1 β [Figure 3.6], and TNF- α and IL-6 [Figure 3.7] using specific immunoassays.

The production of the pro-inflammatory cytokines, IL-12p70 [Figure 3.4A] and to a lesser extent IL-12p40 [Figure 3.4B] were both decreased in the supernatant of PUFA-treated DC following LPS stimulation compared to the DMSO control group. The n-3 PUFAs, EPA (p<0.001) and DHA (p<0.001), had the greatest suppressive effect on IL-12p70 production, but cytokine production was also significantly reduced after culture with CLA (p<0.001). The saturated fatty acid, LA, had no effect on LPS-induced IL-12p70 production here. In Figures 3.2 to 3.4, 50 μ M of lauric acid is shown to have inconsistent effects on LPS-induced IL-12p70 production from DC. This may be attributable to batches of fatty acids differing in potency, as this was found with some of the other fatty acids, or perhaps because LA was less soluble in media and was sometimes found to float on the surface rather than dissolve into solution. Care was taken to avoid this by ensuring LA was warmed before being added into the cells.

Although decreased levels of IL-12p40 were found in the supernatant of all fatty acid-treated DC, the PUFAs DHA ($p < 0.05$) and ALA ($p < 0.01$) reached statistical significance, as did the non-PUFA, lauric acid ($p < 0.01$). Similarly DHA was also the only PUFA capable of significantly suppressing IL-23p19 production ($p < 0.001$) [Figure 3.5A]. In contrast to this, Figure 3.5B shows that EPA ($p < 0.01$) and CLA ($p < 0.001$) increased proinflammatory IL-1 β production in DC treated with LPS as did the saturated fatty acid LA ($p < 0.05$).

The production of IL-10, an anti-inflammatory or regulatory cytokine, was substantially increased in PUFA-treated DC following LPS stimulation [Figure 3.6]. EPA had the most profound effect on IL-10 production, but all PUFA significantly increased levels of IL-10 following DC activation ($p < 0.001$). The saturated fatty acid control, LA, had no effect on IL-10 production.

The levels of TNF- α [Figure 3.7A] and IL-6 [Figure 3.7B] remained relatively unchanged regardless of fatty acid treatment. Again, LA did not significantly affect the production of these cytokines.

3.2.4 PUFA MODULATE CELL SURFACE MARKER EXPRESSION IN IMMATURE AND LPS-STIMULATED DENDRITIC CELLS

GM-CSF was used to differentiate bone marrow cells into DC over 7 days. During this time, cells were concurrently cultured with DMSO (vehicle control), an n-3 PUFA (EPA, DHA, or ALA), an n-6 PUFA (CLA), or saturated fatty acid control (LA). The fatty acid-treated DC (1×10^6 cells/ml) were then cultured for a further 24 hours in the presence or absence of 100 ng/ml LPS. Control and LPS-stimulated cells were subsequently stained with fluorochrome-labelled monoclonal antibodies for numerous cell surface markers (i.e., CD11c, CD40, CD80, CD86, MHCII, CD48, IL-10R, and CCR5) [See Table 2.1].

PUFA were able to reduce the levels of key surface markers even in immature cells [Figure 3.8]. Every PUFA treatment resulted in a noticeable reduction of surface MHCII. This was accompanied by a general decrease in CD40, CD80, and CD86. EPA and DHA were also able to increase the expression of the IL-10 receptor. Contrastingly the saturated fatty acid, LA, increased CD40, CD86 and MHCII in immature cells.

As expected, LPS enhanced the surface expression of CD40, CD80 and CD86 as seen in the first row of **Figure 3.9** where control DMSO cells are shown by the filled histogram and LPS-treated cells are overlaid with a black line. There was also a slight increase in MHCII and IL-10R expression but no change in CCR5 or CD48 levels. It is important to note here that LPS did not cause as significant an increase in MHCII expression in DC as one might expect, and the changes in co-stimulatory marker expression could also be considered quite minor. This is possibly due to the presence of LPS in the foetal calf serum used in the cell culture media, which, if present, would instigate the maturation process in DC before LPS-stimulation and result in smaller changes in cell surface marker expression following the addition of exogenous LPS. Each batch of FCS bought in from Gibco was routinely tested for LPS contamination using an endotoxin kit (Sigma Aldrich), and the FCS was only used if the concentration of LPS was below the levels of detection. However, even minute amounts of LPS can initiate DC maturation and the endotoxin kit may not have been sensitive enough to detect this. This is an on-going major problem with DC work.

The fatty acids, EPA, DHA, CLA, and to a lesser extent, ALA, were able to inhibit some of the LPS-induced changes in cell surface marker expression. EPA and DHA had parallel effects on most of the cell surface markers analysed: Following LPS stimulation the n-3 PUFA reduced levels of CD40, CD80, CD86, and principally, MHCII expression, whilst increasing IL-10R levels compared to DMSO-treated cells. A comparable, but lesser inhibition was recorded when cells were incubated with the n-6 PUFA, CLA: There was a decrease in CD80, CD86, MHCII, and CCR5, and these changes were also observed after ALA treatment. The saturated fatty acid (LA) had no significant effect on DC maturation following LPS stimulation.

Tables 3.1 and **3.2** summarise the results shown in **Figures 3.8** and **3.9** respectively.

3.2.5 EPA AND CLA INHIBIT DC CHEMOTAXIS

CCL19, also known as MIP-3 β , is a ligand for CCR7. CCR7 is upregulated on the surface of mature DC and helps direct them to T cell areas of draining lymph nodes where they interact with and activate naïve T cells (Hansson *et al.*, 2006).

The migration of DC towards the chemokine MIP-3 β was significantly inhibited by culturing cells with EPA or CLA before LPS activation ($p < 0.001$) [Figure 3.10]. The presence of EPA and CLA during cell culture rendered DC less responsive to the chemokine CCL19. Interestingly the other n-3 PUFA, DHA, did not have an inhibitory effect on migration. As expected the saturated fatty acid significantly upregulated DC chemotaxis towards CCL19 ($p < 0.001$).

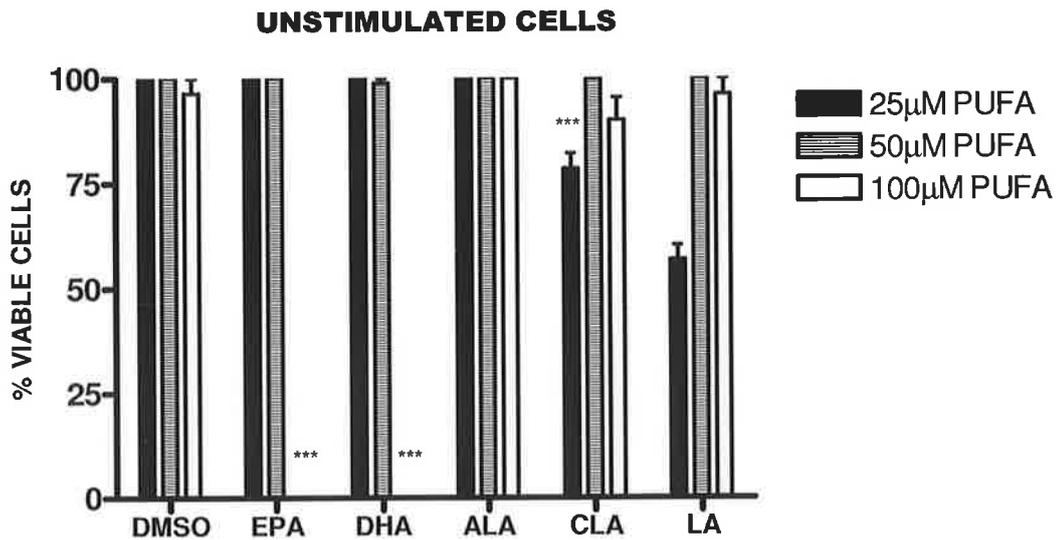


FIGURE 3.1

The concentration of fatty acids used during the course of in vitro cell culture does not significantly affect the viability of dendritic cells (DC)

A dose response assay was carried out in bone marrow-derived DC to assess the possible toxicity of increasing concentrations of fatty acids: Bone marrow-derived dendritic cells (DC) were incubated for a total of 7 days with the specified concentrations (25, 50 and 100 μM) of DMSO (vehicle control), EPA, DHA, ALA, CLA, LA and SA. After 7 days cellular viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA).

Results are expressed as a percentage of untreated cells and are mean ± SE of quadruplicate assays and represent two independent experiments.

***p<0.001 vs DMSO vehicle control determined by one-way ANOVA test

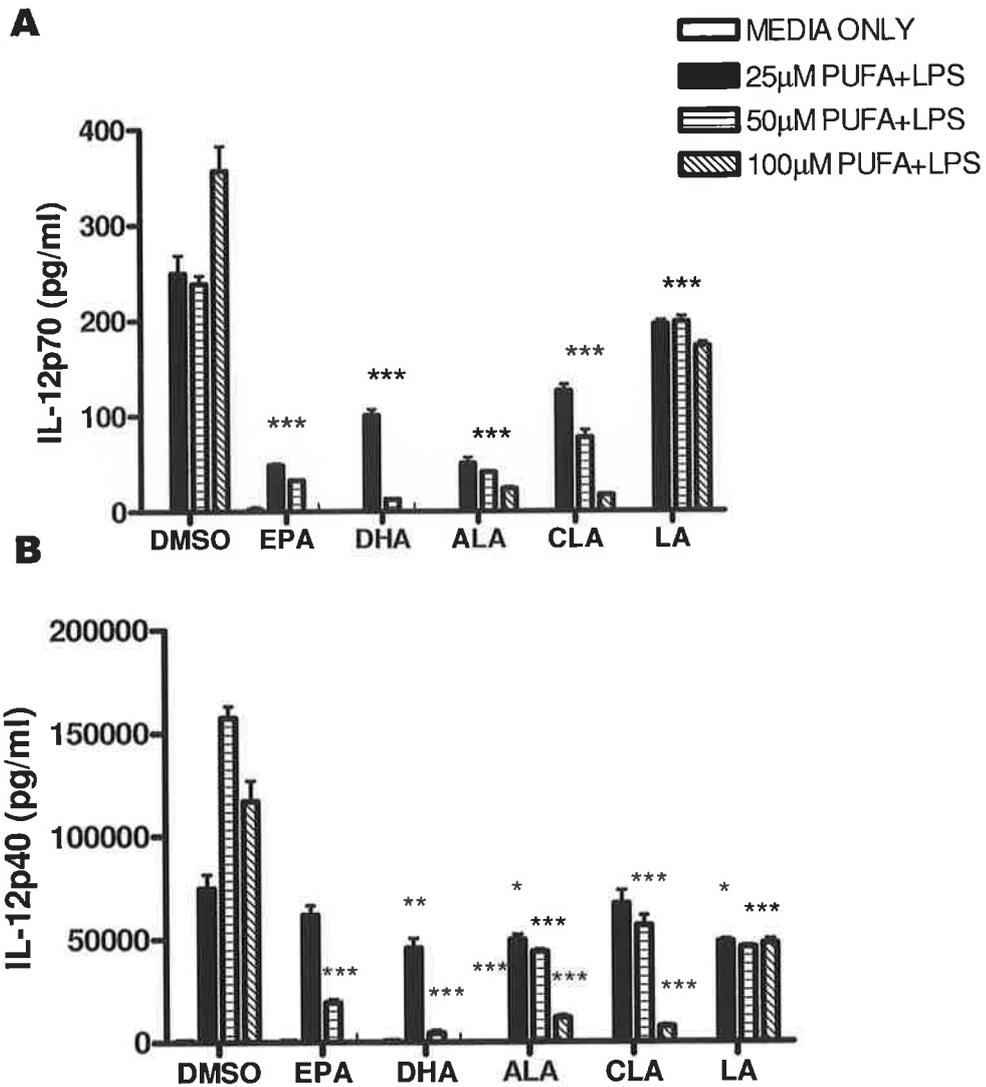


FIGURE 3.2

The addition of 25, 50 and 100µM of PUFA to DC inhibits the production of IL-12p70 [A] and IL-12p40 [B] in a dose dependant manner.

A dose response assay was carried out in DC to ascertain the effects of increasing concentrations of fatty acids on inflammatory cytokine production. The three PUFA concentrations used inhibited IL-12p70 and p40 production in DC stimulated with 100ng/ml LPS. As a dose of 100µM of EPA and DHA was found to be cytotoxic (see Figure 3.1), therefore it was not possible to generate any ELISA data for these treatment groups.

Levels of IL-12p40 and p70 were measured using specific immunoassays. Results are mean ± SE of quadruplicate assays and represent three independent experiments.

***p<0.001, **p<0.01, *p<0.05 vs DMSO vehicle control determined by one-way ANOVA test

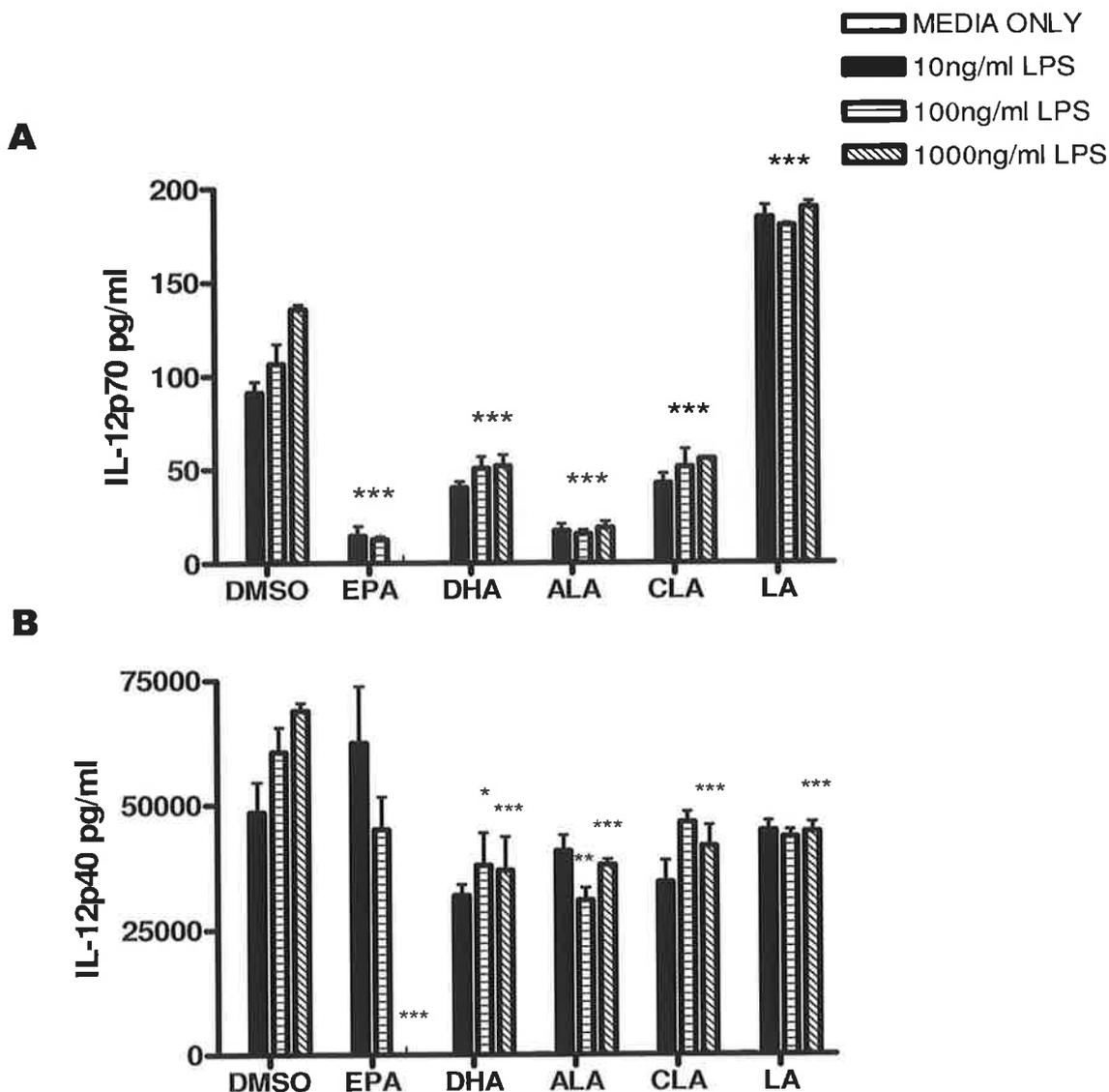


FIGURE 3.3

PUFA are able to inhibit cytokine production in DC after stimulation with three increasing concentrations of LPS (10ng, 100ng, 1µg).

A dose response assay was carried out in DC to ascertain the effects of increasing concentrations of LPS on PUFA-induced inhibition of inflammatory cytokine production. PUFA were able to inhibit IL-12p70 [A] and IL-12p40 [B] production in DC stimulated with three concentrations of LPS. The fatty acids and DMSO were used at the following concentrations: DMSO 50µM, EPA 25µM, DHA 25µM, ALA 25µM, CLA 50µM or LA 50µM

Levels of IL-12p70 and p40 were measured using specific immunoassays. Results are mean ± SE of quadruplicate assays and represent three independent experiments.

***p<0.001, **p<0.01, *p<0.05 vs DMSO vehicle control determined by one-way ANOVA test

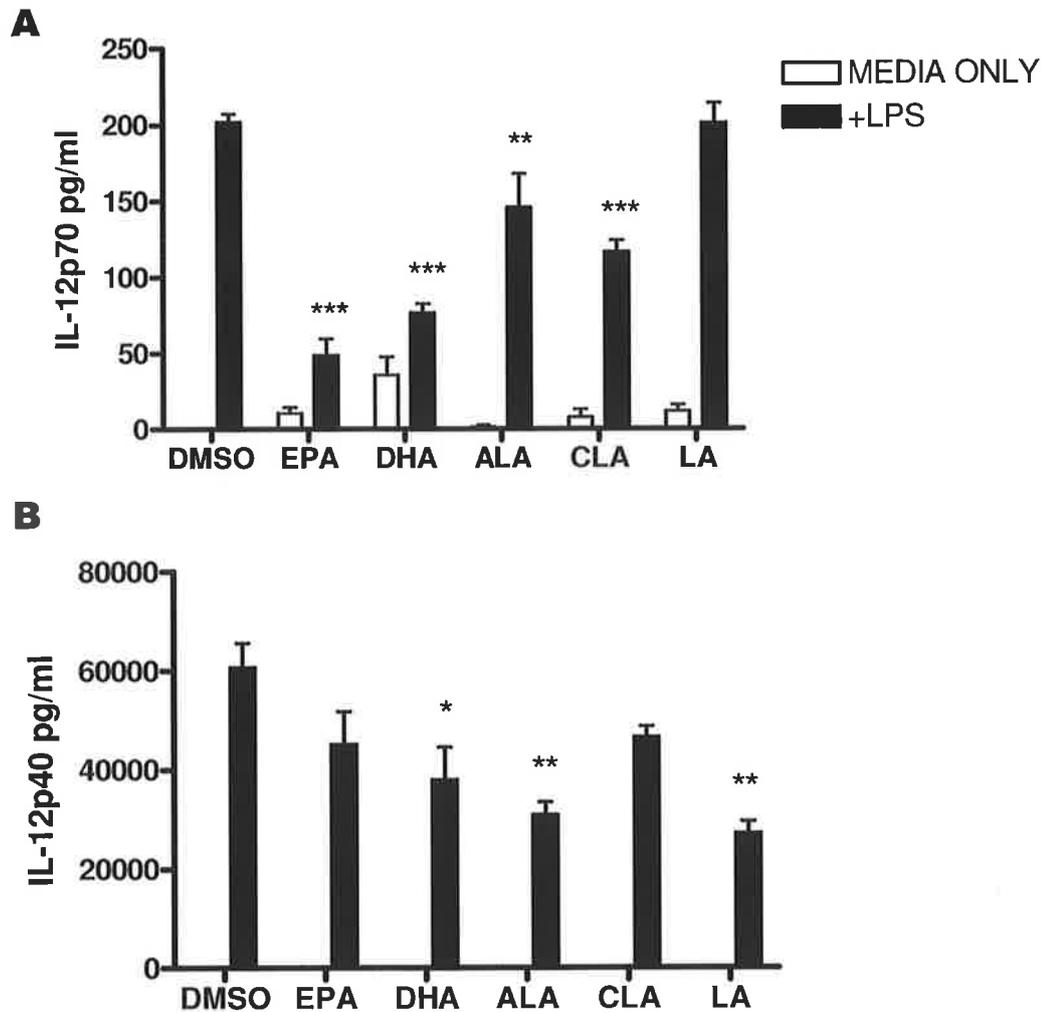


FIGURE 3.4

Bone marrow-derived DC incubated with PUFA inhibit LPS-induced IL-12p70 [A] and IL-12p40 [B] production

Immature DC were differentiated with GM-CSF and concurrently exposed to DMSO (vehicle control), polyunsaturated fatty acids (PUFA) - EPA, DHA, ALA, CLA – or a saturated fatty acid control (LA) for 7 days. The subsequent immature DC (1×10^6 cells/ml) were stimulated *in vitro* with LPS and supernatants recovered after 24 hours. Levels of IL-12p70 and IL-12p40 were measured using specific immunoassays. Results are mean \pm SE of quadruplicate assays and represent three independent experiments.

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs DMSO vehicle control determined by one-way ANOVA test

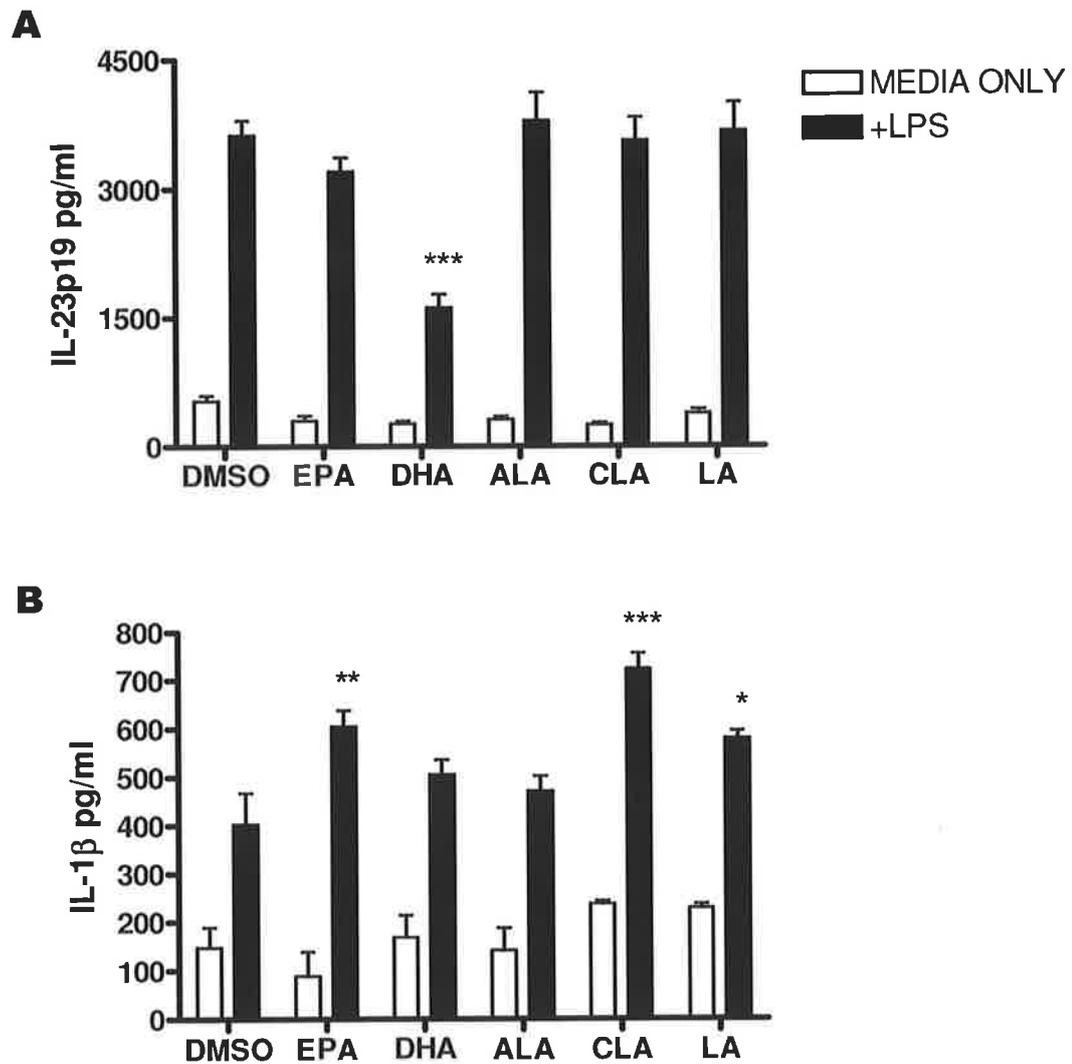


FIGURE 3.5

Individual PUFA have differing effects on the production of LPS-induced IL-23p19 [A] and IL-1β [B]

Immature DC were differentiated with GM-CSF and concurrently exposed to DMSO (vehicle control), polyunsaturated fatty acids (PUFA) - EPA, DHA, ALA, CLA – or a saturated fatty acid control (LA) for 7 days. The subsequent immature DC (1×10^6 cells/ml) were stimulated *in vitro* with LPS and supernatants recovered after 24 hours. Levels of IL-23p19 [A] and IL-1β [B] were measured using specific immunoassays.

Results are mean \pm SE of quadruplicate assays and represent three independent experiments.

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs DMSO vehicle control determined by one-way ANOVA test

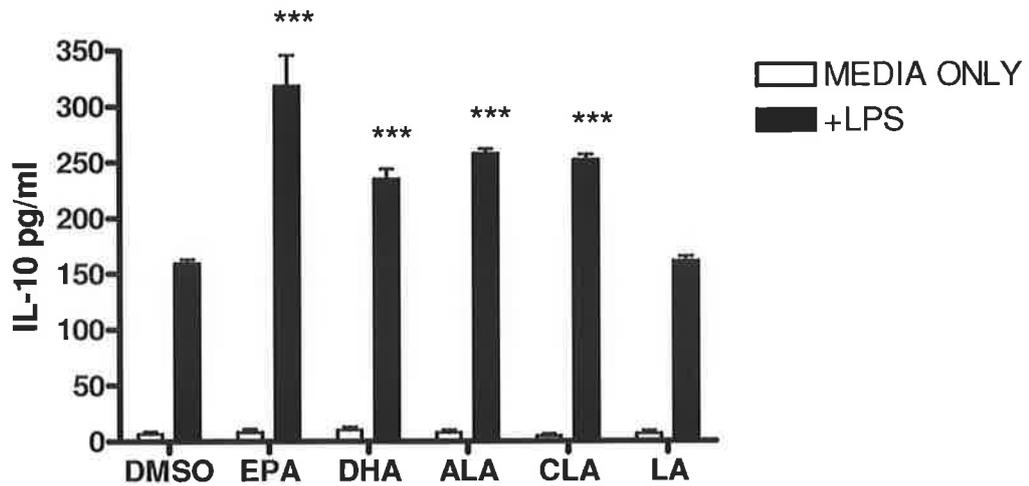


FIGURE 3.6

PUFA-treated DC up-regulate IL-10 production following LPS stimulation

Immature DC were differentiated with GM-CSF and concurrently exposed to DMSO (vehicle control), polyunsaturated fatty acids (PUFA) - EPA, DHA, ALA, CLA – or a saturated fatty acid control (LA) for 7 days. The subsequent immature DC (1×10^6 cells/ml) were stimulated *in vitro* with LPS and supernatants recovered after 24 hours. Levels of IL-10 were measured using a specific immunoassay.

Results are mean \pm SE of quadruplicate assays and represent three independent experiments.

*** $p < 0.001$ vs DMSO vehicle control determined by one-way ANOVA test

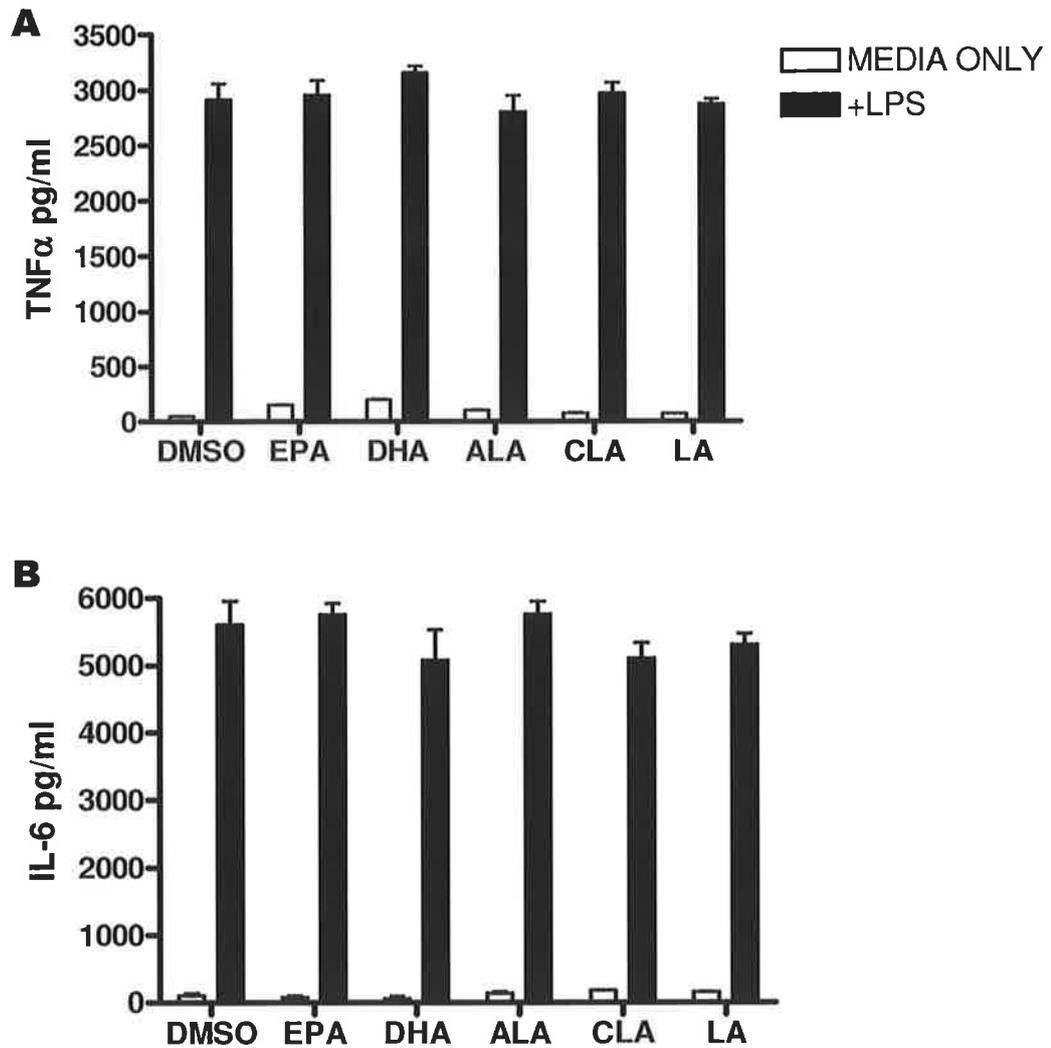


FIGURE 3.7

PUFA have no significant effect on the production of TNF α or IL-6

Immature DC were differentiated with GMCSF and concurrently exposed to DMSO (vehicle control), polyunsaturated fatty acids (PUFA) - EPA, DHA, ALA, CLA - or a saturated fatty acid control (LA) for 7 days. The subsequent immature DC (1×10^6 cells/ml) were stimulated *in vitro* with LPS and supernatants recovered after 24 hours. Levels of TNF α [A] and IL-6 [B] were measured using specific immunoassays

Results are mean \pm SE of quadruplicate assays and represent three independent experiments.

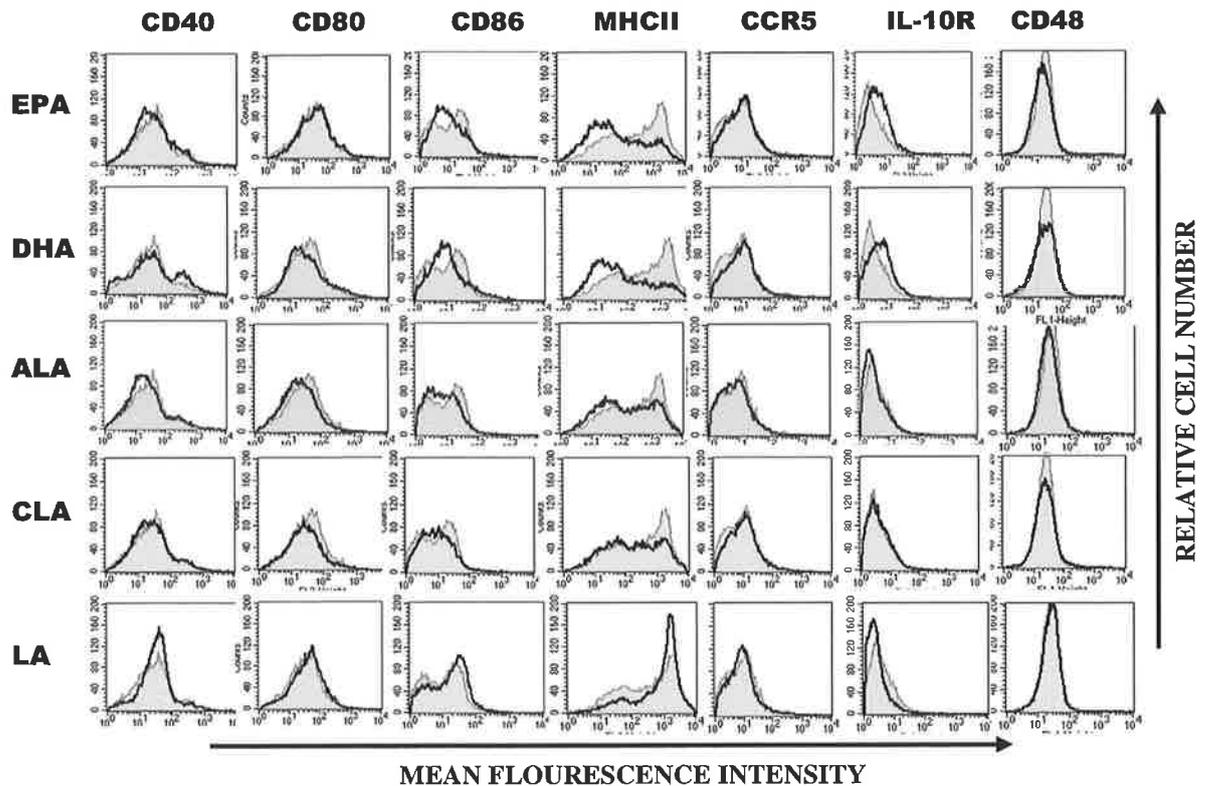


FIGURE 3.8

PUFA modulate the expression of cell surface markers on the surface of immature DC.

Myeloid DC were differentiated with GM-CSF for 7 days in the presence of fatty acids (EPA, DHA, ALA, CLA, or LA) or vehicle control (DMSO), before cells were washed and stained with antibodies specific for CD40, CD80, CD86, MHCII, CCR5, IL-10R, and CD48, or with isotype matched controls. Results of immunofluorescence analysis are shown for DMSO-treated DC (filled histogram), overlaid with PUFA-treated DC (thin black line). Profiles are shown for a single experiment and are representative of 3 experiments.

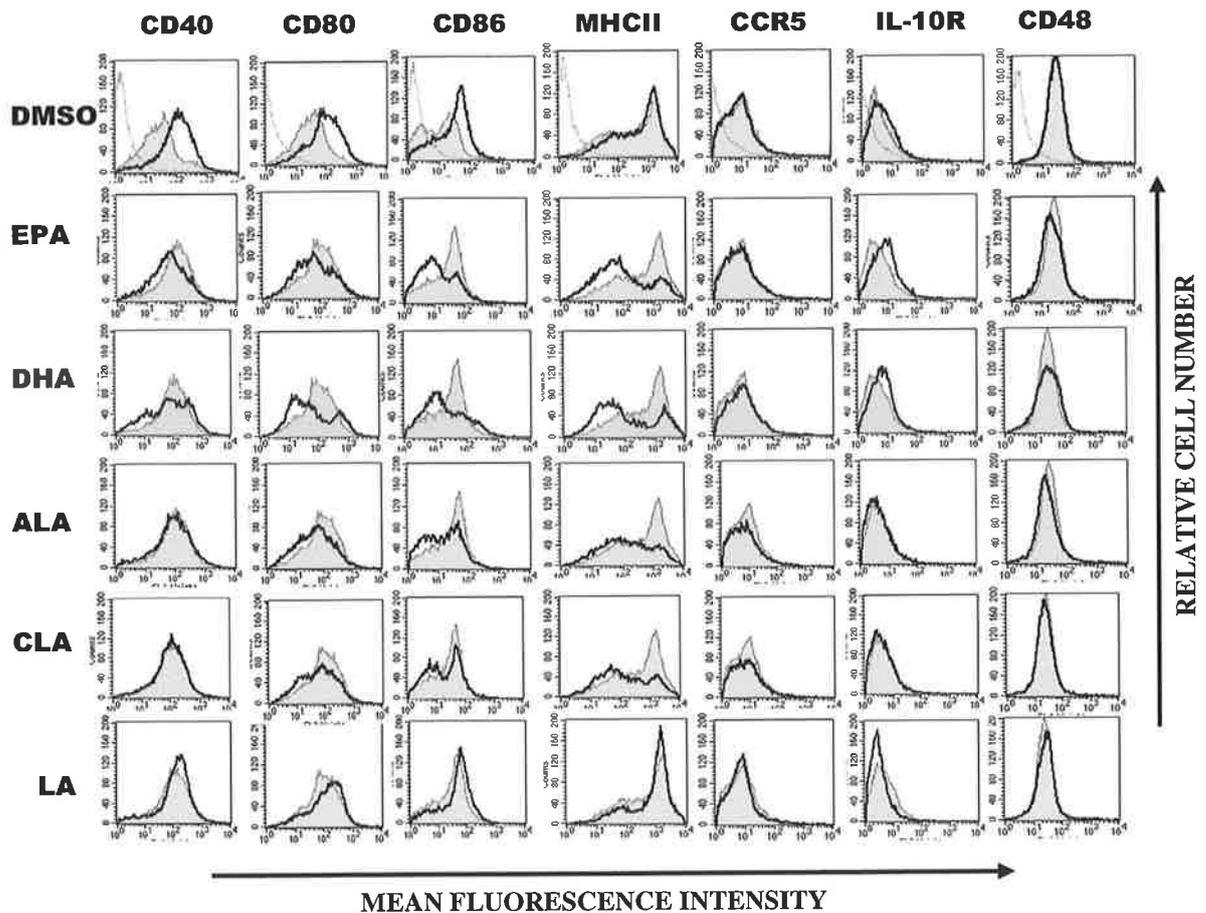


FIGURE 3.9

PUFA modulate the expression of cell surface markers on the surface of LPS-stimulated mature DC.

Myeloid DC were differentiated with GM-CSF for 7 days in the presence of fatty acids (EPA, DHA, ALA, CLA, or LA) or vehicle control (DMSO), before cells were activated with 100ng/ml LPS. After 24 hours cells were washed and stained with antibodies specific for CD40, CD80, CD86, MHCII, CCR5, IL-10R, and CD48, or with isotype matched controls.

The top row of histograms represents DMSO-treated immature cells (filled histogram) overlaid with DMSO-treated and LPS-stimulated cells (black line). This is to show the upregulation of surface markers as DC mature following LPS-activation. Histograms for isotype controls are shown as dotted lines in the first row only.

In rows 2 onwards results of immunofluorescence analysis are shown for LPS-activated cells: DMSO-treated DC +LPS (filled histogram) and PUFA-treated DC +LPS (thin black line). Profiles are shown for a single experiment and are representative of 3 experiments.

The tables below represent the flow cytometry data shown in **Figures 3.8** and **3.9**: A plus (+) symbol shows upregulation of surface marker expression on DC (multiple + symbols indicate a larger positive shift), and a minus (-) signifies a decrease in expression (again multiple - symbols denote a larger decrease). The scores were assigned by examining the histogram shift by eye. A score of zero (0) indicates no observed change.

TABLE 3.1 Summary of the effects of various fatty acids on the expression of cell surface markers on immature DC [Taken from FIGURE 3.8]

+ represents an observed increase in surface marker expression

- denotes a decrease in cell surface marker expression

ZERO (0) represents no observed change

TREATMENT	CD40	CD80	CD86	MHCII	IL-10R	CCR5	CD48
EPA	-	0	--	----	++	+	0
DHA	0	-	--	----	++	+	0
ALA	-	-	-	-	0	0	0
CLA	0	-	-	--	0	+	0
LA	+	0	+	+	-	-	0

TABLE 3.2 Summary of the effects of various fatty acids on the expression of cell surface markers on DC stimulated with LPS [Taken from FIGURE 3.9]

+ represents an observed increase in surface marker expression

- denotes a decrease in cell surface marker expression

ZERO (0) represents no observed change

TREATMENT	CD40	CD80	CD86	MHCII	IL-10R	CCR5	CD48
EPA	--	-	--	----	++	0	-
DHA	--	--	--	----	++	+	-
ALA	0	0	-	--	0	-	-
CLA	0	--	-	----	0	+	0
LA	+	+	+	+	--	0	0

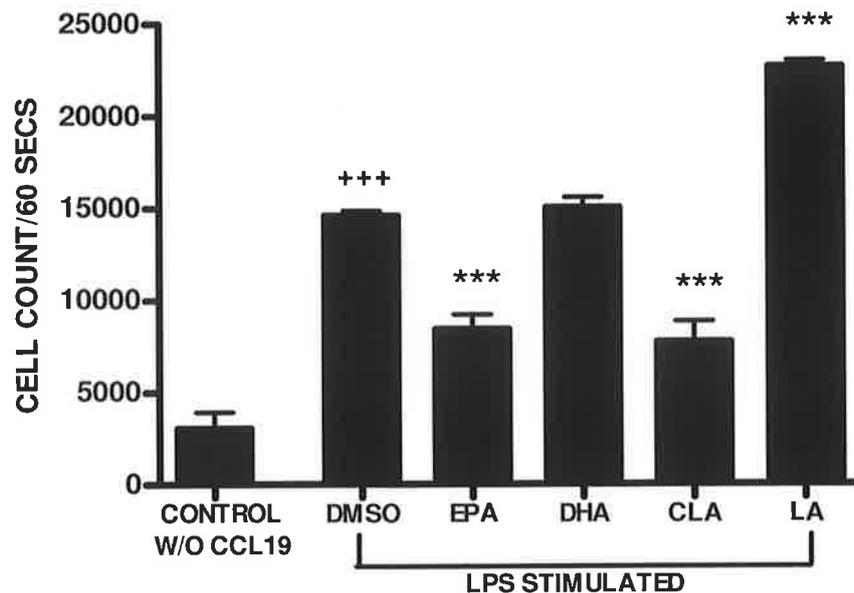


FIGURE 3.10

EPA and CLA reduce the migration of DC towards the chemokine MIP-3 β (CCL19).

DC were cultured in the presence of fatty acids and stimulated with LPS (100ng/ml) before 3×10^5 cells were placed in the upper chamber of a Transwell plate (8.0 μ m). Media containing recombinant CCL19 (100ng/ml) was added to the lower chamber and plates were incubated for 5 hours at 37°C. To determine the number of migrated cells, media from the bottom well was collected and events (cells) counted for 60seconds on a FACsCalibur. To serve as a negative control, unstimulated cells were added to the upper chamber of a Transwell plate and media only (containing no CCL19) was added to the bottom chamber. This was to ensure that DC migration was initiated by the presence of the chemokine.

***p<0.001, **p<0.01, *p<0.05 vs DMSO vehicle control determined by one-way ANOVA test

+++ p<0.001 using an unpaired t-test between control (without CCL19) and LPS-stimulated DMSO groups

3.3 DISCUSSION

The findings of this study demonstrate that polyunsaturated fatty acids (EPA, DHA, and CLA) have the ability to inhibit LPS-induced IL-12 production in DC, whilst enhancing levels of IL-10. Concurrently, PUFA down-regulate the expression of key co-stimulatory molecules on the DC surface both pre- and post-stimulation, i.e., levels of CD40, CD80, CD86, and MHCII were all down-regulated on DC cultured in the presence of EPA and DHA, and to a lesser extent, CLA and ALA. There was also a significant reduction in cell migration towards the chemokine MIP-3 β , which is known to direct mature DC to lymph nodes facilitating their interaction with naïve T cells (Banchereau *et al.*, 2000). The immunomodulatory effects described above were not seen when DC were treated with LA, the saturated fatty acid control (with the exception of IL-12p40 inhibition).

IL-12 is an important link between innate and adaptive immunity as its targets include T cells, DC, and NK cells, and one of its main functions is to direct newly activated T helper cells to a Th1 phenotype (Hasko & Szabo, 1999). IL-12 has been shown to be involved in autoimmune diseases including MS, IBD, and RA, and overproduction is important in inflammatory states such as septic shock (Papadakis & Targan, 2000; Fuss *et al.*, 2006) consequently it represents a potential therapeutic target for the treatment of some inflammatory disorders. The fact that PUFA are able to decrease IL-12 confirms that fatty acids modulate dendritic cells, and also points to the possibility that these PUFA-modified DC may alter subsequent T cell responses given the pivotal role of IL-12 in Th1 development (Trinchieri, 2003). Therefore, the decrease in IL-12 may partly explain the anti-inflammatory effects seen after PUFA dietary supplementation.

IL-23 is another heterodimeric cytokine consisting of the IL-12p40 sub-unit and a unique p19 sub-unit. IL-23 binding to the IL-23R on CD4⁺ T cells promotes their development into Th17 cells which are characterised by the production of IL-17 and IL-6 (Langrish *et al.*, 2005; Kikly *et al.*, 2006). IL-17 has been found to be elevated in many human autoimmune diseases, for example MS and RA, and has therefore been implicated in having a role in tissue destruction (Kikly *et al.*, 2006), and IL-23 has also emerged as a key player in a number of chronic

inflammatory diseases including IBD (Neurath, 2007) and collagen-induced arthritis (Yago *et al.*, 2007).

The IL-23 receptor is not expressed on naïve T cells but is found mainly found on activated T cells (Langrish *et al.*, 2004) and thus it has been suggested that IL-23 is involved in the survival and expansion of already differentiated Th17 cells but not their initial polarisation. It was recently shown that TGF- β and IL-6 upregulate IL-23R expression (Zhang *et al.*, 2007), and may therefore be involved in the generation of Th17 cells in mice. Moreover IL-1 β and TNF- α were also both shown to have a synergistic effect on IL-23-mediated IL-17 production in a model of EAE (Sutton *et al.*, 2006). The limited effect of PUFA on IL-23 production seen here, (with the exception of DHA), coupled with the little or no effect on IL-6 and TNF- α , and augmentative effect on IL-1 β would point to an inconsequential downstream effect of PUFA on Th17 cell development. The more noticeable effect of PUFA on IL-12 production implies a potential effect on subsequent Th1 responses and IFN- γ production. Alterations in ensuing adaptive immune responses with regard to PUFA effects on T cell cytokine production will be discussed in chapter 5.

In contrast to IL-12 and IL-23, IL-10 is considered an anti-inflammatory or regulatory cytokine. It suppresses the production of inflammatory cytokines such as IL-1, IL-6, IL-12, and TNF- α from APC (Papadakis & Targan, 2000), and reduces the secretion of IL-2 and IFN- γ from Th1 cells, as well as inhibiting their proliferation and chemotaxis (Mocellin *et al.*, 2004). Furthermore, IL-10 deficiency in mice results in an overproduction of inflammatory cytokines and leads to the development of chronic inflammatory diseases (Conti *et al.*, 2003) demonstrating its fundamental role in regulating the immune response. PUFA increased LPS-induced IL-10 production in DC, again presenting a possible mechanism used by PUFA to elicit their anti-inflammatory effects. This increase in IL-10 could have subsequent effects on T helper cell differentiation as IL-10 can inhibit Th1 development, or direct T cells to a more regulatory phenotype (Conti *et al.*, 2003).

IL-10 has the ability to decrease IL-12 production (Conti *et al.*, 2003), and so the changed levels of IL-12 induced by PUFA may be due to the presence of increased amounts of IL-10 in the supernatant. The decrease in IL-12 was not tested for IL-10 dependency in this study but PUFA-induced IL-12 suppression

was found to be IL-10-dependent in a previous report by our research group (Loscher *et al.*, 2005). The increase in IL-10 production was also made more significant by the recorded increase in IL-10 receptor (IL-10R) expression in PUFA-treated DC, which was seen more prominently in the n-3 PUFA, EPA and DHA. This increase in IL-10R expression has not been previously reported for n-3 PUFA-treated DC but was recorded with CLA in the publication from our lab cited above. The increased receptor expression may create a positive feedback loop rendering the DC more responsive to IL-10 and subsequently enhancing their production of the cytokine. Given the potent regulatory and anti-inflammatory effects of IL-10, this upregulation in production and receptor expression could be important for how PUFA mediate their beneficial effects. Further to its effects on cytokine production, IL-10 can also alter surface marker expression, and has been shown to inhibit the full maturation of DC by down-regulating ICAM-1, CD80, and CD86 expression (de Jong *et al.*, 2005). IL-10 also has the ability to block the translocation of peptide-MHCII complexes to the DC plasma membrane (Banchereau *et al.*, 2000) all of which culminate in impaired T cell responses. Contrastingly, IL-12 can upregulate MHCII, CD80 and CD86 expression on APC (Bastos *et al.*, 2004). Therefore the observed changes in cytokine production, with regard to IL-10 and IL-12 in PUFA-treated DC, could also account for the recorded alterations in cell surface marker expression.

The ligation of CD80 and CD86 on APC with CD28 on T cells provides the most potent costimulatory signal to T cells resulting in proliferation and cytokine production (Bhatia *et al.*, 2006). Furthermore, CD40-CD40L interaction not only activates APC but also enhances T cell activation (van Kooten & Banchereau, 2000). This, coupled with the fact that over-expression of costimulatory molecules has been reported in the inflamed tissue of patients with MS, RA and IBD (Liu *et al.*, 2001b; Maerten *et al.*, 2003) signifies that costimulatory markers are potential therapeutic targets for treating inflammatory disorders, and modulating these markers on the DC surface would have serious consequences on T cell responses. In the present study, it was found that culturing DC with PUFA decreased the surface expression of CD40, but more significantly, CD80 and CD86 expression, both pre- and post-stimulation with LPS. The expression of MHCII was also markedly down-regulated by PUFA before and after LPS

activation. Since MHCII is required for antigen presentation, and DC are the only APC capable of activating naïve T cells specifically because they express MHCII together with the necessary surface molecules for T cell co-stimulation, the fact that PUFA decrease both MHCII and co-stimulatory molecules on the only cell capable of activating them suggests PUFA treatment would have downstream effects on T cell responses. It would also be reasonable to assume that the decrease in DC chemotaxis seen here would have serious consequences on T cell responses considering the DC must migrate to T cell areas in order to activate naïve T cells and initiate adaptive immunity. PUFA-induced changes in T cell responses will be examined in chapter 4.

The results obtained here with regard to cytokine and maturation marker alterations have been reported for a number of other molecules. For example, cholera toxin (CT) increased LPS-induced IL-10 and decreased IL-12 production in bone-marrow derived DC (Lavelle *et al.*, 2003). The researchers report however that CT increased the surface levels of CD80 and CD86, but had an inhibitory effect on CD40 expression. A similar finding was conveyed by Ross and colleagues (2004) who reported that the adenylate cyclase toxin from *Bordetella pertussis* (the bacteria responsible for whooping cough) was able to simultaneously increase IL-10, and decrease TNF- α and IL-12p70 secretion. Again the toxin increased levels of CD80, CD86, and MHCII in immature DC. These findings suggest that the changes in co-stimulatory marker expression following PUFA treatment may not be dependent on either the increase in IL-10, or the decrease in IL-12 production, since similar changes in cytokine production did not have comparable effects on co-stimulatory marker expression to the two reports cited above.

The immunomodulatory effects of PUFA reported in this study are in agreement with many other publications, for example, Zeyda and co-workers (2005) saw a reduction in TNF- α , IL-12p40, and CD40 mRNA levels following the treatment of human-monocyte-derived DC with PUFAs of n-3 (EPA) and n-6 series (AA or arachidonic acid). Whereas bone-marrow derived DC had decreased expression of CD40, CD80, CD86, and MHCII after the addition of DHA in a study by Weatherill *et al.*, (2005). A very recent study by Wang and associates (2007) reported a reduction in CD80, CD86, and MHCII expression, in addition to

diminished TNF- α and IL-12 production from human monocyte-derived DC following treatment with the n-3 PUFA, EPA and DHA.

Since the most potent determinant of T-helper cell fate appears to be the cytokine milieu present during the differentiation process, and because co-stimulation is vital for efficient T cell activation, both cytokines and cell surface markers represent important targets for immunomodulation, and using natural therapies such as PUFA is an attractive option. The added finding that PUFA (particularly EPA and CLA) can inhibit the chemotaxis of DC to T cells, reiterates the possibility of an altered T cell response following dietary intervention with PUFA. The effects of the PUFA-induced changes recorded here in DC on consequential T cell activation and the adaptive immune response will be examined in subsequent chapters.

CHAPTER 4

EFFECTS OF PUFA- MODULATED DC ON ADAPTIVE IMMUNE RESPONSES

4.1 CHAPTER 4 INTRODUCTION

Dendritic cells are the only cells capable of activating naïve T cells. The subsequent differentiation of naive CD4⁺ T cells into subsets of T helper cells is a pivotal process with major implications for host defence and the pathogenesis of immune-mediated disease (Kapsenberg, 2003; de Jong *et al.*, 2005).

For the last 20 years, effector T cell responses have been restricted to either a Th1 or Th2 phenotype based on their cytokine production profiles: Th1 cells produce large quantities of interferon- γ (IFN- γ), whereas Th2 cells produce IL-4, IL-5 and IL-13. Th1 cells are considered pro-inflammatory and are highly effective at clearing intracellular pathogens, whereas Th2 cells are associated with the clearance of parasitic infections (de Jong *et al.*, 2005). A heightened Th1 response is often seen in some chronic inflammatory and autoimmune disorders, including Crohn's disease (Agnello *et al.*, 2003). However, very recently, a novel subset of Th cells, named Th17 cells, have also been implicated in the pathogenesis of diseases previously attributed to an excessive Th1 response, for example in rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis (Kolls & Linden, 2004; Kikly *et al.*, 2006). Th17 cells predominantly secrete IL-17 (Harrington *et al.*, 2006) and IL-17 functions as a classic effector of innate immunity with actions similar to those of TNF- α or IL-1 β (Yujing Bi, 2007). IL-23 serves to expand previously differentiated Th17 cell populations, but IL-6 and transforming growth factor- β (TGF- β) have been shown to induce the differentiation of Th17 cells from naive precursors (Bettelli *et al.*, 2007), and IL-1 and TNF- α are also thought to be involved in promoting the Th17 subset (Sutton *et al.*, 2006).

To become activated, T helper cells must first encounter their TCR-specific antigen coupled to an MHCII molecule on a DC. A second interaction involving costimulatory receptors (CD28, CD40L) on T cells and their ligands (CD80/CD86, CD40) on APC is required for optimal T cell activation (Bhatia *et al.*, 2006). Contrastingly, an inhibitory signal can be delivered to a T cell when CTLA-4 on its surface is ligated by either CD80 or CD86 on an APC (Alegre *et al.*, 2001; Schneider *et al.*, 2006). Modifications in the expression of any of these influential surface molecules can markedly alter the process of T cell activation, seen for example, when the absence of a co-stimulatory signal renders

T cells anergic (Kapsenberg, 2003). Given that we demonstrated clear effects of PUFA on DC cytokine production and cell surface marker expression, we investigated whether PUFA-modulated DC could influence subsequent adaptive immune responses. Whilst EPA and DHA were previously shown to have immunomodulatory properties, in the following chapter we only examined the effects of CLA as there is little or no data published on the effects of this fatty acid on adaptive immunity making it the most novel PUFA to investigate. The following study firstly examines the effects of CLA-treated DC on T cell cytokine production in an *in vitro* co-culture model. And secondly, to assess the potential immunomodulatory properties of PUFA-altered DC in a more *in vivo* situation, DC were cultured with CLA and activated with KLH (*in vitro*) before being injected into the footpad of naïve mice in an adoptive transfer study. Alterations in antigen-specific T cell responses in cells isolated from the spleens and lymph nodes of the recipient mice were then examined.

4.2 RESULTS

4.2.1 PUFA-MODULATED DC (IRRADIATED AND NON-IRRADIATED) CAN ALTER THE CYTOKINE PRODUCTION PROFILES OF CD4⁺ T CELLS

Figure 4.1 clearly shows that pre-treating DC with PUFA alters subsequent T helper cell responses. DC cultured for 7 days with CLA and activated with OVA peptide were co-incubated with CD4⁺ T cells isolated from the spleens of D011.10 mice which express a TCR specific for a peptide within the ovalbumin (OVA) molecule (OVA (323-339)). When this OVA antigen is presented by DC complexed to MHCII (together with costimulation), the naive CD4⁺ T cells become activated and proliferate (Pompos & Fritsche, 2002). **Figure 4.1** shows that T cells activated with irradiated CLA-treated DC produce significantly less IFN- γ ($p < 0.05$), less IL-17 ($p < 0.05$), and less IL-4 ($p < 0.01$) on day 10 of culture. Unexpectedly, IL-10 was not detected in the supernatant of any the co-culture experiments.

Irradiating DC renders them unable to secrete protein. Here irradiation did not influence the ability of DC to suppress T cell cytokine production, as results were consistent when using both irradiated and non-irradiated DC. However, although IL-17 was suppressed with irradiated and non-irradiated DC, results only reached statistical significance when DC were irradiated ($p < 0.05$). The fact that cytokine production was suppressed when DC were unable to produce cytokines themselves (i.e. irradiated) suggests an apparent role for the altered costimulatory molecules on the DC surface (as discussed in chapter 3) in these PUFA-induced immunomodulatory effects.

In contrast to the *in vitro* data, the saturated fatty acid LA, had a slight inhibitory effect on cytokine production; LA decreased IFN- γ ($p < 0.05$ - irradiated group only) and IL-4 ($p < 0.01$ - irradiated and $p < 0.05$ - non-irradiated group). However, the inhibition of these cytokines was not to the same extent as that seen with CLA and LA was not able to inhibit the production of IL-17.

4.2.2 THE ADDITION OF LPS RENDERS PUFA LESS ABLE TO INHIBIT CYTOKINE PRODUCTION FROM T HELPER CELLS.

We found that cytokine production from T cells was inhibited by CLA-treated DC when activated by OVA alone. To investigate whether PUFA could still suppress cytokine production from T cells when stimulated with a stronger Th1-inducing stimulus, LPS (100ng/ml) was added to DC at the same time as OVA before culture with naïve CD4⁺ T cells, and supernatant assayed for the presence of various cytokines on day 4 and following a second round of activation on day 10 of co-culture.

CLA-treated DC inhibited the OVA-specific production of cytokines from T cells, i.e. IFN- γ , IL-17, IL-2 and IL-4 were all down-regulated on day 10 [Figures 4.2 - 4.5]. The cytokine expression patterns were found to be very different at the two time-points assayed in the co-culture experiment. ELISA results obtained from supernatant recovered on day 10, after T cells had been primed for a second time, showed a more robust response signified by higher cytokine production. This would be more indicative of a true immune response. The less robust response recorded on day 4 of the co-culture still saw CLA-treated DC reduce the OVA-specific production of IL-2 ($p < 0.05$), IL-4 ($p < 0.05$) and IL-17 (not significantly) from T cells. Results from supernatant recovered on day 10 of co-culture reiterate our findings from previous experiments, and show that pre-treating DC with CLA down-regulates the subsequent T cell production of IFN- γ ($p < 0.05$), IL-17, IL-2 ($p < 0.001$), and IL-4 ($p < 0.05$). Less IL-17 was produced on day 10 when DC were pre-incubated with CLA and activated with OVA, however this did not reach statistical significance using a one-way ANOVA test. Statistical analysis of data using an unpaired t-test comparing the DMSO- and CLA-OVA treated groups showed that the means were significantly different ($p < 0.001$).

The additive stimulation of cells with LPS rendered CLA unable to modulate T cell responses as cytokine production both at day 4 and day 10 remained relatively unchanged in the CLA-treated group compared to the DMSO control. Only the production of IL-2 was significantly suppressed on day 4 by the presence of CLA ($p < 0.001$) after OVA and LPS activation.

The saturated fatty acid control, LA, had conflicting effects on cytokine production in the different treatment groups. In the OVA-activated groups, LA increased the production of all the cytokines assayed correlating with the *in vitro* DC data. However upon the addition of LPS, LA decreased IFN- γ and increased IL-17 production at both time-points, whilst suppressing IL-2 on day 4 but augmenting it on day 10. Levels of IL-4 remained largely unchanged. It is important to note at this point that stimulating cells with OVA and LPS resulted in considerable cell proliferation. When viewed under a microscope, wells that had been treated with OVA and LPS appeared completely over-grown with cells and the supernatant was far more discoloured than in OVA-only wells. The erratic pattern of cytokine production in the different treatment groups recorded after OVA and LPS activation could be a result of this over-stimulation, perhaps due to excessive cellular activation and/or increased apoptosis of DC and/or T cells.

4.2.3 PUFA-INDUCED INHIBITION OF CD4⁺ T CELL CYTOKINE PRODUCTION IS PARTIALLY REVERSED BY THE ADDITION OF THE SPECIFIC ERK INHIBITOR U0126

We previously reported that the PUFA-induced changes in DC cytokine production (particularly IL-10) were found to involve the ERK MAPK pathway (previous published observations). Therefore we assessed whether the CLA-induced changes in T cell cytokine production also involved ERK. **Figure 4.6** shows that the production of IFN- γ , IL-17 and IL-2 are all induced following the activation of T cells from OVA-transgenic mice with OVA-stimulated DC. Again DC pre-treated with CLA were able to inhibit the production of IFN- γ ($p < 0.05$), IL-17 ($p < 0.05$), and most significantly IL-2 ($p < 0.001$). Addition of the ERK inhibitor (U0126) to DC 1 hour prior to OVA stimulation partially reversed these inhibitory effects, as the CLA-induced suppression of IFN- γ , IL-17, and IL-2 were no longer statistically significant. The data here reiterates the possibility of an ERK-dependent mechanism used by PUFA to elicit their immunosuppressive actions.

4.2.4 ADOPTIVELY TRANSFERRED PUFA-MODULATED DC ALTER ANTIGEN-SPECIFIC CYTOKINE PRODUCTION FROM CELLS ISOLATED FROM THE LOCAL LYMPH NODES AND SPLEENS OF RECIPIENT MICE.

Bone marrow-derived DC were cultured in the presence of 50 μ M DMSO, CLA and LA for 6 days before being stimulated with KLH (10 μ g/ml) or medium alone for a further 24 hours. After being extensively washed, 10⁵ cells were injected into the footpads of recipient BALB/c mice in 25 μ l PBS (5 animals per group). Local (popliteal) lymph nodes and spleens were removed and processed 7 days later. Spleen cells (2x10⁶/ml) or popliteal lymph node cells (1x10⁶/ml) were cultured with medium alone, KLH (2 μ g/ml, 10 μ g/ml, and 50 μ g/ml), or PMA (20ng/ml) and α CD3 (1 μ g/ml). Supernatant was collected after 72 hours and concentrations of IL-17 [Figure 4.7], IL-10 [Figure 4.8], IL-2 [Figure 4.9], IFN- γ [Figure 4.11], and IL-4 [Figure 4.12] were measured using specific immunoassays. Alterations in the proliferation of lymph node cells were also examined by [³H]-thymidine incorporation and results shown in Figure 4.10. The lymph node data is more representative of the potential effects of CLA-modulated DC on T cell responses *in vivo* considering that cells were injected into the footpad of mice and the popliteal lymph nodes are the local draining lymph nodes. Transferred DC would more than likely migrate to popliteal lymph nodes where they would encounter and possibly induce lymphocytes to produce cytokines. Spleens cells were also examined for any changes in cytokine production to assess whether PUFA-modulated DC could have a more systemic effect. The changes occurring in the spleen were less notable than those in the lymph node for all of the cytokines examined.

Treating DC with CLA before footpad transference inhibited basal IL-17 levels in the lymph nodes (Figure 4.7A; p<0.01) and also suppressed IL-17 production from lymphocytes following KLH stimulation (2 μ g p<0.01; 10 μ g p<0.001; 50 μ g p<0.001). CLA-treated DC pre-activated with KLH also inhibited IL-17 production when cells were re-stimulated with 10 μ g KLH *ex vivo* (p<0.01). Unexpectedly, LA-treated DC also down-regulated IL-17 production from lymph node cells in a similar manner to CLA. LA has consistently been found to decrease the production of the p40 sub-unit from DC in our previous data. The

p40 sub-unit is found in both IL-23 and IL-12, and considering the pivotal role of IL-23 in directing a Th17 response, a decrease in the availability of p40 may limit IL-23 production and perhaps explain the LA-generated suppression of IL-17 production seen here. Of interest, is the finding that CLA treatment decreased IL-17 production from splenocytes. Statistical analysis using an unpaired t-test revealed significant differences in IL-17 production between the DMSO/KLH and CLA/KLH treatments. CLA suppressed IL-17 production in unstimulated splenocytes ($p < 0.001$) and in cells stimulated with $2\mu\text{g}$ KLH ($p < 0.05$). LA did not have an inhibitory effect on IL-17 production in the spleen [Figure 4.7B].

The production of IL-10 from lymph node cells was increased following the transfer of CLA-treated DC in control cells ($p < 0.001$), and in cells stimulated with KLH at a concentration of $2\mu\text{g}$ ($p < 0.001$), $10\mu\text{g}$ ($p < 0.001$), and $50\mu\text{g}$ ($p < 0.05$), but there was no change in the levels of IL-10 following KLH-specific activation [Figure 4.8A]. Again LA induced a comparable increase in IL-10 production to CLA in the lymph nodes, and in fact was able to upregulate KLH-specific IL-10 in the spleen (Figure 4.8B; $50\mu\text{g}$ $p < 0.05$).

Statistical analysis using an unpaired t-test revealed significant differences in IL-2 production when KLH-stimulated DC were cultured with CLA in comparison to those cultured with DMSO before adoptive transfer [Figure 4.9A]. Unstimulated lymphocytes recovered from popliteal lymph nodes produced less IL-2 ($p < 0.01$) in the CLA/KLH treatment group, as did cells plated with $10\mu\text{g}$ KLH ($p < 0.05$). The proliferation data obtained from lymph node cells substantiate the IL-2 cytokine data [Figure 4.10]: cell proliferation was suppressed in control cells from mice receiving CLA-treated DC ($p < 0.05$), and the proliferation of lymph node cells in the CLA/KLH group were also inhibited following stimulation with $10\mu\text{g}$ KLH ($p < 0.05$). There were no significant differences in IL-2 production from spleen cells isolated from the different treatment groups [Figure 4.9B].

The secretion of IFN- γ remained relatively unchanged in lymph node cells and splenocytes despite the considerable CLA-induced reduction of IFN- γ production recorded in the co-culture model [Figure 4.11]. Levels of IL-4 did not fluctuate to any great extent between treatment groups in either the lymph nodes or spleens [Figure 4.12].

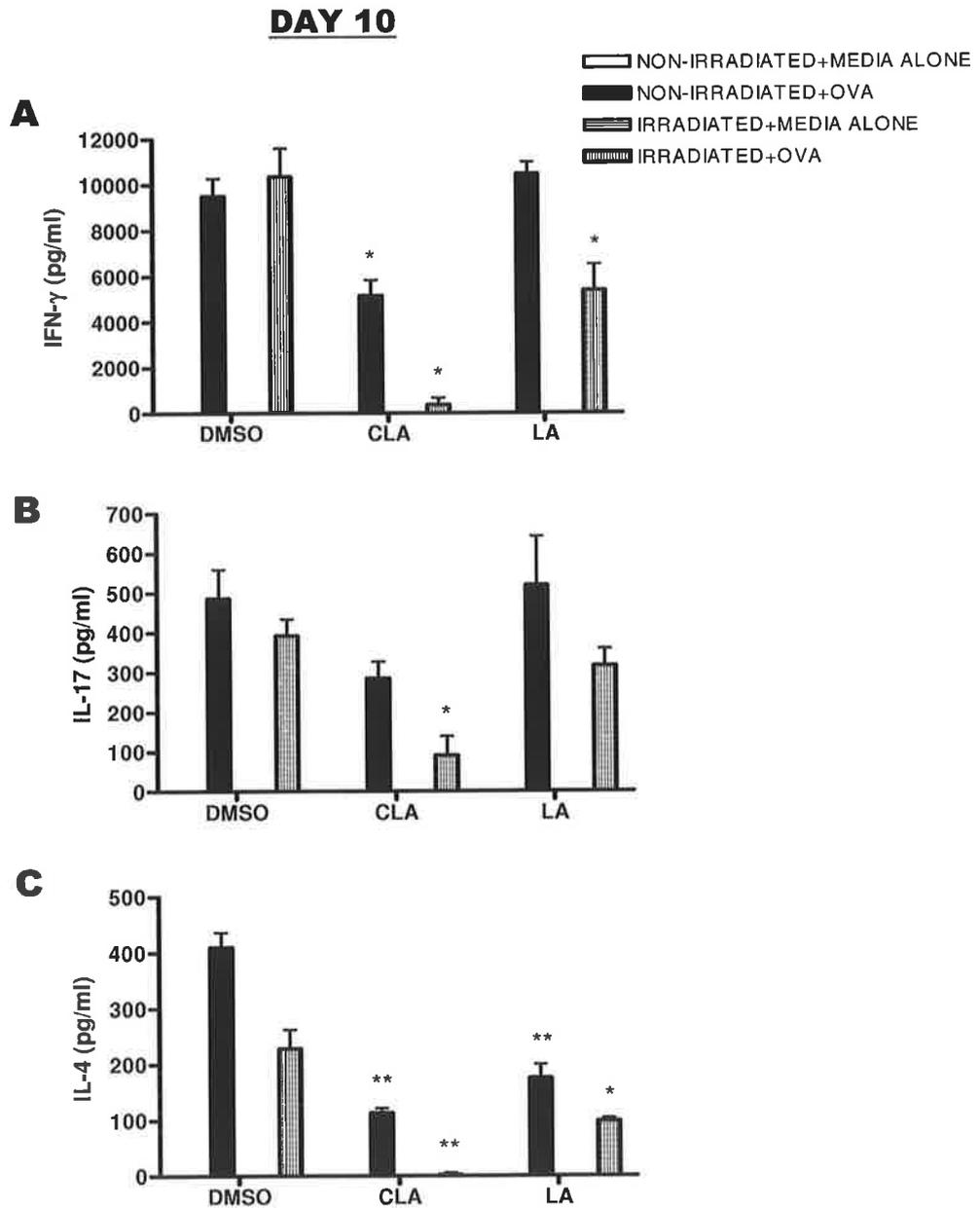


FIGURE 4.1

PUFA-modulated DC can inhibit subsequent T cell cytokine production.

DC were treated with DMSO (control), CLA (50 μ M), or LA (50 μ M) for 6 days before activation with OVA peptide (5 μ g/ml). After 24hours DC (2x10⁵/ml) were added to CD4⁺ T cells (2x10⁶/ml) purified from the spleens of OVA D011.10 transgenic mice. Irradiation of DC took place prior to this step, rendering the cells less able to secrete protein. Fresh PUFA-treated pre-activated DC were added on day 7 plus rIL-2 (10U/ml), and supernatants removed on day 10. Samples were analysed for levels of IFN- γ [A], IL-17 [B], and IL-4 [C]. Results are means \pm SE of triplicate assays and represent three independent experiments.

**p<0.01, *p<0.05 vs DMSO vehicle control determined by one-way ANOVA test

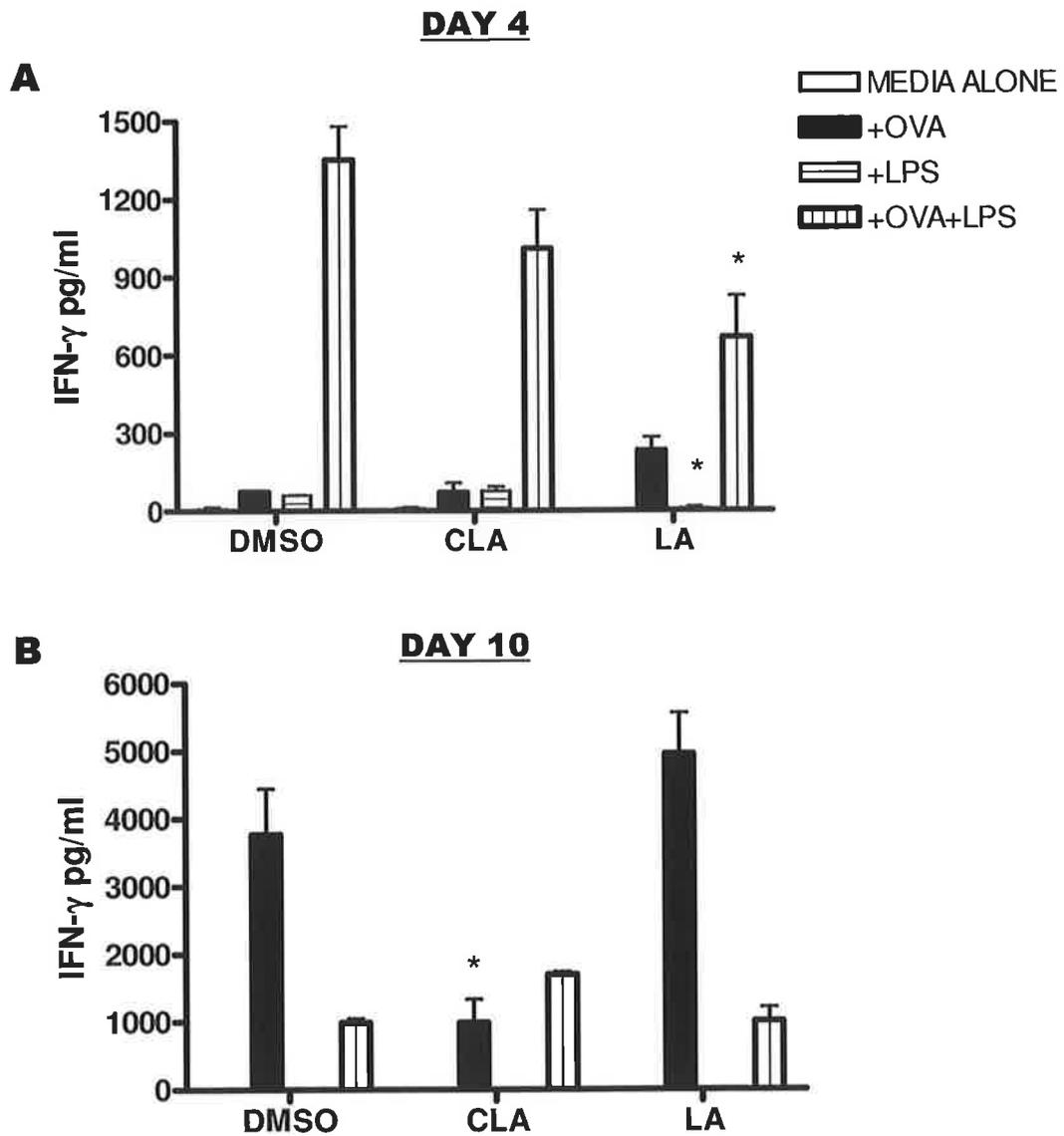


FIGURE 4.2

PUFA-modulated DC are less able to inhibit cytokine production from CD4⁺ T cells when activated by OVA and LPS combined.

DC incubated with DMSO, CLA or LA were treated for 24hours with LPS (100ng/ml), OVA (5µg/ml), or a combination of the two (OVA/LPS) before being irradiated and co-cultured with CD4⁺ OVA transgenic T cells. Supernatant was assessed for levels of IFN-γ on day 4 [A] and day 10 [B] of co-culture.

Results are means ± SE of duplicate assays and represent 2 independent experiments

*p<0.05 vs. DMSO vehicle control determined by one-way ANOVA test

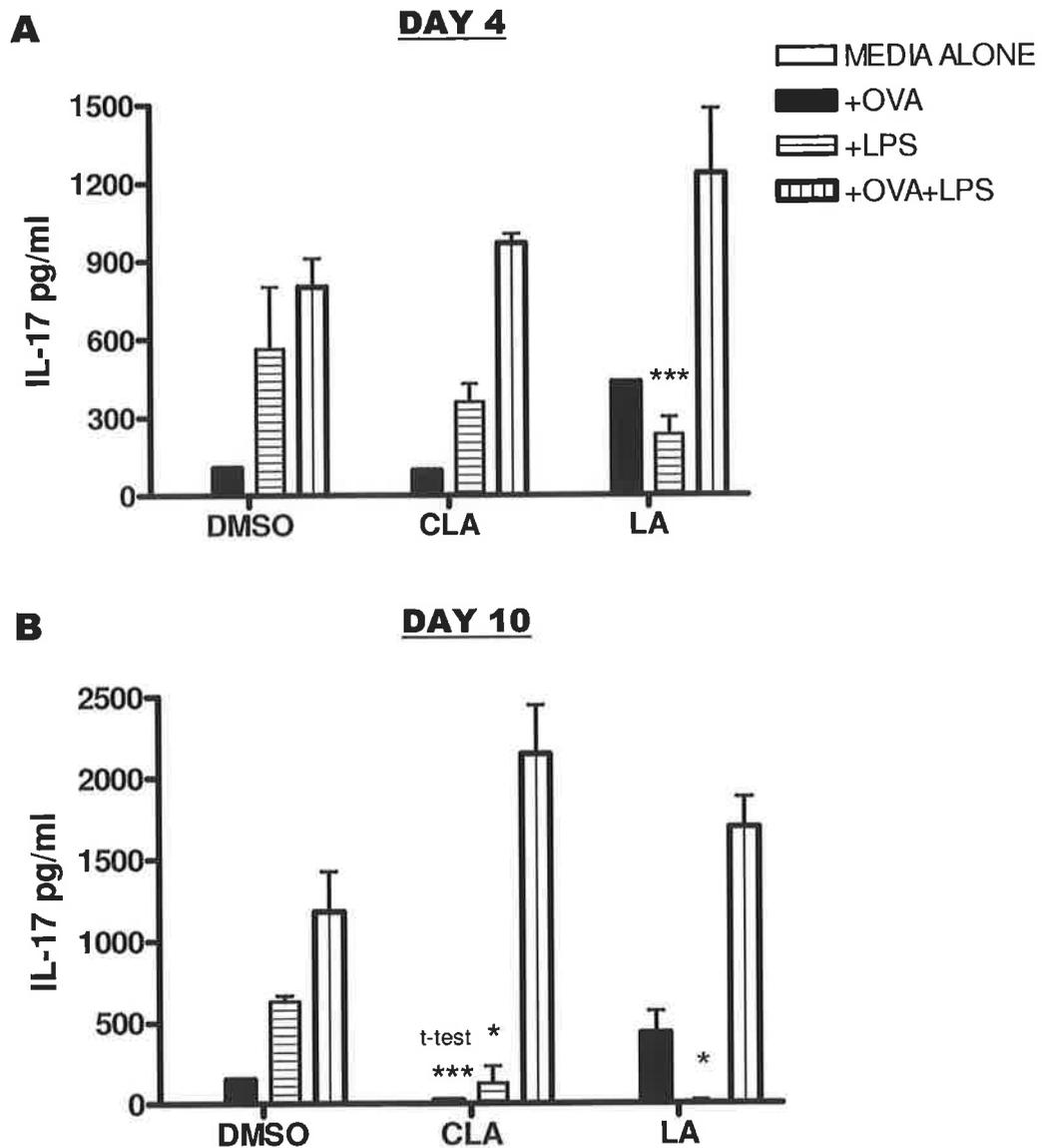


FIGURE 4.3

PUFA-modulated DC are less able to inhibit cytokine production from CD4⁺ T cells when activated by OVA and LPS combined.

DC incubated with DMSO, CLA or LA were treated for 24hours with LPS (100ng/ml), OVA (5µg/ml), or a combination of the two (OVA/LPS) before being irradiated and co-cultured with CD4⁺ OVA transgenic T cells. Supernatant was assessed for levels of IL-17 on day 4 [A] and day 10 [B] of co-culture.

Results are means ± SE of duplicate assays and represent 2 independent experiments

***p<0.001, *p<0.05 vs DMSO vehicle control determined by one-way ANOVA test

Statistical analysis was carried out between DMSO/OVA and CLA/OVA groups using an unpaired t-test (p<0.001) where indicated.

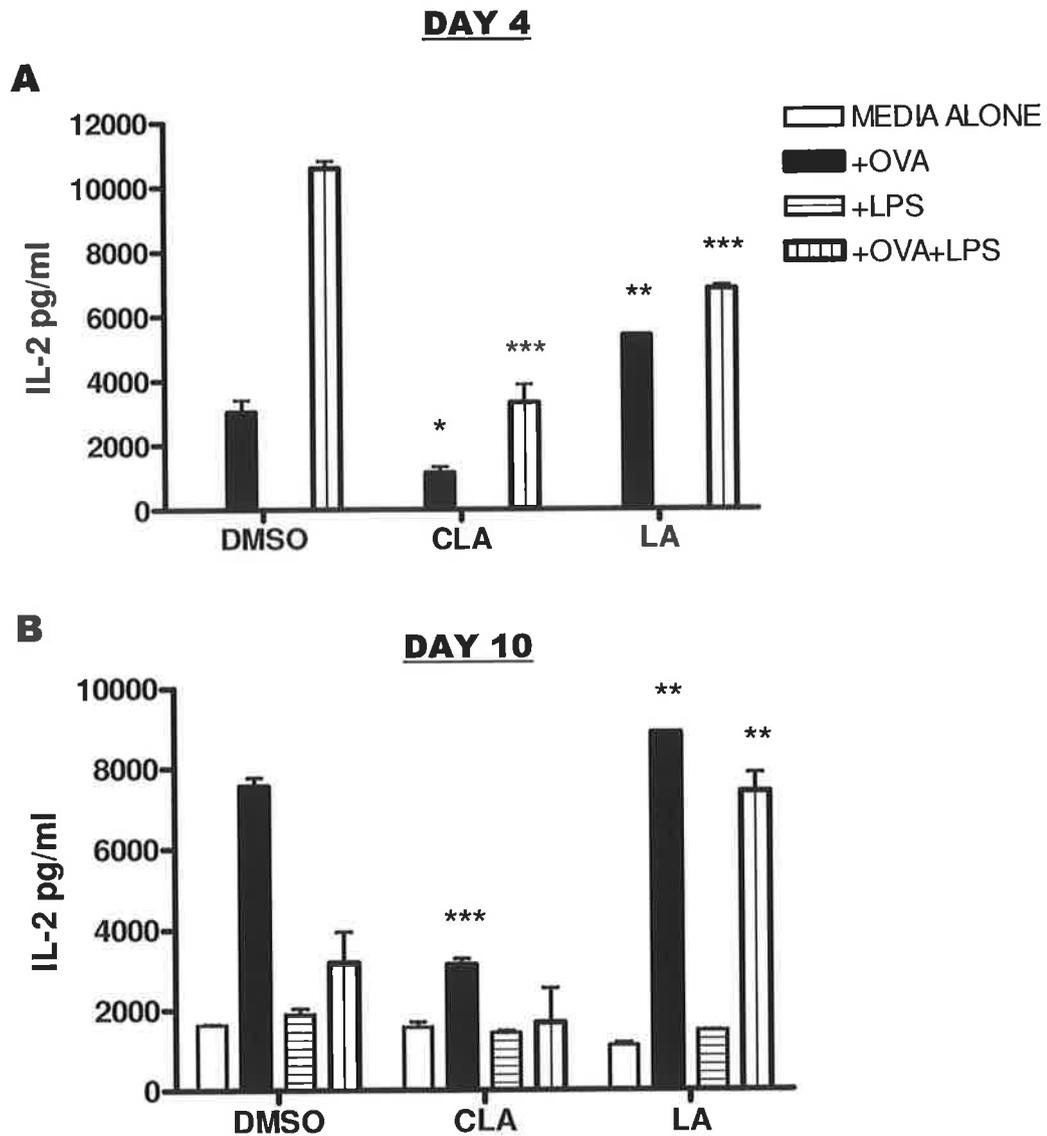


FIGURE 4.4

PUFA-modulated DC are less able to inhibit cytokine production from CD4⁺ T cells when activated by OVA and LPS combined.

DC incubated with DMSO, CLA or LA were treated for 24hours with LPS (100ng/ml), OVA (5μg/ml), or a combination of the two (OVA/LPS) before being irradiated and co-cultured with CD4⁺ OVA transgenic T cells. Supernatant was assessed for levels of IL-2 on day 4 [A] and day 10 [B] of co-culture.

Results are means ± SE of duplicate assays and represent 2 independent experiments

***p<0.001, **p<0.01, *p<0.05 vs DMSO vehicle control determined by one-way ANOVA test

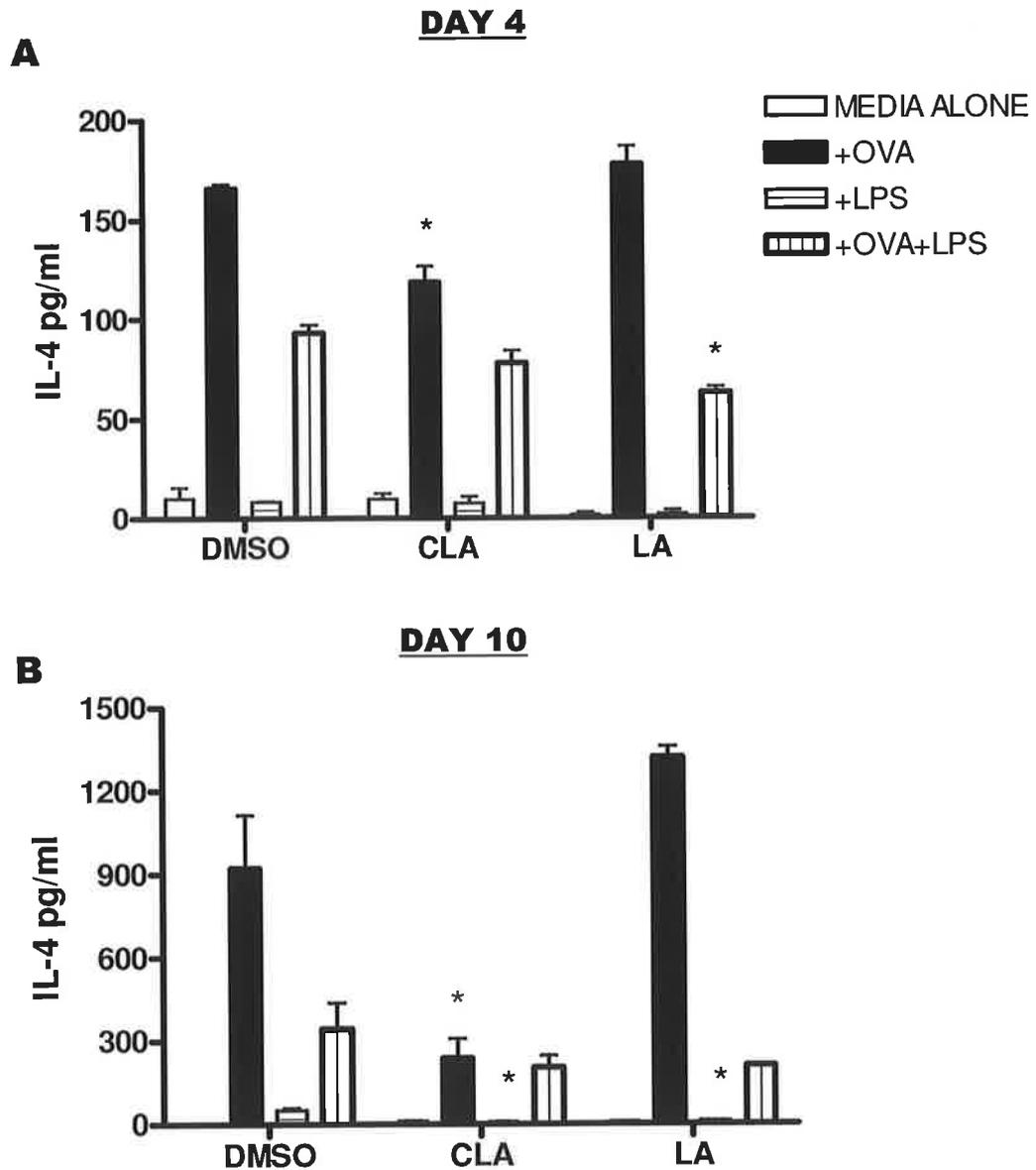


FIGURE 4.5

PUFA-modulated DC are less able to inhibit cytokine production from CD4⁺ T cells when activated by OVA and LPS combined.

DC incubated with DMSO, CLA or LA were treated for 24hours with LPS (100ng/ml), OVA (5 μ g/ml), or a combination of the two (OVA/LPS) before being irradiated and co-cultured with CD4⁺ OVA transgenic T cells. Supernatant was assessed for levels of IL-4 on day 4 [A] and day 10 [B] of co-culture.

Results are means \pm SE of duplicate assays and represent 2 independent experiments

*p<0.05 vs. DMSO vehicle control determined by one-way ANOVA test

DAY 4

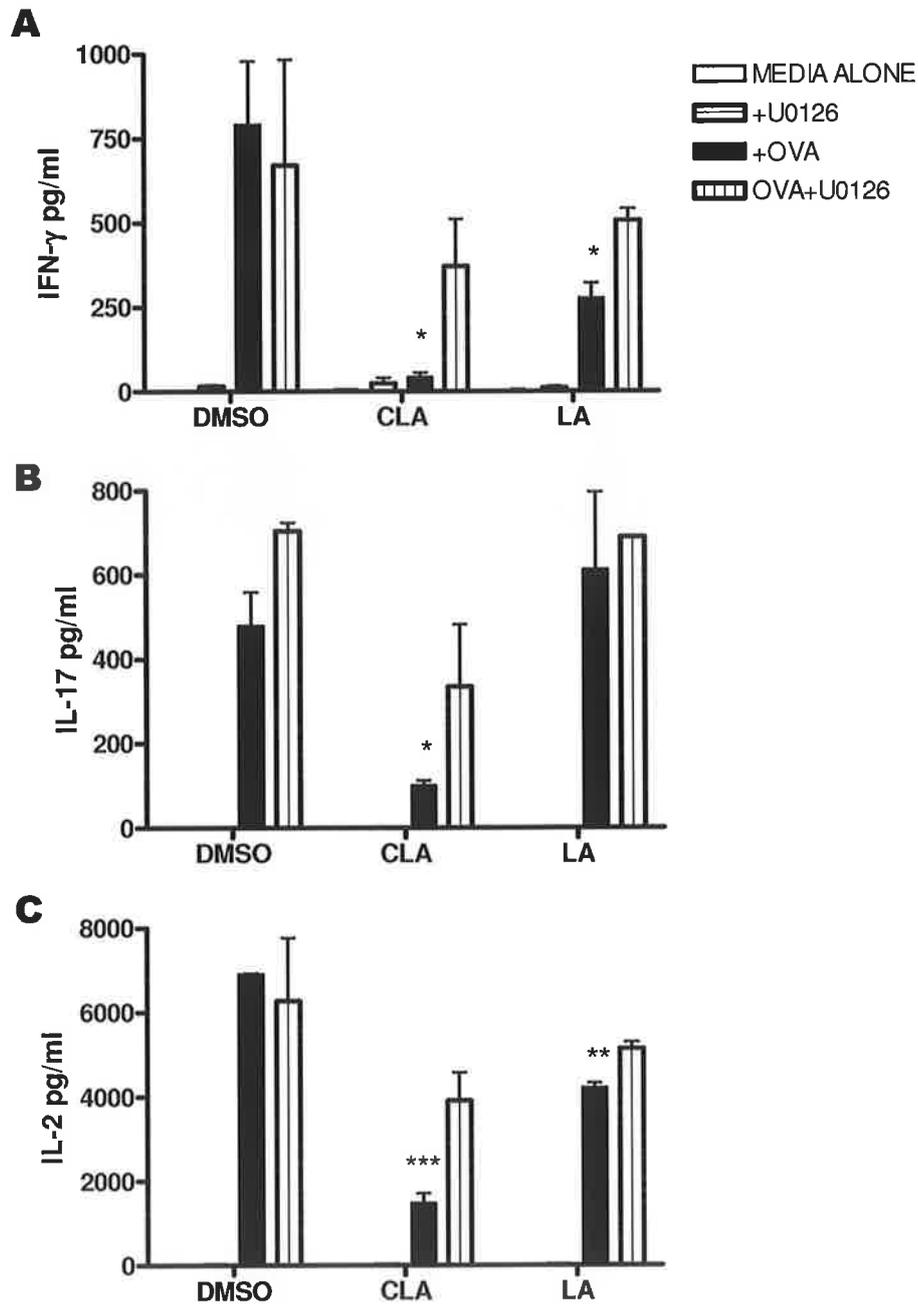


FIGURE 4.6

The ERK inhibitor U0126 partially reverses the inhibitory effects of PUFA on DC-mediated CD4⁺ T cell cytokine production.

PUFA-treated irradiated DC were activated with OVA peptide and co-cultured with naïve T helper cells from OVA transgenic mice. Where indicated the ERK inhibitor U0126 (5 μ M) was added 1 hour prior to DC activation. Supernatant was removed on day 4 of the co-culture experiment for cytokine analysis: IFN- γ [A], IL-17 [B] and IL-2 [C]. Results are means \pm SE of duplicate assays and represent two independent experiments.

***p<0.001, **p<0.01, *p<0.05 vs DMSO vehicle control determined by one-way ANOVA test

IL-17

LYMPH NODE

□ MEDIA ALONE
 ■ 2µg/ml KLH
 ▨ 10µg/ml KLH
 ▩ 50µg/ml KLH
 ▤ PMA/αCD3

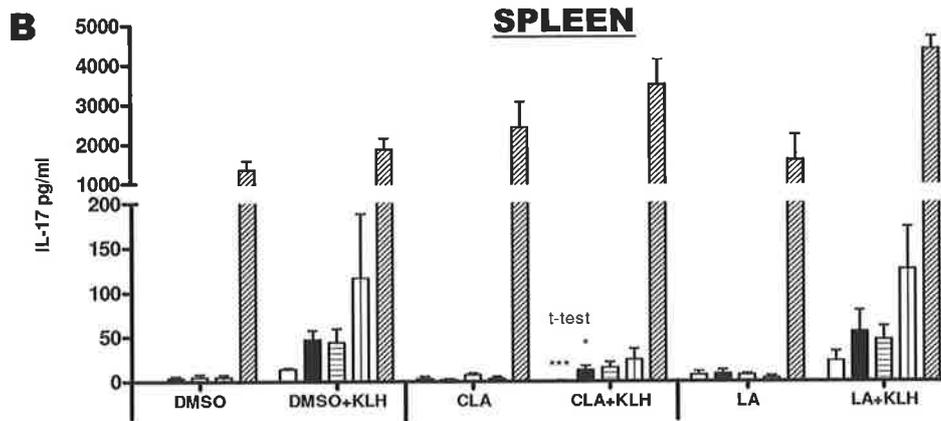
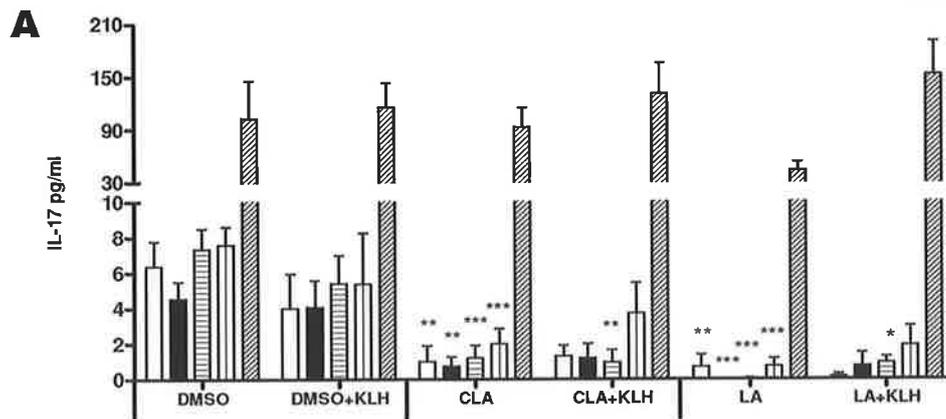


FIGURE 4.7

Adoptively transferred, fatty acid-modulated DC alter antigen-specific IL-17 production from the lymph node and spleen cells of recipient mice

DC were cultured with DMSO, CLA or LA (50µM) for 6 days. On day 6, cells were stimulated with KLH (10µg/ml) or media alone, harvested after a further 24 hours and then injected into the footpads of BALB/c mice (10⁵ cells per footpad). After 7 days, local lymph nodes (popliteal) were removed and processed to give a 1x10⁶ cells/ml. Spleens were also removed and splenocytes adjusted to give 2x10⁶ cells/ml. Cells were added in triplicate to a 96-well plate with increasing concentrations of KLH (2-50µg/ml) and were also stimulated with αCD3 (1µg/ml) and PMA (20ng/ml) as a positive control. Supernatant was removed after 72 hours and levels of IL-17 measured in the lymph nodes [A] and spleen [B]. Results are means ± SE of triplicate assays from 5 separate mice.

***p<0.001, **p<0.01, *p<0.05 vs DMSO vehicle control determined by one-way ANOVA test. Statistical analysis was carried out between DMSO/KLH and CLA/KLH groups using an unpaired t-test (where indicated).

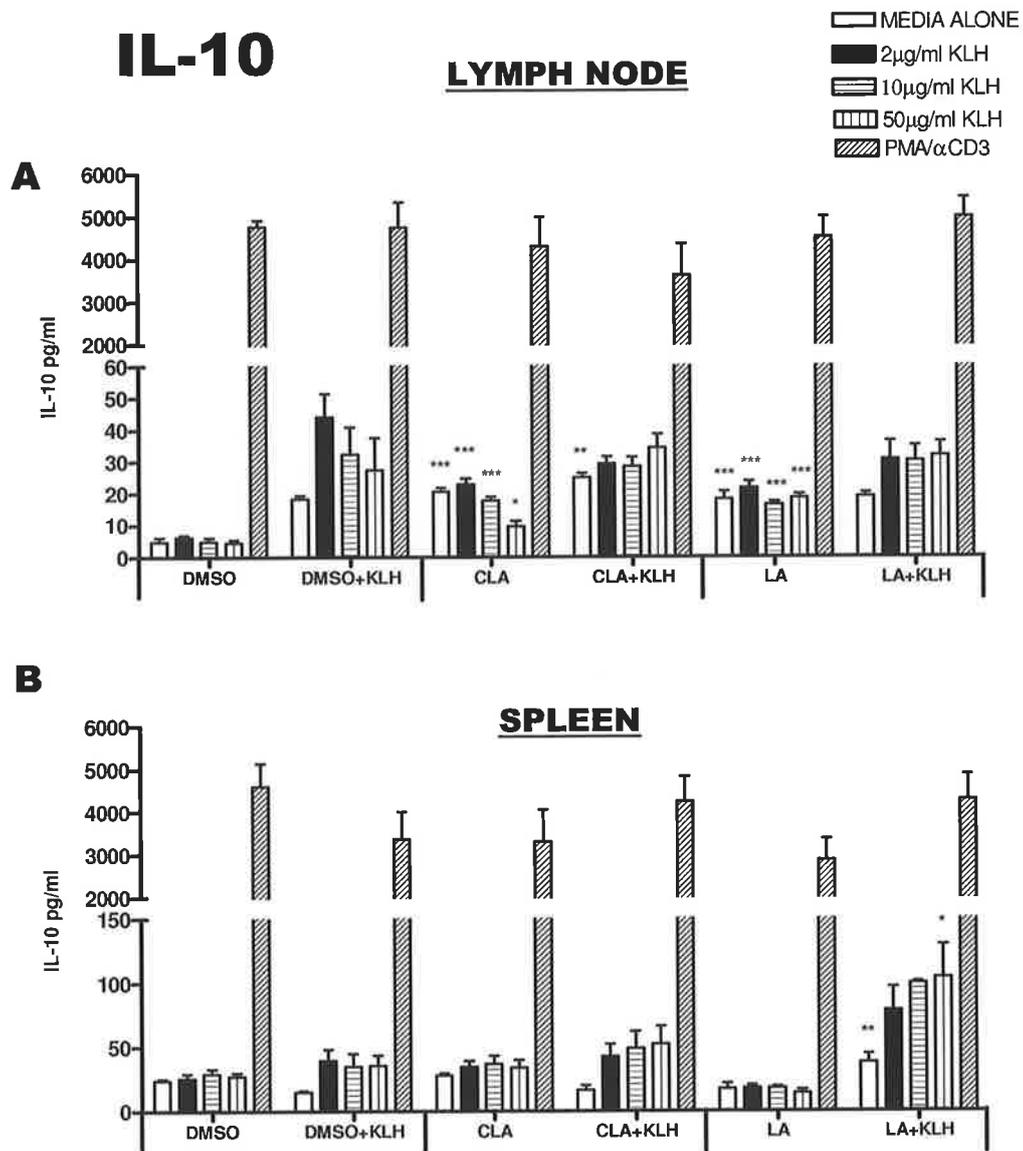


FIGURE 4.8

Adoptively transferred, fatty acid-modulated DC alter antigen-specific IL-10 production from the lymph node and spleen cells of recipient mice

DC were cultured with DMSO, CLA or LA (50µM) for 6 days. On day 6, cells were stimulated with KLH (10µg/ml) or media alone, harvested after a further 24 hours and then injected into the footpads of BALB/c mice (10⁵ cells per footpad). After 7 days, local lymph nodes (popliteal) were removed and processed to give a 1x10⁶ cells/ml. Spleens were also removed and splenocytes adjusted to give 2x10⁶ cells/ml. Cells were added in triplicate to a 96-well plate with increasing concentrations of KLH (2-50µg/ml) and were also stimulated with αCD3 (1µg/ml) and PMA (20ng/ml) as a positive control. Supernatant was removed after 72 hours and levels of IL-10 measured in the lymph nodes [A] and spleen [B]. Results are means ± SE of triplicate assays from 5 separate mice.

***p<0.001, **p<0.01, *p<0.05 vs DMSO vehicle control determined by one-way ANOVA test

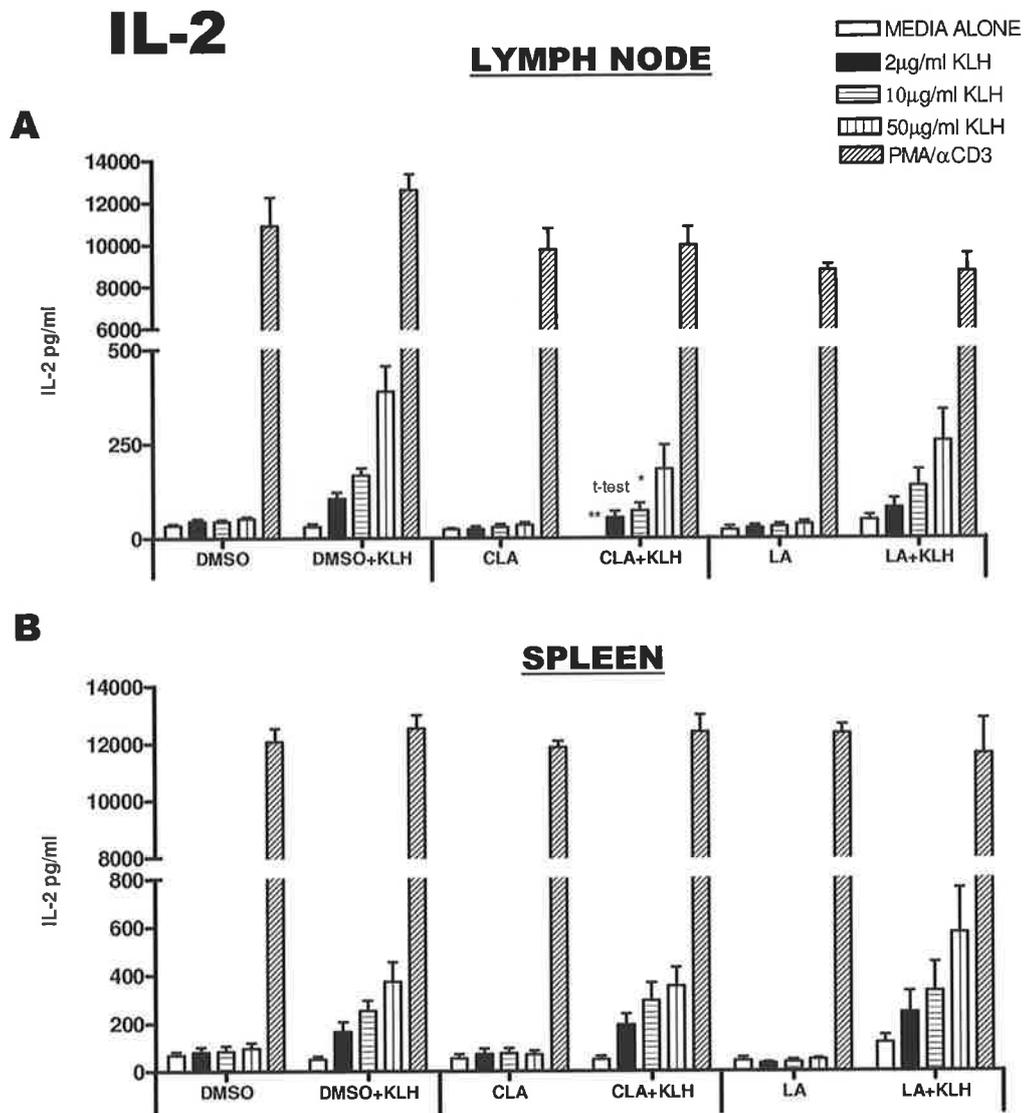


FIGURE 4.9

Adoptively transferred, fatty acid-modulated DC alter antigen-specific IL-2 production from the lymph node and spleen cells of recipient mice

DC were cultured with DMSO, CLA or LA (50µM) for 6 days. On day 6, cells were stimulated with KLH (10µg/ml) or media alone, harvested after a further 24 hours and then injected into the footpads of BALB/c mice (10^5 cells per footpad). After 7 days, local lymph nodes (popliteal) were removed and processed to give a 1×10^6 cells/ml. Spleens were also removed and splenocytes adjusted to give 2×10^6 cells/ml. Cells were added in triplicate to a 96-well plate with increasing concentrations of KLH (2-50µg/ml) and were also stimulated with αCD3 (1µg/ml) and PMA (20ng/ml) as a positive control. Supernatant was removed after 72 hours and levels of IL-2 measured in the lymph nodes [A] and spleen [B]. Results are means ± SE of triplicate assays from 5 separate mice.

**p<0.01, *p<0.05 vs DMSO vehicle control determined by one-way ANOVA test. Statistical analysis was carried out between DMSO/KLH and CLA/KLH groups using an unpaired t-test (where indicated).

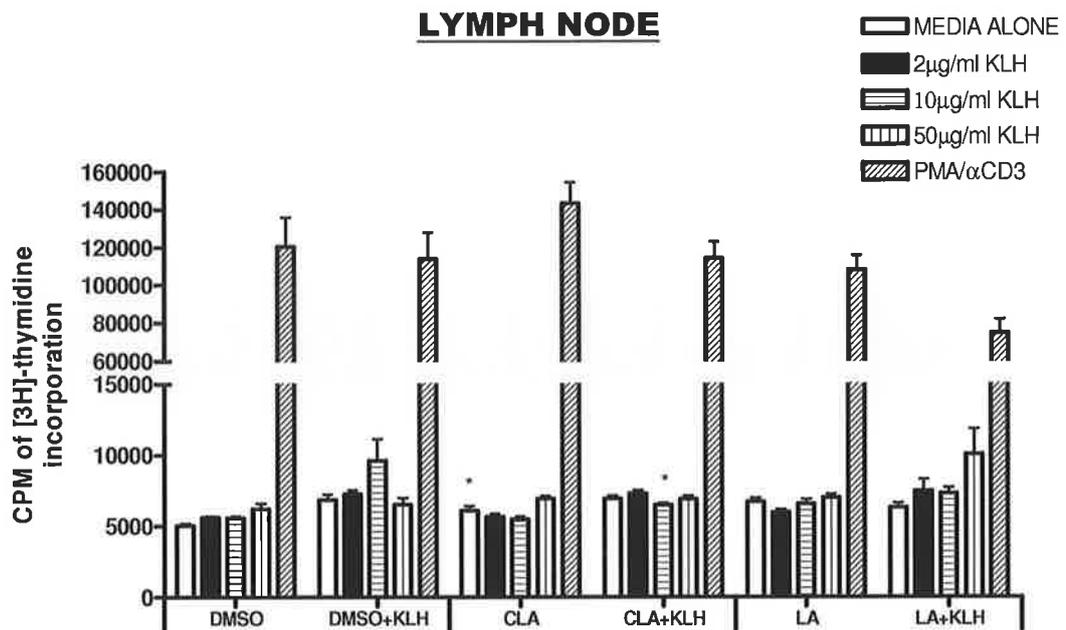


FIGURE 4.10

Adoptively transferred, fatty acid-modulated DC only have a slight effect on the antigen-specific proliferation of lymph node cells isolated from recipient mice

DC were differentiated from bone marrow cells in the presence of DMSO, CLA or LA (50µM) for 6 days. On day 6, cells were stimulated with KLH antigen (10µg/ml) or medium alone and after a further 24 hours, cells were harvested and injected into the footpads of BALB/c mice (5 mice per treatment group, 10⁵ cells per footpad).

After 7 days, local lymph nodes (popliteal) were removed and processed to give a 1x10⁶ cells/ml cell suspension. Cells were then added in triplicate to a 96-well plate with increasing concentrations of KLH (2-50µg/ml) and were also stimulated with αCD3 (1µg/ml) and PMA (20ng/ml) as a positive control. Supernatant was removed after 72 hours and 1µCi/well of [³H]-thymidine added to each well in 200µl of media. Plates were cultured for an additional 4 hours, after which cells were harvested and proliferation assessed by [³H]-thymidine incorporation on a scintillation counter (expressed as counts per minute or cpm). Results are means ± SE of triplicate assays from 5 separate mice.

*p<0.05 vs. DMSO vehicle control determined by one-way ANOVA test

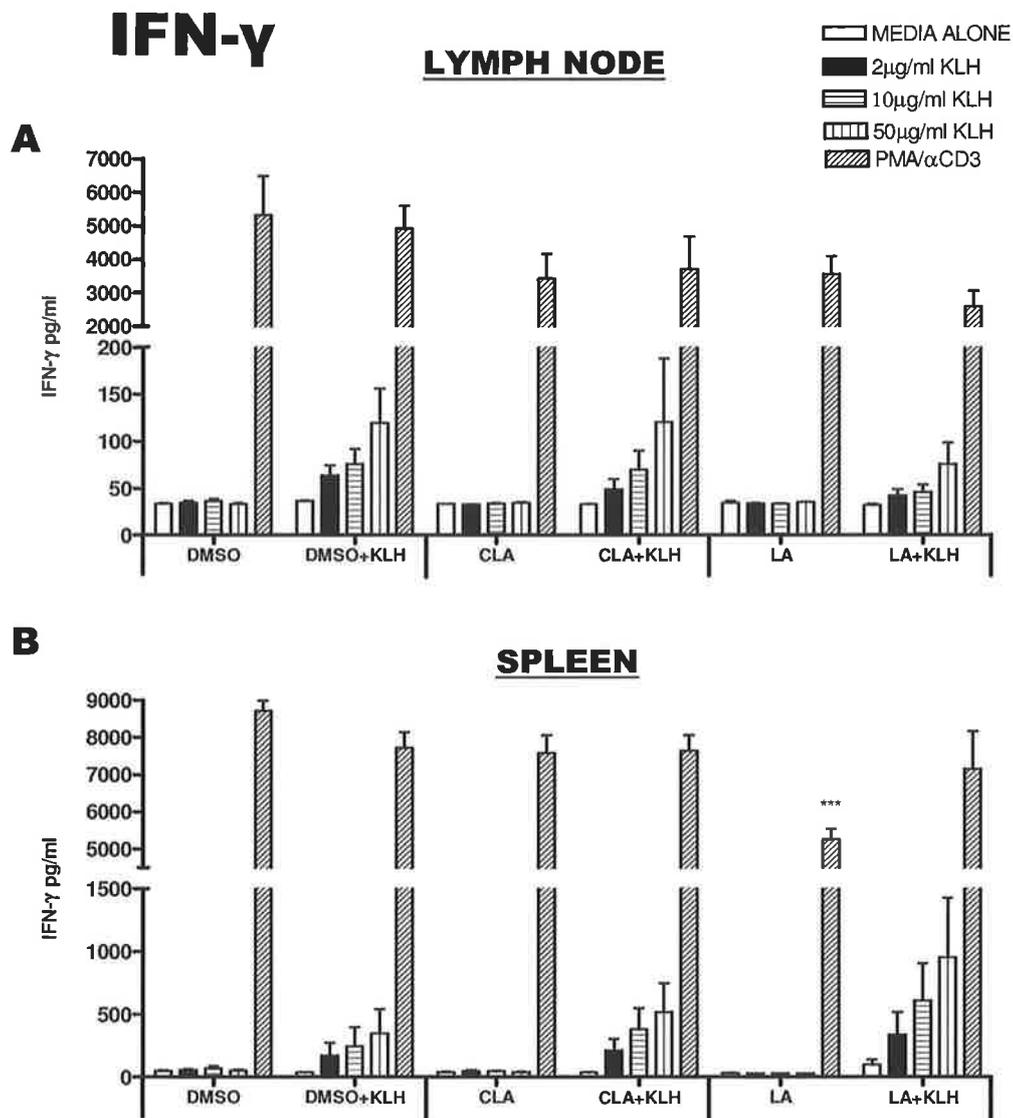


FIGURE 4.11

Adoptively transferred, fatty acid-modulated DC have no significant effect on the antigen-specific production of IFN- γ from the lymph node and spleen cells of recipient mice

DC were cultured with DMSO, CLA or LA (50 μ M) for 6 days. On day 6, cells were stimulated with KLH (10 μ g/ml) or media alone, harvested after a further 24 hours and then injected into the footpads of BALB/c mice (10⁵ cells per footpad). After 7 days, local lymph nodes (popliteal) were removed and processed to give a 1x10⁶ cells/ml. Spleens were also removed and splenocytes adjusted to give 2x10⁶ cells/ml. Cells were added in triplicate to a 96-well plate with increasing concentrations of KLH (2-50 μ g/ml) and were also stimulated with α CD3 (1 μ g/ml) and PMA (20ng/ml) as a positive control. Supernatant was removed after 72 hours and levels of IFN- γ measured in the lymph nodes [A] and spleen [B]. Results are means \pm SE of triplicate assays from 5 separate mice.

***p<0.001 vs. DMSO vehicle control determined by one-way ANOVA test

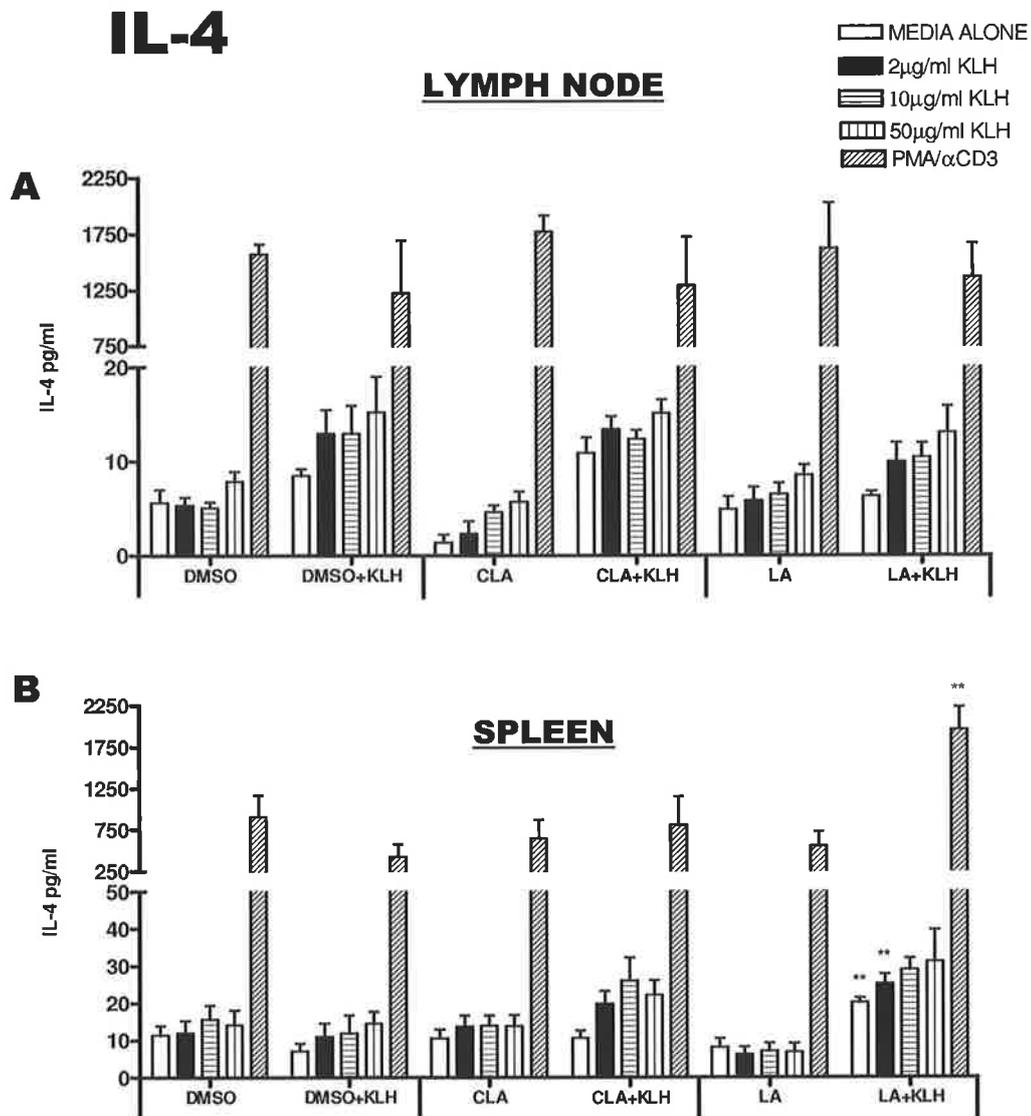


FIGURE 4.12

Adoptively transferred, fatty acid-modulated DC have little effect on the antigen-specific production of IL-4 from the lymph node and spleen cells of recipient mice

DC were cultured with DMSO, CLA or LA (50µM) for 6 days. On day 6, cells were stimulated with KLH (10µg/ml) or media alone, harvested after a further 24 hours and then injected into the footpads of BALB/c mice (10^5 cells per footpad). After 7 days, local lymph nodes (popliteal) were removed and processed to give a 1×10^6 cells/ml. Spleens were also removed and splenocytes adjusted to give 2×10^6 cells/ml. Cells were added in triplicate to a 96-well plate with increasing concentrations of KLH (2-50µg/ml) and were also stimulated with αCD3 (1µg/ml) and PMA (20ng/ml) as a positive control. Supernatant was removed after 72 hours and levels of IL-4 measured in the lymph nodes [A] and spleen [B]. Results are means ± SE of triplicate assays from 5 separate mice.

**p<0.01 vs. DMSO vehicle control determined by one-way ANOVA test

4.3 DISCUSSION

Results here provide evidence that PUFA-modulated dendritic cells have consequences on the ensuing adaptive immune response. This was seen when CLA-modified DC inhibited the production of IFN- γ , IL-17, IL-4 and IL-2 by CD4⁺ T cells in a co-culture model suggesting that PUFA-modulated APC can subvert both Th1 and Th17 responses by reducing IFN- γ and IL-17 production, respectively. Additional immunomodulatory effects of CLA on adaptive immunity were seen when adoptively transferred DC pre-treated with CLA inhibited IL-17 production in lymphocytes isolated from the lymph nodes and spleens of recipient mice, as well as reducing the level of IL-2 produced by lymph node cells and increasing IL-10 production.

Results obtained from the co-culture model were very consistent, particularly on day 10 of the experiment following the second round of T cell stimulation. Levels of IFN- γ were consistently and significantly suppressed. IFN- γ defines a Th1 response and its pro-inflammatory effects at the site of tissue inflammation are well established. One of its major roles is to activate macrophage and DC, leading to augmented phagocytosis, increased MHC I and II expression, and induction of IL-12 production (Szabo *et al.*, 2003). However, attempts to define the role of IFN- γ in autoimmunity by the administration of neutralizing antibodies have yielded conflicting results. In some systems disease pathology was accelerated, for example, the absence of a competent IFN- γ response enhanced disease severity in EAE and collagen-induced arthritis (Rosloniec *et al.*, 2002; Kelchtermans *et al.*, 2007). Contrastingly, some substances have had their therapeutic efficacy attributed to their inhibitory effect on IFN- γ : A herbal acetoside isolated from *Plantago lanceolata L.* was found to significantly decrease IFN- γ production from cells isolated from the MLN of colitic mice and this reduction correlated with ameliorated disease pathology (Hausmann *et al.*, 2007): Infliximab, a chimeric monoclonal antibody that neutralizes both soluble and membrane-bound TNF- α , was used as a treatment in a model of psoriasis (chronic inflammatory skin disease) and was found to dose-dependently impair T cell proliferation and IFN- γ release, both of which were suggested to be the immunomodulatory mechanisms which lead to disease remission (Bedini *et al.*, 2006). It was only in the co-culture model that we saw a significant reduction in

IFN- γ production. In the adoptive transfer experiment we saw no differences in IFN- γ production between control, PUFA and saturated fatty acid groups. This may be solely due to the different culture systems used, as PUFA have been shown to have contrasting effects on distinct cell types. For example, Petursdottir and Hardardottir (2007) found that dietary fish oil had an inhibitory effect on TNF- α and IL-10 production from splenic T cells but increased their secretion by splenic macrophage.

As mentioned above, the presence or absence of IFN- γ has been shown to have distinct effects in different disease models, and although generally considered a pro-inflammatory cytokine a lack of IFN- γ has been shown to exacerbate some inflammatory situations. Irmeler and colleagues (2007) showed that disease pathology was greatly increased in IFN- γ knock-out mice in a model of antigen-induced arthritis. And in an autoimmune model of graft-versus-host disease, Lohr and associates (2006) found that IL-17 was the cytokine responsible for tissue inflammation in this setting whereas IFN- γ had a protective effect. The presence of CLA in both the co-culture system and the adoptive transfer experiment significantly suppressed IL-17 production. IL-17 is mainly secreted by the recently discovered Th17 cell subset. Pathogenic Th17 cells have been detected in a number of inflammatory disorders, including EAE and RA, and their presence has been linked to some of the pathology previously attributed to a disproportionate Th1 response (Kikly *et al.*, 2006; Sutton *et al.*, 2006; Zhang *et al.*, 2006b). IL-17 acts *in vitro* and *in vivo* as a potent inflammatory cytokine, coordinating tissue inflammation by inducing the expression of proinflammatory cytokines, (such as IL-1, IL-6, IL-8, and TNF- α) which mediate immune cell infiltration and tissue destruction (Langrish *et al.*, 2004). This places Th17 and their IL-17 products as potential therapeutic targets. Numerous studies have evaluated the potential benefit of neutralising IL-17 to alleviate inflammation. For example, IL-17 is frequently detected in the inflamed colon of trinitrobenzenesulfonic acid (TNBS)-induced colitic mice, and Zhang and co-workers (2006b) found that IL-17R knockout mice were significantly protected against TNBS-induced weight loss, IL-6 production, and colonic inflammation, and this protection occurred even though there were comparable levels IL-23 and higher levels of IL-12p70 and IFN- γ in IL-17R knockout mice compared with wild-type controls. The findings here of comparable levels of IL-23, but higher

levels of IFN- γ and IL-12p70, which still protected against disease, reiterate that not all pro-inflammatory cytokines have to be down-regulated to provide protection against disease, and seeing as PUFA have the ability to suppress IL-12, IFN- γ and IL-17 in different culture settings may mean they could afford protection in different inflammatory situations.

It is well documented that the cytokines produced by each of the T helper subsets inhibit the development of the other T helper lineages; Th1 cell development is inhibited by IL-4, Th2 by IFN- γ , and Th17 by both IFN- γ and IL-4. Because of this, it would be reasonable to assume that a decrease in IFN- γ or IL-17 would coincide with a rise in IL-4. This however was not the case in the co-culture model where all three cytokines were suppressed by CLA. The production of IL-4 was reduced following CD4⁺ T cell activation with CLA-treated DC compared to the vehicle control, and although generally considered a Th2 cytokine, under certain circumstances IL-4 synergises with IL-12 to induce production of IFN- γ by DC (Wurtz *et al.*, 2004). Therefore the effect of IL-4 is dependant on the maturational stage of the APC target; the presence of IL-4 at early stages of DC activation can inhibit IFN- γ production, whereas IL-4 can actually increase IFN- γ production in mature cells (Bastos *et al.*, 2004). Another interesting finding was the fact that CLA was able to inhibit IL-17 production from T cells (both in the co-culture and adoptive transfer experiment) but did not have an inhibitory effect on the production of cytokines that drive a Th17 response in DC, i.e. we found that IL-1, IL-6, TNF- α and IL-23 secretion was either unaffected or slightly increased in CLA-treated DC following LPS stimulation *in vitro* (see chapter 3), all of which contribute to the development of Th17 cells (Sutton *et al.*, 2006; Kryczek *et al.*, 2007; Yujing Bi, 2007). To our knowledge the effects of PUFA on IL-17 production has not been previously investigated. However, the therapeutic efficacies of other compounds that reduce IL-17 production have been documented: Cyclosporine A (CSA), a widely used immunosuppressive drug, has various biological effects on T cells, one of which is the ability to inhibit IL-15-induced IL-17 production in CD4⁺ T cells from patients with rheumatoid arthritis (Cho *et al.*, 2007). The researchers in this study discovered that the inhibitory effect of CSA on IL-17 production partially depended on the paralleled increase in IL-10, since neutralizing anti-IL-10 antibodies were able to partially reverse this inhibition. We did not detect any IL-10 in the co-culture

model. There was however, a significant increase in IL-10 and decrease in IL-17 in the CLA group of the adoptive transfer study. It would be interesting to ascertain whether the decrease in IL-17 was IL-10 dependent, especially considering we have already shown that the suppression of IL-12 from DC is dependent on the increase in IL-10 production *in vitro* (Loscher *et al.*, 2005).

We have previously reported an ERK-dependent mechanism utilised by CLA to increase IL-10 production, where the amount of ERK and extent of ERK phosphorylation was increased in cells treated with CLA (Loscher *et al.*, 2005). ERK is widely expressed and involved in a number of cellular processes, including, cell proliferation, differentiation and apoptosis (Kolch, 2000). It has been documented that ERK activation is associated with the inhibition of DC maturation (An *et al.*, 2002; Boisleve *et al.*, 2005) therefore its activation could explain the decreased maturational state of the PUFA-treated DC (seen in chapter 3) and the subsequent suppression of cytokine production in T cells described in this study, and also the reversal of cytokine modulation upon addition of the ERK inhibitor (U0126) in the co-culture experiment.

There have been a number of studies published on the effects of PUFA on lymphocytes, with proliferation and IL-2 production shown to be markedly altered: CLA was found to decrease mitogen-induced cell activation in a dose-dependent manner in human T lymphocytes (Tricon *et al.*, 2004); Beppu and co-workers (2006) compared the growth inhibitory effects of CLA on human colon cancer cell lines and found that cell proliferation was inhibited. Feeding mice a diet enriched with (n-3) PUFA reduced the *in vitro* antigen-stimulated production of IL-2 and subsequent proliferation of naive CD4⁺ T lymphocytes (Pompos & Fritsche, 2002). Some studies have found either no effect or augmentation of cytokine production following dietary intervention with PUFA; cytokine production from the PBMCs of 10 volunteers given EPA or DHA for 2 months had unaltered IL-4 production and raised levels of IL-10, IFN- γ and TNF- α (Gorjao *et al.*, 2006). Luongo and colleagues (2003) cultured Jurkat T cells with an equal mixture of two CLA isomers (cis9,trans11 and trans10,cis12) for 72 hours before activation with α CD3/PMA, and CLA was found to have an inhibitory effect on cell proliferation but increased IL-2 and IFN- γ production. Alternatively, others report a more immunosuppressive action of PUFA on lymphocytes; Albers and co-workers (2002) used a sensitisation model to elicit

adaptive immune responses in mice and compare the impact of nutrients on immune functions. The n-3 PUFA diet halved the concentration of TNF- α produced by splenocytes compared to the n-6 PUFA and saturated fatty acid diets but there was no significant difference in IFN- γ or IL-4 production in ConA-stimulated lymph node cells or spleen cells between diet groups. Lin and colleagues (2007) found that fish oil administered before caecal ligation and puncture (CLP) in a model of sepsis attenuated the inflammatory response by enhancing the production of IL-4 but decreasing IFN- γ production in PMNs. And finally, Fritsche *et al.*, (2000) fed animals a fish oil diet before injection with *Listeria monocytogenes*, and found that the n-3 PUFA diet decreased circulating IFN- γ and IL-12p70 24 hours post-infection. Interestingly, there was also a recorded decrease in IFN- γ receptor expression on immune cells (25-35% reduction), which would decrease IFN- γ responsiveness and possibly lead to reduced IL-12 biosynthesis. Therefore the findings here are corroborated and conflicted by previously published studies, again possibly due to the different cell types assayed and methods of cellular activation used.

In conclusion, the findings here suggest that PUFA have the ability to restrict adaptive T helper cell responses that are considered the pathogenic driving forces behind most autoimmune and inflammatory diseases, i.e. Th1 and Th17 responses. Further investigation as to whether these efficacious immunomodulatory effects are mirrored *in vivo* are warranted and will be explored in chapter 6.

CHAPTER 5

THE INVOLVEMENT OF TRANSCRIPTION FACTORS, PPAR γ & NF κ B, IN THE MODULATORY EFFECTS OF PUFA ON DENDRITIC CELLS

5.1 CHAPTER 5 INTRODUCTION

Reports indicate that there are four broad targets for the anti-inflammatory effects of PUFA. These are modified eicosanoid production, changes in cytokine biosynthesis, altered membrane composition and function, and modifications in gene expression (Shaikh & Edidin, 2006). It is now well documented that fatty acids can affect gene expression by binding to and activating a novel family of nuclear receptors known as peroxisomal proliferator-activated receptors (PPARs) (Fritsche, 2006). There are three known isoforms of PPAR; PPAR α , PPAR δ , and PPAR γ , but PPAR γ is the predominant isoform expressed on cells of the myeloid line (Daynes & Jones, 2002; Fritsche, 2006) and is therefore the isoform considered in the following study. PUFA are reported to be natural ligands of PPAR γ (Sampath & Ntambi, 2005), and since PPAR γ activation has been shown to decrease the production of pro-inflammatory cytokines (Faveeuw *et al.*, 2000; Nencioni *et al.*, 2002) and alter co-stimulatory marker expression (Klotz *et al.*, 2007), it has been suggested that PUFA may act through PPAR to exert their immunosuppressive actions (Li *et al.*, 2005). However, while PUFA can function as ligands for PPARs, little is known about which of their immunomodulatory properties are actually PPAR γ -dependent. In this study, we firstly examined the outcome of PPAR γ activation on DC using the specific PPAR γ ligand, rosiglitazone (RSG), and found its suppressive effects on DC cytokine production and surface marker expression to be distinctly similar to the results we recorded following PUFA treatment in chapter 3. Since the n-3 PUFA, EPA and DHA, were previously shown to suppress IL-12 and IL-23 production in a similar manner to RSG, we looked at whether the expression of PPAR γ was altered in DC following EPA and DHA treatment. Furthermore, using a specific PPAR γ inhibitor, we examined whether the effects of PUFA on DC maturation, i.e. cytokine and surface marker suppression, were mediated through the activation of PPAR γ .

As well as possessing anti-inflammatory effects itself, PPAR activation has also been shown to have antagonistic activity on various other transcription factors including NF κ B and STAT (Chung *et al.*, 2000; Chinetti-Gbaguidi & Staels, 2007). Since the production of many cytokines is regulated at the transcriptional level by NF κ B (Hanada & Yoshimura, 2002) and because NF κ B activation aids

DC maturation as characterised by an increase in co-stimulatory molecule and MHCII expression (Banchereau *et al.*, 2000), it was proposed that alterations in total NF κ B levels and/or its nuclear translocation could represent an alternative pathway exploited by PUFA to bring about the previously observed changes in DC *in vitro*.

NF κ B is a ubiquitous and largely pro-inflammatory transcription factor with a pivotal role in regulating the expression of many chemokines, adhesion molecules, and cytokines (Li *et al.*, 2005), therefore we firstly examined total cellular levels of NF κ B in PUFA-treated DC. Inactive NF κ B is sequestered in the cytoplasm by a family of inhibitory proteins, the most common of which are I κ B α , I κ B β and I κ B ϵ (Li & Verma, 2002). The binding of pathogens or cytokines to cellular receptors activate distinct signalling pathways that eventually converge on the activation of an I κ B kinase (IKK) which initiates I κ B phosphorylation. Phosphorylated I κ B is degraded, freeing NF κ B and allowing it to translocate to the nucleus and bind to target genes (Hanada & Yoshimura, 2002). Since I κ B α regulates transient NF κ B activation (Li & Verma, 2002) and because the most common transcriptionally active form of NF κ B is a heterodimer of the p50 subunit associated with p50 (Hatada *et al.*, 2000) we also quantified changes in I κ B α and p50 in the nuclear and cytosolic protein fractions of PUFA-treated dendritic cells. Given that the inhibitory effect of PPAR γ on IL-12 involves its interaction with NF κ B (Chung *et al.*, 2000) we also examined changes in the interaction between the two transcription factors, PPAR γ and NF κ B using confocal microscopy.

The results obtained using the n-3 PUFA suggested that their actions on DC maturation were PPAR γ -independent. Therefore we explored whether the n-6 PUFA, CLA (also shown to have immunosuppressive actions similar to those of RSG) functioned through PPAR γ , particularly because CLA has previously been reported to exert some of its beneficial effects through PPAR γ activation (Bassaganya-Riera & Hontecillas, 2006; Bocca *et al.*, 2007; Bozzo *et al.*, 2007). Again we examined whether alterations in DC cytokine production and surface marker expression were PPAR γ -dependent. The activation of PPAR γ has also been shown to inhibit the migration of DC (Angeli *et al.*, 2003) and neutrophil (Napimoga *et al.*, 2008). Given the fact that we found PUFA were able to

impede DC migration in chapter 3, we went on to examine whether CLA utilises PPAR γ to exert this modulatory effect.

The Western blot and confocal microscopy experiments represented by figures 5.5, 5.9 and 5.11 were carried out in conjunction with Clare Reynolds in the Nutrigenomics Research Group, UCD.

5.2 RESULTS

5.2.1 RSG INHIBITS THE MATURATION PROCESS OF DC FOLLOWING LPS STIMULATION

Rosiglitazone is a selective ligand and activator of PPAR γ , and the activation of PPAR has been shown to have anti-inflammatory effects (Yaqoob, 2003; Szatmari *et al.*, 2006; Klotz *et al.*, 2007). PUFA are also considered ligands for PPAR and therefore PPAR activation has been proposed as a possible mechanism for their immunosuppressive actions. Here we investigated the effects of PPAR γ activation on the process of DC maturation. DC were cultured in the presence of DMSO or RSG (5 μ M) for 7 days before LPS activation. After 24 hours, supernatant was removed and the concentration of various cytokines measured using specific immunoassays. **Figure 5.1** shows that RSG has a significant inhibitory effect on LPS-induced cytokine release in DC: The production of IL-12p40 ($p < 0.01$), IL-12p70 ($p < 0.01$) and IL-23 ($p < 0.05$) were all significantly reduced when cells were cultured with RSG. There was a slight recorded increase in IL-10 production (not statistically significant) [**Figure 5.2A**], an increase in IL-1 β ($p < 0.05$) [**Figure 5.2B**], and no change in either IL-6 or TNF α [**Figure 5.3**].

The effect of PPAR γ activation (using RSG) on the upregulation of surface markers following the addition of LPS was also examined. **Figure 5.4** provides evidence that RSG does not appear to have any major effect on surface marker expression in resting DC [**ROW 1**]. However, following LPS stimulation [**ROW 2**], RSG inhibits the upregulation of CD80, CD86, MHCII and CCR5, with no major alterations in IL-10R or CD48 expression. Furthermore, when DC are cultured with RSG together with the specific PPAR γ inhibitor (GW9662), these inhibitory effects were completely reversed.

5.2.2 EPA AND DHA INCREASE PPAR γ EXPRESSION

The suppressive effects of EPA and DHA on LPS-induced cytokine production from DC seen in chapter 3 were comparable to results recorded when DC were cultured with the specific PPAR γ ligand, RSG. Since PPAR γ has been shown to exert anti-inflammatory effects when activated (Faveeuw *et al.*, 2000; Gosset *et*

al., 2001) and because EPA and DHA are known ligands of PPAR γ (Fritsche, 2006), we examined whether PPAR γ was involved in the anti-inflammatory effects of these n-3 PUFA. We first looked at expression levels of the transcription factor following PUFA treatment and found that EPA and DHA significantly increased PPAR γ expression in LPS-stimulated DC (**Figure 5.5**; $p < 0.05$, $p < 0.001$ respectively), and interestingly, EPA also increased the basal levels of PPAR γ expression in DC prior to LPS stimulation ($p < 0.05$).

5.2.3 MODULATION OF CYTOKINE PRODUCTION BY EPA AND DHA ARE PPAR γ -INDEPENDENT

PPAR γ has been previously shown to inhibit LPS-induced changes in DC (Nencioni *et al.*, 2002). Since we have demonstrated that both EPA and DHA increase cellular levels of PPAR γ and modulate LPS-induced cytokine production by DC, we investigated whether the effects of EPA and DHA on cytokine production were mediated by their activation of PPAR γ . In these experiments DC were pre-treated as before with DMSO, EPA, DHA or LA and then incubated with the specific PPAR γ antagonist, GW9662, for 1 hour prior to activation with LPS. Consistent with the data in chapter 3, EPA and DHA significantly suppressed IL-12p70 ($p < 0.001$) and enhanced IL-10 production ($p < 0.01$ & $p < 0.05$ respectively) [**Figure 5.6**]. Following the addition of GW9662, EPA and DHA retained the ability to significantly inhibit LPS-induced IL-12p70 production by DC ($p < 0.001$; **Figure 5.6A**), and continued to enhance LPS-induced IL-10, although only the EPA-treated group reached statistical significance ($p < 0.01$; **Figure 5.6B**). These data suggest that modulation of LPS-induced cytokine production by EPA and DHA is independent of PPAR γ activation.

5.2.4 EPA AND DHA MODULATE EXPRESSION OF CELL SURFACE MARKERS ON DC VIA A PPAR γ -INDEPENDENT MECHANISM

Several studies have indicated that PPAR γ activation may be involved in regulating the LPS-induced increase of DC cell surface maturation markers (Szatmari *et al.*, 2006; Klotz *et al.*, 2007). Since n-3 PUFA have previously been

shown to inhibit DC maturation and the associated increase in co-stimulatory markers (Zeyda *et al.*, 2005), we explored the possibility that while PPAR γ is not involved in the modulation of cytokines by EPA and DHA, it may play a role in mediating the PUFA-induced effects on DC maturation, especially given the fact that the inhibitory effects on DC maturation markers were similar when cells were cultured with PUFA (as seen in chapter 3) and RSG. **Figure 5.7** shows that treating DC with EPA and DHA suppresses the expression of CD86 and MHCII, and DHA also decreases CD40 expression. These effects were still observed in the presence of the PPAR γ inhibitor indicating that these changes are not dependent on PPAR γ activation.

Figure 5.8 demonstrates that activating DC with LPS for 24 hours induces maturation via the up-regulation of CD40, CD86, CD80, MHCII and CD48 (top panel). Pre-treatment of DC with EPA and DHA resulted in significant suppression of LPS-induced expression of CD80, CD86, MHCII, and in the case of EPA a small reduction in CD40 expression. This suppression was maintained in the presence of the PPAR γ inhibitor demonstrating that these effects were also independent of PPAR γ activation. The saturated fatty acid control LA, had no suppressive effect on expression of these markers and indeed slightly enhanced expression of CD40, CD80 and CD86 in LPS-stimulated DC. The levels of CD48 were relatively unchanged by any of the treatments. Interestingly, both EPA and DHA had the ability to upregulate the expression of the IL-10R both pre- and post-LPS stimulation, possibly rendering the DC more responsive to the increased levels of IL-10 possibly creating a positive feedback loop.

5.2.5 EPA AND DHA DECREASE TOTAL CELLULAR LEVELS OF NF κ B AND ITS ACTIVATION

Considering the key role of NF κ B in regulating DC maturation (Banchereau *et al.*, 2000) we explored the possibility that fatty acids may alter levels of NF κ B and/or its activation. We firstly quantified the concentration of total cellular NF κ Bp65 in control and fatty acid-treated DC [**Figure 5.9**] and found that both EPA and DHA significantly decreased total cellular NF κ Bp65 levels in resting DC ($p < 0.001$) and in LPS-stimulated DC ($p < 0.001$). In order for activation to occur, NF κ B must be released from I κ B proteins in the cytoplasm and

translocate to the nucleus where it binds to target DNA (Hanada & Yoshimura, 2002), therefore we went on to examine the levels of NF κ Bp65 and I κ B α in nuclear and cytosolic protein fractions of PUFA-treated cells. Western blot analysis of cytosolic protein fractions revealed that EPA and DHA significantly decreased cytosolic NF κ Bp65 in unstimulated cells (**Figure 5.10A** $p < 0.05$). Stimulation with LPS did not alter the ability of PUFA to down-regulate NF κ Bp65 in the cytosol as shown in **Figure 5.10A** where EPA and DHA both decreased cytosolic NF κ Bp65 at 2 hours and significantly at 5 hours ($p < 0.001$) post-LPS treatment. **Figure 5.10B** represents the relative amounts of I κ B α found in the cytosolic fractions of PUFA-treated DC. EPA and DHA were able to suppress I κ B α levels in resting DC ($p < 0.001$), and at 2 hours ($p < 0.01$ and $p < 0.001$) and 5 hours ($p < 0.05$) post-LPS stimulation. Nuclear fractions were analysed for NF κ Bp65 and I κ B α but these proteins were undetectable in these samples possibly due to their complete absence or presence below the level of detection.

5.2.6 PUFA MODULATE THE INTERACTION OF PPAR γ WITH NF κ B

Previous reports have suggested that PPAR γ physically interacts and regulates NF κ B activation (Chung *et al.*, 2000). Since we have already shown that EPA and DHA modify NF κ Bp65 activation and elevate PPAR γ expression, we employed confocal microscopy (in conjunction with Clare Reynolds, UCD) to examine the levels of PPAR γ and NF κ B in the nucleus and cytosol of PUFA-treated DC and looked at the differences in the co-localization of these transcription factors [**Figure 5.11**]. EPA- and DHA-treated DC showed significantly less cytosolic NF κ Bp65 expression 5 hours post-LPS stimulation (**Figure 5.11D**: $p < 0.01$ and $p < 0.05$, respectively) and nuclear NF κ Bp65 2 hours post-LPS stimulation (**Figure 5.11E**: $p < 0.05$ and $p < 0.01$, respectively). DC cultured with DHA had significantly greater cytosolic PPAR γ levels at 0 hours (**Figure 5.11F**; $p < 0.05$) compared to the DMSO control. There was also an increase in cytosolic PPAR γ expression 5 hours post LPS-stimulation in DC treated with EPA and DHA but these findings were not significant [**Figure 5.11F**]. Nuclear PPAR γ expression was significantly increased 5 hours post-LPS

stimulation in EPA treated DC compared to the vehicle control (**Figure 5.11G**; $p < 0.01$). An important aspect of the confocal analysis was to study the co-localization of the transcription factors to address the potential physical interactions between NF κ Bp65 (green) and PPAR γ (red), wherein co-localization appears yellow (data graphed in **Figure 5.11H**). The degree of co-localization was calculated using Volocity 4 software which uses the Pearson Correlation. Clearly, co-localization mostly occurs in the nucleus, indicating that PPAR γ may interact directly with NF κ Bp65 following nuclear translocation. Interestingly EPA- and DHA-treated DC showed significantly greater association between PPAR γ and NF κ B 5 hours after LPS-stimulation ($p < 0.001$, $p < 0.01$, respectively: **Figure 5.11H**). This increased interaction was also observed in resting cells treated with DHA ($p < 0.01$). These data suggest that the interaction between PPAR γ and NF- κ Bp65 could be responsible for the functional effects of EPA and DHA.

5.2.7 THE IMMUNOMODULATORY EFFECTS OF CLA ARE NOT PPAR γ -DEPENDENT

Besides EPA and DHA, CLA was also shown to have suppressive effects on DC maturation and cytokine production (see chapter 3). Previous studies have attributed the immunoprotective effects of CLA to its activation of PPAR γ (Bassaganya-Riera & Hontecillas, 2006; Ringseis *et al.*, 2006), therefore we investigated whether this n-6 PUFA used PPAR γ activation to elicit its suppressive effects on DC maturation given that the n-3 PUFA did not. Here, instead of adding the PPAR γ antagonist to cells 1 hour prior to LPS activation, we exposed cells to both PUFA and the PPAR γ inhibitor (GW9662) for the entire 7 days of culture to ensure PPAR γ activation was blocked from the outset. Following LPS stimulation for 24 hours, supernatant was collected and cytokine levels determined. Alternatively, resting and LPS-stimulated cells were stained with antibodies specific for CD40, CD80, C86, MHCII, CCR5, IL-10R and CD48. Results show that CLA suppresses the production of IL-12p40 (**Figure 5.12A**, $p < 0.05$), and IL-12p70 (**Figure 5.12B**, $p < 0.05$) and that these suppressive effects were not reversed in the presence of the PPAR γ inhibitor, GW9662. Levels of IL-10 remained relatively unchanged [**Figure 5.12C**], as did IL-6

[**Figure 5.13B**] and TNF α [**Figure 5.13C**], whereas CLA augmented the production of IL-1 β [**Figure 5.13A**]. These results corroborate our previous findings and data suggests that PUFA-induced changes in DC cytokine production occur independently of PPAR γ .

The same conclusion can be drawn from the results presented in **Figure 5.14**. In unstimulated cells [**Row 1**], CLA is shown to down-regulate MHCII and CCR5 expression whilst slightly enhancing levels of the IL-10R. Following the addition of LPS, the upregulation of CD80 and CD86, and most notably, MHCII and CCR5 are inhibited by CLA [**Row 2**]. None of these CLA-induced changes were reversed by the specific PPAR γ inhibitor GW9662, providing further evidence of an alternative mechanism used by CLA to elicit its immunomodulatory actions.

5.2.8 CLA DOES NOT USE PPAR γ TO INHIBIT DC MIGRATION

PUFA are known ligands of PPAR, and PPAR levels are upregulated following PUFA treatment, so there must be a reason as to why PUFA upregulate this transcription factor. PPAR γ activation has been shown to suppress DC migration to draining lymph nodes (Angeli *et al.*, 2003) and we showed in chapter 3 that PUFA inhibit DC migration to CCL19 (a chemokine that directs DC to the T-cell rich areas of lymph nodes), therefore we speculated that perhaps CLA activates PPAR γ to alter the ability of DC to migrate towards CCL19/MIP-3 β . We performed a chemotaxis assay to determine whether PPAR γ activation was used by CLA to inhibit the movement of DC towards the lymph node chemokine, CCL19.

DC were treated with DMSO, CLA and RSG +/- the PPAR inhibitor, GW9662, for a total of 7 days, after which cells were stimulated with LPS for a further 24 hours. Cells (3×10^5) were then transferred to the upper chamber of a Transwell plate and media containing CCL19 (100ng/ml) added to the bottom well. Plates were incubated for 5 hours and the number of cells migrating towards CCL19 in each sample was determined by recording the number of events acquired on a FACsCalibur in 60 seconds. Results in **Figure 5.15** clearly show that the addition of CCL19 to the bottom chamber of the Transwell plate induces cell migration ($p < 0.001$). Treating cells with both CLA and RSG significantly

inhibited DC chemotaxis ($p < 0.001$). However, blocking PPAR γ activation only reversed this inhibition in cells that had been treated with RSG. The migration of cells that had been treated with CLA and the PPAR γ inhibitor was still significantly inhibited ($p < 0.001$). Therefore, results suggest that CLA does not employ PPAR γ to alter DC migration.

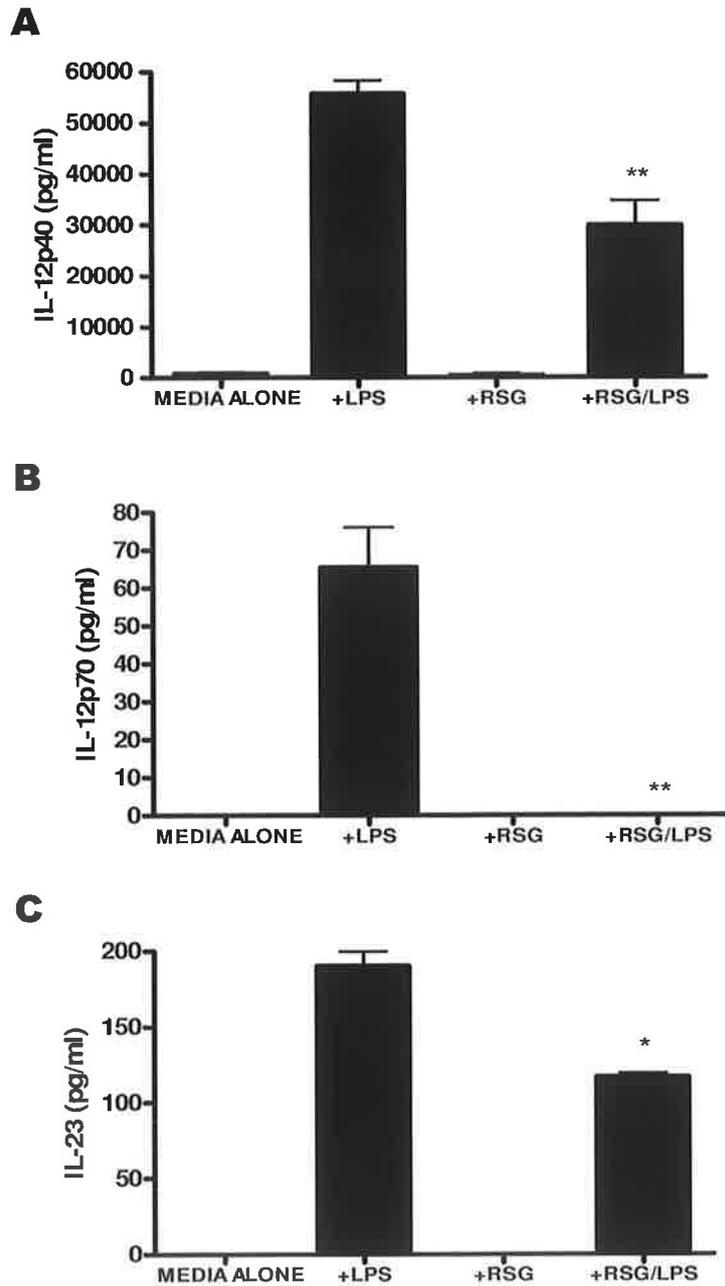


FIGURE 5.1

RSG significantly inhibits the secretion of IL-12p40, IL-12p70 and IL-23 from dendritic cells following activation with LPS

DC were cultured with either DMSO (control) or the PPAR γ ligand, rosiglitazone (5 μ M), for 7 days. Cells were then harvested and stimulated with LPS (100ng/ml). Supernatant was removed 24 hours later and the levels of IL-12p40 [A], IL-12p70 [B] and IL-23 [C] quantified using specific immunoassays. Results are means \pm SE of triplicate assays and represent three independent experiments.

An unpaired t-test was carried out to determine whether differences between the control/LPS and RSG/LPS groups were statistically significant (**p<0.01, *p<0.05)

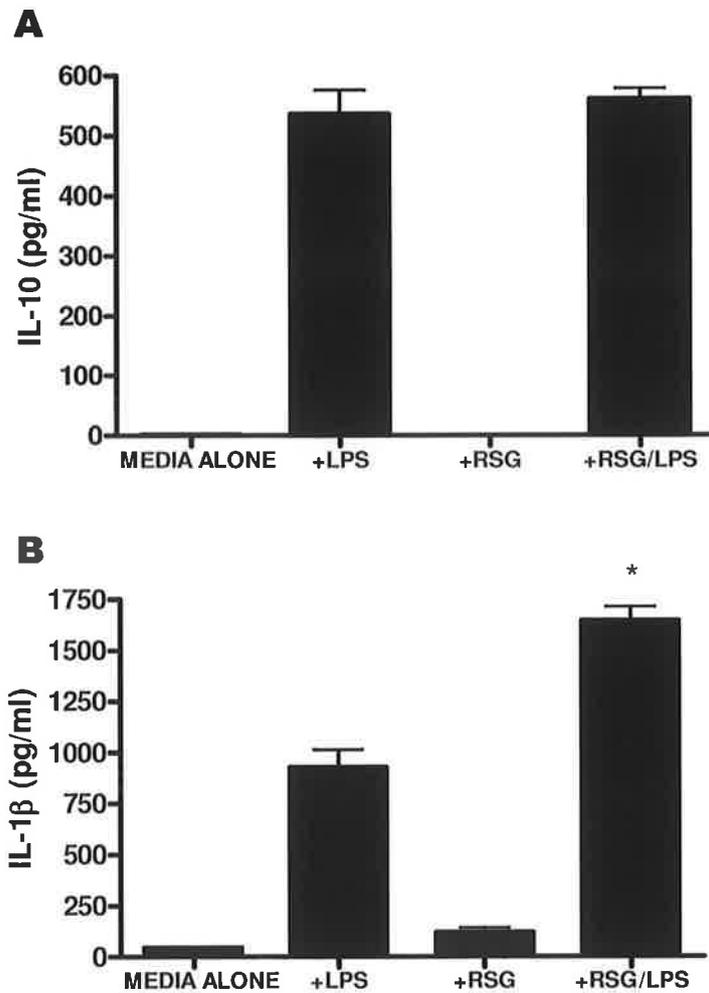


FIGURE 5.2

RSG has little effect on IL-10 production, but significantly enhances the secretion of IL-1 from dendritic cells following LPS activation

DC were cultured with either DMSO (control) or the PPAR γ ligand, rosiglitazone (5 μ M), for 7 days. Cells were then harvested and stimulated with LPS (100ng/ml). Supernatant was removed 24 hours later and the levels of IL-10 [A] and IL-1 β [B] quantified using specific immunoassays. Results are means \pm SE of triplicate assays and represent three independent experiments.

An unpaired t-test was carried out to determine whether differences between the control/LPS and RSG/LPS groups were statistically significant (* $p < 0.05$)

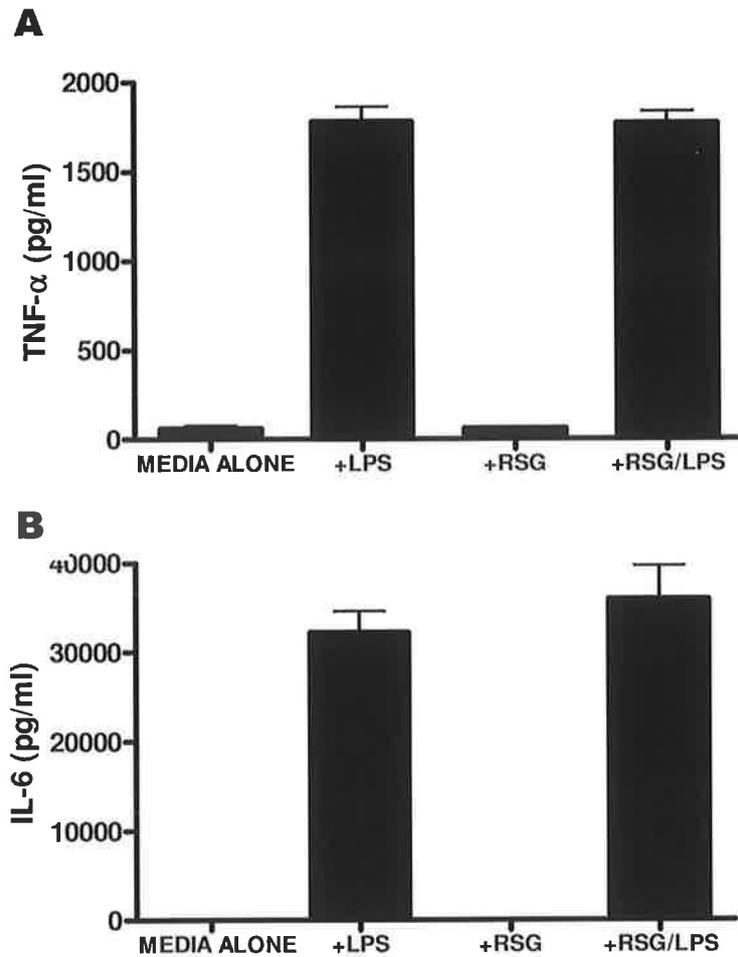


FIGURE 5.3

RSG has no significant effect on TNF- α or IL-6 production from dendritic cells following LPS stimulation

DC were cultured with either DMSO (control) or the PPAR γ ligand, rosiglitazone (5 μ M), for 7 days. Cells were then harvested and stimulated with LPS (100ng/ml). Supernatant was removed 24 hours later and the levels of TNF- α [A] and IL-6 [B] quantified using specific immunoassays. Results are means \pm SE of triplicate assays and represent three independent experiments.

An unpaired t-test was carried out to determine whether differences between the control/LPS and RSG/LPS groups were statistically significant.

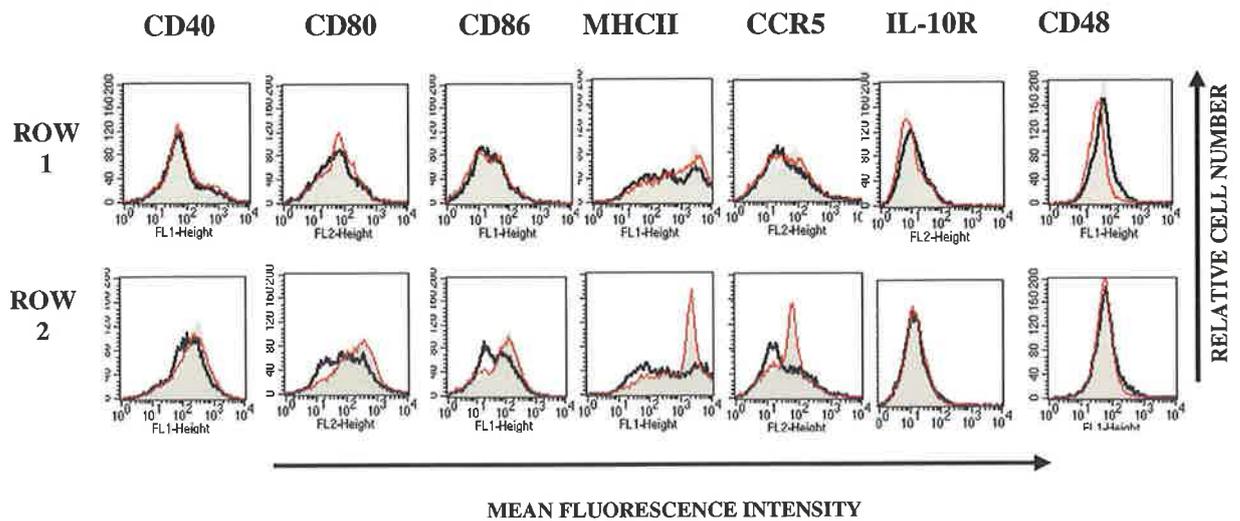


FIGURE 5.4

RSG inhibits the LPS-induced maturation of DC and this inhibition is reversed by the specific PPAR γ inhibitor, GW9662

Bone marrow derived DC were cultured in the presence of DMSO (control), RSG (5 μ M), or RSG and the PPAR γ inhibitor GW9662 (10 μ M) for 7 days.

Cells were either immediately washed and stained with antibodies specific for certain surface markers [ROW 1], or stimulated with LPS (100ng/ml) for 24 hours then washed and stained [ROW 2]. The antibodies used were specific for CD40, CD80, CD86, MHCII, CCR5, IL-10R and CD48 (cells were also stained with isotype-matched controls).

Results of immunofluorescence analysis are shown for DMSO-treated DC (filled histogram), RSG-treated DC (black line), and RSG-treated DC that had also been incubated with the PPAR γ inhibitor (red line). Profiles are shown for a single experiment and are representative of 3 experiments.

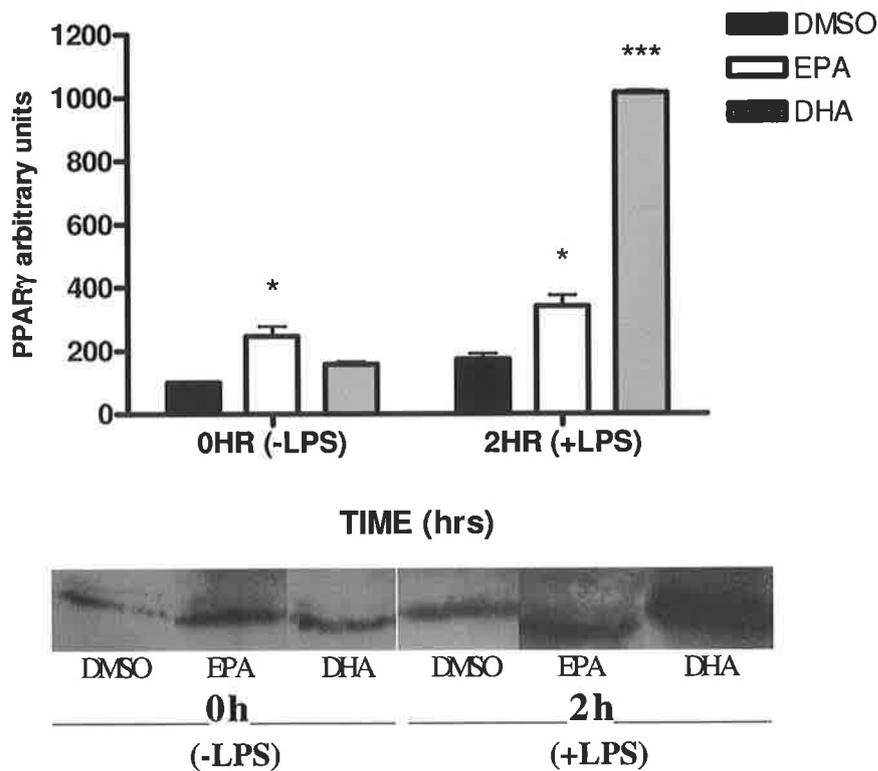


FIGURE 5.5

EPA and DHA modulate total cellular levels of PPAR γ

DC treated for 7 days with either DMSO (vehicle control), EPA (25 μ M) or DHA (25 μ M) were either left unstimulated (0 hours) or stimulated with LPS (100ng/ml) for 2 hours. Total cellular levels of PPAR γ were determined by Western blot analysis as described in the methods section. Briefly: Proteins and pre-stained protein markers were separated by SDS-PAGE and blotted onto PVDF transfer membrane. Following blocking, washing and antibody incubation steps, protein complexes were visualized with supersignal (Pierce, USA) and membranes exposed to film for 1-10 min and processed using an Agfa X-ray processor. Protein bands were quantified using the GeneSnap acquisition and GeneTools analysis software (GeneGenius Gel Documentation and Analysis System, Cambridge, UK) and graphed results are expressed as a fold change relative to control (DMSO). Results are mean \pm SE of triplicate assays and represent three independent experiments.

A representative immunoblot is shown below the graphed data

Lane 1: 0hr DMSO, Lane 2: 0hr EPA, Lane 3: 0hr DHA, Lane 4: 2hr DMSO, Lane 5: 2hr EPA, Lane 6: 2hr DHA.

***p<0.001, *p<0.05 vs. DMSO vehicle control determined by one-way ANOVA test

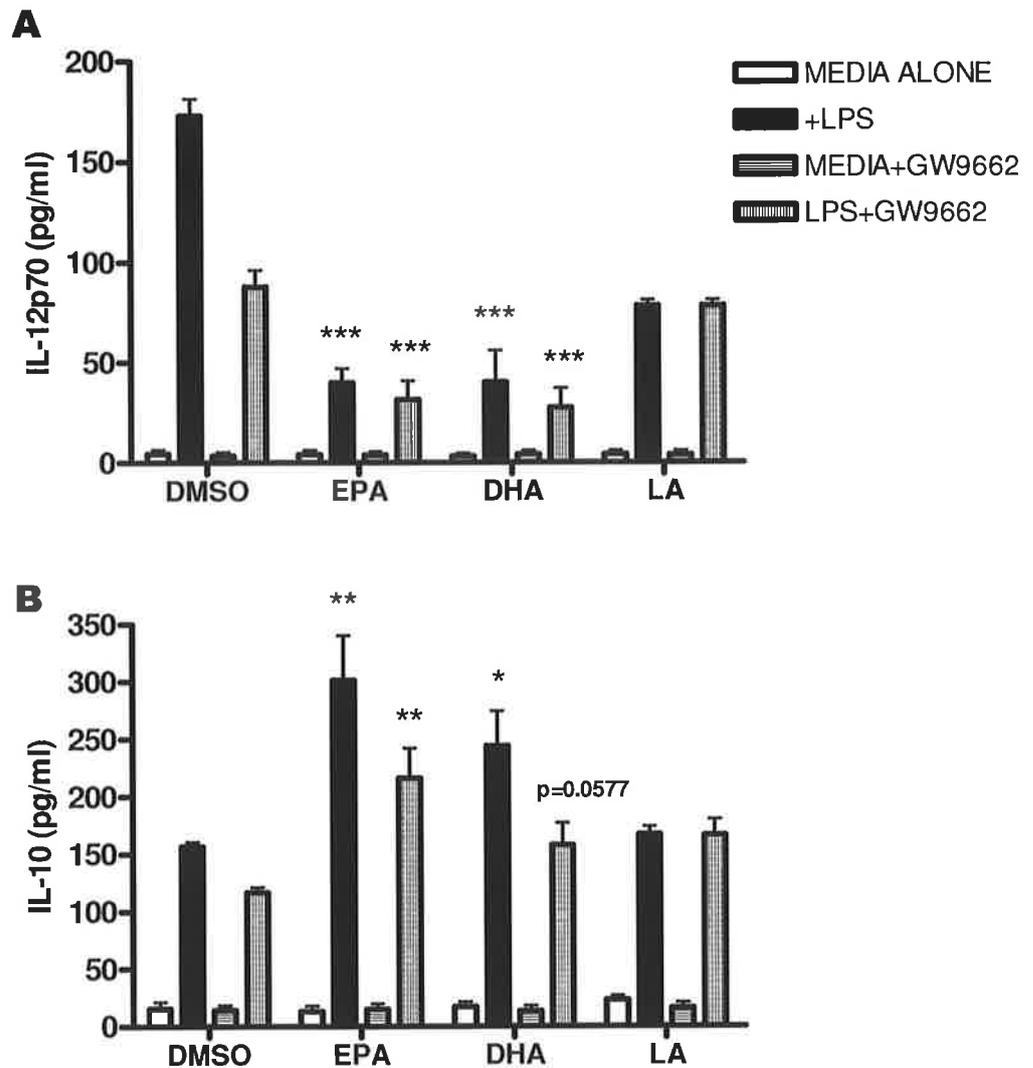


FIGURE 5.6

The PPAR γ inhibitor GW9662 does not reverse PUFA-induced changes in IL-12p70 and IL-10 production in DC

Fatty acid-treated DC were exposed to the PPAR γ inhibitor, GW9662 (10 μ M), for 1 hour prior to LPS-stimulation (100ng/ml). Supernatant was removed 24 hours later and the levels of IL-12p70 [A] and IL-10 [B] assessed. Results are means \pm SE of quadruplicate assays and represent three independent experiments.

***p<0.001, **p<0.01, *p<0.05 vs. DMSO vehicle control determined by one-way ANOVA test

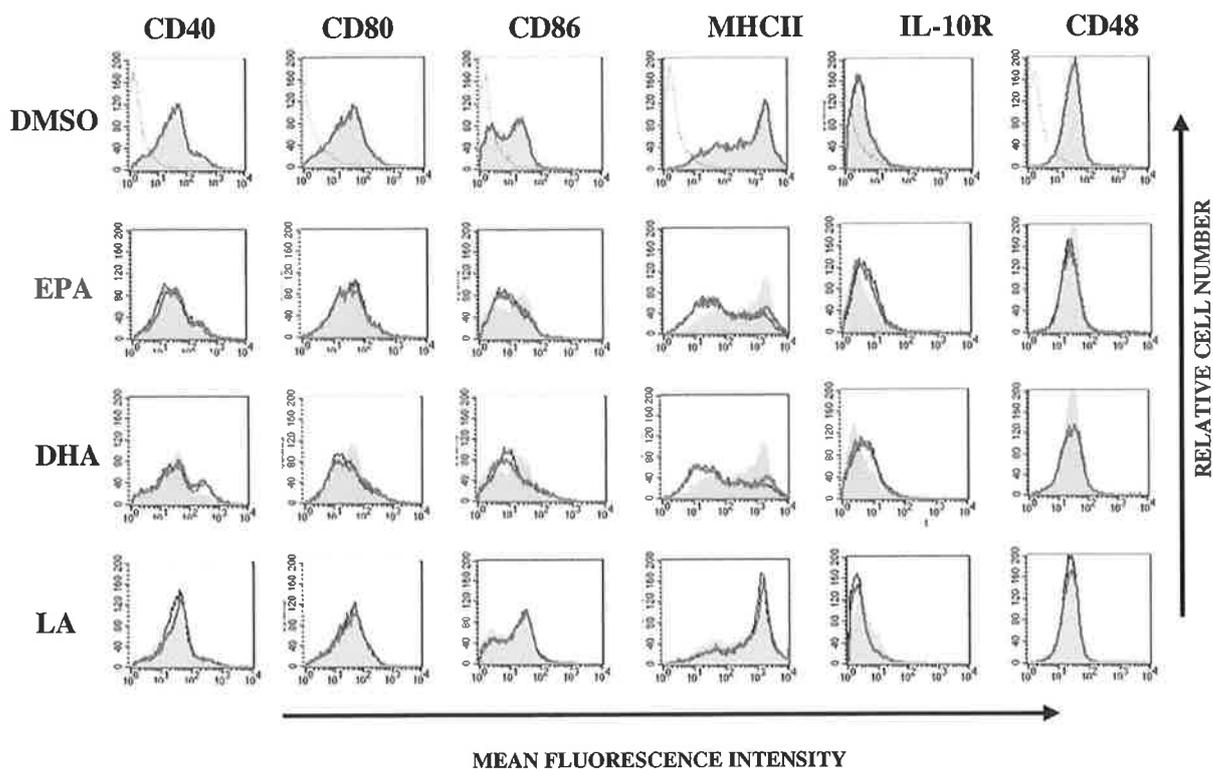


FIGURE 5.7

EPA and DHA modulate the expression CD86, MHCII and IL-10R on the surface of immature DC independently of PPAR γ

Myeloid DC were differentiated with GM-CSF for 7 days in the presence of fatty acids (EPA, DHA, or LA) or vehicle control (DMSO), before being exposed to the specific PPAR γ inhibitor, GW9662 (10 μ M). After 24 hours, cells were washed and stained with antibodies specific for CD40, CD80, CD86, MHCII, IL-10R, and CD48, or with isotype-matched controls. Results of immunofluorescence analysis are shown for DMSO-treated DC (filled histogram), PUFA-treated DC (thin black line), and treated DC incubated with the PPAR γ inhibitor (solid grey line). Histograms for isotype controls are shown as dotted lines in the first row only. Profiles are shown for a single experiment and are representative of 3 experiments.

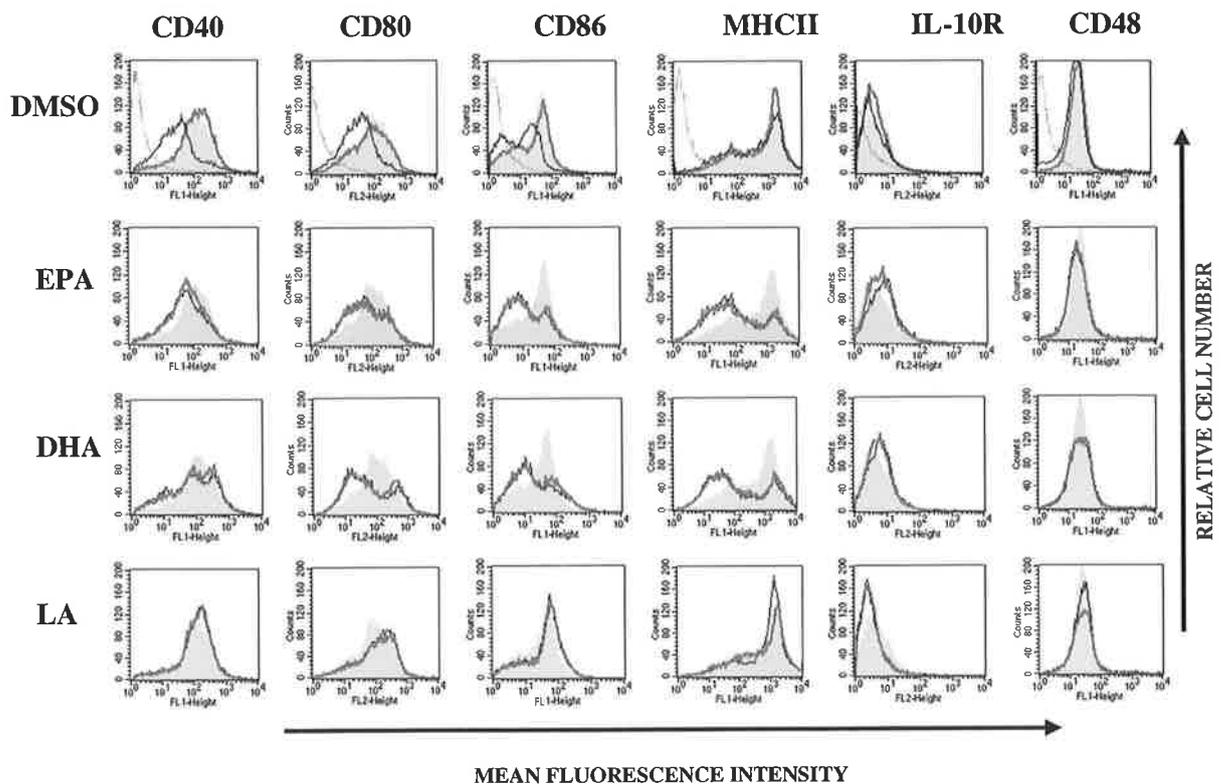


FIGURE 5.8

EPA and DHA modulate the expression of CD40, CD80, CD86, MHCII and IL-10R on the surface of LPS-stimulated DC independently of PPAR γ

Myeloid DC were differentiated with GM-CSF for 7 days in the presence of fatty acids (EPA, DHA or LA) or vehicle control (DMSO). Immature DC were exposed to the specific PPAR γ inhibitor, GW9662 (10 μ M) for one hour prior to LPS stimulation (100ng/ml). Following incubation for 24 hours, cells were washed and stained with antibodies specific for CD40, CD80, CD86, MHCII, IL-10R, and CD48, or with isotype-matched controls. Results of immunofluorescence analysis are shown for DMSO/LPS-treated DC (filled histogram), PUFA/LPS-treated DC (thin black line), and fatty acid/LPS- or DMSO/LPS-treated DC exposed to the PPAR γ inhibitor (solid grey line). Histograms for isotype controls are shown as dotted lines in the first row only. Profiles are shown for a single experiment and are representative of 3 experiments.

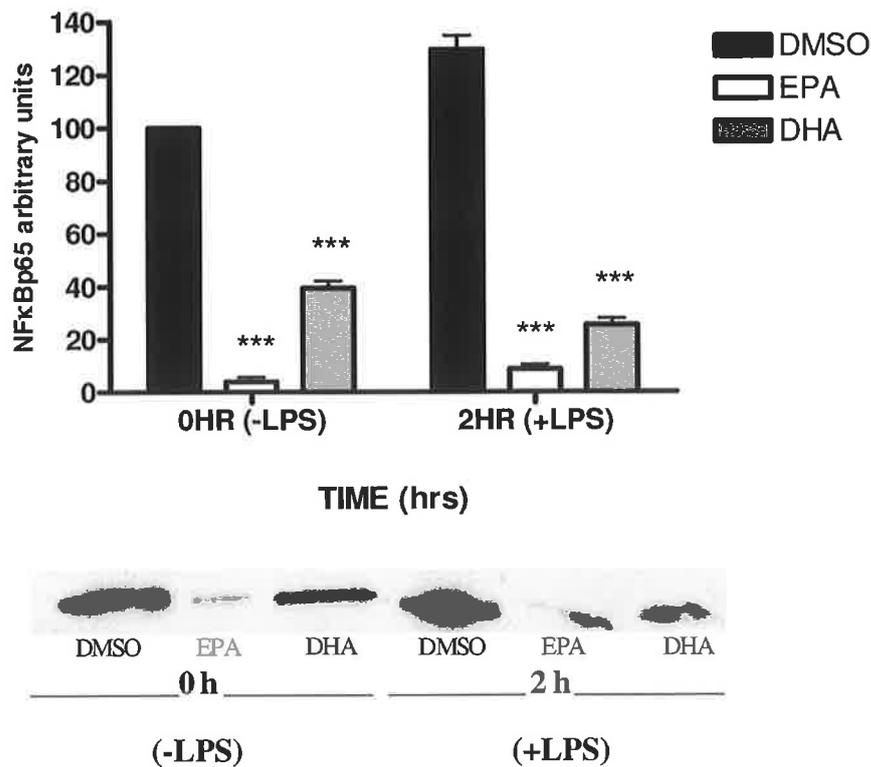


FIGURE 5.9

EPA and DHA modulate total cellular levels of NFκBp65

DC treated for 7 days with either DMSO (vehicle control), EPA (25 μM), or DHA (25 μM) were either left unstimulated (0hr) or stimulated with LPS (100 ng/ml) for 2 hours. Total cellular levels of NFκBp65 were determined by Western blot analysis as described in the methods. Briefly: Proteins and pre-stained protein markers were separated by SDS-PAGE and blotted onto PVDF transfer membrane. Following blocking, washing and antibody incubation steps, protein complexes were visualized with supersignal (Pierce, USA) and membranes were then exposed to film for 1-10 min and processed using an Agfa X-ray processor. Protein bands were quantified using the GeneSnap acquisition and GeneTools analysis software (GeneGenius Gel Documentation and Analysis System, Cambridge, UK) and results are expressed as a fold change relative to control (DMSO). Results are mean ± SE of triplicate assays and represent three independent experiments.

A representative immunoblot is shown below the graphed data

Lane 1: 0hr DMSO, Lane 2: 0hr EPA, Lane 3: 0hr DHA, Lane 4: 2hr DMSO, Lane 5: 2hr EPA, Lane 6: 2hr DHA.

***p<0.001 vs. DMSO vehicle control determined by one-way ANOVA test

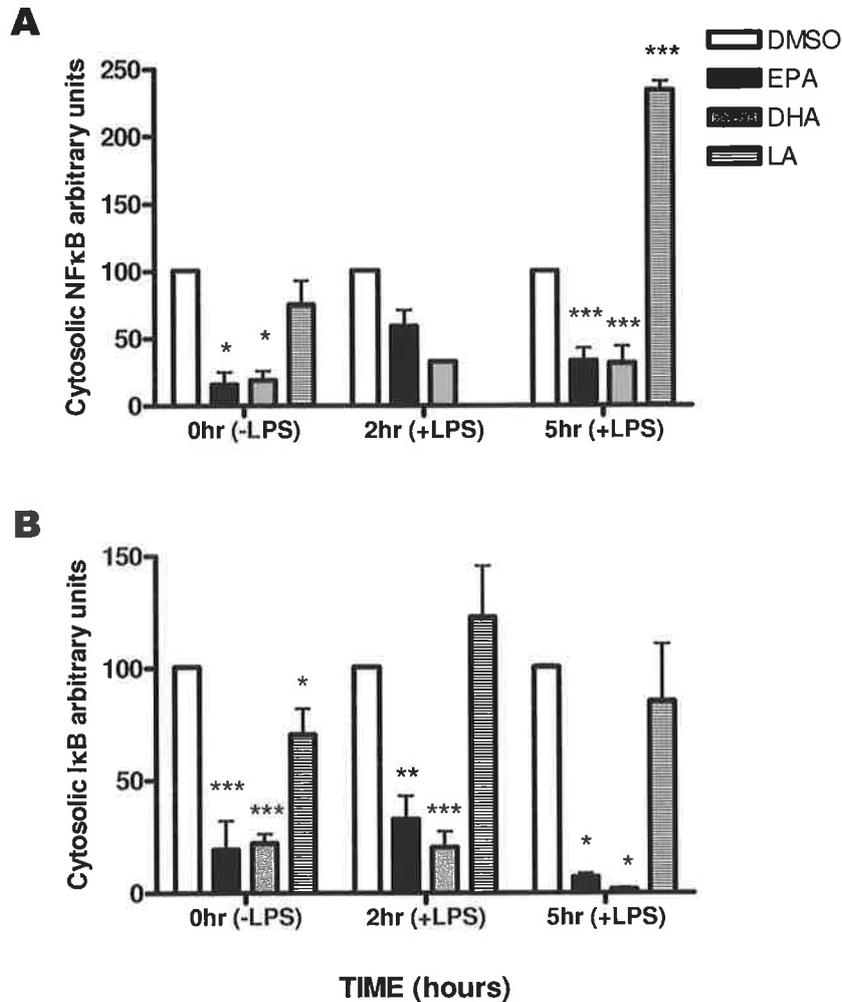


FIGURE 5.10

EPA and DHA modulate NFκB activity

DC were treated for 7 days with either DMSO (vehicle control), EPA (25 μM), DHA (25 μM), or LA (50μM) and either left unstimulated (0hr) or stimulated with LPS (100 ng/ml) for 2 and 5 hours. Cells were harvested and nuclear and cytosolic fractions were extracted as described in the methods. Expression of cytosolic NFκBp65 [A] and cytosolic IκBα [B] (no nuclear protein was detected) were measured by Western blot analysis: Briefly, proteins and pre-stained protein markers were separated by SDS-PAGE and blotted onto PVDF transfer membrane. Following blocking, washing and antibody incubation steps, protein complexes were visualized with supersignal (Pierce, USA). Membranes were then exposed to film (from 1 min to overnight exposure) and processed using an Amersham film Hyperprocessor. Protein bands were quantified using the densitometry program on the Syngene gel analysis documentation system and results are expressed as a fold change relative to control (DMSO). Results are mean ± SE of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01, *p<0.05 vs. DMSO control determined by one-way ANOVA test

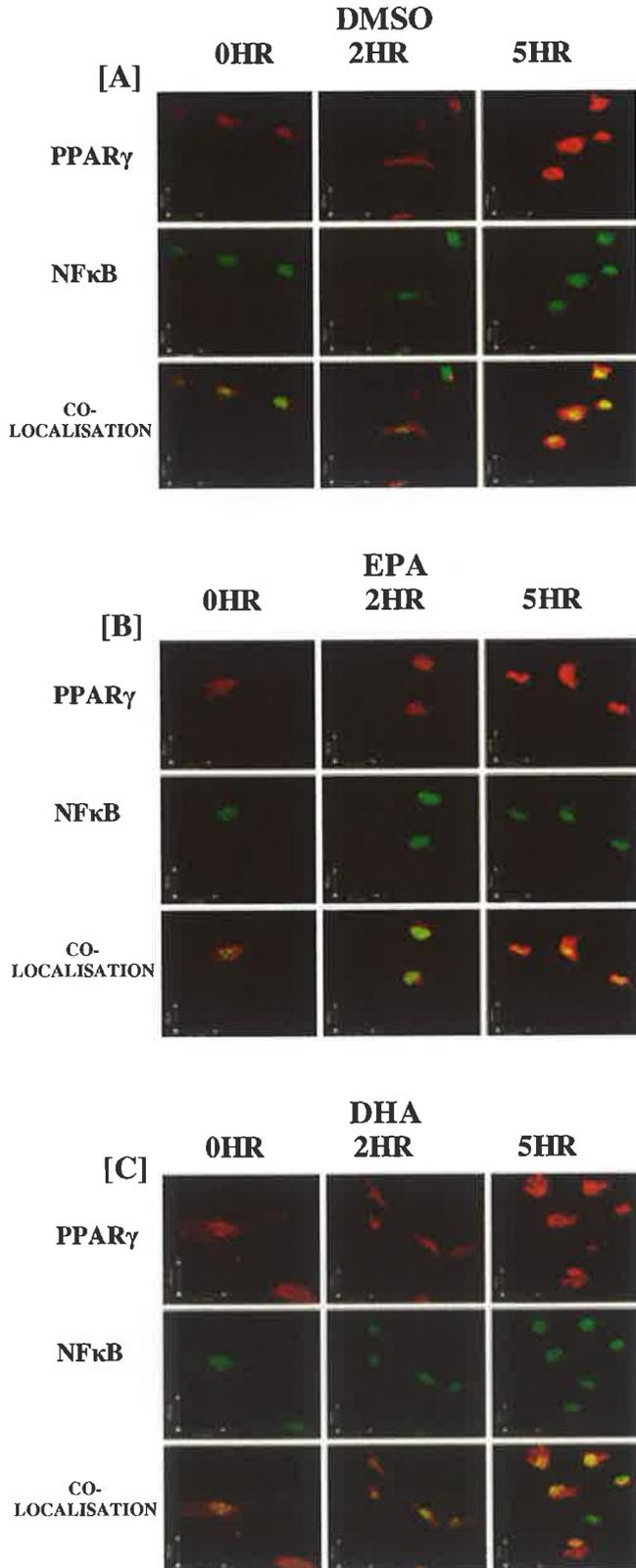


FIGURE 5.11

EPA and DHA modulate the expression of PPAR γ and NF κ Bp65 and alter their interaction

Fatty acid-treated DC were either left unstimulated (0 hr) or stimulated with LPS (100ng/ml) for 2 and 5 hours before being fixed and permeabilised.

Cells were first incubated with primary antibodies for NF κ Bp65 and PPAR γ then incubated with secondary antibodies conjugated to either FITC (green used for NF κ B), or Rhodamine (red used for PPAR γ). Overlap and therefore co-localisation of the two fluorochromes (and transcription factors) is shown in yellow and the degree of co-localisation was calculated using the Pearson's Correlation coefficient (Volocity 4 Software). Examples of the confocal images obtained are shown here for DMSO (vehicle control) [A], EPA [B], DHA [C].

[See next page for graphed data obtained from confocal images]

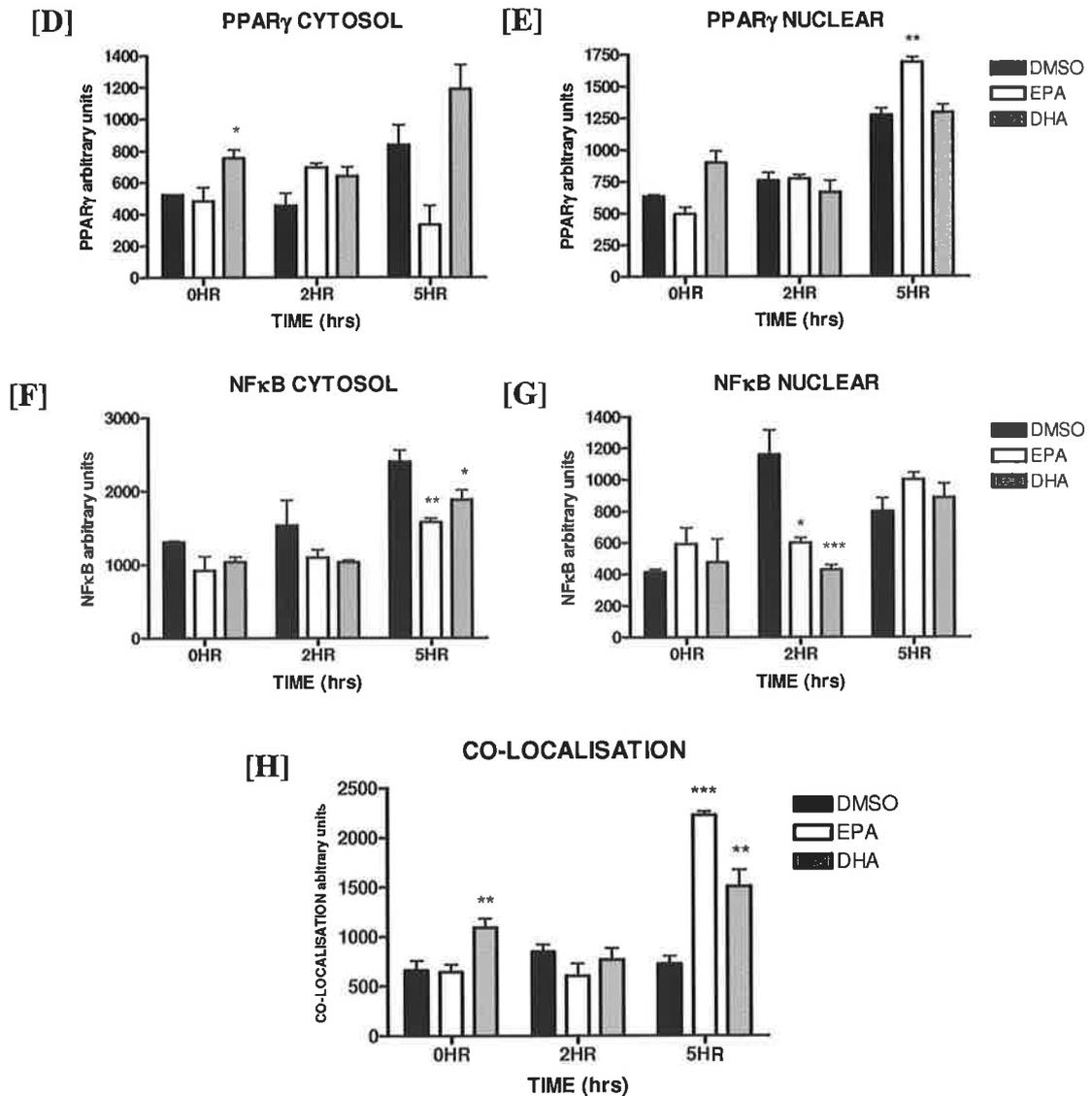


FIGURE 5.11

EPA and DHA alter the expression and co-localisation of the transcription factors, PPAR γ and NF κ B, in DC

Figures 5.11 A-C on the previous page show example images obtained during confocal microscopy and detail the methods used to obtain these images. The figures here graphically represent these confocal images and show the mean value of immunofluorescence intensity for NF κ Bp65 [D&E] and [F&G] PPAR γ in both nuclear and cytosolic cellular regions of PUFA-treated DC. Furthermore, co-localisation of the two transcription factors was calculated using Pearsons Correlation (calculated using Volocity 4 software) [H].

Multiple-labelled immunofluorescence images of DC treated with DMSO, EPA or DHA and either unstimulated, or stimulated with LPS 2 or 5 hrs were captured using a BioRad MRC 1024 confocal microscope. Confocal analysis parameters were identical for each image with 8-15 non-overlapping sections per slide. Images were then acquired using Volocity 4 3D imaging software (Improvision Software, Coventry UK). The amount of NF- κ Bp65, PPAR γ and amount of co-localisation in DC was quantified using Volocity 4 software (Improvision Software).

***p<0.001, **p<0.01, *p<0.05 vs. DMSO vehicle control determined by one-way ANOVA test

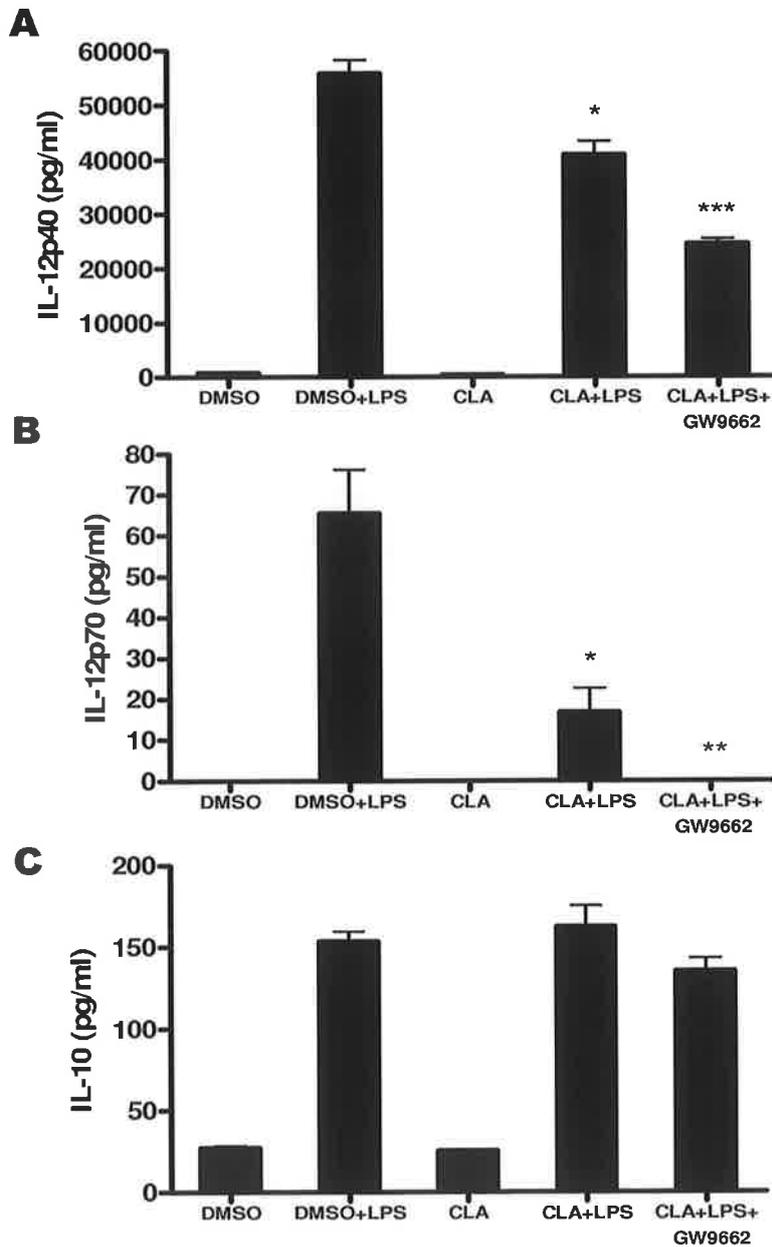


FIGURE 5.12

The modulatory effects of CLA on LPS-induced cytokine production in DC are not PPAR γ -dependent.

DC were cultured with either DMSO (control), CLA (50 μ M), or CLA plus the PPAR γ antagonist, GW9662 (10 μ M) for 7 days. Cells were then harvested and stimulated with LPS (100ng/ml). Supernatant was removed 24 hours later and the levels of IL-12p40 [A], IL-12p70 [B] and IL-10 [C] quantified using specific immunoassays. Results are means \pm SE of triplicate assays and represent three independent experiments.

An unpaired t-test was carried out to determine firstly whether differences between the control/LPS and CLA/LPS groups were statistically significant, and then whether differences were still different after the addition of the PPAR γ inhibitor, i.e. control/LPS vs. CLA/LPS/GW9662 (***p<0.001, **p<0.01, *p<0.05)

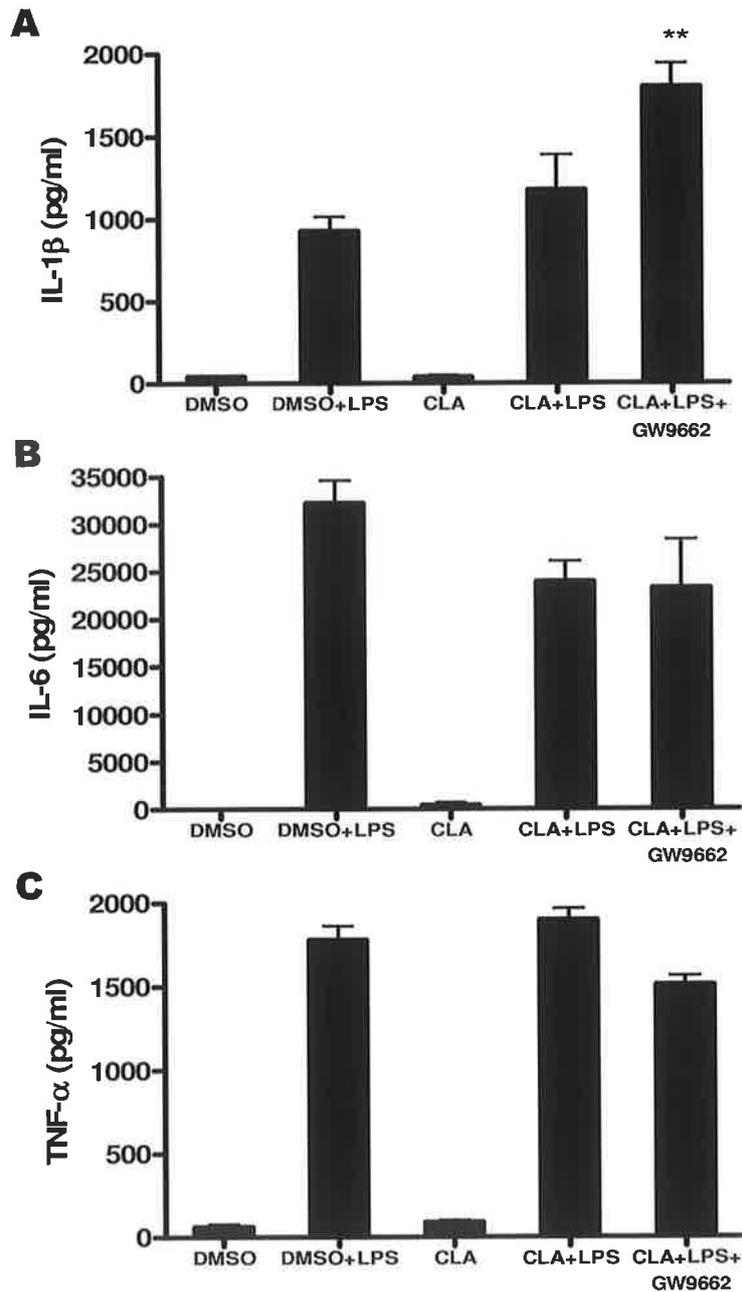


FIGURE 5.13

The modulatory effects of CLA on LPS-induced cytokine production in DC are not PPAR γ -dependent.

DC were cultured with either DMSO (control), CLA (50 μ M), or CLA plus the PPAR γ antagonist, GW9662 (10 μ M) for 7 days. Cells were then harvested and stimulated with LPS (100ng/ml). Supernatant was removed 24 hours later and the levels of IL-12p40 [A], IL-12p70 [B] and IL-10 [C] quantified using specific immunoassays. Results are means \pm SE of triplicate assays and represent three independent experiments.

An unpaired t-test was carried out to determine firstly whether differences between the control/LPS and CLA/LPS groups were statistically significant, and then whether differences were still different after the addition of the PPAR γ inhibitor, i.e. control/LPS vs. CLA/LPS/GW9662 (**p<0.01)

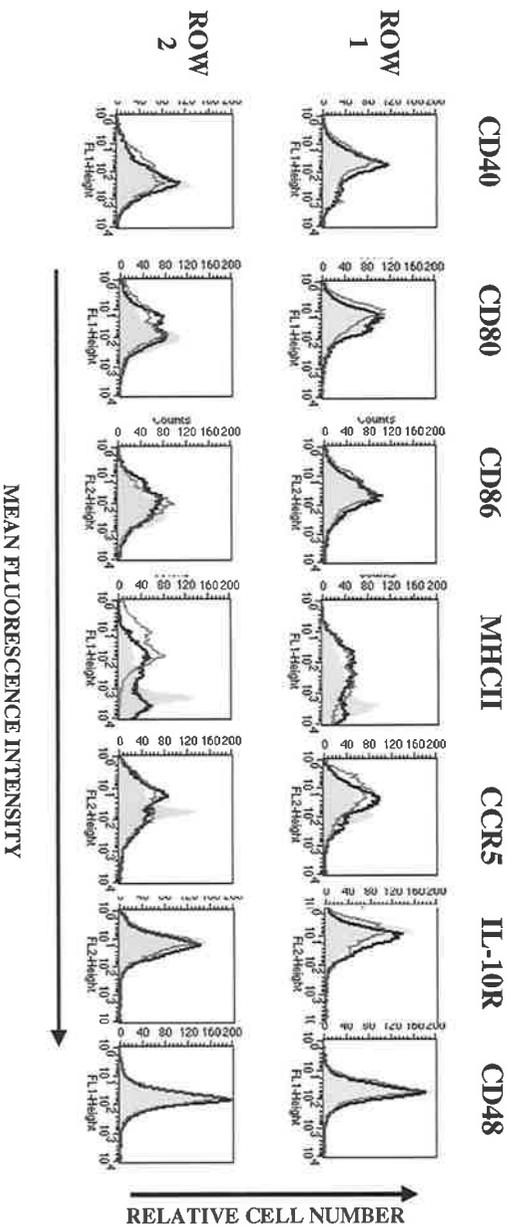


FIGURE 5.14

CLA modulates the expression of CD80, CD86, MHCII, CCR5 and IL-10R on the surface of LPS-stimulated DC independently of PPAR γ

Bone marrow derived DC were cultured in the presence of DMSO (control), CLA (50 μ M), or CLA and the PPAR γ inhibitor GW9662 (10 μ M) for 7 days.

Cells were either immediately washed and stained with antibodies specific for certain surface markers [ROW 1], or stimulated with LPS (100ng/ml) for 24 hours then washed and stained [ROW 2]. The antibodies used were specific for CD40, CD80, CD86, MHCII, CCR5, IL-10R and CD48 (cells were also stained with isotype-matched controls).

Results of immunofluorescence analysis are shown for DMSO-treated DC (filled histogram), CLA-treated DC (black line), and CLA-treated DC that had also been incubated with the PPAR γ inhibitor (red line). Profiles are shown for a single experiment and are representative of 3 experiments.

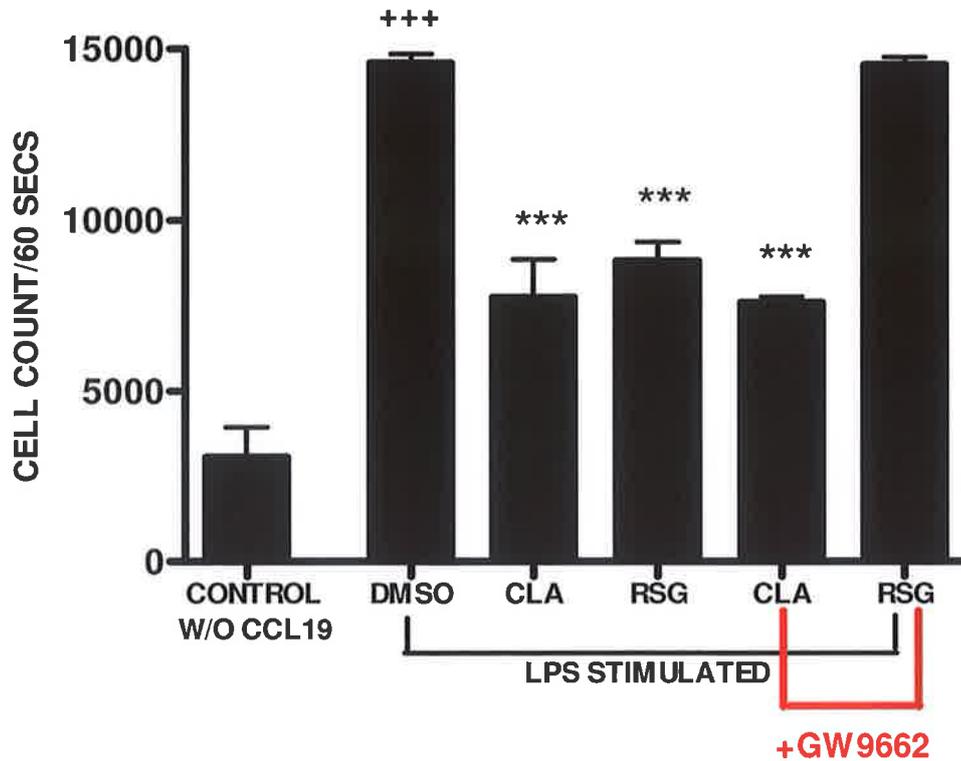


FIGURE 5.15

CLA and RSG inhibit the migration of DC towards the chemokine MIP-3 β (CCL19). The CLA-induced inhibition is not reversed by the PPAR γ inhibitor, GW9662

DC were cultured in the presence of DMSO (vehicle control), and CLA and RSG +/- the PPAR γ antagonist (GW9662) for 7 days. Cells were then stimulated with LPS (100ng/ml) for 24 hours before 3×10^5 cells were placed in the upper chamber of a Transwell plate (8.0 μ m). Media containing recombinant CCL19 (100ng/ml) was added to the lower chamber and plates were incubated for 5 hours at 37°C. To determine the number of migrated cells, media from the bottom well was collected and events (cells) counted for 60 seconds on a FACsCalibur.

As a negative control, unstimulated cells were placed into the upper well of a Transwell plate and media alone (without CCL19) added to the bottom chamber to ensure that the DC migration observed was initiated by the presence of the chemokine. Results are means \pm SE of triplicate assays and represent 2 separate experiments.

***p<0.001 vs. DMSO vehicle control determined by one-way ANOVA test
 +++ p<0.001 using an unpaired t-test between control (without CCL19) and LPS-stimulated DMSO groups

5.3 DISCUSSION

PPAR γ is widely expressed by a number of immune cells, and can affect various aspects of innate and adaptive immunity through specific effects on individual cell populations. For example, PPAR γ negatively regulates the functions of macrophage and monocytes by decreasing their oxidative burst (von Knethen & Brune, 2002) and suppressing inflammatory cytokine production (Jiang *et al.*, 1998). In the adaptive immune system, PPAR γ has been shown to regulate T helper cell responses (Clark *et al.*, 2000) and decrease IFN- γ and IL-2 production by mitogen-activated splenocytes (Cunard *et al.*, 2002):

Here we investigated the role of PPAR γ in DC. Several studies have already shown that activating PPAR γ in DC using ligands such as PGJ₂, rosiglitazone and pioglitazone, results in the down-regulation of DC activation and maturation, characterised by a suppression of IL-12 production and decreased expression of key co-stimulatory molecules (Skorokhod *et al.*, 2004; Appel *et al.*, 2005; Szatmari *et al.*, 2006). Our results corroborate these findings and show that RSG inhibits the production of IL-12p40 and IL-12p70 from LPS-stimulated DC and also prevents the maturation process by down-regulating surface levels of CD80, CD86, MHCII and CCR5 following LPS stimulation, which were reversed following the addition of the PPAR γ antagonist, GW9662. We also report for the first time that RSG suppresses IL-23 production from DC. IL-23 plays a major role in Th17 cell development and thus IL-17 production (Kikly *et al.*, 2006), and Th17 cells have been implicated as being key pathogenic effector cells in a number of inflammatory diseases, including MS, RA and IBD (Langrish *et al.*, 2005; Zhang *et al.*, 2007). Suppressing IL-23 has been shown to be therapeutically beneficial in these diseases: For example, Langrish and colleagues (2005) reported that IL-23-deficient mice are resistant to the development of EAE, CIA and IBD. Therefore, RSG- or PPAR γ activation-induced IL-23 suppression may be beneficial in these inflammatory disorders.

To date little is known as to the effect of PUFA on PPAR γ activation with regard to DC function. Therefore we examined whether the immunomodulatory effects of long chain PUFA on DC are mediated by PPAR γ , firstly looking at the n-3 PUFA (EPA and DHA) and then the n-6 PUFA, CLA. The data clearly demonstrates that all PUFA suppress LPS-induced IL-12p70 and enhance IL-10

production, which correlates with the findings of others who have shown that EPA and DHA suppress IL-12p70 and IL-12p40 in murine and human DC respectively (Weatherill *et al.*, 2005; Zeyda *et al.*, 2005).

IL-12 is produced by innate immune cells and is a key cytokine in the development of Th1 responses, and as such IL-12 has been implicated in the pathogenesis of infectious, inflammatory and autoimmune diseases, including inflammatory bowel disease (Liu *et al.*, 2001a; Trinchieri, 2003). Our findings are consistent with studies that demonstrate that PUFA can inhibit Th1 responses: Feeding mice a fish oil diet rich in EPA and DHA reduced circulating and splenic IL-12p40 and IFN- γ production in mice following infection with *Listeria monocytogenes* (Fritsche *et al.*, 1999) and CLA was shown to be beneficial in a murine model of colitis which is considered a predominantly Th1-mediated disease (Bassaganya-Riera *et al.*, 2004).

The suppression of IL-12 by PPAR γ ligands such as troglitazone, rosiglitazone and PGJ2, is well established (Faveeuw *et al.*, 2000; Nencioni *et al.*, 2002; Klotz *et al.*, 2007) and confirmed here. The effect of PPAR γ ligands on IL-23 production has not been previously investigated, and was also found to be inhibited by RSG. Given that EPA and DHA, like RSG, suppress the production of IL-12 and IL-23 by DC (DHA in particular), we examined whether this correlated with changes in PPAR γ expression. We found that both EPA and DHA increased cellular PPAR γ protein expression in DC pre- and post-LPS stimulation. This has been shown in other cell types where EPA and DHA were found to increase PPAR γ mRNA and cellular PPAR γ protein in human renal tubular cells and adipocytes respectively (Chambrier *et al.*, 2002; Li *et al.*, 2005). However, although expression levels of PPAR γ were increased, the specific PPAR γ inhibitor (GW9662) failed to reverse the anti-inflammatory effects of EPA, DHA, or CLA on DC cytokine production, i.e. the PUFA were still able to reduce IL-12 and enhance IL-10 production (although CLA had little effect on IL-10 here).

Most of the studies that examine the effects of PPAR γ ligands on DC function have reported no effect on IL-10 production. However, a very recent paper reported that RSG upregulates the production of IL-10 from monocyte-derived DC but only when present in nanomolar concentrations (Thompson *et al.*, 2007). Micromolar amounts of RSG had no effect on IL-10 production from DC which

agrees with the findings here, and implies that the concentration of PPAR ligand used is of great importance. IL-10 is a potent anti-inflammatory cytokine that inhibits innate immune cell IL-12, TNF- α , and IL-1 production (Conti *et al.*, 2003) and IL-10 was found to be upregulated by PUFA in this study. Similar findings have been reported previously: CLA enhanced IL-10 production in whole blood (Ly *et al.*, 2005) and T cells (Song *et al.*, 2003). Of note here is the finding that EPA, DHA and CLA enhanced IL-10R expression. Increased receptor expression would enhance cellular responses to the increased levels of IL-10 secreted by PUFA-treated DC, creating a positive feedback loop. Therefore it was not surprising that the PUFA-induced increase in IL-10 was not reversed by the specific PPAR γ inhibitor, GW9662. The suppressive effects of PUFA on IL-12 production were not reversed in the presence of the PPAR γ inhibitor either, and this finding is in agreement with a recent publication that showed the suppressive effects of EPA and DHA on macrophage IL-12 to be PPAR γ independent (Zhang & Fritsche, 2004).

DC maturation is a crucial step that facilitates the development of immature cells into potent antigen presenting cells. As well as producing cytokines, they increase the expression of MHCII and co-stimulatory molecules, including CD40, CD80, CD86, which facilitates their interaction with, and activation of, naïve T cells (Kapsenberg, 2003). Previous reports have shown that the activation of PPAR γ by troglitazone, 15d-PGJ2 and rosiglitazone suppressed CD80 but enhanced CD86 following DC activation (Gosset *et al.*, 2001; Nencioni *et al.*, 2002). Our results showed a suppression of CD80, CD86, MHCII and CCR5 following RSG treatment, effects which were completely reversed in the presence of the PPAR γ antagonist. Similar to RSG, PUFA also had potent inhibitory effects on DC maturation markers and we examined whether these changes were PPAR γ -dependent. Treatment of DC with EPA, DHA and CLA significantly suppressed the up-regulation of co-stimulatory markers following LPS activation but these changes were not reversed in the presence of the PPAR γ inhibitor. The modulation of DC maturation status has been shown to affect subsequent T helper cell activation resulting in the development of CD4⁺ T helper cell anergy (Klotz *et al.*, 2007). Therefore the effects of PUFA and RSG could have downstream effects on the adaptive

immune system, albeit through divergent mechanisms. This should be investigated in future experiments.

The expression of PPAR γ is upregulated during T cell activation (Clark *et al.*, 2000), and ligand binding has been shown to inhibit the production of IFN- γ and IL-2 from T cells (Cunard *et al.*, 2002). Therefore PUFA may actually exert their effects on T cells via a PPAR γ mechanism rather than the APC. It has been suggested previously that PPAR γ exerts its anti-inflammatory effects by attenuating the ability of DC to prime T cells (Klotz *et al.*, 2007) but others have shown it to act directly at the level of the T cell (Clark *et al.*, 2000). Considering the suppression of IL-12 (Appel *et al.*, 2005) and inhibition of IFN- γ (Cunard *et al.*, 2002) seen following PPAR γ activation, it would be interesting to see whether the RSG- and PUFA-induced inhibition of IL-23 found here would have subsequent effects on IL-17 production from T cells via PPAR γ activation. This is of particular interest because results in chapter 4 showed that CLA-treated DC had an inhibitory effect on IL-17 and IFN- γ production from CD4⁺ T cells.

The inhibitory effect of PPAR γ on IL-12 has been shown to involve its interaction with NF κ B (Chung *et al.*, 2000) and members of the NF κ B family of transcription factors are important for the activation and function of DC (Appel *et al.*, 2005). Therefore the effects of EPA and DHA on levels of NF κ Bp65, and the interaction between NF κ B and PPAR γ were examined. Treatment of DC with both EPA and DHA resulted in a decrease in total cellular, cytosolic and nuclear NF κ Bp65 protein in resting and LPS-stimulated DC. Similar reports showed EPA and DHA to suppress NF κ B activity in THP-1 macrophage (Weldon *et al.*, 2007), RAW264 macrophage (Lee *et al.*, 2003), pancreatic tumor cells (Ross *et al.*, 2003), human neutrophils (Fickl *et al.*, 2005) and Jurkat T cells (Denys *et al.*, 2005). However, few studies have examined the interaction between PPAR γ and NF κ B. Previous reports have demonstrated that c-rel, a member of the NF κ B family, regulates IL-12 expression in DC (Grumont *et al.*, 2001) and interestingly PPAR γ activation in DC down-regulates c-rel activation (Appel *et al.*, 2005). These studies suggest that PPAR γ may exert its inhibitory effect on IL-12 production via the NF κ B pathway. Given our evidence that EPA and DHA activated PPAR γ and suppressed NF κ B, we explored the interaction between the two transcription factors in DC. Confocal microscopy results clearly

demonstrated that EPA and DHA increased the physical association between PPAR γ and NF κ B in DC.

The PUFA-induced decrease in NF κ B activity we report here provides perhaps the best explanation as to how PUFA exert their effect on IL-12. One other study examined the potential role of PPAR γ in mediating suppressive effects on IL-12 in DC. It reported that a malarial pigment, Hemozoin, inhibited the differentiation and maturation of human dendritic cells via a PPAR γ -dependent mechanism (Skorokhod *et al.*, 2004). However, their inhibitor studies only focused on the DC activation marker CD83 and no data was made available on cytokine production or expression of co-stimulatory molecules. It is of importance to note that while this molecule exerted its effects on DC maturation via a PPAR γ -dependent pathway, it had no effect on the activation of NF κ B (Skorokhod *et al.*, 2004).

The fact that PUFA upregulate the expression of PPAR γ implies that there must be a function for this increase. A role for PPAR γ has been suggested in migration: Angeli and co-workers (2003) showed that the activation of PPAR γ by rosiglitazone specifically impaired the departure of Langerhans cells from the epidermis to draining lymph nodes; Appel and associates (2005) found that adding troglitazone to monocyte-derived DC completely blocked their migration towards CCL19, and Napimoga's research group (2008) showed that administration of another PPAR γ ligand, 15d-PGJ(2), decreased leukocyte rolling and adhesion to inflamed tissue. Given the suppression of migration we saw following PUFA treatment in chapter 3, we used a chemotaxis assay to assess whether this was through a PPAR γ -dependent mechanism. Both CLA and RSG significantly reduced the amount of cells migrating towards CCL19; however only cells treated with RSG had their migratory capacity restored upon the addition of the PPAR γ inhibitor. The chemotaxis of CLA-treated cells was still significantly reduced. Therefore, PUFA-induced alterations in migration also appear to be PPAR γ -independent.

Our inhibitor studies demonstrate that PUFA exert their modulatory effects on cytokine production, surface marker expression and migration even in the presence of GW9962, a specific PPAR γ inhibitor. Therefore these PUFA-induced effects are proposed as PPAR γ -independent. Given that NF κ B plays a key role in regulating the expression of cytokines and surface molecules

(Banchereau *et al.*, 2000), the suppression of NF κ B activation evident following EPA and DHA treatment may explain how PUFA exert their effects. Future work should examine whether similar alterations in NF κ B expression and activation are seen following CLA treatment.

These data indicate that while PUFA may be ligands for PPAR γ , their activation of this transcription factor does not mediate their effects on DC, and therefore does not explain their potent anti-inflammatory effects in these cells.

CHAPTER 6

IN VIVO EFFECTS OF A CLA-ENRICHED DIET

6.1 CHAPTER 6 INTRODUCTION

Results in previous chapters have shown that PUFA are able to inhibit DC maturation *in vitro* and down-regulate proinflammatory cytokine production from CD4⁺ T cells in a co-culture system. Furthermore, some promising results were seen in the adoptive transfer experiment, so in the final chapter we examined the *in vivo* effects of CLA by using a PUFA-incorporated diet in a number of different settings: Firstly, to ascertain whether the changes seen in *in vitro* in both T cells and DC were mirrored *in vivo*, alterations in cytokine production and cell surface marker expression from CD4⁺ splenic T cells and bone marrow-derived dendritic cells isolated from animals fed a CLA-supplemented diet were examined. Secondly, to determine the impact of a PUFA-incorporated diet on endotoxin-induced proinflammatory cytokine production *in vivo*, we assessed alterations in circulating cytokine levels in CLA-fed animals in a murine model of septic shock. Lipopolysaccharide is a cell wall component of gram-negative bacteria that can induce the production of a large quantity of proinflammatory cytokines into the circulation, such as IFN- γ and TNF- α , and these cytokines are what eventually cause septic shock (Furusako *et al.*, 2001). Therefore, therapies that down-regulate these circulating cytokines would prove beneficial in treating this disorder. Dietary intervention studies have been conducted previously in this model and were shown to have efficacious results: Fritsche and co-workers (1999) fed mice a fish oil-supplemented diet for 4 weeks, after which mice were injected with *Listeria monocytogenes*. Plasma levels of IL-12p70 and IFN- γ measured 24 hours later were both greatly reduced in animals fed the fish oil diet. Another study by Lai and colleagues (2005) recorded a suppression in circulating IL-6 and TNF- α in pigs fed a CLA-supplemented diet before LPS challenge.

The third *in vivo* model used to examine the effects of a CLA-supplemented diet in this study was a murine model of IBD. IBD is the collective term for UC and CD, and a disease that affects millions of people worldwide. It is characterised by a chronic uncontrolled inflammation of the intestinal mucosa thought to be due to an abnormal response to antigens of normal intestinal flora (Papadakis & Targan, 2000). Histologically, mucosal accumulation of leukocytes is a characteristic feature of IBD, and the activation of T cells and macrophage has

been regarded as a crucial factor in its pathogenesis (Ogawa *et al.*, 2004). Several animal models have been developed to help research this inflammatory disease and although they have limitations, they resemble some important immunological and histopathological aspects of IBD in humans (Elson *et al.*, 2005). One such model, and the model considered in this study, is the dextran sodium sulphate (DSS) chemically-induced model of colitis. Feeding DSS polymers in the drinking water of mice causes a reproducible acute colitis. Symptoms include weight loss, shortening of the colon, neutrophil infiltration and epithelial changes including fibrosis and ulceration. The initial acute phase of inflammation is followed by a slow regeneration of the colonic epithelium and a concurrent chronic inflammatory process associated with high mucosal levels of IFN- γ and IL-4 (Elson *et al.*, 2005; Wirtz *et al.*, 2007). It is thought that DSS is directly toxic to gut epithelial cells which leads to a disrupted epithelial barrier thereby increasing the hosts exposure to normal intestinal flora (Melgar *et al.*, 2005).

A dietary intervention study using a CLA-supplemented feed was used to assess the potential benefits of PUFA in the diet in a porcine DSS colitis model and CLA was found to have favourable protective effects on intestinal tissue damage (Bassaganya-Riera *et al.*, 2004). In another study, intestinal inflammation was induced in IL-10 knockout mice by inoculation with complex intestinal microflora (CIF) and/or cultures of *Enterococcus faecalis*. Mice fed an EPA-enriched diet had an approximate 40% reduction in colonic histopathology compared to animals fed the control (oleic acid) diet (Roy *et al.*, 2007). Therefore, data suggests that increased PUFA in the diet has beneficial effects in models of IBD and septic shock. Given the fact that the treatment of many inflammatory/autoimmune diseases, including IBD and RA, rely on broad-spectrum anti-inflammatory drugs such as steroids, there is an obvious deficit in specific immune-targeted and less harmful treatments for these increasingly prevalent diseases. Perhaps as our knowledge and understanding of the inflammatory process expands and the exact mechanism of action of PUFA becomes clearer, nutritional intervention may be an optional additive therapy for these debilitating conditions.

6.2 RESULTS

6.2.1 A CLA-SUPPLEMENTED DIET ALTERS *EX VIVO* CYTOKINE PRODUCTION FROM BONE MARROW-DERIVED DC

Animals 4-6 weeks of age were fed either a CLA-supplemented diet (1% (w/w)), or a control diet (1% (w/w) linoleic acid) for 5 weeks. Bone marrow-derived DC were cultured as described in *section 2.3.2* (there was no exogenous addition of fatty acids during culture). Cells were then stimulated with 100ng/ml LPS for 24 hours. Subsequent cytokine analysis of supernatant revealed that dietary CLA inhibits LPS-induced production of IL-12p70 ($p < 0.001$) and IL-12p40 ($p < 0.01$), whilst enhancing IL-23p19 production ($p < 0.001$) by DC [**Figure 6.1**]. There was little change in TNF- α production [**Figure 6.2A**], whilst both IL-1 ($p < 0.01$) and IL-6 (not significantly) were upregulated in DC isolated from CLA-fed animals compared to those fed the control diet [**Figure 6.2B-C**]. All these findings are in agreement with the *in vitro* results presented in chapter 3. However, there was a surprising decrease in IL-10 secretion [**Figure 6.3**] which contradicts results obtained when DC were cultured with PUFA *in vitro* (discussed in chapter 3).

The top panel of histograms in **Figure 6.4** shows the LPS-induced upregulation of cell surface markers in DC from animals fed the control diet, confirming a normal maturation process in these cells, i.e. the upregulation of CD40, CD80, CD86 and MHCII. Similar results were seen in DC isolated from CLA-fed mice (data not shown). Rows 2 and 3 compare expression patterns of co-stimulatory molecules on DC from the control group (filled histogram) and CLA-fed group (black line). Row 2 represents unstimulated cells, where a slight decrease in MHCII and CCR5 expression can be seen in DC isolated from animals fed a PUFA-supplemented diet. Once cells are stimulated with LPS, the presence of CLA in the diet appears to slightly increase the expression of CD40 and IL-10R, whilst decreasing MHCII and CCR5 (row 3). The remaining markers remain largely unchanged.

6.2.2 FEEDING ANIMALS A CLA-SUPPLEMENTED DIET ALTERS EX VIVO CYTOKINE PRODUCTION FROM CD4⁺ T CELLS

Following dietary supplementation for 5 weeks, CD4⁺ T cells were isolated from the spleens of fed animals and activated *ex vivo* with α CD3/CD28 for 3 days. Supernatant was removed and assessed for cytokine production. **Figure 6.5** shows that T cells from animals fed a CLA-incorporated diet produce significantly more IFN- γ ($p < 0.001$) and slightly less IL-17 (not significant) following *ex vivo* activation compared to animals fed a control diet. The anti-inflammatory cytokines IL-10 and IL-4 [**Figure 6.6**] were significantly upregulated in supernatant from the CLA-fed animals ($p < 0.001$), whereas IL-2 production was slightly increased ($p < 0.01$) [**Figure 6.7A**]. [³H]-thymidine incorporation was used to measure differences in T cell proliferation and **Figure 6.7B** shows that there was no difference in the activation-induced proliferation of T cells between diet groups.

Flow cytometry did not reveal any major alterations in T cell surface marker expression when comparing the control and CLA diet [**Figure 6.8**]. Activation with α CD3/CD28 resulted in a modest upregulation of CD28, CTLA-4 and CCR5 and most notably, the activation marker CD25 on spleen cells (top panel). The presence of CLA in the diet did not modify any of the surface markers analysed on activated splenic CD4⁺ T cells when compared to the expression profiles of cells from control-fed animals.

6.2.3 CIRCULATING INFLAMMATORY CYTOKINES ARE REDUCED FOLLOWING CLA DIETARY SUPPLEMENTATION IN A MURINE MODEL OF SEPTIC SHOCK

Animals were fed either a control or CLA-supplemented diet for 5 weeks (10 mice per diet group). 5 mice from each group then received an iv injection of LPS (3 μ g) into their tail vein. Blood was collected 6 hours after endotoxin challenge and the levels of pro-inflammatory cytokines assayed in the plasma using specific immunoassays.

Animals fed a CLA-supplemented diet prior to intravenous challenge with LPS had less circulating IFN- γ and IL-12p40 than those animals fed a control (linoleic acid) diet. Outwardly, animals appeared less energetic, more hunched and their

fur was more bristled in the control diet-fed group suggesting they had a more severe reaction to the circulating endotoxin than the animals fed a CLA-incorporated diet. This was confirmed with cytokine analysis: **Figure 6.9** shows that animals from the CLA-fed group produced significantly less IFN- γ and IL-12p40 ($p < 0.05$) 6 hours-post LPS injection. Circulating levels of IL-12p70 and IL-1 β were also shown to be reduced in animals fed a CLA-supplemented diet but this inhibition did not reach statistical significance [**Figure 6.10**]

6.2.4 A CLA-SUPPLEMENTED DIET SIGNIFICANTLY PROTECTS AGAINST THE DEVELOPMENT OF DSS-INDUCED COLITIS

2 groups of C57BL/6 mice were fed an α -linolenic control diet, while 1 group received a diet rich in CLA (6 animals per group). Weights and food consumption were monitored for 1 week prior to the induction of colitis and were found to be distinctly similar between sets of animals. On day 7, DSS (3% w/v) was introduced to the drinking water of one group of animals on each diet and after 5 days of DSS normal water was replaced. The third group of mice (fed the control diet) had normal drinking water for the duration of the experiment to serve as a control. All mice were weighed daily and assessed for outward signs of colitis (rectal bleeding, loose stools and diarrhoea) and a scoring system used for the comparative assessment of inflammation.

Results clearly show that feeding animals a CLA-supplemented diet confers some protection against the development of chemically induced colitis. **Figure 6.11A** shows the daily recorded weights of animals in the 3 treatment groups. The weights of control fed animals that did not receive DSS remained stable and even increased slightly during the study. Both groups of animals that were challenged with DSS (on day 7) started to lose weight approximately 2 days later, and steadily lost weight until the experiment was terminated on day 13 (7 days-post DSS challenge). **Figure 6.11B** shows the percentage weight loss per day from days 9-13 of the experiment when DSS-induced weight loss was most apparent. Colitic animals in the CLA-fed group clearly lost less weight than animals fed the control diet on each of these days, however differences only reached statistical significance on days 9 ($p < 0.05$) and 13 ($p < 0.01$). The protective effect of CLA is also illustrated in **Figure 6.12A** where the difference

in total percentage weight loss (initial vs. end weight) between the CLA and control-fed animals was found to be very significant ($p < 0.001$). It is important to note that all animals in the control-fed DSS group were dead by day 13 of the colitis study, which explains the rapid decline in weight from day 12 to 13 in that group. 4 of the animals in the CLA-fed group receiving DSS survived and were sacrificed on day 13. This equates to a 66.7% survival rate in animals fed a CLA-supplemented diet compared to 0% survivors in the control fed group. All animals remained healthy in the group that did not receive DSS. The percentage survival of animals in the 3 experimental groups is shown in **Figure 6.12B**.

Animals were assigned a daily score of 0-4 as a means of comparatively assessing inflammation and disease development. Scores were allocated by determining inflammation-associated bleeding (i.e. blood in the faeces) and stool consistency. **Figure 6.13** shows that clinical symptoms of colitis appeared earlier in control animals than in those fed the CLA-supplemented diet. By day 13 all animals in the control DSS-challenged group were dead and were assigned a score of 4, whereas the end score for animals in the CLA group only reached a score of 3.

The weights and lengths of colons from mice in the three experimental groups were measured at the end of the study [**Figure 6.14**]. Colon length was decreased in animals with colitis from both diet groups, but shortening was slightly less significant in the CLA group ($p < 0.01$) compared to the control fed animals ($p < 0.001$). Unexpectedly there was a significant decrease in colon weight in the colitic mice. There would normally be a decrease in colon length concurrent with an increase in colon weight following DSS challenge due to oedema and mucosal thickening. (Ogawa *et al.*, 2004). However, this was not observed here.

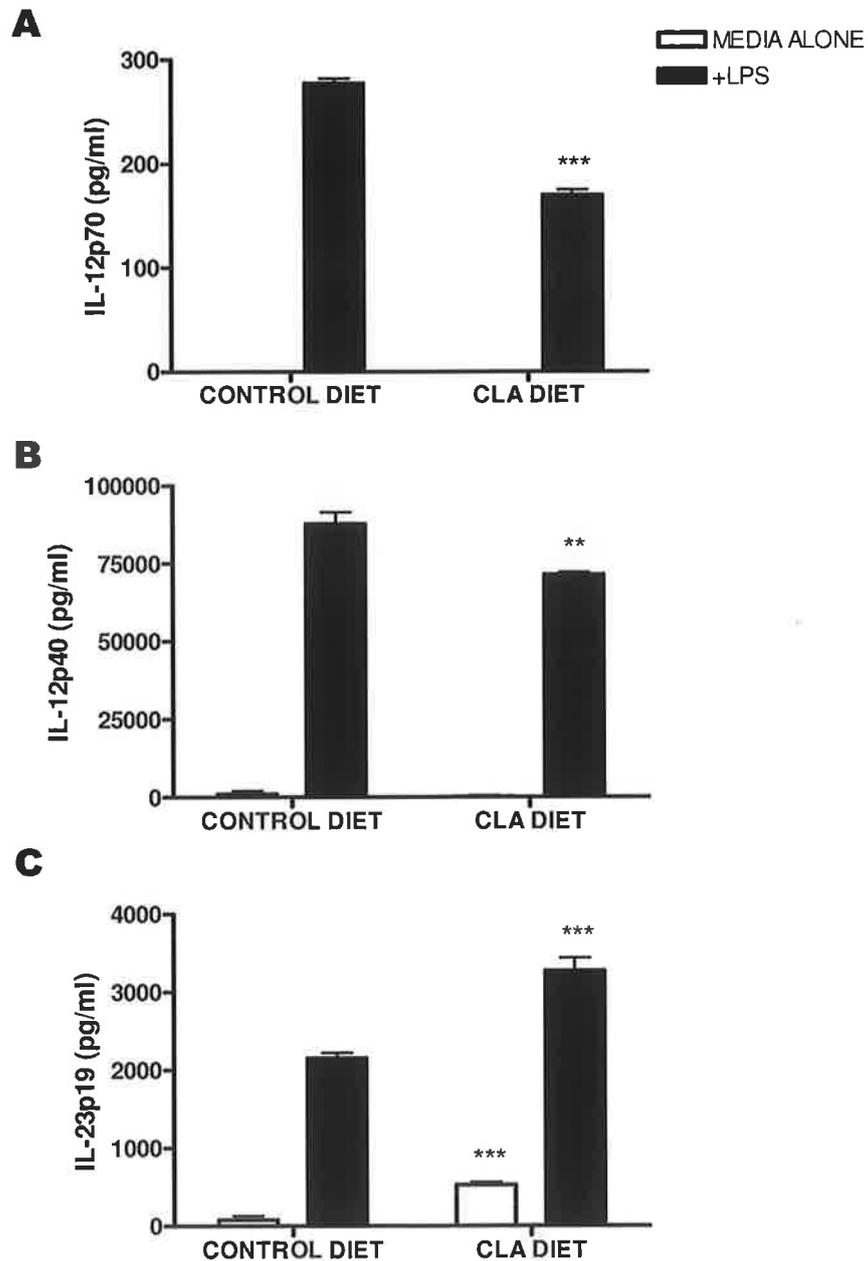


FIGURE 6.1

Feeding animals a CLA-incorporated diet alters LPS-induced cytokine release from bone marrow-derived DC.

Animals were fed either a 1% (w/w) CLA-incorporated diet or a 1% (w/w) linolenic acid-supplemented control diet for 5 weeks. DC were differentiated (*ex vivo*) from bone marrow cells with GM-CSF for 7 days, and following LPS-stimulation for 24 hours, supernatants were removed and assessed for levels of IL-12p70 [A], IL-12p40 [B], and IL-23p19 [C] using specific immunoassays.

Results are means \pm SE of triplicate assays from five separate mice.

Statistical analysis was carried out using an unpaired t-test to determine whether differences between the control and CLA diets were significant (***) $p < 0.001$, **) $p < 0.01$)

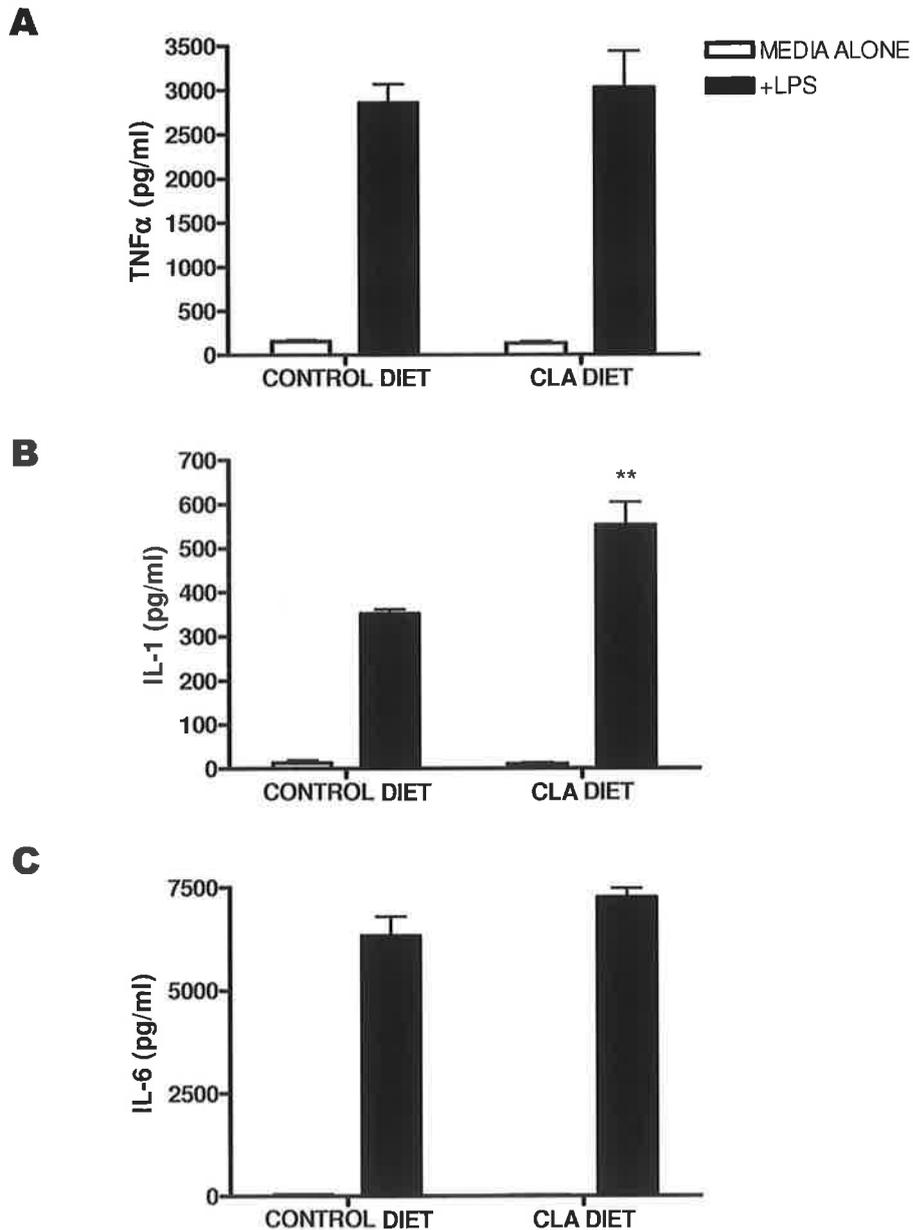


FIGURE 6.2

Feeding animals a CLA-incorporated diet alters LPS-induced IL-1 β release from bone marrow-derived DC, but has no effect on TNF- α or IL-6 production.

Animals were fed either a 1% (w/w) CLA-incorporated diet or a 1% (w/w) linoleic acid-supplemented control diet for 5 weeks. DC were differentiated (*ex vivo*) from bone marrow cells with GMSCF for 7 days, and following LPS-stimulation for 24 hours, supernatants were removed and assessed for levels of TNF- α [A], IL-1 β [B] and IL-6 [C] using specific immunoassays.

Results are means \pm SE of triplicate assays from five separate mice.

Statistical analysis was carried out using an unpaired t-test to determine whether differences between the control and CLA diets were significant (** $p < 0.01$)

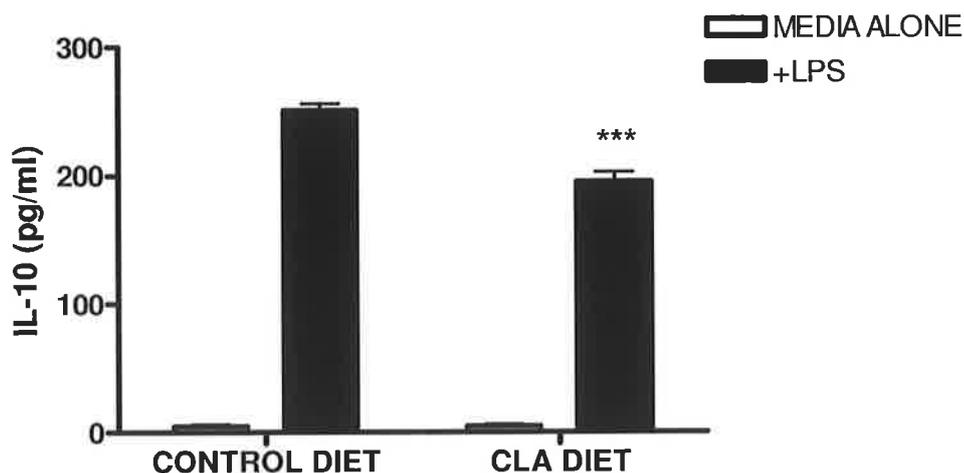


FIGURE 6.3

Feeding animals a CLA-incorporated diet suppresses the LPS-induced release of IL-10 from bone marrow-derived DC.

Animals were fed either a 1% (w/w) CLA-incorporated diet or a 1% (w/w) linoleic acid-supplemented control diet for 5 weeks. DC were differentiated (*ex vivo*) from bone marrow cells with GM-CSF for 7 days, and following LPS-stimulation for 24 hours, supernatants were removed and assessed for levels of IL-10 using specific immunoassays.

Results are means \pm SE of triplicate assays from five separate mice.

Statistical analysis was carried out using an unpaired t-test to determine whether differences between the control and CLA diets were significant (***) $p < 0.001$

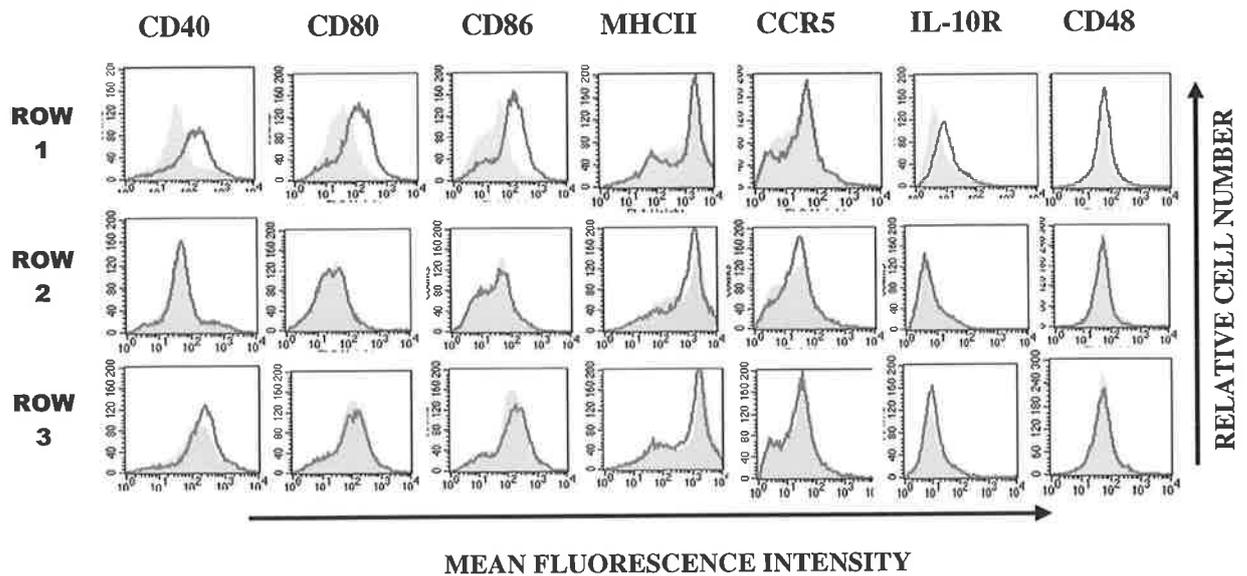


FIGURE 6.4

Feeding animals a CLA-incorporated diet does not affect the expression of surface markers on bone marrow-derived DC.

Animals were fed a control diet or CLA-incorporated diet for 5 weeks before DC were differentiated in the presence of GM-CSF for 7 days, then cultured for a further 24 hours +/- LPS (100ng/ml). After 24 hours cells were washed and stained with antibodies specific for CD40, CD80, CD86, MHCII, CCR5, IL-10R and CD48, or with isotype-matched controls. Samples were gated on CD11c⁺ cells to ensure a pure population of DC were examined and results of immunofluorescence analysis are shown. Profiles are shown for a single experiment and are representative of 2 experiments with 5 animals per diet group

In **ROW 1**, unstimulated DC from animals fed the control diet (filled histogram) are overlaid with LPS-activated DC from the same animals to show normal upregulation of maturation markers (black line).

ROW 2 compares unstimulated cells from the two diet groups – control-fed DC are represented by the filled histogram, with DC from the CLA-fed animals overlaid with a black line.

ROW 3 shows LPS-stimulated cells from the control-fed group (filled histogram) and the CLA-fed group (black line)

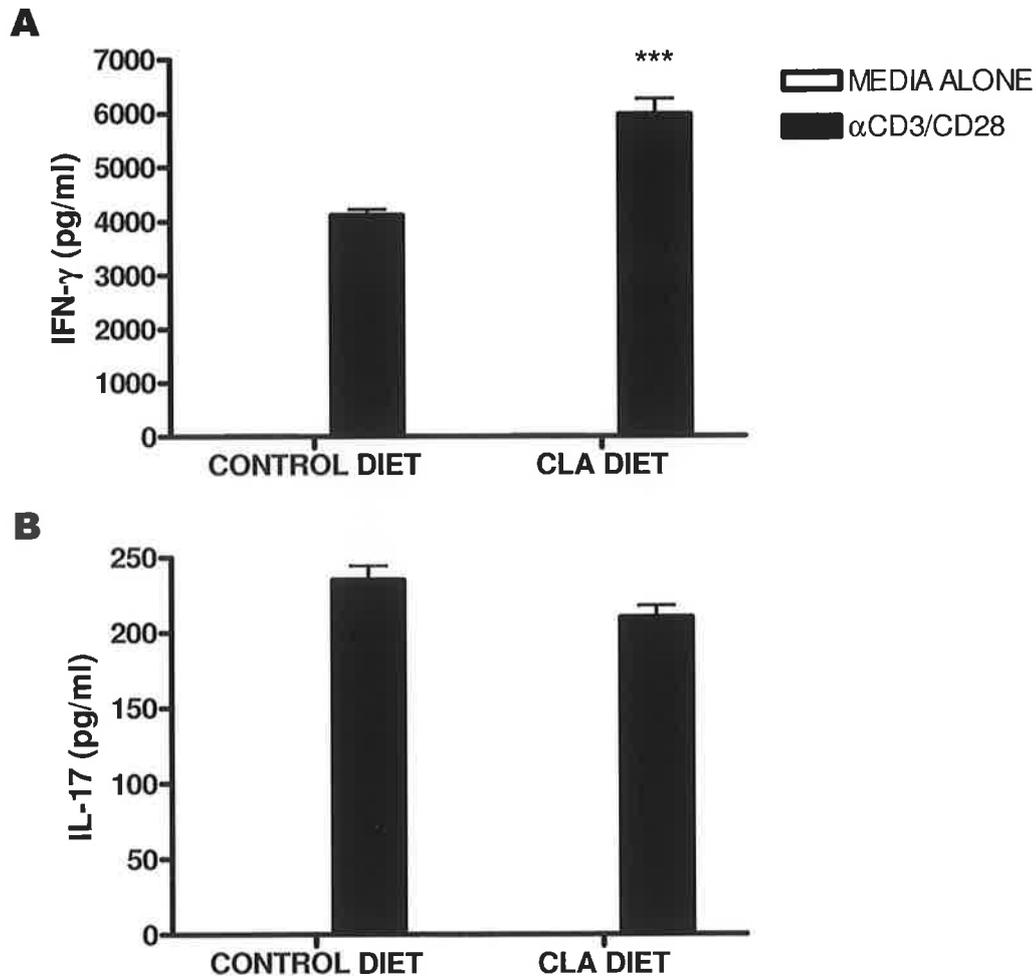


FIGURE 6.5

Feeding animals a CLA-incorporated diet alters ex vivo cytokine release from splenic CD4⁺ T cells stimulated with α CD3/CD28

Animals were fed either a 1% (w/w) CLA-incorporated diet or a 1% (w/w) linoleic acid-supplemented control diet for 5 weeks. CD4⁺ T cells were isolated from the spleens of fed animals and activated *ex vivo* with plate bound α CD3 (5 μ g/ml), soluble CD28 (10 μ g/ml), and recombinant IL-2 (10U/ml) for 3 days before supernatant was removed and assessed for levels of IFN- γ [A], and IL-17 [B] using specific immunoassays.

Results are means \pm SE of triplicate assays from five separate mice.

Statistical analysis was carried out using an unpaired t-test to determine whether differences between the control and CLA diets were significant (***)p<0.001)

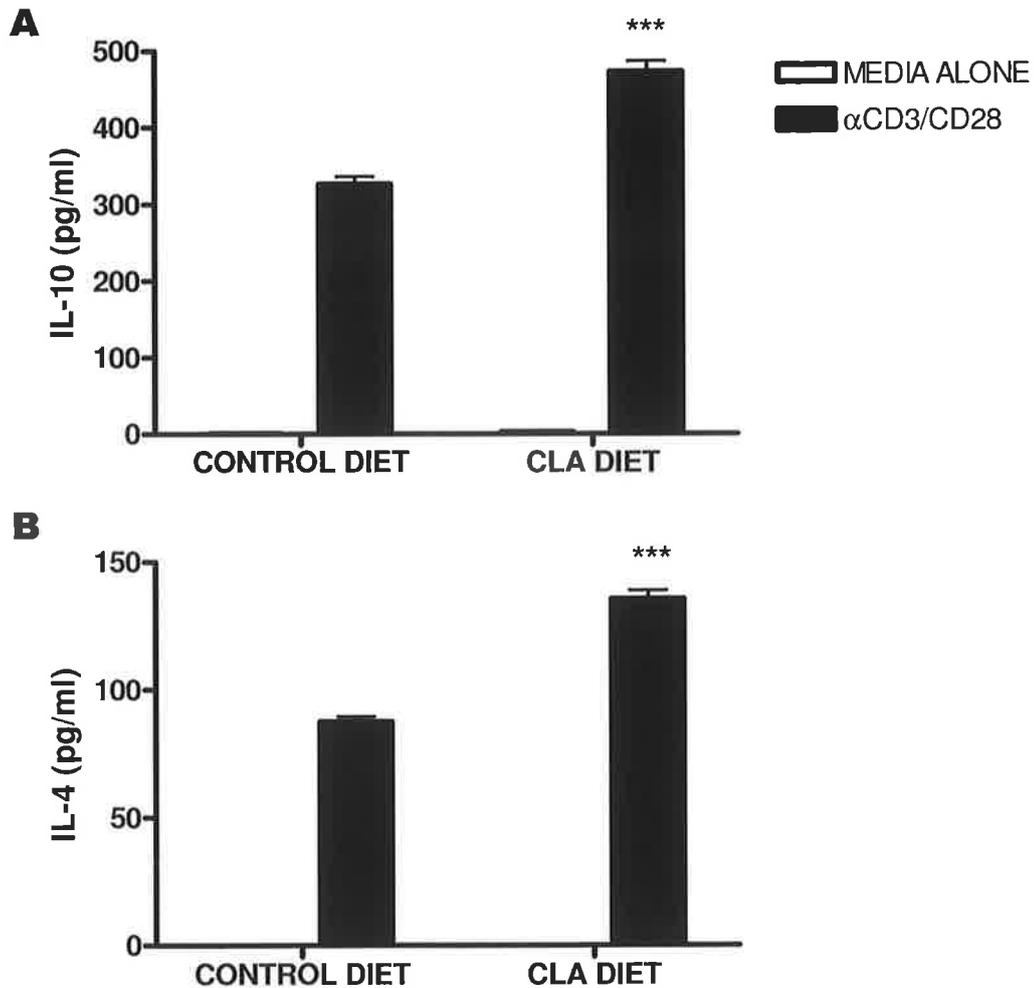


FIGURE 6.6

Feeding animals a CLA-incorporated diet alters ex vivo cytokine release from splenic CD4⁺ T cells stimulated with α CD3/CD28

Animals were fed either a 1% (w/w) CLA-incorporated diet or a 1% (w/w) linoleic acid-supplemented control diet for 5 weeks. CD4⁺ T cells were isolated from the spleens of fed animals and activated *ex vivo* with plate bound α CD3 (5 μ g/ml), soluble CD28 (10 μ g/ml), and recombinant IL-2 (10U/ml) for 3 days before supernatant was removed and assessed for levels of IL-10 [A], and IL-4 [B] using specific immunoassays.

Results are means \pm SE of triplicate assays from five separate mice.

Statistical analysis was carried out using an unpaired t-test to determine whether differences between the control and CLA diets were significant (***) ($p < 0.001$)

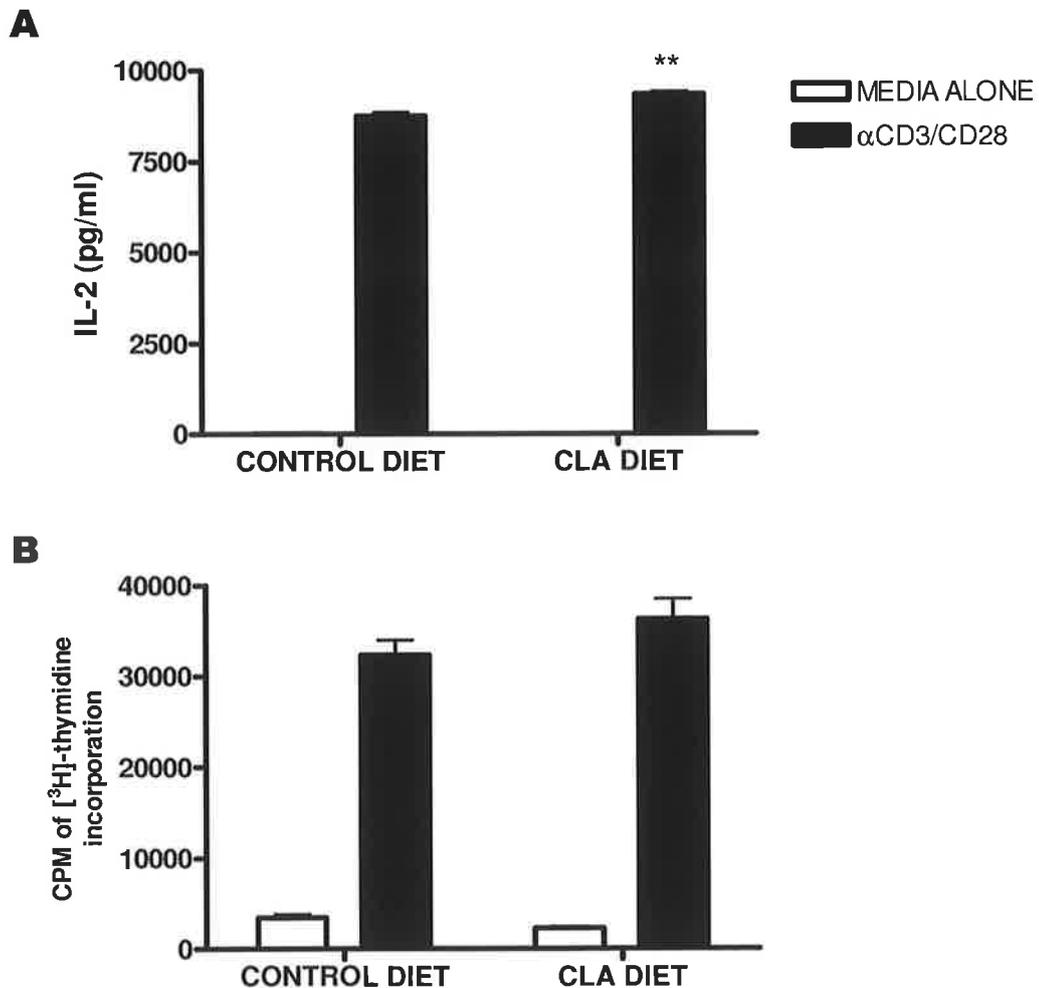


FIGURE 6.7

Feeding animals a CLA-incorporated diet increases ex vivo IL-2 production from splenic CD4⁺ T cells but does not significantly affect proliferation

Animals were fed either a 1% (w/w) CLA-incorporated diet or a 1% (w/w) linoleic acid-supplemented control diet for 5 weeks. CD4⁺ T cells were isolated from the spleens of fed animals and activated *ex vivo* with plate bound α CD3 (5 μ g/ml), soluble CD28 (10 μ g/ml), and recombinant IL-2 (10U/ml) for 3 days before supernatant was removed and assessed for levels of IL-2 using specific immunoassays.

Following supernatant removal 1 μ Ci/well of [³H]-thymidine was added to each well in 200 μ l of media. Cells were cultured for an additional 4 hours, after which cells were harvested and proliferation assessed by [³H]-thymidine incorporation on a scintillation counter. Results are expressed as mean counts per minute (cpm) of [³H]-thymidine incorporation for triplicate cultures of lymphocytes. Results in both graphs are means \pm SE of triplicate assays from five separate mice.

Statistical analysis was carried out using an unpaired t-test to determine whether differences between the control and CLA diets were significant (**p<0.01)

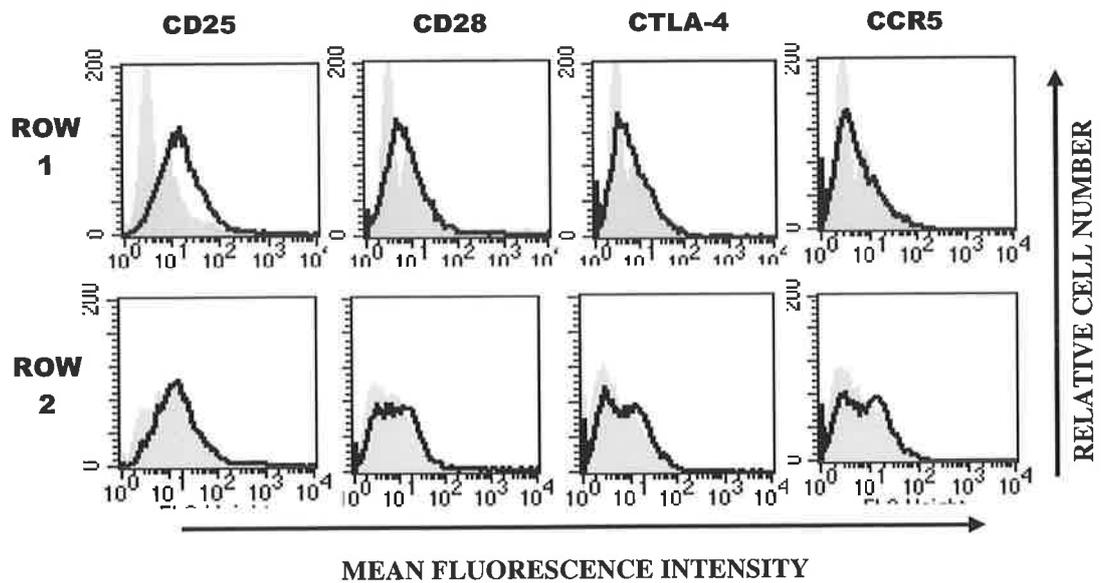


FIGURE 6.8

Feeding animals a CLA-incorporated diet does not affect the expression of surface markers on splenic CD4⁺ T cells.

Animals were fed the control or CLA-supplemented diet for 5 weeks before spleens were removed and processed as previously described. Purified CD4⁺ T cells were activated *ex vivo* with α CD3/CD28 for 72 hours before cells were stained with antibodies specific for CD3, CD4, CD28, CTLA-4 and CCR5. Immunofluorescence analysis is shown for cells gated on a CD3⁺CD4⁺ population.

ROW 1 depicts unstimulated (filled histogram) vs. stimulated cells (black line) from the control fed animals, to show upregulation of surface markers following cell activation.

ROW 2 represents stimulated T cells from animals fed the control diet (filled histogram) and the CLA-diet (black line)

Profiles are shown for a single experiment and are representative of 2 experiments with 5 animals per diet group

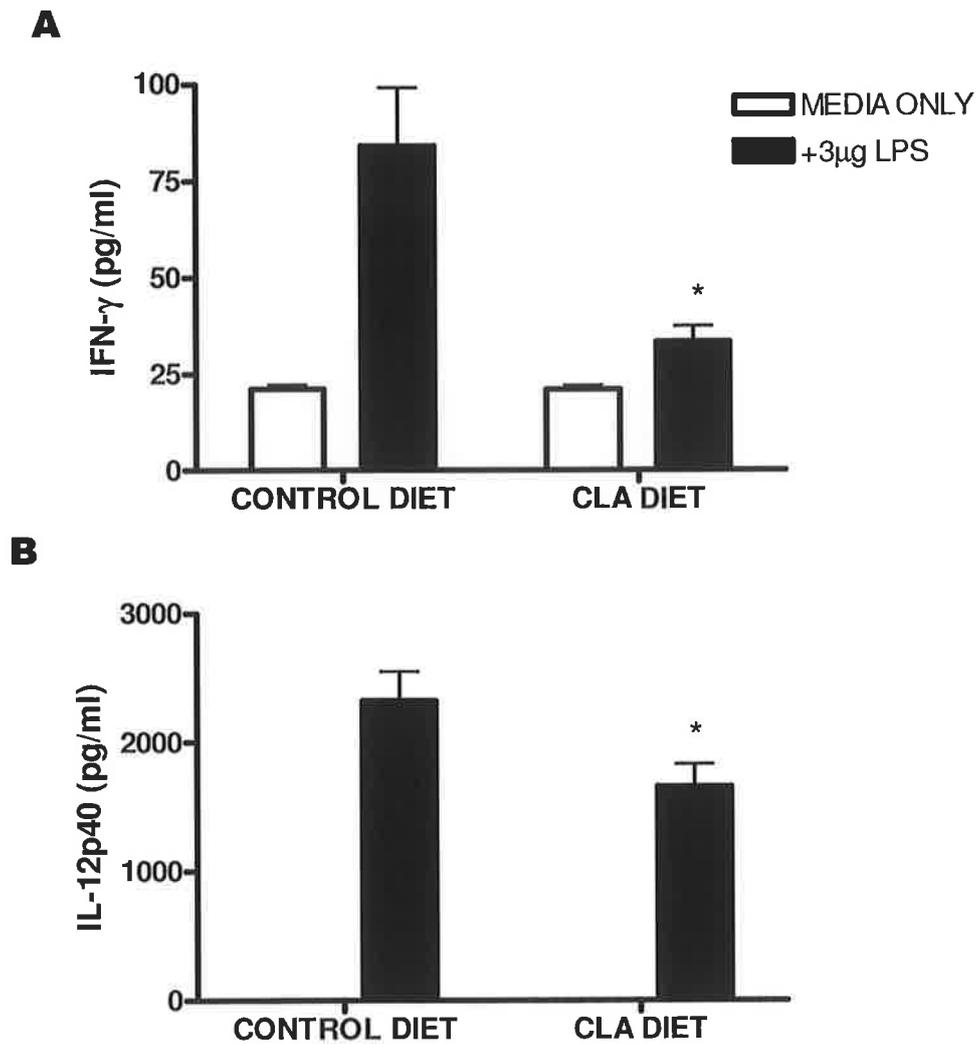


FIGURE 6.9

Feeding animals a CLA-rich diet decreases levels of circulating inflammatory cytokines following intravenous endotoxin challenge

Animals were fed either the control or CLA-supplemented diet for 5 weeks. Half the animals from each group (5 mice) were then injected intravenously with 3µg LPS. Blood was collected 6 hours later and assessed for levels of IFN-γ [A] and IL-12p40 [B].

Results are means ± SE of duplicate assays from five separate mice.

Statistical analysis was carried out using a Mann Whitney U-test to determine whether differences between the control and CLA diets were significant (*p<0.05)

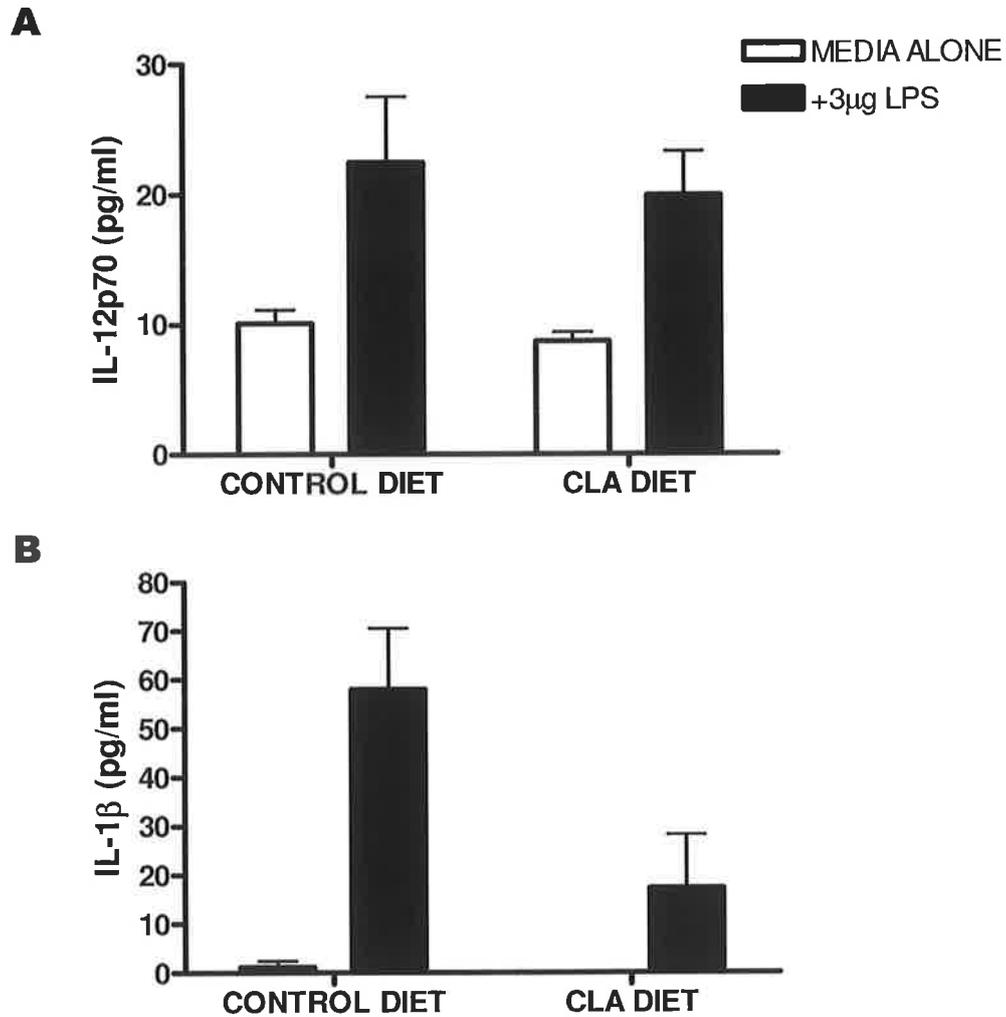


FIGURE 6.10

Feeding animals a CLA-rich diet decreases levels of circulating inflammatory cytokines following intravenous endotoxin challenge

Animals were fed either the control or CLA-supplemented diet for 5 weeks. Half the animals from each group (5 mice) were then injected intravenously with 3µg LPS. Blood was collected 6 hours later and assessed for circulating levels of IL-12p70 [A] and IL-1β [B].

Results are means ± SE of duplicate assays from five separate mice.

Statistical analysis was carried out using a Mann Whitney U-test to determine whether differences between the control and CLA diets were significant

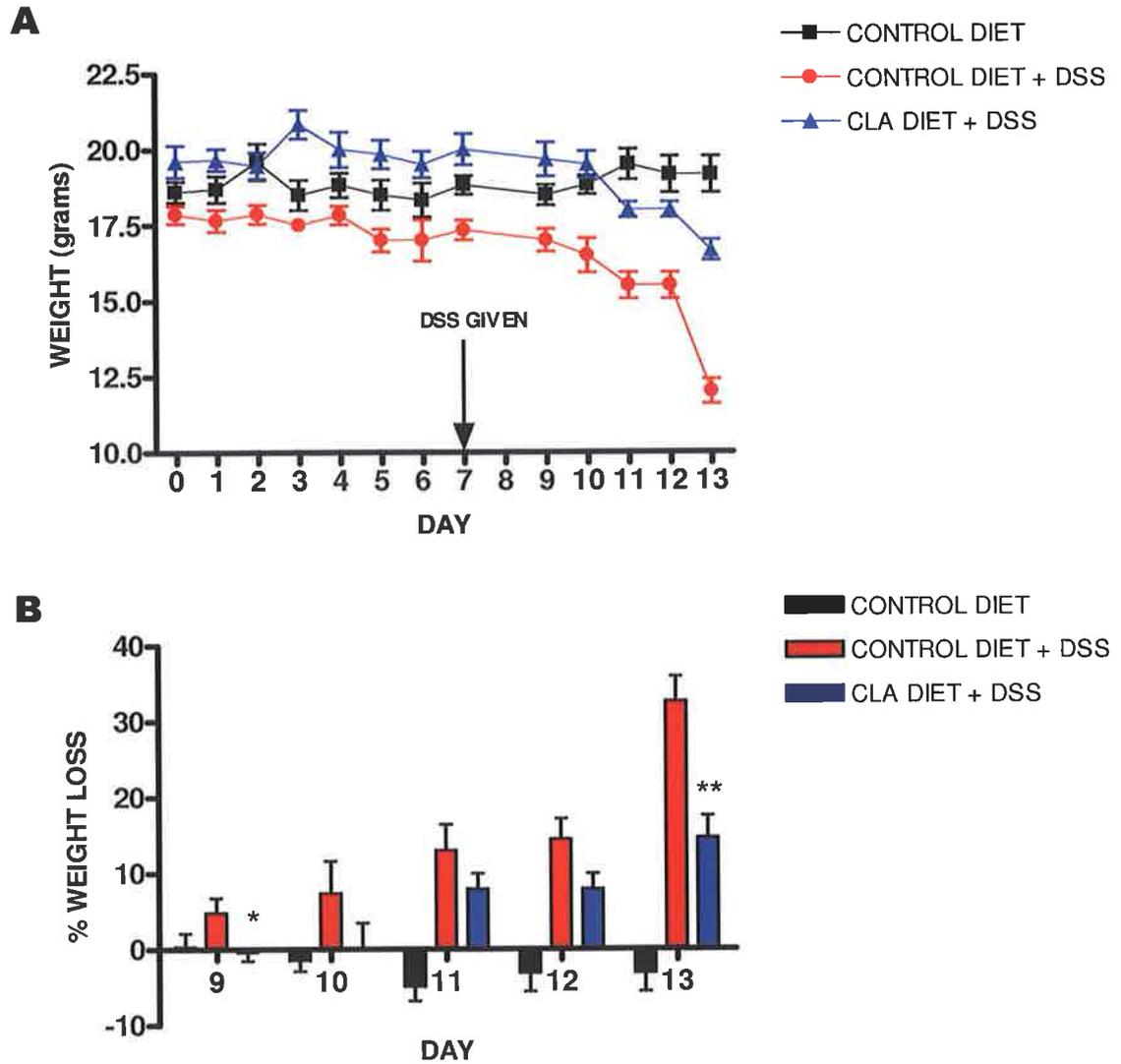


FIGURE 6.11

The weight loss associated with disease progression during DSS-induced colitis is prevented by feeding animals a CLA-supplemented diet

Animals were fed either a control diet (linoleic acid) or CLA-incorporated diet for 5 weeks prior to the addition of DSS (3% w/v) in their drinking water. After 5 days of DSS, normal drinking water was replaced. Animals were weighed daily and checked for outward signs of colitis. The control group were fed the control diet and provided with normal drinking water for the duration of the experiment.

Weights are shown \pm SE for animals in each of the 3 treatment groups [A]. The percentage weight loss, comparing initial weight with weight on days 9-13 of the experiment was calculated for surviving animals in each of the treatment groups [B]. Statistical analysis was carried out using an unpaired t-test to determine whether differences in weight loss between the control and CLA diets were significant in the DSS animals (** $p < 0.01$, * $p < 0.05$).

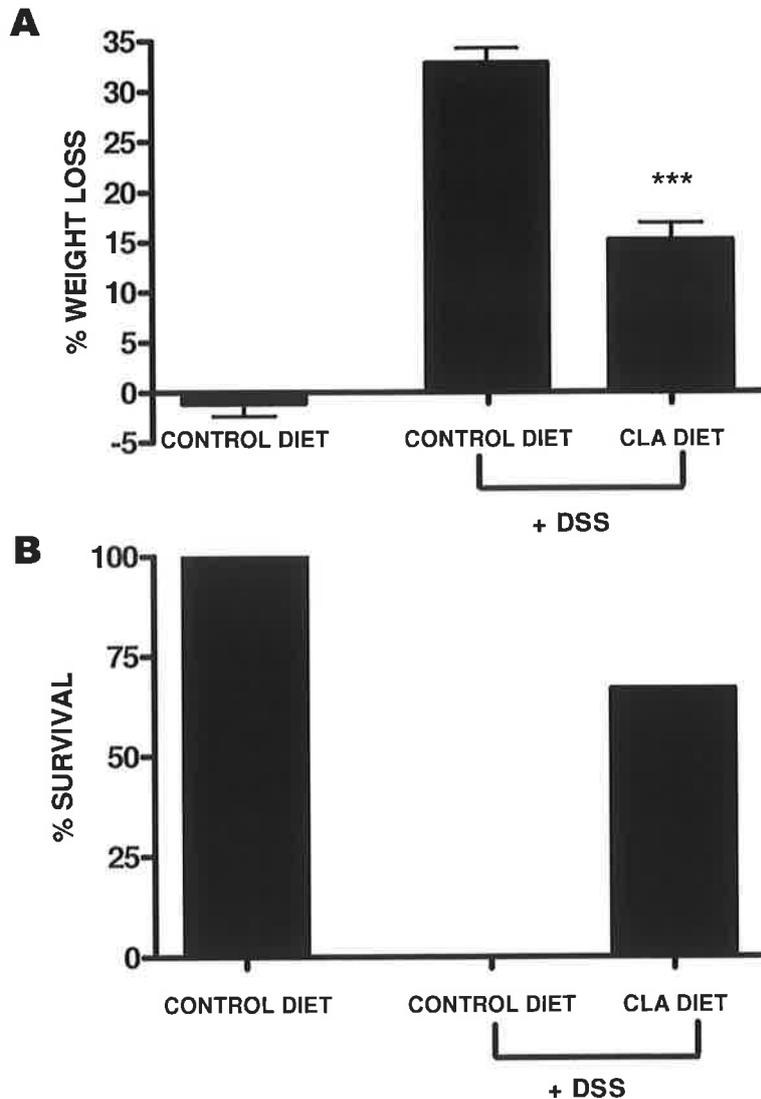


FIGURE 6.12

Feeding animals a CLA-supplemented diet inhibits the weight loss and mortality associated with DSS-induced colitis

Animals were fed either a control diet (linoleic acid) or CLA-incorporated diet for 5 weeks prior to the addition of DSS (3% w/v) in their drinking water. After 5 days of DSS, normal drinking water was replaced. Animals were weighed daily and checked for outward signs of colitis. The control group were fed the control diet and provided with normal drinking water for the duration of the experiment.

The percentage weight loss, comparing initial weight with weight at the termination of the experiment on day 13 was calculated for surviving animals in each of the treatment groups [A]. Statistical analysis was carried out using an unpaired t-test to determine whether differences in weight loss between the control and CLA diets were significant in the DSS animals (***) $p < 0.001$. A one-way ANOVA test showed that both diet groups challenged with DSS lost a significant amount of weight compared to the non-DSS group ($p < 0.001$)

The percentage of animals surviving in each treatment group until day 13 of the experiment was also calculated and shown as % survival in graph [B].

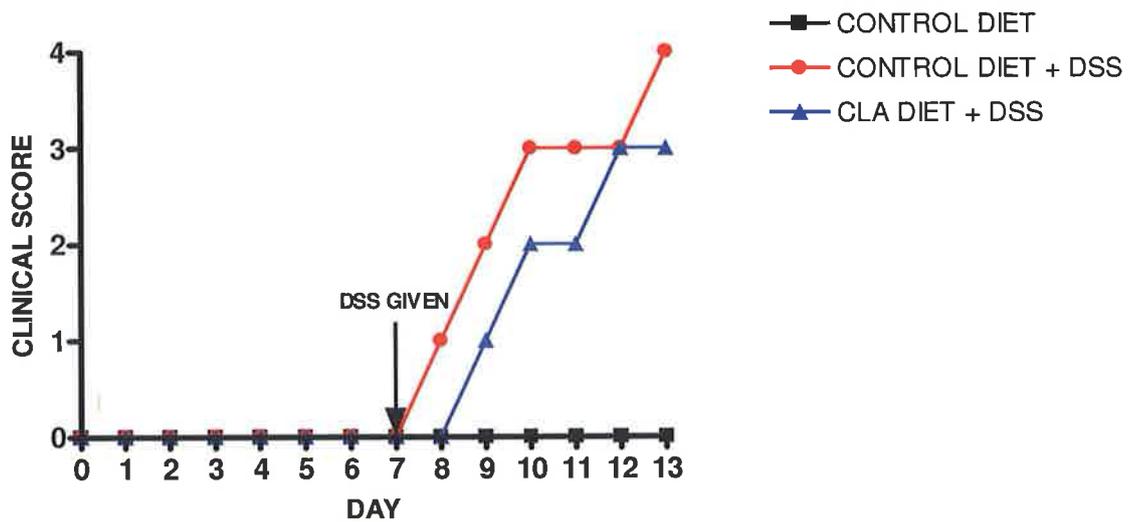


FIGURE 6.13

Feeding animals a CLA-supplemented diet delays the appearance of clinical symptoms associated with disease development in DSS-induced colitis

Mice were fed either a control (linoleic acid) or CLA-incorporated diet for 5 weeks prior to the addition of DSS (3% w/v) in their drinking water. After 5 days of DSS, normal drinking water was replaced. A third group were fed the control diet and did not receive DSS.

Animals were weighed daily and checked for outward signs of colitis using the scoring system detailed in the method (*section 2.9.2*) and shown in **Table 2.5** below. Inflammation-associated bleeding was assessed by examination of blood in the stool and stool consistency judged on a daily basis to assign a score of 0-4 for the treatment groups.

TABLE 2.5 *Scoring system for the comparative analysis of intestinal bleeding*

SCORE	% WEIGHT LOSS	STOOL CONSISTENCY
0	0	Solid, no blood
1	1-5	Semi-solid, blood tinged
2	5-10	Semi-solid – Fluid, blood present
3	10-20	Blood Fluid
4	>20	Mouse sacrificed

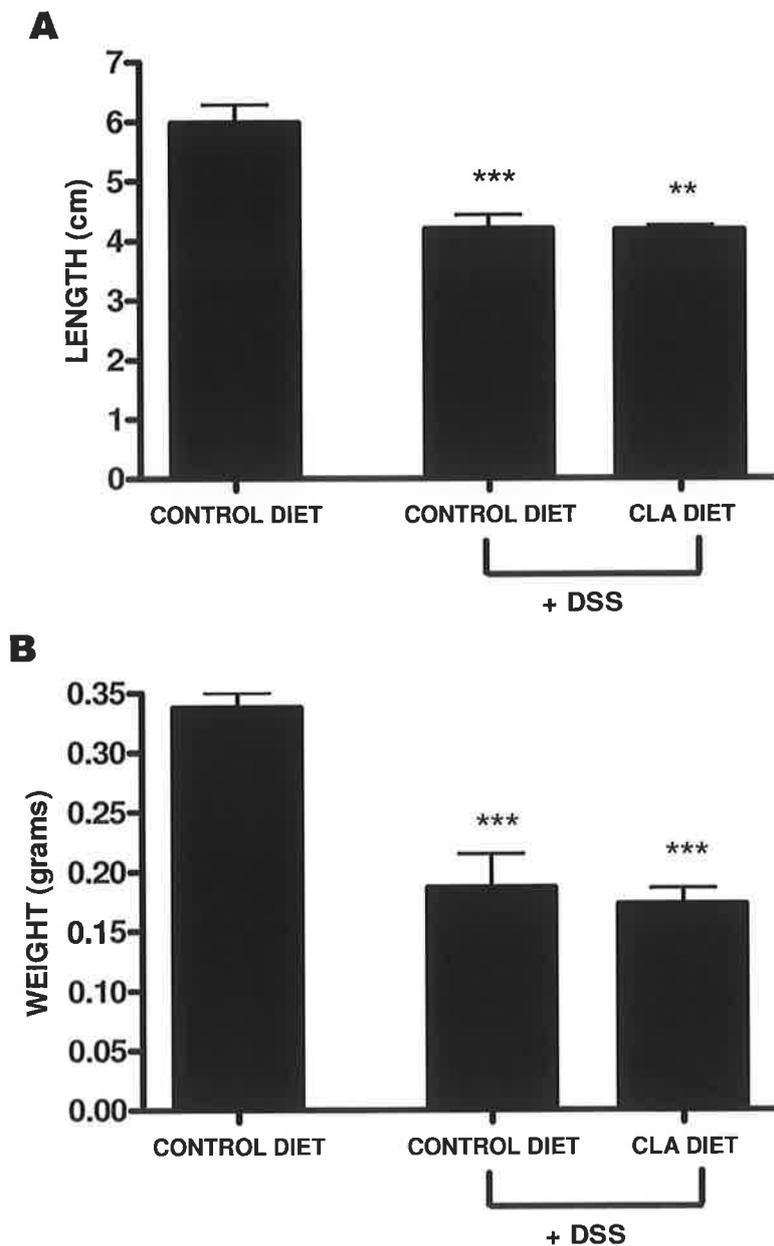


FIGURE 6.14

A CLA-enriched diet does not affect the colon length or weight of colitic mice
 C57BL/6 mice were fed a control or CLA-supplemented diet prior to the addition of 3% w/v DSS in their drinking water for 5 days to induce colitis. Normal drinking water was replaced after this period and animals weighed on a daily basis and assessed for signs of colitis. Once the experiment was terminated on day 7-post DSS challenge, colons were removed, weighed (wet weight) [A] and the length [B] measured to assess any differences between diet groups. Results represent means \pm SE of 6 mice (control diet), 3 mice (control/DSS group), and 4 mice (CLA/DSS group). Statistical analysis was carried out using a one-way ANOVA test to ascertain whether differences in data was significant *** $p < 0.001$, ** $p < 0.01$ for control vs. DSS challenged animals

6.3 DISCUSSION

In previous chapters we focused on the immunomodulatory actions of PUFA *in vitro* and generated some very interesting and novel results. In this final chapter, as proof of principle, we employed a CLA-incorporated diet to assess whether the anti-inflammatory actions of CLA could be mirrored *in vivo*. In the endotoxin-shock assay, two of the systemic inflammatory and pathogenic markers known to be upregulated by injecting LPS were inhibited by dietary CLA: Animals fed the CLA-incorporated diet had significantly less circulating levels of IL-12p40 and IFN- γ compared to mice fed the control (linoleic acid) diet. Levels of IL-12p70 and IL-1 β were also reduced but differences were not found to be statistically significant.

An animal model of IBD was used as the second inflammatory situation to address the potential *in vivo* immunomodulatory actions of CLA. Animals fed the CLA-incorporated diet were significantly protected from DSS-induced pathology (assessed by weight loss and inflammation scores) and mortality compared to those fed the control diet. Results from both the LPS-shock and IBD models provide evidence that the anti-inflammatory effects of CLA we recorded *in vitro* are reflected in two read-outs of inflammation *in vivo*.

To establish whether the PUFA-induced changes in cytokine production and surface marker expression we saw *in vitro* were paralleled *in vivo*, we examined the same parameters in bone marrow-derived DC and splenic CD4⁺ T cells isolated from fed animals and activated them *ex vivo* (with either LPS (DC) or α CD3/ α CD28 (CD4⁺ T cells)). The cytokine production profiles of LPS-stimulated DC isolated from CLA-fed animals corroborate the majority of the results we recorded *in vitro*; i.e. suppression of IL-12p40 and IL-12p70, increased secretion of IL-1 β and IL-23p19, and no change in IL-6 or TNF- α . However, there was a much-unanticipated decrease in IL-10 production from these cells. Results of cytokine production from fed T cells were also surprising. We recorded an increase in IFN- γ production and IL-2. However we found a lower production of IL-17 and raised levels of IL-10 and IL-4 in T cells from CLA-fed animals. This still presents an anti-inflammatory profile owing to the increased presence of anti-inflammatory/regulatory cytokines rather than a suppression of pro-inflammatory ones as we saw *in vitro*. A study by Yang and

Cook (2003) found that CLA decreased TNF- α production from RAW macrophage *in vitro*. They then conducted a dietary study where animals were fed a CLA-supplemented diet for 6 weeks. The PUFA incorporated diet had no effect on TNF- α production *in vivo* and splenocytes isolated from these animals actually produced higher amounts of IL-2 compared to control-fed animals which corroborate our findings, and show significant differences between results obtained *in vitro* and *in vivo*.

One explanation for our anomalous results could be the fact that T cells isolated from fed animals were activated with α CD3 and α CD28 (receptor-mediated activation) rather than it being antigen-specific activation as in the co-culture experiment. We showed in chapter 4 that PUFA down-regulate key co-stimulatory molecules on DC which may be a major contributing factor associated with the ability of PUFA to alter adaptive immune responses. This was signified in the co-culture experiments where DC were irradiated and rendered less able to secrete protein, but still significantly down-regulated cytokine production from T cells, thus implicating the observed changes in DC surface marker expression as the driving force behind the recorded alterations in T cell cytokine production. Here, the abundance of CD28 ligation when T cells were stimulated *ex vivo* would not mimic a true *in vivo* environment in a dietary intervention study because, if our *in vitro* data is correct, PUFA induce a down-regulation of CD80 and CD86, both of which ligate CD28 and are involved in T cell activation (Manzotti *et al.*, 2006). A lack of CD80 and CD86 would decrease T cell activation as a lack of co-stimulation has been shown to induce T cell anergy (Bacchetta *et al.*, 2005). There was no significant difference in the costimulatory marker expression of DC isolated from fed animals. It is difficult to evaluate data from fed DC, as these bone marrow-derived cells were not directly exposed to PUFA, as the cells were *in vitro* and in the co-culture experiments. In a feeding study, the cells directly exposed to dietary PUFA would include those in the gastrointestinal tract. Therefore, examining alterations in lamina propria dendritic cells isolated from fed animals would provide a truer picture of the effects of a PUFA-incorporated diet.

Given that the dendritic cells used in the co-culture model were irradiated and we thought that their actions on T cell responses were attributable to altered co-stimulatory marker expression, we examined the corresponding co-stimulatory

and/or inhibitory molecules on fed T cells (CD28, CTLA-4, CCR5 and CD25). Engagement of CD28 with B7 ligands delivers a positive signal, resulting in T cell proliferation and cytokine production. Conversely, CTLA-4 (CD152), a homologue of CD28, is upregulated on the surface of activated T cells and competes with CD28 to bind CD80 and CD86. Binding attenuates T cell responses by down-regulating T cell activation, facilitating apoptosis and suppressing cytokine production (Collins *et al.*, 2005; Manzotti *et al.*, 2006). Furthermore, blockade of CTLA-4 signalling using neutralising antibodies was found to promote the expansion of antigen-specific T cells and to enhance IL-2 and IFN- γ (Liu *et al.*, 2001b). CCR5 is a chemokine receptor preferentially expressed on Th1 cells and functions to direct cells to sites of inflammation (Oppermann, 2004). Finally, CD25 is used as a T cell activation marker (Rutella & Lemoli, 2004). We did not find any significant differences in the cell surface markers analysed on CD4⁺ T cells isolated from the spleens of animals fed the CLA-diet compared to those on the control diet. There was no change in CD25 expression and therefore T cell activation. Cytokine analysis revealed no change in IL-2 production, and there was a slight increase in cell proliferation (assayed by thymidine incorporation), all of which suggest that CLA does not impede T cell activation or proliferation but rather alters their phenotype, probably through changes in APC antigen presentation. Again, perhaps a truer picture of the immunomodulatory effects of a PUFA-rich diet on T cells would be gained by isolating T cells from the mesenteric lymph nodes of fed animals. A number of dietary intervention studies have reported PUFA-induced alterations in T cells: EPA and DHA upregulated early CTLA-4 protein expression in murine CD4⁺ T cells (Ly *et al.*, 2006). And EPA was shown to suppress CD25 expression 24 hours after stimulation with PMA in human blood peripheral mononuclear cells (PBMC) (Terada *et al.*, 2001) and human Jurkat T cells (Zeyda *et al.*, 2003). Another study by Zhang and colleagues (2006a) found that cells isolated from the spleen and lymph nodes of animals fed a fish oil diet for 2 weeks did not differ in their surface expression of CD25. There was however, a marked difference in cell numbers between diet groups and it was hypothesised that fish oil suppressed the clonal expansion of differentiated T cells. This relates to most of the research in to the *ex vivo* or *in vitro* effects of PUFA on T cells which has been more concerned with cell proliferation rather than cytokine production. For

example, EPA suppressed human T cell proliferation (Terada *et al.*, 2001) and in a study by Tricon and co-workers (2004), CLA was found to decrease mitogen-induced cell activation in a dose-dependent manner in human T lymphocytes measured by the expression of CD69, a T cell activation marker. Results here contradict our findings, where we saw no obvious changes in T cell proliferation, IL-2 production or cellular activation. It is difficult to draw comparisons between our co-culture data and *ex vivo* stimulated cells from fed animals, just as it is difficult to directly compare our data with published dietary studies where cells are activated differently, because different modes of activation have been shown to alter the outcome of a response. For example, Ly and associates (2006) reported that T cells from mice fed an n-3 PUFA diet produced less IL-2 but only when activated by α CD3/CD28 and not with α CD3/PMA. Activating T cells with recombinant CD3/CD28, which is considered receptor-mediated, is still a crude method of stimulating T cells compared to a true *in vivo* situation. The effects we have shown PUFA to have on DC, and then the subsequent effects these PUFA-modulated DC have on T cell responses, suggest that fatty acids may alter the ability of APC to present antigen and co-stimulate T cells rather than have a direct effect on the T cells themselves.

DC from fed animals produced less IL-12 but increased amounts of IL-23, and these two cytokines have been shown to cross-regulate each other (Becker *et al.*, 2006). Furthermore, T cells from fed animals produced increased amounts of IFN- γ but reduced IL-17, and IFN- γ is known to inhibit IL-17 production (Kikly *et al.*, 2006). Indeed, disease exacerbation in the absence of IFN- γ is often associated with an uncontrolled Th17 response (Lohr *et al.*, 2006; Irmeler *et al.*, 2007).

Alterations in IL-17 production following CLA treatment are almost certainly not due to changes in the cytokines that drive Th17 cell differentiation. There was an increase in IL-23p19, IL-1, and IL-6 production from fed DC, all of which are involved in directing a Th17 response (Harrington *et al.*, 2006; Sutton *et al.*, 2006; McGeachy *et al.*, 2007), but IL-17 production was suppressed in T cells from fed animals, and IL-17 was also decreased in the co-culture and adoptive transfer assays. In this way, perhaps CLA inhibits IFN- γ by suppressing IL-12 production from DC, whilst controlling IL-17 production from T cells but having no inhibitory effect on the cytokines that direct Th17 differentiation (i.e. IL-1,

IL-6 and IL-23). These disparate actions on different cell types ties in with previous findings in our lab, where we demonstrated that PUFA are able to inhibit TNF- α production in J774 macrophage, but have little effect on TNF- α production in DC. Others have also found PUFA to have different effects on different cell types: Petursdottir and Hardardottir (2007) demonstrated that dietary fish oil decreased TNF- α and IL-10 production by splenic T cells but had the complete reverse effect on the production of these cytokines by splenic macrophage.

Injecting mice with endotoxin leads to a systemic inflammatory response and an excessive production of proinflammatory cytokines, including TNF- α , IL-1, IL-6, IL-12, and IFN- γ , which contribute significantly to lethality in septic shock syndromes (Belladonna *et al.*, 2006). Associated symptoms include hypothermia, fever, anorexia (i.e., decreased food intake), and cachexia (decreased body weight) (Dinarello & Cannon, 1993; Hasko & Szabo, 1999). Here we found that mice fed a CLA-incorporated diet challenged with LPS produced significantly less IFN- γ and IL-12p40 compared to controls. IFN- γ is considered the prototypical proinflammatory Th1 cytokine that has a role in both innate and adaptive immunity. One of its major roles is to activate macrophage and DC, leading to increased phagocytosis and cytokine production (Szabo *et al.*, 2003). It also induces the release of TNF- α and IL-1 from APC, and upregulates the production of pro-inflammatory chemokines augmenting the inflammatory process (Hill & Sarvetnick, 2002). IFN- γ promotes IL-12 production from APC creating a positive feedback loop between the Th1 cytokines and enhancing the Th1 response (Hasko & Szabo, 1999). IL-12 is also an important link between innate and adaptive immunity as its targets include T cells, DC, and NK cells, and one of its main functions is to direct newly activate T helper cells to a Th1 phenotype (Hasko & Szabo, 1999). In T cells IL-12 synergises with IL-2, TCR-CD3 signalling, and CD28 ligation, to rapidly induce IFN- γ production (Trinchieri, 2003). IL-12 directs the development of Th1 cells which produce IFN- γ which augments IL-12 production creating a cumulative positive feedback effect. Following CLA supplementation, we saw a decrease in peripheral IFN- γ and IL-12p40 which makes sense considering one augments the production of the other. There was also reductions in IL-12p70 and IL-1 β but results were not found to be statistically significant, whereas circulating TNF- α was below the

levels of detection. The observed decrease in IL-12 and IFN- γ seen here not only confirms our *in vitro* data, but also validates the relevance of our *in vitro* findings in an *in vivo* situation. Decreasing the levels of these damaging cytokines has been proved beneficial in previous studies; Belladonna and colleagues (2006) found that survival in *Pseudomonas aeruginosa*-challenged mice was associated with a dramatic decrease in circulating levels of TNF- α and IFN- γ . Sempowski and co-workers (1999) also used an LPS-shock model to investigate the *in vivo* role of CD7 in systems dependent on IFN- γ . CD7 is an immunoglobulin superfamily molecule involved in T and NK cell activation and cytokine production, and CD7-deficient animals have antigen-specific defects in IFN- γ production. The researchers found that 67% of CD7-deficient mice survived LPS injection, whereas 19% of control C57BL/6 mice survived LPS challenge. Again survival was associated with decreased serum IFN- γ and TNF- α . Unexpectedly, we did not find any TNF- α in the serum samples of LPS-challenged animals. This was also the case in a study conducted by Albers and associates (2002) where they used a contact sensitiser, dinitrochlorobenzene (DNCB), to elicit adaptive immune responses in BALB/c mice. They found that serum collected 6 hours after sensitisation did not contain any TNF- α . Perhaps blood samples should have been taken at earlier time-points considering cytokines such as TNF- α and IL-1 are both produced earlier. This was reported by Langenkamp and colleagues (2000) who found TNF and IL-1 in the supernatant 3-4 hours after LPS-stimulation.

There have been numerous reports as to the beneficial effects of dietary intervention with PUFA in LPS-shock models: Fritsche and colleagues (1999) fed C3H/HeN mice either lard (low PUFA), soybean oil (n-6 PUFA), or menhaden fish oil and corn oil (n-3 PUFA) diet for 4 weeks, after which mice were injected ip with *Listeria monocytogenes*. Plasma levels of IL-12p70 and IFN- γ were measured 24 hours later by ELISA, and akin to our results, both were greatly reduced in the fish oil fed animals. Furthermore they found that the n-6 diet actually increased IFN- γ mRNA in spleen cells, which also corroborate our findings from fed animals. Another study by Lai and associates (2005) found that pigs fed a CLA-supplemented diet had lower amounts of circulating IL-6 and TNF- α , and increased amounts of IL-10 following LPS challenge.

It would be interesting to see whether any of the PUFA-induced anti-inflammatory effects we recorded *in vitro* go on to provide protection against the development of DSS-induced colitis we saw in CLA-fed animals. Feeding animals a CLA-incorporated diet dramatically reduced weight loss and mortality in the IBD model compared to animals fed the control diet. In C57BL/6 mice, DSS challenge induces the production of IL-1, IL-12p40, IL-12p70, IL-17, and IFN- γ in the inflamed colon (Melgar *et al.*, 2005). Histopathology reveals a loss of crypts, reduction of goblet cells, ulcerations and a moderate infiltration of inflammatory cells to the mucosa, and oedema in the submucosa. Cellular infiltration correlates with increased colon wet weight (~50% increase has been recorded) whereas colon length decreases as the disease develops. Additionally, MLNs enlarge and the caecum decreases in size (Melgar *et al.*, 2005). We did not find any significant differences in colon length or weight between the diet groups, so we can speculate that CLA did not protect against disease development by preventing cellular infiltration.

Perhaps the consistent PUFA-induced decrease in IL-12 we have recorded is the key to the protective effects of CLA we saw in the colitis study, as neutralising IL-12 has shown promise in the treatment of IBD: Mannon and colleagues (2004) administered an anti-IL-12 monoclonal antibody to patients with active CD, and clinical improvements were associated with a suppression of IL-12, IFN- γ and TNF- α production by colonic mononuclear cells isolated from the lamina propria. Others have reported a lack of IFN- γ to confer protection against colitis: DSS-treated WT mice exhibited a robust production of IFN- γ in the gut, a remarkable loss of body weight, as well as high rate of mortality (60%). In striking contrast, IFN- γ -deficient mice did not develop DSS-induced colitis, as indicated by the maintenance of body weight and a survival rate of 100% (Ito *et al.*, 2006a). So the decrease in IFN- γ we found in the co-culture experiment and following LPS-challenge in CLA-fed animals could also be proposed as a possible reason for the reduction in the severity of colitis seen in CLA-fed mice. An alternative therapeutic mechanism to IL-12 or IFN- γ suppression could be the upregulation of IL-10 we have recorded following PUFA treatment. The protective effect of IL-10 is apparent in STAT3-deficient mice that have a deficiency in IL-10 function and develop spontaneous enterocolitis (Strober *et al.*, 2007). IL-10 knockout (KO) mice also spontaneously develop colitis. Yen

and co-workers (2006) backcrossed IL-10-KO mice with mice lacking only IL-12 (p35^{-/-}) or only IL-23 (p19^{-/-}). Similar to the IL-10-deficient animals, IL-12p35/IL-10-KO mice developed signs of colitis whereas the IL-10/p19-double-KO mice remained disease free, suggesting that IL-23, through the actions of IL-17 (Sheibanie *et al.*, 2007), and not IL-12, is required for the manifestation of chronic intestinal inflammation. Indeed the newly discovered Th17 subset of effector cells have been implicated as being responsible for many inflammatory disorders previously attributed to Th1 cells, including EAE, RA and IBD (Langrish *et al.*, 2005; Sutton *et al.*, 2006; Yen *et al.*, 2006; Hirota *et al.*, 2007). We found that IL-17 was inhibited by CLA in both the co-culture and adoptive transfer experiments which provides another possible mechanism used by PUFA to elicit their anti-inflammatory actions.

Neither the IL-12/IFN- γ nor the IL-23/IL-17 axis may be the sole driving force behind IBD, but they may have cumulative effects, particularly because levels of IL-12 and IL-23, as well as the downstream effector cytokines, IL-17 and IFN- γ , have all been found to be elevated in Crohn's disease sufferers (Nielsen *et al.*, 2003; Fuss *et al.*, 2006). And because blocking both the p40 and p19 sub-units has been shown to be more beneficial than blocking them individually (Neurath, 2007).

Data suggests multiple components of the innate and adaptive immune system contribute to aspects of the disease process in IBD, and a number of the immunomodulatory actions of PUFA we recorded both in T cells and DC could help provide some protection against disease development. Others have shown PUFA supplementation to have therapeutic efficacy both in IBD and in other inflammatory diseases. For example, animals were fed a control or fish oil diet and contact dermatitis or atopic dermatitis induced by using two methods of dinitrofluorobenzene (DNFB) application. DNFB-induced irritation was lessened in FO-fed animals, as was oedema and vasodilation. And acute and T cell-mediated responses were prevented by the administration of dietary n-3 PUFA (Sierra *et al.*, 2006). Butz and co-workers (2007) found dietary CLA reduced inflammation and delayed the onset of disease in a collagen-induced model of arthritis. And Bassaganya-Riera and associates (2004) found that feeding animals CLA ameliorated the severity of DSS-induced colitis by reducing weight loss and lessening disease activity.

The data here provides evidence that CLA has beneficial immunomodulatory effects *in vivo*. It seems reasonable to assume that some of the anti-inflammatory actions of PUFA recorded *in vitro* are of importance *in vivo* in view of the observed protective effects provided by a CLA-supplement diet both in the model of septic shock and in the DSS-induced model of colitis. To verify this hypothesis, further investigations into the alterations induced by PUFA on a cellular level, particularly in the DSS model, are warranted.

CHAPTER 7

GENERAL DISCUSSION

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The outcome of an immune response is a complicated process involving numerous cell types and mediators and dysregulation at any stage of cellular activation can have serious consequences. Under most circumstances, inflammation is a normal physiological and beneficial response that protects us from invading microorganisms and infectious agents. However, excessive inflammation can lead to extensive tissue damage, disability and even death. A number of human chronic diseases are attributed to a sustained inflammatory response, including RA, IBD, MS and atherosclerosis (Andreaskos *et al.*, 2004). The main medical treatments for these disorders are steroids and immunosuppressive agents which non-specifically reduce immunity and inflammation and can have serious side-effects (Nakamura *et al.*, 2006). Advances in our understanding of the inflammatory process have led to the development of therapies which selectively inhibit inflammatory mediators, such as cytokines and cell surface molecules. Due to their widely accepted immunomodulatory actions, extensive research is being undertaken to establish the mechanisms used by PUFA to elicit their anti-inflammatory effects and to reveal their potential as therapeutic agents for the treatment of autoimmune disorders. This study has uncovered a number of pathways PUFA may use to elicit their immunomodulatory effects:

Dendritic cells are considered the most potent antigen presenting cells possessing the unique ability to activate naïve T cells. In this study we found that DC exposed to PUFA *in vitro* produced significantly less IL-12 following LPS stimulation and we also reported lower levels of circulating IL-12 *in vivo* when animals were fed a CLA diet before endotoxin challenge. Since IL-12 has such a significant role in initiating and maintaining an inflammatory response (Watford *et al.*, 2003) its inhibition has proved beneficial in treating inflammatory disorders (Zhang *et al.*, 2007). In addition to suppressing pro-inflammatory IL-12, PUFA exposure enhanced the production of IL-10 from DC *in vitro*. IL-10 is known for its regulatory and immunosuppressive actions and administration of IL-10 to patients with Crohn's disease has been reported to reduce bowel inflammation (Mocellin *et al.*, 2004). Since IL-12 is involved in initiating Th1 cell development, and because IL-10 promotes the differentiation of T regulatory

cells, the PUFA-induced suppression of IL-12 and enhancement of IL-10 we recorded implied that fatty acids may have consequential effects on T cell responses, therefore we investigated the down-stream effects of PUFA on adaptive immunity. Ours is the first study to show that PUFA-modulated DC have subsequent effects on T cell responses, and results showed a reduction in IFN- γ , IL-17, IL-2 and IL-4 production from these T cells.

Many autoimmune and inflammatory conditions, such as MS, RA, and IBD, have been documented as predominantly Th1-mediated disorders associated with the overproduction of IFN- γ by T cells (Zhang, 2007). We recorded a CLA-induced reduction in IFN- γ production from CD4⁺ T cells in the co-culture model and mice fed a CLA-incorporated diet had reduced levels of circulating IFN- γ in the LPS-shock model. Our findings are corroborated by dietary studies where laboratory animals fed PUFA-enriched diets produced less IL-1, IL-2, IL-6 and IFN- γ (Stulnig, 2003). Given the widely accepted pro-inflammatory role of IFN- γ , suppression of this cytokine may aid the resolution of inflammation. Indeed clinical trials involving fontolizumab, a monoclonal antibody directed against human IFN- γ , has shown some efficacy in treating patients with CD (Nakamura *et al.*, 2006).

Another pathogenic subset of T cells, namely Th17 cells, have also been detected in a number of inflammatory disorders, including EAE, RA and IBD, and their presence has been linked to some of the pathology previously attributed to a disproportionate Th1 response (Kikly *et al.*, 2006; Sutton *et al.*, 2006; Zhang *et al.*, 2006b). This places Th17 and their IL-17 products as potential therapeutic targets. The effect of PUFA on IL-17 production has not been previously investigated and we found that IL-17 was significantly reduced by CLA in the co-culture model and in the adoptive transfer experiment. The CLA-induced decrease in IFN- γ production was a likely result considering the consistent decrease in IL-12 we observed following PUFA treatment. However, the fact that IL-17 was also significantly reduced was surprising because the production of cytokines involved in promoting a Th17 response (i.e. IL-1, IL-6, IL-23 and TNF- α) remained relatively unchanged by CLA. This provides evidence that the altered co-stimulatory markers observed on the PUFA-treated DC *in vitro* have huge consequences on the phenotype of the developing T cells. Numerous studies have evaluated the benefit of neutralising IL-17 to alleviate inflammation.

For example, IL-17 is frequently detected in the inflamed colon of trinitrobenzenesulfonic acid (TNBS)-induced colitic mice. Zhang and colleagues (2006b) found that IL-17R knockout mice were significantly protected against TNBS-induced weight loss, IL-6 production and colonic inflammation, and this protection occurred even though there were comparable levels IL-23 and higher levels of IL-12p70 and IFN- γ in IL-17R knockout mice compared with wild-type animals.

Individually, neither IL-12/IFN- γ (Th1) nor IL-23/IL-17 (Th17) may be the sole driving force behind IBD (or other inflammatory diseases), they probably have cumulative effects, particularly because increased levels of both IL-12 and IL-23 and their down-stream effectors have been found in Crohn's disease sufferers (Nielsen *et al.*, 2003; Fuss *et al.*, 2006) and because blocking both the p40 and p19 sub-units has been shown to be more beneficial than blocking them individually (Neurath, 2007). Therefore the fact that CLA was shown to inhibit both IFN- γ and IL-17 production is a favourable attribute and could be what afforded significant protection in the PUFA-fed animals in the colitis study.

Another beneficial effect of PUFA is that although we saw a reduction in T cell cytokine production, CLA did not seem to change the activation status or subsequent expansion of T cells, i.e. expression of CD25 (T cell activation marker) and thymidine incorporation (T cell division) was unaltered by feeding a CLA diet. We also found the proliferation of lymph node cells from mice in the CLA treatment group of the adoptive transfer experiment was relatively unchanged. This suggests an immunomodulatory role rather than an immunosuppressive one for PUFA. There is contrasting evidence surrounding the effects of fatty acids on cell proliferation. Many studies have reported a PUFA-induced decrease in lymphocyte proliferation (Pompos & Fritsche, 2002; Tricon *et al.*, 2004; Ly *et al.*, 2005), whereas a recent human trial detected enhanced lymphocyte proliferation in response to n-3 PUFA supplementation (Gorjao *et al.*, 2006). As discussed in chapter 6, the method of T cell activation greatly influences the outcome of a response. For example, a study by Ly and colleagues (2006) reported that T cells from mice fed an n-3 PUFA diet produced less IL-2 but only when activated by α CD3/CD28 and not when activated with α CD3/PMA. This makes comparing results from different studies fairly difficult if different modes of cellular activation were used, but it also helps to explain the

unexpected results we obtained when splenic CD4⁺ T cells were isolated from animals fed a CLA-incorporated diet and stimulated *ex vivo*. In contrast to the *in vitro* co-culture data, we did not observe a PUFA-induced suppression of IFN- γ or a significant reduction in IL-17 production following the α CD3/ α CD28 stimulation of T cells from fed animals. However there was an increase in anti-inflammatory IL-10 and IL-4. These unexpected results are perhaps due to the lack of direct PUFA exposure, or due to the crude method of cellular activation. Results from the co-culture model provide evidence that PUFA act at the level of the APC rather than influencing T cell responses directly. We can make this assumption because the DC in the co-culture model were irradiated and therefore less able to secrete cytokines. Although preliminary results showed that DC could suppress IFN- γ and IL-17 production pre- and post-irradiation, the fact that the PUFA-treated DC could still inhibit the T cell-derived pro-inflammatory cytokines when they could not secrete cytokines themselves suggests that PUFA modulate adaptive responses through alterations in antigen presentation and/or T cell co-stimulation. Therefore, activating T cells with recombinant antibodies rather than PUFA-modulated APC would not mimic a true *in vivo* situation following dietary intervention.

The hypothesis that PUFA alter APC rather than T cells is compounded by the significant reduction in MHCII expression we recorded when DC were exposed to PUFA *in vitro*. Our findings agree with previous publications; (Hughes *et al.*, 1996; Chen *et al.*, 2005) and imply that these DC would be impaired in their ability to present antigen to T cells. Indeed this was reported in a study by Sanderson and co-workers (1997) who found that DC isolated from rats fed an n-3 PUFA diet had reduced MHCII on their surface and consequently could not present antigen to KLH-sensitised responder spleen cells. MHCII is upregulated by many inflammatory mediators, particularly cytokines like IFN- γ . Lee and associates (2006) reported that PAMPS, such as LPS, which bind to TLRs, are also capable of inducing MHCII, but instead of using the master regulator, CIITA, they activate NF κ B to upregulate MHCII. It would be interesting to learn whether the decrease in MHCII expression seen in PUFA-treated DC is due to the decrease in total NF κ B and NF κ B activation we also saw in these cells.

To fully activate a naïve T cell, dendritic cells present antigen coupled to MHCII and also express co-stimulatory markers on their surface (CD40, CD80/CD86)

which ligate corresponding receptors on T cells (CD40L, CD28 respectively). Co-stimulation lowers the activation threshold facilitating T cell proliferation, and cytokine production, whereas the absence of co-stimulation can lead to T cell anergy or cell death (Kapsenberg, 2003). Examination of surface marker profiles in PUFA-treated DC showed a collective decrease in CD80, CD86, CD40 and MHCII expression. The over-expression of B7 molecules has been reported in the inflamed tissues of patients suffering from MS, RA and IBD (Liu *et al.*, 2001b) and researchers have also reported increased expression of CD40 and CD40L in the inflamed ileum of CD sufferers (Danese *et al.*, 2004). Targeting co-stimulatory molecules as a therapeutic approach in inflammatory bowel disease has been reviewed by Maerten and colleagues (2003). Examples include a study where the use of anti-CD40L antibodies in TNBS-induced colitis (a model for Th1-mediated Crohn's disease) effectively prevented mucosal inflammation and decreased IFN- γ production by lamina propria T helper cells (Danese *et al.*, 2004). And where blocking CD80 signalling using a CD80-CAP (competitive antagonist peptide) suppressed established inflammation in TNBS-induced colitis (Rajaraman Eri, 2008). The down-regulation of co-stimulatory markers we recorded in DC *in vitro* may provide a pathway used by PUFA to provide therapeutic benefit in inflammatory disease, and perhaps help explain how the CLA diet afforded protection against disease development in the DSS colitis study. *Ex vivo* analysis of the corresponding co-stimulatory/inhibitory markers on CD4⁺ T cells from animals fed a PUFA-incorporated diet did not reveal any significant findings. Again this suggests that PUFA alter T cell differentiation but not the T cell itself. It should also be taken into account that these splenic cells were not directly exposed to PUFA and a truer picture would be obtained by examining the surface marker expression of T cells isolated from the MLNs of fed animals. The same can be said about the DC from fed animals. These DC were isolated from animals given dietary CLA but the cells were differentiated for 7 days without any exogenous PUFA. Even though we found that the DC produced significantly less IL-12 following LPS stimulation, there was no difference in co-stimulatory marker or MHCII expression on these cells, probably because they were not directly exposed to PUFA. To assess the effects of dietary PUFA on DC, intestinal lamina propria cells should be examined. This would be of particular interest in light of the fact that the protective effects

of dietary CLA we saw in the colitis model may be due to effects on dendritic cells. DC have been widely implicated in the pathogenesis of IBD: 5 times more DC have been found in the colon (Cruickshank *et al.*, 2004) and 15-36 times more DC have been found in the MLN (Stagg *et al.*, 2003) of colitic mice. Bilsborough and Viney (2004) found DC in colitic animals had increased expression of CD40, CD80, CD86 as well as higher production of IL-12p40 and IL-23p19. Berndt and colleagues (2007) found that direct stimulation of BMDC with DSS increased the release of several cytokines and chemokines. Therefore DC could be one of the cell types responsible for initiating inflammation during DSS-induced colitis. Perhaps some of the immunomodulatory effects of PUFA we saw in DC *in vitro* afforded protection from disease development *in vivo*. This is a possibility considering many of the parameters we found altered in DC by PUFA have shown therapeutic efficacy in studies on inflammatory disease, and this is further implied because fatty acids, especially long-chain, are transported through epithelial cells in the microlymphatics which is where resident DC can be found and is therefore an opportunity for DC to be exposed to fatty acids in the diet (Tsuzuki *et al.*, 2006).

Although the anti-inflammatory effects of PUFA have been well documented, their molecular mode of action remains relatively unknown. Therefore we explored a number of signalling pathways PUFA may exploit to carry out their modulatory actions:

The ERK kinase signalling pathway is triggered during DC maturation (Banchereau *et al.*, 2000), and we and others have shown that PUFA use the ERK pathway to exert some of their modulatory effects on immune cells (Denys *et al.*, 2001; Loscher *et al.*, 2005). A study by Li and colleagues (2007) showed that inhibiting ERK activation during LPS-induced DC maturation, enhanced CCR7 expression and their chemotaxis towards CCL19. The fact that we have found the suppressive effects of CLA on T cells and DC partially reversed by inhibiting ERK suggests that perhaps a PUFA-induced alteration in ERK activation impedes DC migration to draining lymph nodes and could account for the altered chemotaxis towards CCL19 that we observed here. The effect of ERK inhibition on the expression of CCR7 and migration of PUFA-treated DC towards CCL19 should be investigated.

In acute DSS-induced colitis a number of NFκB-dependent inflammatory cytokines are upregulated. Herfarth and colleagues (2000) examined the effects of gliotoxin, a fungal metabolite known to inhibit NFκB activity, in a DSS model of colitis. They found that gliotoxin dose-dependently down-regulated colonic inflammation. Neurath and co-workers (1998) also found that down-regulating NFκBp65 resulted in a striking reduction in the production of various important proinflammatory cytokines by macrophage from patients with IBD. They suggested that targeting NFκB family members may be a novel molecular approach for the treatment of IBD, particularly because of the overexpression of NFκB by macrophage in IBD patients and because the anti-inflammatory activity of glucocorticoids frequently used for treatment of chronic intestinal inflammation has been attributed to their inhibitory effect on NFκB activity. This pinpoints another potential beneficial effect of PUFA in an inflammatory situation considering we observed a reduction in NFκB availability and activation in DC treated with EPA and DHA. An interesting observation was recorded by Zhao and co-workers (2007). They used a human colonic epithelial cell-line, HCT116, and these cells, like primary colonic epithelial cells, do not express TLR2 or TLR4, but are activated by signalling through NOD receptors. They found that NFκB activation and IL-8 production were both reduced in these cells following their treatment with EPA or DHA compared to controls. So if IBD sufferers have mutations in NOD2 which leaves them more susceptible to disease (Cobrin & Abreu, 2005), and the above study shows that PUFA signal through NOD to decrease NFκB in colonic epithelial cells, is there a possibility that some patients would be more susceptible to PUFA therapy?

PUFA are considered natural PPAR ligands (Sampath & Ntambi, 2005), furthermore, fatty acids have been shown to both increase PPAR expression and activate them (Bassaganya-Riera *et al.*, 2004). We found that PPARγ was upregulated and its association with NFκB increased in EPA and DHA-treated DC. However, the PUFA-induced actions on DC, with regard to altered cytokine production, cell surface marker expression and migration, were not reversed by the addition of the PPARγ inhibitor and were therefore deemed PPARγ-independent. However, the significant induction of PPARγ expression in DC after culture with PUFA must have a purpose and should be investigated further.

Collectively, results presented here suggest that CLA (and possibly the n-3 PUFA) modify cytokine production and surface marker expression in DC which leads to altered antigen presentation and/or co-stimulation, which then modulates the cytokine production profile of T cells. The T cell response does not appear to be directly switched off by CLA since CD25 expression, proliferation and IL-2 production were largely unaltered. The migration of DC towards the lymphoid chemokine, CCL19, was also inhibited following PUFA treatment, as was the activation and total cellular levels of NF κ B. Effects on cytokine production, surface marker expression and migration all appear to be PPAR γ -independent. The immunomodulatory effects of PUFA went on to show efficacy in two *in vivo* inflammatory models: Firstly, circulating IFN γ and IL-12 were reduced in CLA-fed animals following the administration of endotoxin, and secondly, mice were protected from the development of DSS-induced colitis by feeding a CLA-supplemented diet. All of these findings advocate PUFA as prospective therapeutic tools.

Studies have revealed that human beings evolved on a diet with a ratio of omega-6 to omega-3 fatty acids of approximately 1. In Western diets, the ratio is 15/1-16/1. A high omega-6 to omega-3 ratio promotes the pathogenesis of many diseases, including cardiovascular disease, and inflammatory and autoimmune disorders, whereas a higher concentration of omega-3 exerts immunosuppressive effects (Simopoulos, 2006). Indeed, IBD affects approximately 0.1% of the Western population (Uhlig *et al.*, 2006) with a far lower prevalence of IBD found in populations consuming high n-3 fatty acid fish (Wild *et al.*, 2007). This provides further evidence for the potential use of fatty acid-supplementation in resolving chronic inflammatory diseases.

CHAPTER 8

BIBLIOGRAPHY

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CHAPTER 9

APPENDIX

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CELL CULTURE MEDIA

RPMI 1640	500 ml
5% (v/v) Heat inactivated Foetal Calf Serum (FCS)	25ml
Penicillin/streptomycin/L-glutamine Culture Cocktail (Gives a final concentration of 2 mM L-glutamine, 100 µg/ml penicillin and 100 U/ml streptomycin)	10 ml

10X PHOSPHATE BUFFERED SALINE (PBS)

Na ₂ HPO ₄ .2H ₂ O (8 mM)	23.2 g
KH ₂ PO ₄ (1.5 mM)	4 g
NaCl (137 mM)	160 g
KCl (2.7 mM)	4 g
Make up to 2L pH to 7.4 with 5M NaOH	

FACS BUFFER

2% (v/v) FCS
0.05% (w/v) NaN ₃
PBS

TRIS BUFFERED SALINE (TBS) pH 7.6

Tris base	2.422 g
NaCl	8.766 g
Dissolve in 1L dH ₂ O pH to 7.6 with conc. HCl	

2N H₂SO₄

H ₂ SO ₄ (36 N)	11.1 ml
dH ₂ O	88.9 ml

PROTEIN PURIFICATION BUFFERS:

1M HEPES (pH 7.9)	11.92g into 50ml
1M MgCl ₂	74.55g into 50ml
1M KCl	3.73g into 50ml
1M NaCl	2.92g into 50ml
0.5M PMSF	0.3484g (into 4ml acetone)
0.5M EDTA	9.306g into 50ml
Glycerol	25% (v/v) = 12.5ml into 50ml 20% (v/v) = 10ml into 50ml

SAMPLE BUFFER

0.5mM Tris HCl pH 6.8
10% (v/v) Glycerol
10% (w/v) Sodium dodecyl sulphate (SDS)
5% (v/v) β-mercaptoethanol
0.05% (w/v) Bromophenol Blue

SEPARATING GEL (10%)

33% (w/v) Bisacrylamide (30% stock)
1.5M Tris-HCl pH8.8
1% (w/v) SDS
0.5% (w/v) Ammonium persulphate
dH ₂ O
0.1% (v/v) TEMED

STACKING GEL

6.5% (v/v) Acrylamide/Bisacrylamide (30% stock)
0.5M Tris-HCl pH6.8
1% (w/v) SDS
0.5% (w/v) Ammonium persulphate
dH ₂ O
0.1% (v/v) TEMED