Dietary fatty acids modulate inflammatory cytokine production through activation of MAP kinases

Masters Thesis

MSc

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DECLARATION OF AUTHORSHIP

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Abstract

Polyunsaturated fatty acids (PUFA) have therapeutic effects in inflammatory diseases, however the specific mechanisms by which they exert these effects have not yet been defined. Several studies have shown involvement of toll like receptor TLR2 and TLR4 in some of these inflammatory diseases shown to be inhibited by PUFA. In order to determine how PUFA exert their beneficial effects in these diseases it is important to examine how PUFA modulate the expression and activation of TLR2 and TLR4 and their downstream signalling pathways. This study investigates the effects of a number of PUFA, including conjugated linoleic acid (CLA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), on MAP kinase signaling pathways and cytokine production in macrophages following activation of these cells through these TLRs. Macrophages are key cells involved in activation of innate immune responses. This study used the J774 murine macrophage cell line as a model. The results demonstrate that PUFA exert distinct effects on both pro-inflammatory and anti-inflammatory cytokine production depending on whether the cell is activated via TLR2 or TLR4. Thus, providing evidence that the effects of PUFA on immune cell function are highly dependent on the mode of activation of the cell. Furthermore, individual PUFA exert these effects through differential regulation of MAP kinase signalling proteins. Activation of ERK was found to be critical for mediating the effects of PUFA on cytokine production, with p38 being involved to a lesser extent. Given that these fatty acids can change the way in which an immune cell responds to activation through TLRs, further research must be undertaken in order to fully

elucidate the benefits of dietary supplementation with fatty acids and indeed to determine any drawbacks associated with their use.

Objectives

Dietary PUFA interact with multiple components of the immune system and modulate the balance of pro- and anti-inflammatory cells. TLR 2 and TLR 4 activate a common signalling pathway that results in the induction of inflammatory cytokines. We hypothesised that dietary PUFA could alter the response of immune cells following activation through TLRs and that this was mediated by MAPK activation. The main aim of this study was to examine the effects of various n-3 and n-6 PUFA on murine macrophage activation and function, which were determined by measuring cytokine secretion, cell surface marker expression and intracellular signalling. We measured these responses following activation of the macrophages through TLR4 and TLR2.

Abbreviations

AA	Arachidonic Acid
APC	Antigen Presenting Cell
BSA	Bovine Serum Albumin
CLA	Conjugated Linoleic Acid
COX	Cyclooxygenase
DC	Dendritic Cell
DHA	Docosahexaenoeic Acid
DMSO	Dimethyl Sulphoxide
dsDNA	Double Stranded Deoxyribonucleic Acid
EFA	Essential Fatty Acids
EPA	Eicosapentanoeic Acid
ERK	Extra-Cellular Signal Regulated Kinases
FCS	Foetal Calf Serum
GLA	Gamma Linoleic Acid
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor
GPCR	Guanine Nucleotide Exchange Factor
HIV	Human Immuno-defiency Virus
HSP	Heat shock protein
IBD	Inflammatory Bowel Disease
ICAM	Inter Cellular Adhesion Molecule
IFN	Interferon
IGF	Insulin-like Growth Factor
IL	Interleukin
INOS	Inducible NO-Synthase
IRAK	Interleukin 1 receptor associated kinase
JNK	c-Jun N-terminal Kinase
KCl	Potassium Chloride
LA	Linoleic Acid
LAT	Latency Associated Transcript
LFA	Lymphocyte Function Associated
LNA	Alpha Linoleic Acid
LOX	Lipooxygenase
LPS	Lipopolysaccharide
LRR	Leucine Rich Repeat
LTA	Lipoteichoic Acid
MAPK	Mitogen Activated Protein Kinase
MEK	MAP/ERK Kinase
MHC	Major Histocompatability Complex
MKK	MAP Kinase Kinase
MKKK	MAP Kinase Kinase Kinase
MKPS	MAPK Phosphatases
MyD88	Myeloid Differentiation primary response gene 88
NK	Natural Killer
NO	Nitric Oxide
PAMP	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline

PDGF	Platlet Derived Growth Factor
PKC	Protein Kinase C
PMN	Polymorphonuclear Cells
PRR	Pattern Recognition Receptor
PUFA	Polyunsaturated Fatty Acid
RA	Rheumatoid Arthritis
SA	Stearic Acid
SAPK	Stress Activated Protein Kinase
SDS	Sodium Dodecyl Sulphate
STAT	Signal transducers and activators of transcription
TAK	Transforming Growth Factor β-Activated Kinase
TBS	TRIS Buffered Saline
TCR	T cell Receptor
TGF	Transforming Growth Factors
TIR	Toll/IL-1 Receptor
TLR	Toll Like Receptor
TMB	Tetramethyl-benzidine
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TRAF	TNF Receptor Associated Factor
TRAM	Toll-receptor-associated molecule
TRIF	TIR-domain-containing adaptor inducing IFN-beta
VCAM	Vascular cell adhesion molecule

Chapter 1
General Introduction

1.0 General Introduction

1.1 Immunity:

Immunity refers to the state of protection from infectious disease; there are two types; innate immunity and adaptive immunity. Innate immunity is comprised of a set of disease resistant mechanisms, which are unspecific for particular pathogens (Janeway, 2005). Phagocytic cells like macrophages play an important role in many aspects of innate and adaptive immunity and link these two differing responses (Janeway, 2005). Adaptive immunity however displays high specificity in relation to pathogen and has a remarkable property in immunological memory (Janeway, 2005).

1.2 Innate Immune System:

The innate immune system is widely believed to constitute an evolutionary defence strategy and one which is the dominant immune system found in fungi, plants, insects and in multicellular organisms (Janeway, 2005). The main functions of the vertebrate innate immune system are recruitment of immune cells to sites of infection and inflammation; activation of the complement cascade to identify bacteria; activate cells and clearance of dead cells and antibody complexes; the identification and removal of foreign molecules present in organs, tissues, blood and lymph fluids by utilising specialised white blood cells and the activation of the adaptive immune system through antigen presentation (Lewis CE, 1999). The strategy of innate immune recognition is based on the detection of constitutive and conserved products of microbial metabolism. Many metabolic pathways and individual gene products are unique to micro-organisms and absent from host cells (Lewis CE, 1999). Some of these pathways are involved in housekeeping functions and their products are

conserved among micro-organisms of a given class and are essential for their survival. For example, lipopolysaccharide (LPS), lipoproteins, peptidoglycan and lipoteichoic acids (LTAs) are all molecules made by bacteria, but not by eukaryotic cells (Lewis CE, 1999). Therefore, these products can be viewed as molecular signatures of microbial invaders, and their recognition by the innate immune system can signal the presence of infection. One important aspect of innate recognition is that its targets are not absolutely identical between different species of microbes (Janeway, 2005). However, although there are several strain- and species-specific variations of the fine chemical structure, these are always found in the context of a common molecular pattern, which is highly conserved and invariant among microbes of a given class. For example, the lipid-A portion of LPS represents the invariant pattern found in all Gram-negative bacteria and is responsible for the pro-inflammatory effects of LPS, whereas the O-antigen portion is variable in LPS from different species of bacteria and is not recognized by the innate immune system (Lewis CE, 1999).

Because the targets of innate immune recognition are conserved molecular patterns, they are called pathogen-associated molecular patterns (PAMPs). Accordingly, the receptors of the innate immune system that recognize PAMPs are called pattern-recognition receptors (PRR) (Janeway, 2005). PAMPs have three common features that make them ideal targets for innate immune recognition. First, PAMPs are produced only by microbes, and not by host cells. Therefore, recognition of PAMPs by the innate immune system allows the distinction between 'self' and 'microbial non-self '. Second, PAMPs are invariant between microorganisms of a given class. This allows a limited number of germ-line-encoded PRRs to detect the presence of any microbial infection. So, recognition of the conserved lipid-A pattern in LPS, for

example, allows a single PRR to detect the presence of almost any Gram-negative bacterial infection. Third, PAMPs are essential for microbial survival. Mutations or loss of PAMPs are either lethal for that class of microorganisms, or they greatly reduce their adaptive fitness. Therefore, 'escape mutants' are not generated. These properties of PAMPs indicate that their recognition must have emerged very early in the evolution of host-defense systems. (Janeway, 2005)

Indeed, many PAMPs are recognized by the innate immune systems not only of mammals, but also of invertebrates and plants. PAMPs are actually not unique to pathogens and are produced by both pathogenic and non-pathogenic microorganisms. In fact, none of the gene products that are unique to pathogens — the so-called 'virulence factors' — are known to be recognized by the mammalian innate immune system (Medzhitov, 2001). This means that PRRs cannot distinguish between pathogenic and commensal microorganisms. This distinction, however, is vitally important. We live in constant contact with commensal microflora, and continuous activation of inflammatory responses by commensals would have potentially lethal consequences for the host. This, however, does not occur under normal physiological conditions. The exact mechanisms that allow the host to 'tolerate' non-pathogenic microorganisms are largely unknown. Presumably, compartmentalization (for example, confinement of microflora to the luminal side of intestinal epithelium), as well as anti-inflammatory cytokines, such as transforming growth factor-β (TGF-β) and interleukin (IL)-10, have an important role in this process (Medzhitov, 2001).

The innate immune system uses various PRRs that are expressed on the cell surface, in intracellular compartments, or secreted into the blood stream and tissue fluids. The

principal functions of PRRs include: opsonization, activation of complement and coagulation cascades, phagocytosis, activation of pro-inflammatory signaling pathways and induction of apoptosis.

1.3 Barriers To Infection:

There are three barriers to infection. These are mechanical, chemical and biological. The first mechanical meaning the physical barrier refers to the epithelial surfaces that form a physical barrier that is very impermeable to most infectious agents. Therefore the skin acts as our first line of defence against invading organisms (Janeway, 2005). Desquamation of skin epithelium helps in the removal of infectious agents that have adhered to the epithelial surfaces. Cilia movement helps in keeping air passages and the GI tract free from micro-organisms etc. Chemical barriers also protect against infection. The skin and respiratory tract secrete anti-microbial peptides such as β-defensins. Lysozyme and phopholipase A in saliva, tears are also antibacterial. Gastric acid and proteases in the stomach serve as powerful chemical defenses against ingested pathogens. Biological barriers such as the commensal flora in the GI tract compete with pathogenic bacteria for food space and can change conditions in the environment such as pH or available iron. This prohibits pathogens to reach sufficient numbers to cause illness. (Janeway, 2005)

Humoral Barriers:

There are also humoral barriers that include inflammation, the complement system and cellular barriers of the innate immune system. Humoral immunity refers to antibody production and the processes that follow including: Th2 activation, cytokine production, germinal centre formation, affinity maturation and memory cell

generation. Humoral immunity also refers to the effector functions of antibodies, which include pathogen and toxin neutralisation, complement activation, opsonin promotion of phagocytosis and pathogen elimination (Janeway, 2005). The complement system is composed of approximately 20 different protein which work together to destroy pathogens and signal other immune cells to the threat. Activation of the complement system results in the production of split products which serve as mediators of inflammation. These mediators act together to induce monocytes and neutrophils to adhere to vascular endothelial cells, extravate and migrate toward the site of complement activation in the tissues (Janeway, 2005). Coagulation is another barrier this prevents bleeding and limits the spread of invading pathogens into the blood-stream.

Cellular Barriers:

Innate Cells:

Innate cells comprise a range of cells, including macrophage, dendritic cells, monocytes, neutrophils and natural killer cells. Dendritic cells (DCs) are potent antigen presenting cells (APCs) that possess the ability to stimulate naïve T cells. They comprise a system of leukocytes widely distributed in all tissues. DCs are derived from bone marrow progenitors and circulate in the blood as immature precursors prior to migration into peripheral tissues. Within different tissues, DCs differentiate and become active in the taking up and processing of antigens, and their subsequent presentation on the cell surface linked to major histocompatibility (MHC) molecules. Upon appropriate stimulation, DCs undergo further maturation and migrate to secondary lymphoid tissues where they present antigen to T cells and

induce an immune response (Janeway, 2005). Natural killer (NK) cells are a form of cytotoxic lymphocyte which constitute a major component of the innate immune system. NK cells play a major role in the host-rejection of both tumors and virally infected cells. They were named "natural killer" because of the initial concept that they do not require activation in order to kill cells which are "missing self" recognition.

Another innate immune cell, the neutrophil, is also known as a polymorphonuclear cell (PMNs) due to the polymorphic shape of its nucleus. The neutrophils main role is in inflammation. In the tissues, neutrophils are active phagocytic cells, like macrophages however, they do not act as antigen presenting cells. Neutrophils are the most abundant type of phagocytes and are the first to site of infection by a process called extravasation. They are attracted into the tissue by chemotactic factors, which include complement proteins, clotting proteins and T cell derived cytokines (see table 4.1). Neutrophils, although not an antigen presenting cell (APC), are most effective at killing ingested micro-organisms and can do this by oxygen dependent or independent pathways (Janeway, 2003). Neutrophils react to pathogen by activating a respiratory burst containing oxidising agents such as hydrogen peroxide and hypochlorite.

Macrophage, greek for big eaters are cells within tissues that originate from specific white blood cells called monocytes. The monocytes and macrophages act in both innate and adaptive immunity (Lewis CE, 1999). Their role is to phagocytose (i.e. engulf and digest) cellular debris and pathogens and to stimulate lymphocytes and other immune cells to respond to pathogen. The macrophage is the major differentiated cell of the mononuclear phagocyte system. This system comprises bone marrow monoblasts and pro-monocytes, tissue macrophage and peripheral blood

monocytes. Macrophage are found in the lymphoid organs, liver, lungs, GI tract, the central nervous system, bone, endothelial cells, fibroblasts, and monocytes. When monocytes enter the tissues and become macrophages they undergo several changes. The cells enlarge and increase the amount of intracellular lysosyme allowing enhanced phagocytosis. In the tissues, macrophages live for months or years and may be motile. Macrophages movement is enhanced using pseudopods, which also act to engulf pathogens and subsequent activation (Lewis CE, 1999).

Activated macrophages have an important role in phagocytosis, they recognize and remove unwanted particulate matter including products of inflammation and invading organisms, antigens and toxins (Lewis CE, 1999). Macrophages also have an important role in the presentation of antigens (as APC's) to T-helper cells and in activating T cells with IL-1 release. Receptors present on the surface of macrophages determine the control of activities, such as growth, differentiation, activation, recognition, endocytosis, migration and secretion. Numerous ligands have been reported as binding to the surface of macrophage (Table 1.0). The first of the macrophage receptors to be identified was those for the Fc region of the IgG molecule and for the cleavage product of the third component of the complement system (C3) (Lewis CE, 1999). The attachment of the Fc portions of Ig molecules to the surface of the macrophage via Fc receptors may trigger various functions such as endocytosis, the generation of transmembrane signals, resulting in the reorganisation of cytoskeletal microfilaments at the site of attachment facilitating phagocytosis and the secretion of potent mediators.

Macrophages not only produce many cytokines they also possess receptors for such cytokines. Individual cytokines or combinations of cytokines interacting with specific receptors modulate the function of macrophages.

Table 1.0: Surface Receptors of monocytes and macrophages

Receptor	Ligand
Fc Receptor Complement Receptors	IgG_{2a}, IgG_{2b}, /IgG_1, IgG_3, IgA, IgE C3b, C3bi, C5a, C1q
Cytokine Receptors	MIF, MAF, LIF, CF, MFF, IL-1, IL-2, IL-3, IL-4, IFN-α, IFN-β, IFN-γ. Colony-stimulating factors (GM-CSF, M-CSF/CSF-1)
Receptors For Peptides and small molecules	H_1, H_2, 5HT, 1,2,5-Dihydroxy Vitamin D3, *N*-formulated peptides, enkephalins/endorphins, substance P, arg-vasopressin.
Hormone Receptors	Insulin, glucocorticosteroids, angiotensin.
Lipoprotein lipid receptors	Anionic low-density lipoproteins, PGE_2, LTB_4, LTC_4, LTD_4, PAG, apolipoproteins B and E (Chylomicron remnants, VLDL).
Receptors for coagulants and anticoagulants	Fibrinogen/Fibrin, coagulation factor VII, α1-antithrombin, heparin.
Others	Cholinergic agonists, α1-adrenergic agonists, β2-adrenergic agonists.

1.4 Activation, Antigen Processing And Presentation:

Mononuclear phagocytes and neutrophils provide a first line defense against microbial invasion. While the neutrophil is a more efficient phagocyte when the particle load is great or much larger than in relation to the cell macrophages become more effective. Macrophages move towards microbial particles guided by a gradient of chemotactic factors. Engulfment of microbial particle occurs through use of pseudopods and the recognition of PAMP's by PRR's by the macrophage (Lewis CE, 1999). The main PRR's found on macrophage are the toll-like receptors (TLRs) and receptors for the Fc portion of antibodies. Upon engulfment the microbial particle is taken within a membrane-bound structure called a phagosome. The phagosome then enters the endocytic processing pathway eventually fusing with a lysosome. Lysosomes contain lysozyme and hydrolytic enzymes which digest the microbial particle. These digested particles can then either interact with MHC II molecules and finally presented to T_H Cells or be released for further opsonisation (Lewis CE, 1999).

There are two types of activation for macrophages, classically activated and alternatively activated macrophages. Macrophages become classically activated by exposure to two signals, the first is the cytokine IFN-γ which primes the macrophage but does not activate (Lewis CE, 1999). The second signal is tumor necrosis factor (TNF) or an inducer of TNF. The physiologically relevant second signal is usually the result of TLR ligation inducing production of endogenous TNF within the macrophage upon contact with a stimulus e.g. LPS (Luster et al., 2005a). Upon activation significant changes in cellular morphology and secretory profile of the cell occurs. A variety of chemokines including, IL-8/CXCL8, IP-10/CXCL10, MIP-1α/CCL3, MIP-1ß/CCL4, and RANTES/CCL5, are secreted and act as

chemoattractants for immature DC's, neutrophils, natural killer cells and activated T cells. Several pro-inflammatory cytokines are also released including IL-1ß, IL-6, and TNF-α (Duffield, 2003, Gordon, 2003, Ma et al., 2003, Mosser, 2003). TNF-α also contributes to the pro-apoptotic activity of classically activated macrophage. The release of these molecules is important in host defence and in direction of adaptive immune system, but unchecked massive infiltration of leukocytes and flooding of tissue with inflammatory mediators and pro-apoptotic factors can inflict serious tissue injury (Lewis CE, 1999).

Tissue destruction by chronic inflammation has also been associated with tumour development and type 1 autoimmune diseases. Alternatively activated macrophages do not require priming, IL-42 or IL-13 can act as efficient stimuli (Doherty et al., 1993). The binding of these to the respective antigens is followed by pinocytosis of soluble antigen which is then loaded onto MHC class II molecules and displayed to Tcells (Conner and Schmid, 2003). The alternatively activated macrophage also changes its cellular morphology and secretory profile upon activation. Leukocytes are attracted by the macrophage through its release of chemokines which include MDC/CCL22, 30,31 PARC/CCL18, 32,33 and TARC/CCL17 (Imai et al., 1999). Inflammation is negated by the release of factors such as IL-1ra, IL-10 and TGF-β (Mosser, 2003). The alternatively activated macrophage also secretes PDGF, IGF that promote cell proliferation. These secreted molecules work to resolved inflammation and promote wound repair due to the anti-inflammatory, fibrotic and proliferative activites. Alongside its beneficial effects, the alternatively activated macrophage has been involved in allergy and asthma (Duffield, 2003, Gordon, 2003).

The link between innate and adaptive immunity is "managed" by CD4+ lymphocytes or T_H Cells. These CD4+ cells express T-Cell receptors (TCR's) that recognise antigen bound to the MHC II molecules. Activation of these naïve T helper cells causes release of cytokines which influence the activity of not only the antigen presenting cells that activated the T helper cells but many other cell types. Several types of effector CD4+ T helper cell responses can be induced by a professional APC (i.e. macrophage, dendritic cells, NK cells), these have been given the designation Th1 and Th2. Both of these eliminate different types of pathogens. The Th1 response is characterised by the production of IFN-γ which activates the bactericidal activities of macrophages and induces B cells to make opsonizing antibodies leading to cell-mediated immunity (Luster et al., 2005a). The Th2 response is characterised by the release of IL-4 which results in the activation of B cells to make neutralizing antibodies leading to humoral immunity (Luster et al., 2005a).

A distinct subset of the T helper cells, Th17 has been identified. Th17 cells produce IL-17, TNF-α and IL-6. IL-17 is a potent inflammatory cytokine and is involved in inducing expression of proinflammatory cytokines, IL-6 and TNF, and chemokines (such as KC, MCP-1 and MIP-2) (Bettelli et al., 2007). This subset is regulated by TGF-β, IL-6 and IL-23 and inhibited by IL-4 and IFN-γ the cytokine involved in macrophage priming. IL-17 stimulates fibroblasts, endothelial cells and macrophages to produce pro-inflammatory mediators IL-1, IL-6, TNF-α and chemokines. IL-17 also activates enhanced granulocytes, macrophages, NK cells and CD8 Tcells.

Regulatory Tcells or T_{reg} are a specialised subpopulation of T cells that suppress activation of the immune system maintaining homeostasis and tolerance to self

antigens (Roncarolo and Levings, 2000). These Treg cells are also only activated when bound to its antigen on the MHC class II molecule of an APC for which it is specific. Besides naturally occurring $CD4^+$ $CD25^+$ Treg cells, other $CD4^+$ Treg cells include Tr-1 cells secreting interferon (IFN)-γ and IL-10, and Th3 cells secreting high levels of transforming growth factor (TGF)-β, IL-4, and IL-10 (Roncarolo and Levings, 2000).

1.5 Adaptive Immunity

The adaptive immune system is composed of highly specialised systemic cells and processes, which eliminate pathogenic challenges. The adaptive immune system is activated by the innate immune system which is the first line of defence against invading pathogens (Janeway, 2005). The major functions of the adaptive immune system include recognition of specific "non-self" antigens in the presence of "self" antigens during antigen presentation. The main cells of the adaptive immune system are the B and T lymphocytes. T lymphocytes are a major source of cytokines (Janeway, 2005). There are two main subsets of T lymphocytes, distinguished by the presence of cell surface molecules known as CD4 and CD8. T lymphocytes expressing CD4 are also known as helper T cells. This subset can be further subdivided into Th1 and Th2, and the cytokines they produce are known as Th1-type cytokines and Th2-type cytokines. Th1 cells produce IFN-γ, IL-2, TNF-α and lymphotoxin. IFN-γ is the main Th1 cytokine (Janeway, 2005). The Th2-type cytokines include IL 4, IL 5 and IL 13, which are associated with the promotion of IgE and interleukin-10, which has more of an anti-inflammatory response. In excess, Th2 responses will counteract the Th1 mediated microbicidal action.

As discussed earlier macrophage are an important antigen presenting cell and require two signals for activation. One is IFN-γ and the other can come from varied means e.g. LPS but is required to sensitise the macrophage to respond to IFN-γ. Effector T_H1 cells can deliver both signals. IFN-γ is a characteristic cytokine produced by armed T_H1 cells whereas the CD40 ligand expressed by T_H1 cells provides the sensitisation through contact with CD40 on macrophage (Janeway, 2005). This causes T_H1 cells to secrete IFN-γ, this subsequently binds to the IFN-γ receptors on macrophage causing activation. This activation causes the secretion of TNF-α, IL-1, and IL-12. TNF-α and IL-1 promote inflammation to recruit phagocytic leukocytes while lL-12 enables naive Tcells to differentiate into T_h1 cells (Janeway, 2005).

1.6 Cytokines

Cytokines are small, secreted proteins which mediate and regulate immunity, inflammation, and haematopoiesis (Janeway, 2005). They must be produced *de novo* in response to an immune stimulus. They generally act over short distances and short time spans and at very low concentration. They act by binding to specific membrane receptors, which then signal the cell via second messengers, often tyrosine kinases, to alter gene expression (Medzhitov, 2001). Responses to cytokines include increasing or decreasing expression of membrane proteins (including cytokine receptors), proliferation, and secretion of effector molecules. Cytokine is a general name; other names include lymphokine, monokine, chemokine, and interleukin. Cytokines act in an autocrine, paracrine and endocrine manner. It is common for different cell types to secrete the same cytokine or for a single cytokine to act on several different cell types. Cytokines are made by many cell populations, but the predominant producers are

helper T cells (Th) and macrophages (Lewis CE, 1999). The largest group of cytokines stimulates immune cell proliferation and differentiation. This group includes interleukin 1 (IL-1), which activates T cells; IL-2, which stimulates proliferation of antigen-activated T and B cells; IL-4, IL-5, and IL-6, which stimulate proliferation and differentiation of B cells; Interferon gamma (IFN–γ), which activates macrophages; and IL-3, IL-7 and granulocyte monocyte colony-stimulating factor (GM-CSF), which stimulate haematopoiesis (Janeway, 2005). Cytokines are also grouped into pro-inflammatory and anti-inflammatory cytokines. Anti-inflammatory cytokines include: IL-4, IL-6, IL-10 and TGF-ß (Lalani et al., 1997). Pro-inflammatory cytokines include: IL-1β, IL-12, and TNF-α. See table 1.1 for list of cytokines and their biological function.

Table 1.1 Cytokines: sources and functions

Name	Source	Function
IL-1	Macrophages	Small amounts induce acute phase reaction, large amounts induce fever.
IL-2	TH1-cells	Stimulates growth and differentiation of T cell response. Can be used in immunotherapy to treat cancer or suppressed for transplant patients.
IL-4	TH2-cells, just activated naive CD4+ cell, memory CD4+ cells	Involved in proliferation of B cells and the development of T cells and mast cells. Important role in allergic response (IgE).
IL-6	Macrophages, TH2-cells	Induces acute phase reaction
IL-10	Monocytes, TH2-cells, mast cells	Inhibits Th1 cytokine production
IL-12	Macrophages	NK cell stimulation, Th1 cells induction. May suppress food allergies.
IL-13	TH2-cells	Stimulates growth and differentiation of B-Cells (IgE), inhibits TH1-cells and the production of macrophage inflammatory cytokines
IL-17	T cells	Induces production of inflammatory cytokines, and maintains inflammation
IL-18	Macrophages	Induces production of Interferon-gamma (IFNγ)

Interleukin 10:

IL-10 is one of the most important anti-inflammatory cytokines found within the human immune response. It is a potent inhibitor of Th1 cytokines, including both IL-2 and IFN-γ. This activity accounts for its initial designation as cytokine synthesis inhibition factor (Howard and O'Garra, 1992, Lalani et al., 1997, Opal et al., 1998). In addition to its activity as a Th2 lymphocyte cytokine, IL-10 is also a potent deactivator of monocyte/macrophage pro-inflammatory cytokine synthesis (Brandtzaeg et al., 1996, Clarke et al., 1998). IL-10 is primarily synthesized by CD4+ Th2 cells, monocytes, and B cells and circulates as a homodimer consisting of two tightly packed 160-amino-acid proteins (Howard and O'Garra, 1992). After engaging its high-affinity 110-kd cellular receptor, IL-10 inhibits monocyte/macrophage-derived TNF-α, IL-1, IL-6, IL-8, IL-12, granulocyte colony-stimulating factor, MIP-1α, and MIP-2α (Brandtzaeg et al., 1996, Gerard et al., 1993, Marchant et al., 1994b). IL-10 inhibits cell surface expression of major histocompatibility complex class II molecules, B7 accessory molecules, and the LPS recognition and signaling molecule CD14 (Opal et al., 1998). It also inhibits cytokine production by neutrophils and natural killer cells. IL-10 inhibits nuclear factor κB (NF-κB) nuclear translocation after LPS stimulation and promotes degradation of messenger RNA for the pro-inflammatory cytokines (Clarke et al., 1998).

In addition to these activities, IL-10 attenuates surface expression of TNF receptors and promotes the shedding of TNF receptors into the systemic circulation (Dickensheets et al., 1997, Joyce et al., 1994). IL-10 is readily measurable in the circulation in patients with systemic illnesses and a variety of inflammatory states (Marchant et al., 1994a, van der Poll et al., 1997). IL-10 is present in sufficient

concentrations to have a physiologic impact on host responses to systemic inflammation. In the case of inflammatory bowel disease (IBD) especially colitis, loss of tolerance against bacterial flora of intestine is the causative agent (Braat et al., 2003). Tolerance against flora requires recognition of antigens by PRRs and presence of regulatory cells and cytokines.

Peptidoglycan activates TLR2. Stimulation of TLR2 triggers association with an adapter protein, myeloid differentiation primary response protein 88 (MyD88), and the recruitment of interleukin1- receptor-associated kinase (IRAK) proteins. TNF- receptor-associated factor 6 (TRAF6) initially binds to this complex and then dissociates to interact with transforming growth factor-β-activated kinase-1 (TAK1), which in association with other proteins leads to the activation and phosphorylation of components of the IKK (inhibitor of NFκB kinase) complex that gives rise to downstream activation of NFκB (p50 and p65 subunits) leading to activation of the inflammatory cytokine cascade (Rakoff-Nahoum et al., 2004). The cytokines and factors produced as a result of activation of pattern-recognition receptors can help initiate or sustain host responses that are anti-inflammatory (eg, interleukin 10 and transforming growth factor β) or pro-inflammatory (eg, TNFα and interleukin 12).

IL-10 plays a critical role in the shaping of immune responses. Produced by activated macrophages and Th2 T cells, IL-10 promotes the development of humoral, Th2 cytokine-driven immune responses (Moore et al., 1993). IL-10 inhibits the development of Th1 immune responses (Fiorentino et al., 1991), by reducing the capacity of macrophages to produce IL-12, a potent inducer of Th1 immune responses (Bohn and Autenrieth, 1996). IL-10 has been proposed to exert a regulatory effect in

intestinal mucosa (Niessner and Volk, 1995, Schreiber et al., 1995). The importance of IL-10 in shaping mucosal immune responses has been demonstrated by the spontaneous onset of inflammation in the IL-10-deficient mouse (Kuhn et al., 1993). IL-10-deficient mice spontaneously develop enterocolitis when housed in conventional environments, but when housed in SPF conditions IL-10-deficient mice develop inflammation limited to the colon, suggesting that resident enteric flora play a role in the development of spontaneous colitis in these mice.

IL-10 has an important role in regulatory network of cytokines controlling mucosal tolerance and is a potent anti-inflammatory therapy in IBD. In patients with ulcerative colitis, the pattern of cytokine expression shows increased expression of IL-5, IL-6, IL-10, and IL-13 (Madsen, 2002). The therapeutic experience of IL-10 in animal models of colitis is very encouraging. *In vitro* studies have shown that exogenous IL-10 can down-regulate the enhanced pro-inflammatory cytokine release from lamina propria mononuclear cells isolated from patients with Crohn's disease (Steidler et al., 2000). Direct administration of the anti-inflammatory cytokine IL10 into localised area has been studied with little therapeutic benefit due to its short half life ex-vivo (van Deventer et al., 1997). Genetically engineered bacteria such as *Lactococcus lactis* expressing IL-10 have also shown reduction in colitis in animal models and humans (Steidler et al., 2000). Schering- Plough has isolated the gene for IL-10 and is producing a recombinant form of the cytokine called tenovil. This has reached phase 3 trials.

IL-12

IL-12 is an interleukin, which is naturally produced by macrophages in response to antigenic stimulation. It is composed of a bundle of four alpha helices. It is a heterodimeric cytokine, which is encoded by two separate genes, IL-12p35 and IL-12p40. The bioactive form of IL-12 is a 75 kDa heterodimer (IL12p70) comprised of independently regulated disulfide-linked 40 kDa (p40) and 35 kDa (p35) subunits. The p40 subunit exists extracellularly as a monomer (IL12p40) or dimer (IL12 $(p40)_2$) and can antagonize the action of IL12p70 (Trinchieri, 1994). The highly coordinated expression of p40 and p35 genes to form IL-12 p70 in the same cell type at the same time is essential for the initiation of an effective immune response. It is known as a T cell stimulating factor, which can stimulate the growth and function of T cells. IL-12 induces IFN-γ synthesis and promotes Th1 cell differentiation whereas the production of IL-10 by macrophages and a subset of B cells antagonizes the activities of IL-12 (Trinchieri, 1994).

Pro-inflammatory cytokines such as IL-12 are strictly regulated. For example IFN-γ, TNF-α and GM-CSF can induce cell production of IL-12. IL-10 as mentioned before is an effective inhibitor of IL-12. IL-12 also has immunoregulatory properties, and plays a vital role in development of immunity to intracellular pathogens. A number of these pathogens including Leishmania, measles virus and human immunodeficiency virus (HIV) have shown subversion of the development of cell-mediated immunity through inhibition of IL-12 production. The interaction of *Leishmania* spp, measles virus, and HIV with macrophages and monocytes results in a marked decrease in IL-12 production (Chougnet et al., 1996). Ligation of phagocytic receptors on macrophage has also shown suppression of IL-12 production. This is achieved by two

distinct mechanisms, one depends on direct inhibition of gene transcription the second relies on inhibitory cytokine production. The production of IL-12 p40 is regulated primarily at the transcriptional level. The IL-12 p40 promoter contains multiple potential transcription-factor binding sites, three of which have been directly implicated in p40 gene regulation (Ma et al., 1997). The ligation of phagocytic receptors on human monocytes or murine macrophages has recently been shown to suppress IL-12 production. CR3 (CD11b/CD18, Mac-1) and the Fc receptors are the two phagocytic receptor classes that have been shown to have the clearest role in down-regulating IL-12 in these systems (Marth and Kelsall, 1997). The ligation of either CR3 or FcgR with monoclonal antibodies or with particulate ligands results in a profound inability of monocytes or macrophages to produce IL-12 in response to a variety of pro-inflammatory stimuli, including LPS, *S. aureus*, and CD40 ligand (Marth and Kelsall, 1997).

IL-12 has shown to be an important bridge between innate and adaptive immunity due to its activation of T cells to a T_H1 cell. Overproduction of the cytokine leads to inflammatory states and it has been shown to be involved in IBD and RA.

TNF-α:

TNFα is another cytokine involved in systemic inflammation and is also a member of a group of cytokines which stimulate the acute phase reaction. TNFα causes cell death, proliferation, differentiation, inflammation, viral replication etc. Its primary role however is in the regulation of immune cells. Dysregulation and overproduction of TNFα has been implicated in a variety of diseases as well as cancer. TNFα is

mainly produced by macrophages although lymphoid cells, mast cells, endothelial cells, fibroblasts and neuronal tissue can also produce it. TNFα attracts neutrophils very potently and helps them to adhere to the endothelial cells for migration. It also stimulates phagocytosis and production of IL-1 oxidant and the inflammatory lipid prostaglandin E2 PGE_2. An increasing concentration of TNFα will cause the cardinal signs of inflammation, heat, swelling, redness and pain. TNFα plays a central role in chronic inflammation (Podolsky, 2002). During activation of the inflammation cascade, antigen-presenting cells such as macrophages cause the activation and differentiation of CD4+ T lymphocytes. T lymphocyte cells differentiate into T helper (Th)-1 and Th-2 cells. Th-1 cells secrete interferon gamma (IFNϒ) and interleukin (IL)-2, IL-12, and IL-18, whereas Th-2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13 (Louis, 2001). Interferon gamma from the Th-1 response stimulates macrophages to produce TNFα.

TNFα is a pro-inflammatory mediator that has been shown to play an integral role in the pathogenesis of IBD. Transcription of the TNFα gene in activated macrophages results in the secretion of TNFα (Podolsky, 2002). Circulating soluble TNFα binds to two TNFα receptors mediating activation of other macrophages, augmentation of the T cell response, and recruitment of neutrophils to local sites of inflammation (Sandborn and Hanauer, 1999). TNFα also up-regulates other pro-inflammatory mediators such as IL-6 and IL-1ß (Louis, 2001). High levels of pro-inflammatory cytokines, including TNFα, in the intestinal mucosa are pivotal in the development of relapses and for sustaining chronic inflammation (Mascheretti et al., 2004). There are several ways of inhibiting TNFα, including binding and neutralizing the molecule by monoclonal antibodies, blocking TNFα production and secretion either directly or

indirectly, and inhibiting soluble TNFα receptors. Thalidomide inhibits TNFα production in monocytes, T lymphocytes, and macrophages. In a rat model of experimental colitis, thalidomide effectively decreased colitis, through the inhibition of TNFα in addition to its anti-angiogenic properties (Kenet et al., 2001).

1.7 Toll-Like Receptors:

Toll-like receptors are a conserved family of receptors involved in the recognition of a wide range of microbial molecules. TLRs comprise a family of type I transmembrane receptors, which are characterized by an extracellular leucine-rich repeat (LRR) domain and an intracellular Toll/IL-1 receptor (TIR) domain. The prototypic receptor Toll was first identified in the fruit fly *Drosophila*. Since then 10 TLR's have been identified in humans and 13 in mice (Janeway, 2006). TLR's are a type of PRR's. Each of the TLR receptors recognizes a small range of conserved molecules from a group of pathogens. These molecules are called pathogen-associated molecular patterns (PAMPs). These PAMPs are common molecular motifs found on pathogens. Toll-like receptors recognize these specific patterns and regulate the activation of both innate and adaptive immunity (Hirschfeld et al., 2001).

Ligands for the TLR's have been established although not fully and are shown in table 1.2. After recognition of microbial pathogens TLRs trigger intracellular signaling pathways that result in the induction of inflammatory cytokines, IFN, and chemokines. TLRs also activate a common signaling pathway that results in the

induction of inflammatory cytokines such as TNFα, IL-6, IL-1β, and IL-12. Macrophages phagocytose bacteria into a phagosome to be degraded. In the absence of TLRs or one of its adaptor molecules MyD88, internalisation of bacteria was delayed and degradation of bacteria was less efficient (Watts, 2004). In macrophages and dendritic cells, the pathogen is exposed to the TLRs. In this way, the TLRs identify the nature of the pathogen and turn on an effector response appropriate for dealing with it. These signaling cascades lead to the expression of various cytokine genes.

Table 1.2 Toll-like Receptor Ligands

Receptor	Ligand(s)	Adaptor(s)	Location
TLR1	Triacyl lipoproteins	MyD88/Mal	Cell surface
TLR2	Lipoproteins, gram-positive peptidoglycan, lipoteichoic acids, fungi, viral glycoproteins	MyD88/Mal	Cell surface
TLR3	DS-RNA, poly I:C	TRIF	Cell compartment
TLR4	LPS, viral glycoproteins, HSP60	MyD88/MAL/TRIF/TRAM	Cell surface
TLR5	Flagellin	MyD88	Cell surface
TLR6	Diacyl lipoproteins	MyD88/Mal	Cell surface
TLR7	Small synthetic compounds, ss-RNA	MyD88	Cell compartment
TLR8	Small synthetic compounds, ss-RNA	MyD88	Cell compartment
TLR9	Unmethylated CpG DNA	MyD88	Cell compartment

TLR 2:

TLR 2 is expressed on microglia, schwann cells, monocytes, macrophage, dendritic cells, polymorphonuclear leukocytes, B and T cells including Tregs. TLR2, in association with TLR1 or TLR6, recognizes various bacterial components, including peptidoglycan, lipopeptide and lipoprotein of Gram-positive bacteria and mycoplasma lipopeptide. There are five subfamilies within the TLR family. The TLR2 subfamily is composed of TLR1, TLR2, TLR6, and TLR10 (Takeuchi et al., 1999). TLR2 recognizes several atypical types of LPS from *Leptospira interrogans* and *Porphyromonas gingivalis*, in contrast to TLR4, which recognizes LPSs from enterobacteria such as *Escherichia coli* and *Salmonella* spp (Hirschfeld et al., 2001). The properties of the atypical LPSs recognized by TLR2 differ structurally and functionally from the enterobacteria LPS recognized by TLR4.

One aspect of TLR2 ligand recognition involves cooperation with other TLR family members, in particular TLR6 and TLR1, which confer discrimination among different microbial components. TLR2 and TLR6 co-immunoprecipitate, suggesting that they physically interact in the cell (Ozinsky et al., 2000). TLR1 has also been reported to associate with TLR2. TLR1/TLR2 heterodimers recognize a variety of bacterial lipopeptides, including the 19 kDa mycobacterial lipoprotein (Takeuchi et al., 2002), meningococcal lipoproteins (Wyllie et al., 2000), and the synthetic lipoprotein structure PAM3CSK4 (Takeuchi et al., 2001). TLR6/TLR2 heterodimers recognize mycoplasma lipoproteins (MALP) and, potentially, peptidoglycan (Imler and Hoffmann, 2001).

CD14, a co-receptor required for LPS recognition and down-stream effects mediated by TLR4 has also been shown to be required for signaling induced by TLR2 ligands. In the case of TLR2, the presence of CD14 enhances the efficiency of recognition by TLR2 to many ligands (Werts et al., 2001). There are a variety of cytokines produced by TLR2 signalling, including TNFα, IL-2, IL-6, IL-12 and MIP-2. TLR2 mediated cellular activation may play a role in protection from pathogens that contain specific TLR2 ligands. For example, TLR2 knockout mice were shown to be more prone to morbidity and mortality due to pneumococcal meningitis. Higher expression of TLR2 was also found in both Crohns disease and ulcerative colitis suggesting that an abnormal mechanism may provide an excess of inflammatory mediators during the active phases of IBD (Canto et al., 2006).

TLR 4:

TLR4 is expressed on monocytes, macrophage, dendritic cells, B cells, mast cells, epithelial cells and endothelial cells. TLR4 is the best studied of the family of receptors. It binds to LPS, as well as host protein molecules released at site of infection and damage, e.g. heat shock protein 60 (HSP60). HSPs such as HSP60 and HSP70 are normally intracellular proteins, but can also become "danger" molecules when released under stress or following excessive apoptosis. They activate macrophages via TLR-2 and TLR-4 to secrete pro-inflammatory cytokines such as TNF-α and IL-12, and to over-express co-stimulatory molecules on APCs (Smiley et al., 2001).

The recognition of LPS requires other molecules in addition to TLR4. LPS binds to LPS-binding protein, and this LPS–LPS-binding protein complex is subsequently recognized by CD14, a glycosylphosphatidylinositol-anchored molecule preferentially

expressed in macrophages and neutrophils. LPS stimulation is followed by increased physical proximity between CD14 and TLR4 (Da Shilva et al, 2001). MD-2 another required molecule associates with the extracellular portion of TLR4 and enhances LPS responsiveness (Akashi et al., 2000). In IBD patients levels of expression of TLR4 are higher than those in normal patients also suggesting like in TLR2 that an abnormal mechanism may provide an excess of inflammatory mediators during the active phases of IBD (Canto et al., 2006). Polymorphisms have also been studied in TLR4 with levels of polymorphs higher in ulcerative colitis and Crohns disease patients compared to control population (Canto et al., 2006). This could account for the persistence of the disease within families.

TLR Signaling

The activation of TLR signaling pathways originates from the cytoplasmic TIR domains. In the signaling pathway downstream of the TIR domain, a TIR domain-containing adaptor, MyD88, was first characterized to play a crucial role. In addition, recent accumulating evidence indicates that TLR signaling pathways consist, at least, of a MyD88-dependent pathway that is common to all TLRs, and a MyD88-independent pathway that is peculiar to the TLR3- and TLR4 signaling pathways(Akira et al., 2001). (See fig 1.0 for signalling pathways).

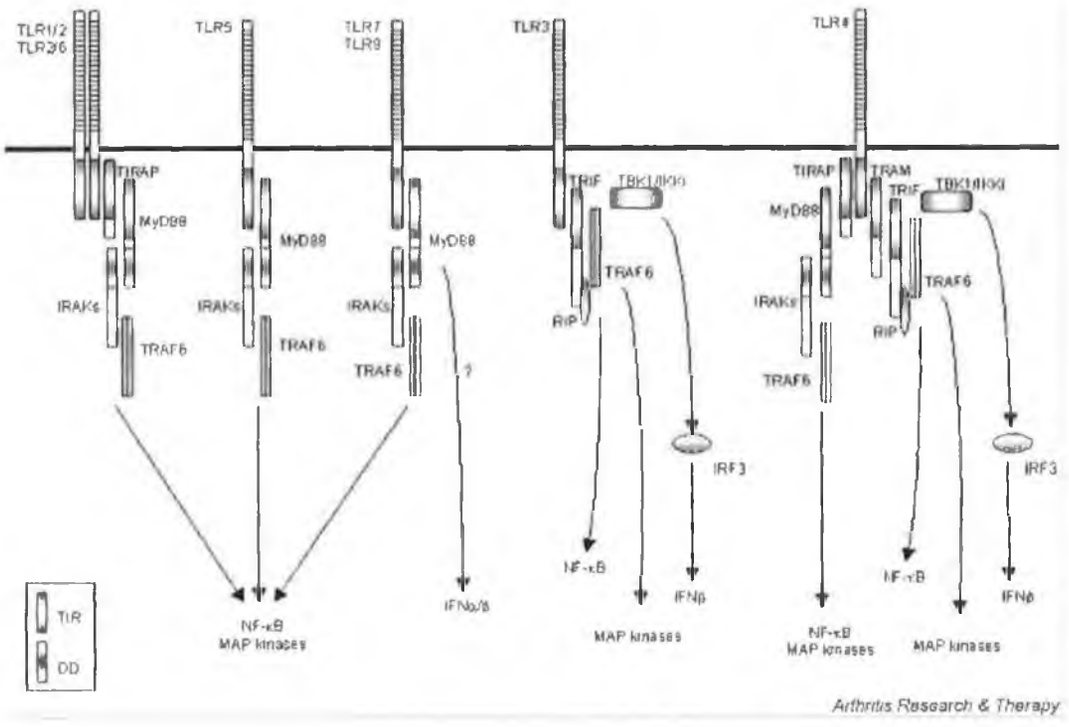

Fig 1.0 TLR pathways (taken from Arthritis Research and Therapy)

MyD88 Dependent Pathway

MyD88 possesses the TIR domain in the C-terminal portion, and a death domain in the N-terminal portion. MyD88 associates with the TIR domain of TLRs. Upon stimulation, MyD88 recruits IL-1 receptor-associated kinase (IRAK) to TLRs through interaction of the death domains of both molecules. IRAK is activated by phosphorylation and then associates with TRAF6, leading to the activation of two distinct signaling pathways, and finally to the activation of JNK and NF-κB as shown in fig 1.0. MyD88 knockout mice showed no responses to the TLR4 ligand LPS in terms of macrophage production of inflammatory mediators, B cell proliferation, or endotoxin shock (Kawai et al., 1999). The cellular responses to the TLR2 ligands peptidoglycan and lipoproteins were abolished in MyD88 knockout mice (Takeuchi et al., 2000). An alternatively spliced variant of MyD88, MyD88s, which lacks the

intermediate domain, has been shown to be induced by LPS stimulation and to inhibit LPS-induced NF-_B activation through inhibition of IRAK activity (Burns et al., 2003, Janssens et al., 2002). Thus, MyD88s may negatively regulate the inflammatory responses triggered by LPS.

MyD88 Independent Pathway

MyD88 knockout mice did not show any production of inflammatory cytokines, such as TNF-α and IL-12, in response to any of the TLR ligands. Studies demonstrated that there is a MyD88-independent pathway as well as a MyD88-dependent pathway in TLR signaling. In the MyD88-independent pathway, LPS stimulation leads to activation of the transcription factor IRF-3, and thereby induces IFN-γ. IFN-γ, in turn, activates Stat1, leading to the induction of several IFN-inducible genes (Doyle et al., 2002, Hoshino et al., 2002, Toshchakov et al., 2002). In addition to the TLR4 ligand, the TLR3 ligand dsRNA has been shown to induce activation of NF-κB in MyD88 knockout cells (Alexopoulou et al., 2001). IRF-3 also associates with IKK's, which induce NF-κB through phosphorylation.

1.8 Mitogen Activated Protein Kinases

MAP kinases, currently numbering over a dozen, have provided a focal point for remarkably rapid advances in our understanding of the control of cellular events by receptors for growth factors and cytokines. Mitogen-activated protein (MAP) Kinases are serine / threonine specific protein kinases that respond to extracellular stimuli (mitogens) and regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival / apoptosis (Akira et al, 2001). Extra-cellular stimuli

lead to activation of a MAP kinase via a signaling cascade composed of MAP kinase, MAP kinase kinase (MKK), and MAP kinase kinase kinase (MKKK). Negative regulation of MAPK activity is effected primarily by MAPK phosphatases (MKPs). Four distinct groups of MAPK's have been characterised in mammals. Extra-cellular signal regulated kinases (ERKs) (Valledor et al., 2000). The ERKs also known as classical MAP kinases signaling pathway are preferentially activated in response to growth factors and regulates cell proliferation and cell differentiation. c-Jun N-terminal kinases (JNKs), also known as stress activated protein kinases (SAPKs). p38 Isoforms, both JNK and p38 signaling pathways are responsive to stress stimuli such as cytokines, UV irradiation, heat shock, and osmotic shock and are involved in cell differentiation and apoptosis. ERK5 has been the recent MAPK found and is activated by growth factors and by stress stimuli and it participates in cell proliferation (Valledor et al., 2000). ERK1, ERK2, JNK, and P38 are all involved in TNFα biosynthesis. All three families of MAPKs have been shown to be activated in macrophages using a variety of stimuli.

TLR4 the signaling receptor for LPS requires the association of TLR 4 with another molecule, MD-2, required for LPS recognition (Akashi et al., 2000). This association with MD-2 is essential for activation of MAPKs and phosphorylation of the transcription factor Elk-1 (Yang et al., 2000). TNFα can also induce activation of macrophage through binding to its receptors (TNFR1 and TNFR2). This binding initiates the association of TNF receptor associated proteins to the cytoplasmic end of TNF receptors leading to the induction of either a death pathway or a survival pathway. (See fig 1.1). When cells are stimulated with activating factors, such as LPS,

macrophages stop proliferating and produce pro-inflammatory cytokines (Valledor et al., 2000).

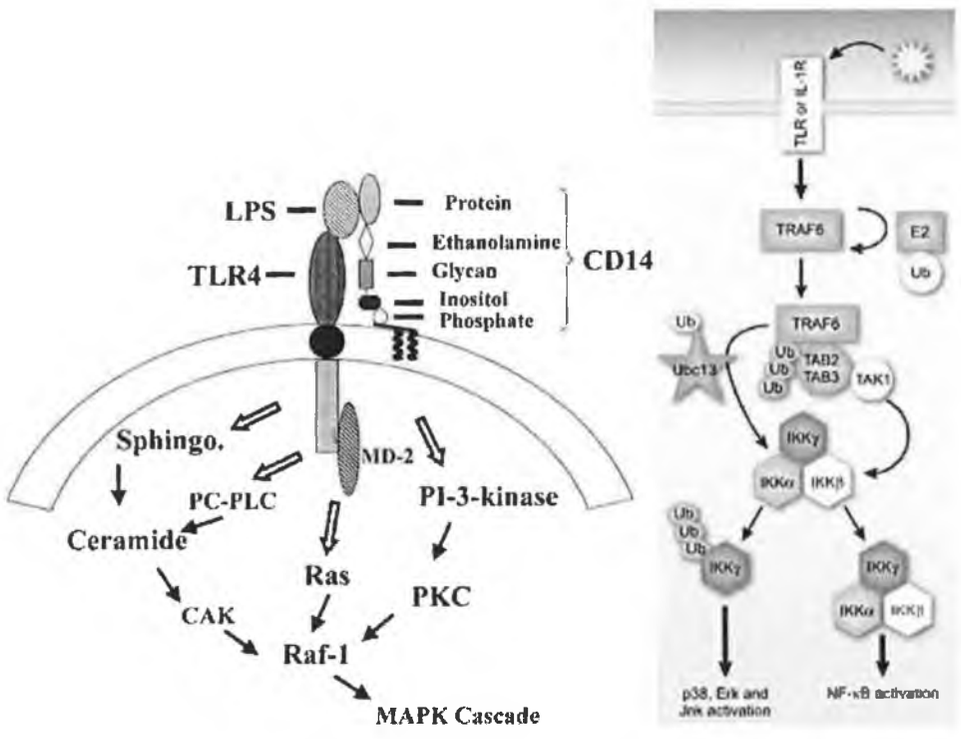

Fig 1.1 MAPK Pathways

ERK1 and ERK2 Cascade:

ERK1 and ERK2 are proteins of 43 and 41 kDa that are nearly 85% identical overall, with much greater identity in the core regions involved in binding of substrates (Boulton et al., 1990, Schieven, 2005). Tyrosine and threonine are phosphorylated to activate the kinases. They are activated by serum, growth factors, cytokines, certain stresses and ligands for G protein-coupled receptors (GPCRs). ERK1 and ERK2 are activated by a MEKS, MEK1 and MEK2 (Crews et al., 1992, Kosako et al., 1992). Both of these MEKs have been shown to fully activate ERK1/2 *in vitro* (Robinson et al., 1996, Zheng and Guan, 1993). Raf isoforms and Mos (MEKK's) are the only

phosphorylators of MEKs in a single cascade. These proteins appear to phosphorylate only two MEK family members, MEK1 and MEK2, placing these MEKKs exclusively in the ERK1/2 MAP kinase cascade (Dent et al., 1992, Force et al., 1994, Kyriakis et al., 1992).

Perhaps the most well defined signaling pathway from the cell membrane to ERK1 and ERK2 is that used by receptor tyrosine kinases (Hunter, 1995, Pawson and Scott, 1997). The cascade plays a central role in the induction of processes such as proliferation, differentiation, development, and under certain conditions also in cell survival, learning, migration and apoptosis. The cascade is first activated by an extracellular stimuli binding to receptors present on the cell surface. Stimulation of these receptors by the appropriate ligand results in an increase in receptor catalytic activity and subsequent autophosphorylation on tyrosine residues. Phosphorylation of these receptors results in the formation of multiprotein complexes whose organization dictates further downstream signaling events (Akira et al, 2001). The signaling within the cell is initiated by activation of small G proteins (e.g. Ras), which transmit the signal further by recruiting the MAP3K tier Raf kinases to the plasma membrane, where they are activated. This is achieved by the recruitment of adaptor proteins, such as Shc and Grb2, to the receptor through interactions between their SH2 domains and phosphotyrosine residues. (Fig 1.2). The guanine nucleotide exchange factor (GEF) then becomes engaged with the complex and induces Ras to exchange GDP for GTP. GTP-ligand bound Ras is capable of directly interacting with a number of effectors, including Raf isoforms, of which the best characterized is Raf-1 (Dent et al., 1992, Moodie et al., 1993). Localization of Raf to the plasma membrane also allows protein kinases such as Src, PKC, and PAK to further modify RAF to increase its activity (Diaz et al., 1997).

ERK activation has been shown to promote IL-10 production and regulate IL-12 production. Studies using ERK inhibitors have shown that ERK activation suppresses IL-12 production in macrophages (Feng et al., 1999), and dendritic cells (Puig-Kroger et al., 2001). ERK activation has been shown to also be essential for IL-10 production in dendritic cells, as inhibition of ERK completely abrogated IL-10 production (Loscher et al., 2005).

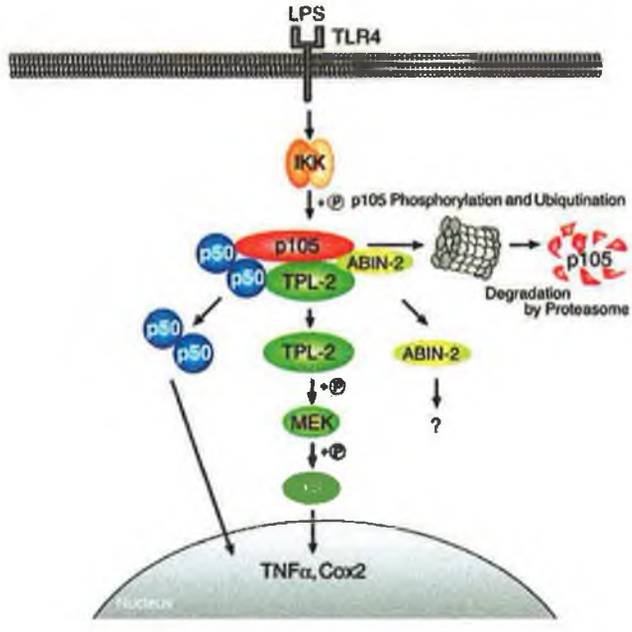

Fig 1.2 ERK Cascade (Taken from www.biochemj.org)

c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK)

JNK/SAPKs are activated upon phosphorylation of two sites, a tyrosine and threonine, like other MAP kinases (Derijard et al., 1994). They are activated by inflammatory cytokines such as IL-1, lymphotoxin-β, TNF, and TGF-β, certain ligands for GPCRs, agents that interfere with DNA and protein synthesis, many other stresses, and to some extent by serum, growth factors, and transforming agents. (Fig 1.3). JNKs are important in controlling programmed cell death or apoptosis. (Derijard et al., 1994). JNK proteins are involved in cytokine production, the inflammatory response, stress-induced and developmentally programmed apoptosis, actin reorganization, cell transformation and metabolism.

JNK has been shown to be involved in IL-1β production. The inhibition of JNKs enhances chemotherapy-induced inhibition of tumor cell growth, suggesting that JNKs may provide a molecular target for the treatment of cancer. JNK inhibitors have also shown promise in animal models for the treatment of rheumatoid arthritis. A selective JNK inhibitor, SP600125, has been found to be mildly anti-inflammatory in the rat adjuvant-induced arthritis, but provides increased protection against bone and cartilage destruction (Han et al., 2001). Of the JNK isoforms, JNK2 is particularly important in arthritis because it is the dominant isoform expressed in synoviocytes. A study utilising an inhibitor which blocked all three isoforms of JNK, marked bone protection was seen in the rat adjuvant arthritis model together with decreased synovial AP1 activation and collagenase-3 gene expression (Hammaker et al., 2003). A JNK2 specific inhibitor in collagen induced arthritis using JNK2 knockout mice showed mild joint protection and a modest decrease in cartilage and bone damage. An

inhibitor which blocks both JNK1 and JNK2 is needed to significantly reduce damage (Hammaker et al., 2003). Two of the MEK family members have been implicated in the JNK/SAPK pathways, these are MKK4 and MKK7. Both MKK4 and MKK7 have the ability to phosphorylate p38 family members *in vitro* and when over-expressed, although JNK/SAPKs are the preferred substrates.

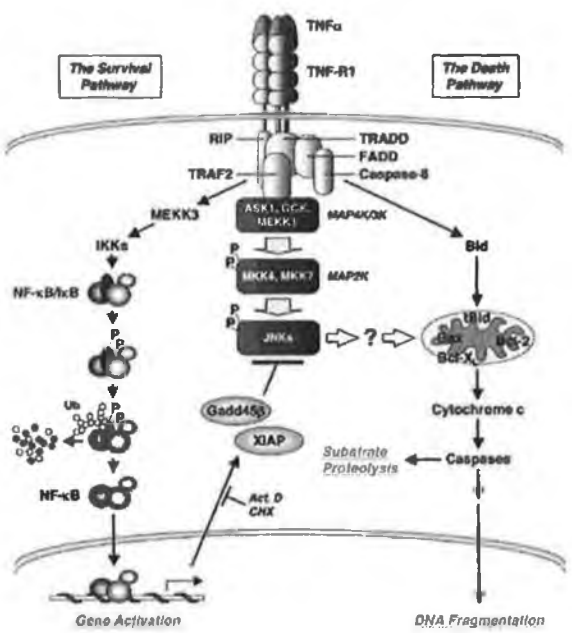

Fig. 1.3 JNK/SAPK Pathway (taken from www.the-scientist.com)

p38

p38 MAP kinase has four isoforms (α,β,γ,δ) and plays an especially important role in the production of cytokines such as interleukin (IL)-1, tumour necrosis factor α (TNFα) and IL-6. p38α was discovered independently in three contexts. It was found as a tyrosine phosphoprotein present in extracts of cells treated with inflammatory cytokines as the target of a pyridinyl imidazole drug that blocked production of tumor

necrosis factor-α (TNFα) and as such was called cytokine-suppressive anti-inflammatory drug-binding protein or CSBP and as a reactivating kinase for MAP kinase-activated protein (MAPKAP) kinase-2 (Han et al., 1994, Lee et al., 1994, Rouse et al., 1994).

The p38 MAPKs regulate the expression of many cytokines. p38 is activated in immune cells by inflammatory cytokines and has an important role in activation of the immune response. Because the p38 MAPKs are key regulators of inflammatory cytokine expression, they appear to be involved in human diseases such as asthma and autoimmunity. Several MAPK p38 inhibitors have been shown to block the production of interleukin-1 (IL-1), tumour-necrosis factor-α (TNF-α) and other pro-inflammatory cytokines (Ono and Han, 2000). A variety of agents including cytokines, hormones, GPCRs, osmotic and heat shock, and other stresses activate p38 family members. (Fig 1.4).

Two MEK family members, MEK3 and MEK6, have high activity toward p38 MAP kinases (Derijard et al., 1995). MEK3 appears to favour phosphorylation of p38α and MEK6 phosphorylates all p38 members (Enslen et al., 2000). Both will also phosphorylate JNK/SAPK isoforms. MKK3 is especially important for TNFα-induced p38 activation and for the p38-mediated synthesis of IL-12 and IFN-γ key cytokines in the pathogenesis of IBD (Wysk et al., 1999). p38α shows the strongest increase in kinase activity among the MAPKs within the inflamed intestinal mucosa of IBD patients (Waetzig and Schreiber, 2003, Waetzig et al., 2002). Thus, p38 has been suggested to play a key role in intestinal inflammation, cytokine production, and T cell activation in IBD. Various animal models suggest that p38 inhibitors such as

SB203580 attenuate disease activity, mortality or reduced at least partially pro-inflammatory cytokine concentrations (Hove et al., 2002). The tremendous interest in p38 MAPK as a therapeutic target for inflammatory diseases stems from an ever-growing body of data demonstrating the importance of the p38 pathway in the cellular response to inflammatory stimuli and the very broad efficacy of p38 inhibitors in preclinical animal models of disease. The relevant target is considered to be p38α, one of the four isoforms of the enzyme (MAPK14, MAPK11, MAPK13, MAPK12 or p38α, β, δ, γ, respectively). P38α is found in leukocytes, epithelial cells, smooth muscle cells, whereas p38δ is more highly expressed in macrophages and p38γ in skeletal muscle (Schieven, 2005).

At this time, all inhibitors under investigation are dual p38α/β inhibitors. In the collagen-induced arthritis or rat adjuvant arthritis models of RA, p38 inhibitors have been shown to reduce the arthritis score, to reduce the expression of cytokines such as IL-1β, and TNFα (Badger et al., 2001). p38 inhibitors have also been reported to be efficacious in models of pain, IBD and asthma (Badger et al., 2001). The p38 inhibitor SB203580 was found to induce a suppression of TNF-α and an increase in IL-10 and inducing an anti-inflammatory response in the inflamed bowel tissue. This inhibitor also down regulated colitis-induced activation of NFκB(Karin and Lin, 2002).

The regulation of TNFα, a key mediator in the inflammatory process in IBD, is interconnected with MAPK. Other pro-inflammatory cytokines, like IL-16, which is up-regulated in IBD, also activate JNKs and p38 (Krautwald, 1998). p38α, JNK1/2, and ERK1/2 were significantly activated in the inflamed colonic mucosa of IBD

patients, with p38α exhibiting the strongest activation in both Crohns Disease and Ulcerative colitis (Krautwald, 1998).

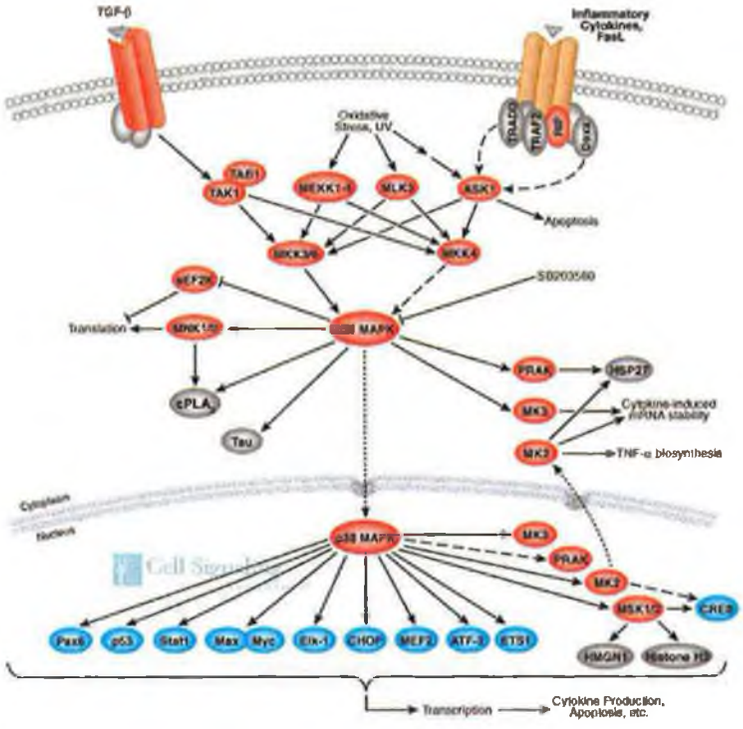

Fig 1.4 P38 MAPK signalling cascade (taken from cell signalling)

1.9 Inflammation and Inflammatory Disease:

Inflammation is the complex biological response of vascular tissues to harmful stimuli. In the absence of inflammation wounds would never heal and tissue destruction would certainly compromise survival of the organism. However, inflammation, which runs unchecked, can also lead to a host of inflammatory

diseases, such as atherosclerosis and rheumatoid arthritis (RA). For this reason inflammation is a tightly controlled process. Inflammation is classified as either acute or chronic. (Janeway, 2006). Acute inflammation is the initial response to harmful stimuli and involves the increased movement of plasma and leukocytes from the blood into the injured tissues.

Neutrophil polymorphs provide the first line of defence of innate immunity to pathogens. Chemokines and eicosanoids cause migration of leukocytes to site of infection, through chemotactic attraction. Neutrophil production in the bone marrow will increase significantly during a period of acute inflammation. Within a few hours, the activated neutrophils will phagocytose pathogens and release inflammatory mediators. (Janeway, 2006). Among these are the macrophage inflammatory proteins (MIP-1α and MIP-1β), which attract circulating macrophages to site of inflammation. These macrophages are activated and have increased phagocytosis and increased secretion of cytokines. Activated macrophages secrete three cytokines, IL-1, IL-6 and TNF-α. All three cytokines induce coagulation. IL-1 induces increased expression of ICAM-1 and VCAM-1. TNF-α and IL-1 also act on macrophage to induce production of chemokines. (Hove et al, 2005)

Chronic inflammation leads to a shift in the type of cells present at site of inflammation and involves the simultaneous destruction and healing of the tissue from the inflammatory process. The accumulation and activation of macrophages is the hallmark of chronic inflammation. Two cytokines in particular IFN-γ and TNF-α play a central role in chronic inflammation. Th1 cells and NK cells release IFN-γ while activated macrophages secrete TNF-α. IFN-γ activates macrophages allowing

increased expression of MHC II molecules and increased cytokine production leading to more effective antigen presentation. (Lewis CE, 1999). In the case of autoimmune diseases pharmaceutical intervention is required, for example patients suffering with ulcerative colitis, corticosteroids are used for reduction in inflammation. Although sometimes effective the side effects can and are severe. They include high blood pressure, onset of diabetes, cataracts and stomach ulcers. These therapies also increase the risk of infection. In nutritional studies the n-3 PUFA in fish oil have been shown to reduce inflammation in patients with ulcerative colitis. For these reasons the interest in inflammation and nutritional therapies has become a major area of research.

1.10 Polyunsaturated Fatty Acids (PUFA)

Dietary polyunsaturated fatty acids (PUFA) have effects on diverse physiological processes impacting normal health and chronic disease, such as the regulation of plasma lipid levels, cardiovascular and immune function, insulin action, and neuronal development and visual function. Ingestion of PUFA will lead to their distribution to virtually every cell in the body with effects on membrane composition and function, eicosanoid synthesis, and signaling as well as the regulation of gene expression. Cell specific lipid metabolism, as well as the expression of fatty acid-regulated transcription factors likely plays an important role in determining how cells respond to changes in PUFA composition.

Structure and Nomenclature:

PUFA belong to the class of simple lipids, as are fatty acids with two or more double bonds in *cis* position. PUFA are named on the number of carbon atoms they contain, the number of double bonds and the location of the first double bond counted from the methyl end of the fatty acid. The location of the first double bond, counted from the methyl end of the fatty acid, is designated by the omega- or n- number. There are two main families of PUFA: n-3 and n-6. These PUFA family are not convertible and have very different biochemical roles. Linoleic acid (n-6) (LA) and alpha-linolenic acid (n-3) (ALA) are two of the main compounds, known as dietary essential fatty acids (EFA) these cannot be synthesized by humans and are as such essential in dietary intake. Conjugated linoleic acid (CLA) is a mixture of positional and geometrical isomers of linoleic acid (C18:2, *cis*-9, *cis*-12), an essential fatty acid for human and animals, and involves a double bond at positions 8 and 10, 9 and 11, and 10 and 12 or 11 and 13.

Sources:

The predominant sources of n-3 PUFA are vegetable oils and fish. Vegetables oils are the major sources of ALA. In particular, ALA is found in the chloroplast of green leafy vegetables, such purslane and spinach, and in seeds of flax, linseed, walnuts, etc. Purslane (*Portulaca olearacea*), is the richest source of ALA of any green leaf vegetable (Simopoulos et al, 1989). Moreover, it is one of the few plants known to be a source of eicosapentaenoic acid (C20:5 n-3). Other sources include nuts and seeds, vegetables and some fruits, which collectively contribute minor quantities of n-3 PUFA to the diet. Fish is the main source of EPA and of docosahexaenoic acid (C22:6 n-3). Vegetables are the main sources of n-6 PUFA. The most important n-6 fatty

acid, LA, is found in large amounts in western diets in corn oil, sunflower oil and soybean oil (Lauritzen et al., 2001). CLA is found naturally in animal tissues and food sources, including ruminant meats, poultry, eggs and dairy products, such as cheeses, milk and yogurt that have undergone heat-processing treatments. The principal dietary isomer of CLA is *cis*-9, *trans*-11 CLA, also known as rumenic acid and RA. This isomer is produced in the rumen of ruminant animals by microbial metabolism of linoleic and linolenic acids. *Cis*-9, *trans*-11 CLA may be absorbed directly or undergo further metabolism. Another CLA isomer, also found in ruminant tissue, is *trans*-10, *cis*-12 CLA. Most of the animal studies to date with CLA have used mixtures of CLA isomers that are mostly *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA in approximately equal amounts.

A decrease in the amount of n-3 PUFA has lead to an imbalance and increase in the ratio of n-6/n-3. Intake of n-3 PUFA is much lower today because of the decrease of fish consumption and the industrial production of animal feeds rich in grains containing n-6 PUFA, leading to production of meat reach in n-6 and poor in n-3 PUFA. The same is true for cultured fish and eggs (Simopoulos and Salem, 1989, van Vliet and Katan, 1990). Even cultivated vegetables contain fewer n-3 PUFA than do plants in the wild (Simopoulos and Salem, 1986).

Effects:

Studies of hunter-gatherer societies indicates that man evolved on a diet that was low in saturated fat and the amounts of n-3 and n-6 PUFA was quite equal. In the last 100-150 years changes have taken place in the food supply, that lead to increases in saturated fat from grain-fed cattle; increases in *trans*-fatty acids from the

hydrogenation of vegetable oils; and enormous increases in n-6 PUFA. The biological functions of the n-6 PUFA are diverse. LA is a structural component in the ceramides of the water barrier of the skin (Lauritzen et al., 2001). AA is the precursor of eicosanoids, the local hormones which participate in physiological and pathophysiological conditions (e.g. parturition initiation, platelet aggregation, renal electrolyte regulation, blastocyte implantation, and activation of immune cells). n-6 PUFA also play a role in signal transduction across cell membranes as second messengers. The understanding of the essentiality of the n-3 PUFA lags behind but is a fast expanding area. The n-3 PUFA can in part substitute for the n-6 PUFA, maybe as a sparring effect, in ameliorating some of the EFAs deficiency symptoms (e.g. growth retardation), but are now considered also to have their own distinct role. The biological functions of dietary n-3 PUFA in the organism are provision of energy and carbon atoms. EPA and DHA also serve as a precursor for n-3 eicosanoids, in general these have a much lower potency than those derived from the n-6 PUFA and are only formed in considerable amounts in tissues at high dietary intakes of EPA and DHA. (Lauritzen et al., 2001).

The effects of n-3 PUFA on the synthesis, bioactivity and metabolic clearance of eicosanoid products accounts for their anti-inflammatory properties. CLA has been shown to modulate the immune system by increasing macrophage killing ability (Hayek et al., 1999). CLA is also proapoptotic, and has anticancer properties.

Eicosanoids:

EFA in the plasma membranes serve as substrates for the enzyme cyclooxygenase (COX) and lipooxygenase (LOX) and are converted into a number of important, very

active, short-lived, hormone-like compounds referred to as "eicosanoids". Eicosanoids are signalling molecules, which are derived from n-3 or n-6 fats. These signaling molecules exert a complex control over many of the bodys systems especially inflammation and immunity. (Lauritzen et al., 2001). They also act as messengers in the central nervous system. The n-6 eicosanoids are generally pro-inflammatory; n-3's are much less so. The amounts of these fats in a person's diet will affect the body's eicosanoid-controlled functions, with effects on cardiovascular disease, triglycerides, blood pressure, and arthritis.

Eicosanoids influence numerous metabolic activities including platelet aggregation, inflammation, hemorrhage, vasoconstriction and vasodilation, blood pressure and immune functions. There are four families of eicosanoids—the prostaglandins, prostacyclins, the thromboxanes and the leukotrienes. For each, there are two or three separate series, derived either from an n-3 or n-6 essential PUFA. (Luster et al., 2005a). These series' different activities largely explain the health effects of n-3 and n-6 fats. Two families of enzymes catalyze fatty acid oxygenation to produce the eicosanoids, cyclooxygenase, or *COX*, which has at least three isoforms, *COX-1, -2, -3* – leading to the prostanoids and, lipoxygenase, in several forms (Luster et al., 2005b). The eicosanoids from AA (Aracadonic Acid) generally promote inflammation. Those from EPA are less inflammatory or even anti-inflammatory. The increased dietary intake of PUFA results in the decreased production of eicosanoid products. Dietary n-3 and GLA counter the inflammatory effects of AA's eicosanoids in three ways along the eicosanoid pathways. *Displacement*—Dietary n-3 decreases tissue concentrations of AA. Animal studies show that increased dietary n-3 results in decreased AA in brain and other tissue. *Competitive inhibition*—DGLA and

EPA compete with AA for access to the cyclooxygenase and lipoxygenase enzymes. So the presence of DGLA and EPA in tissues lowers the output of AA's eicosanoids. *Counteraction*—Some EPA derived eicosanoids counteract their AA derived counterparts.

Anti-inflammatory Effects:

The beneficial effects of n-3 PUFA are derived in part from their effect on the immune system. Metabolism of AA and LA derived from the n-6 PUFA, and of EPA and ALA derived from the n-3 PUFA, leads to the generation of eicosanoids such as prostaglandin's and leukotrienes. The eicosanoids derived from AA and EPA have very similar molecular structures but markedly different biologic effects. For example, the EPA-derived eicosanoids are in general much less potent inducers of inflammation than the AA-derived eicosanoids. Consequently, a predominance of n-6 PUFA will result in a pro-inflammatory status with production of prostaglandin's of the 2 series and leukotrienes of the 4 series. (Luster et al., 2005b). As the relative amount of n-3 PUFA increases, more prostaglandin's of the 3 series and leukotrienes of the 5 series are produced. These eicosanoids are considered to be less inflammatory (Shapiro et al., 1993). A reduction in the amount of the more inflammatory products from AA (PGE2 and LTB4), has been implicated as an underlying mechanism for the anti-inflammatory effects of fish oil (Meydani and Dinarello, 1993).

The immune response also may be altered by changes in the production of immunologic mediators such as cytokines (James et al., 2000). In the case of arthritis supplementation with n-3 PUFA can modulate the expression and activity of

degradative and inflammatory factors that cause cartilage destruction during arthritis. Incorporation of n-3 PUFA into articular cartilage chondrocyte membranes results in a dose-dependent reduction in expression and activity of proteoglycan degrading enzymes (aggrecanases) and expression of inflammation-inducible cytokines (IL-1α and TNF-α) and COX-2, but not the constitutively expressed COX-1 (Curtis et al., 2000).

Studies involving THP-1 monocyte derived macrophages have revealed that DHA suppresses the production of inflammatory cytokines TNFα, IL-1β and IL-6, during LPS stimulation to a greater degree than EPA (Weldon et al., 2007). IL-8, IL-6 and IL-10 expression is lowered by n-3 PUFA and oleate in endothelial cells and monocytes, an effect associated with reduced adhesion molecule expression and lower adhesion capacity of monocytes (Moore et al., 1993). n-3 PUFA particularly EPA have also been shown to decrease IL-12 production, an effect associated with their inhibitory effect on lymphocyte proliferation and decreased expression of IL-12R (Medzhitov, 2001). Pre-treatment of lymphocytes with DHA or EPA (n-3) can reduce the expression of the adhesion molecules L-selectin and LFA-1 (Fiorentino et al., 1991b). Fish oil rich diets (n-3 PUFA) are associated with higher TGF-β1 expression in T cells (Fiorentino et al., 1991a). This growth factor is implicated in immune response modulation.

These findings provide evidence that n-3 PUFA supplementation can specifically affect regulatory mechanisms involved in chondrocyte gene transcription and thus further advocate a beneficial role for dietary fish oil supplementation in alleviation of several of the physiological parameters that cause and propagate arthritic disease. In

Ulcerative Colitis patients LTB4 and PGE2, both products of AA metabolism, are increased. In ulcerative colitis LTB4 is an important mediator of inflammation and has the ability to recruit additional neutrophils from the blood stream into the mucosa, exacerbating the disease process by further increase LTB4 (Stenson et al., 1992). Four months of diet supplementation with fish oil in patients with ulcerative colitis resulted in reductions in rectal dialysate LTB4 levels, improvements in histologic findings and weight gain.

Another important molecule produced by phagocytes is nitric oxide (NO). The production of NO is catalysed in phagocytes by inducible NO-synthase (iNOS), upon cell activation. The transcription of iNOS and the production of NO by macrophages can be inhibited by PUFA. NO is important in the microbicidal function but upon cell hyperstimulation the effects are harmful to the organism resulting in chronic inflammation. PUFAs have an inhibitory effect on phagocytosis but can stimulate the process when directly added to cell cultures these include AA and DHA. n-6 PUFA have been shown to increase IL-1 production whereas n-3 PUFA have shown a decrease (Medzhitov, 2001). CLA has been shown to suppress TNFα, IL-1β, and IL-6 production in IFNγ stimulated RAW macrophages. Animals fed diets rich in n-3 and n-6 PUFA have decreased expression levels of LFA-1, ICAM-1 and CD2 (Bohn and Autenrieth, 1996). Saturated fatty acids induce NFκB activation and COX-2 expression but unsaturated fatty acids inhibit both saturated fatty acid and LPS induced activation of NFκB and COX-2 expression (Fiorentino et al., 1991b). This inhibition of LPS induced NFκB is mediated through suppression of TLR2 and TLR4 signaling pathways (Bohn and Autenrieth, 1996). Suppression of T cell signalling through MAPK pathways has also been elucidated. PUFAs are incorporated into lipid

rafts where they displace LAT (Fiorentino et al., 1991b). This disruption of the subdomain impairs PLCγ and calcium signalling. This reduction in calcium response leads to the interference with the JNK MAPK pathway. The blocking of the pathway interferes with IL-2 production and cell surface expression of IL-2R reducing T cell proliferation.

Chapter 2
Materials and Methods

2.0 Methods and Materials

2.1 Preparation and handling of reagents

Laboratory chemicals and reagents were stored according to manufacturer's instructions. All were of analytical grade and purchased from Sigma-Aldrich Ireland Ltd. (Dublin, Ireland) and BDH Chemicals (VWR International Poole UK) unless otherwise stated. Reagents in powder form were prepared in distilled water (dH_2O), unless otherwise stated. Solid reagents were weighed using an electronic balance Scout Pro (Ohaus Corporation, NY, USA) in disposable plastic weigh-boats. The pH of all solutions was measured using a calibration check microprocessor pH meter (Hanna Instruments Ltd.) calibrated with buffers at pH 4.0, pH 7.0 and pH 10.0. All solutions were stored at room temperature (RT) unless otherwise stated. For transfer of liquid volumes Gilson pipettes were used (Gilson S.A., France). Volumes greater than 1 ml were measured using disposable plastic Pasteur or graduated pipettes (Sarstedt Ltd, Wexford, Ireland). Graduated pipettes were filled using an electronic pipette aid (Drummond, PA, USA). Gloves were used while handling all chemicals and hazardous chemicals were opened only in a fume-hood (1200 standard, chemical systems control Ltd., Ireland).

2.2 Cell culture

All tissue culture was carried out using aseptic techniques in a class II laminar airflow unit (LAF) (Thermo Electron Corporation). The unit was switched on at least 15 min before use and swabbed with 70 % (v/v) ethanol (EtOH) before and after use. Cells were maintained in a 37°C incubator with (5 %) CO_2 (95 %) humidified air (Thermo Electron Corporation OH USA). The LAF and incubator were cleaned thoroughly with 70 % (v/v) EtOH and Vircon (Antec International Suffoc UK) on a weekly basis.

2.2.1 Cell Line

A murine macrophage cell line J774.2 was used exclusively in this study. The J774.2 cell line was a gift from Professor Kingston Mills (Trinity College Dublin, Ireland). The J774.2 cell line was derived from the cell line on the basis of its ability to preferentially metastasise to bone (Lelekakis et al., 1999). The cell line was maintained as a monolayer in Roswell Park Memorial Institute (R.P.M.I) 1640 medium (Gibco-BRL, Paisley, UK), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Gibco-BRL, Paisley, UK), and 2% Penicillin Streptomycin.

2.2.2 Cell line subculture

J774.2 cell monolayers were passaged every three to four days depending on confluency. Cells were examined daily using an inverted microscope (Olympus CKX31, Olympus Corporation, Tokyo, Japan). When cell cultures reached 70-80% confluency they were either used in experiments and / or sub-cultured as follows. RPMI 1640 with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Gibco-BRL, Paisley, UK), and 2% Penicillin-Streptomycin-Glutamine (complete media) was pre-warmed to 37°C. The Flasks were tapped gently to allow the loosely adhered cells to detach from surface of flask into the media. The cell suspension was then transferred to a 50 ml tube using a graduated pipette and centrifuged in a Universal 32R centrifuge (Grant Instruments) at 1200 rpm for 5 min. The supernatant was decanted, and the cell pellet resuspended in 10 ml of complete medium. Cells were counted using a haemocytometer (Hausser Scientific PA USA) and viability assessed by trypan blue dye exclusion (section 2.2.6). Cells were sub-cultured at a ratio of 1:7 or 1:8 depending on confluency by adding 1 ml aliquots of the cell suspension to 19 ml complete RPMI 1640, in a vented 75 cm^2 TC flasks (Nunc Roskilde Denmark).

2.2.3 Preparation of frozen cell stocks

Cell stocks were prepared from cultures that were approximately 70% confluent. Cryoprotectant was 10 % (v/v) dimethylsulphoxide (DMSO Sigma MO USA) and 90 % (v/v) FCS. Cells were harvested as described (2.2.2). A flask of cells was sub-cultured as described in section 2.2.2. The supernatant was removed and the cells were resuspended in 1 ml cryoprotectant and transferred to a 2 ml cryovial (nalgene cryoware, Nunc International). The cryovial was sealed, labelled with permanent marker and placed at -80°C for 24hrs before transferring to a liquid nitrogen tank (Forma Scientific Ltd. Ireland). A large number of master stocks were prepared from the initial cell stock. Secondary stocks were generated from the master stocks and maintained for experimental use. All experiments were carried out with working stocks within thirty passages to reduce experimental error caused by changing cell populations.

2.2.4 Revival of frozen cell lines

Cryovials were carefully removed from the liquid nitrogen tank and then quickly thawed in a water bath at 37°C in order to minimize any damage to cell membranes. The exterior of the cryovial was sterilised with 70 % (v/v) EtOH solution. Cells were then mixed within a sterile 50 ml tube containing 10 ml of ice-cold complete medium by gentle pipetting, and centrifuged at 1200 rpm for 5 min to pellet cells. The supernatant was decanted and the cells were resuspended in 10 ml fresh room temperature complete medium and centrifuged again at 1200rpm for 5 min. This supernatant was decanted and pellet resuspended in 10mls of fresh pre-warmed (37°C) complete media. The cells were then sub-cultured as described in section 2.2.2. Two passages were required before cells could be used in experiments.

2.2.5 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 in colour, when viewed under a microscope. 100µl of cell suspension was mixed with 400µl of trypan blue solution (0.4% (v/v)) in a 1ml eppindorf tube. After ~ 5 min, this solution was applied to a Brightline haemocytometer (Sigma MO USA) and the cells were counted under high-power magnification (×400). Cells inside the central grid containing 25 squares were counted Fig. 2.0. By noting the number of dead cells, the percentage viability of the culture could be determined.

Cell density and % viability were determined using the following formulae:

Cells/ml = (N / 4) x 10 x 10^4

Where, N = mean cell number per field counted, 1 = number of fields counted,

10 = dilution factor, and 10^4 = constant.

% Viability = (number of live cells counted / total number of cells counted) x 100

Only cell preparations with ≥ 90 % viability were used for experiments.

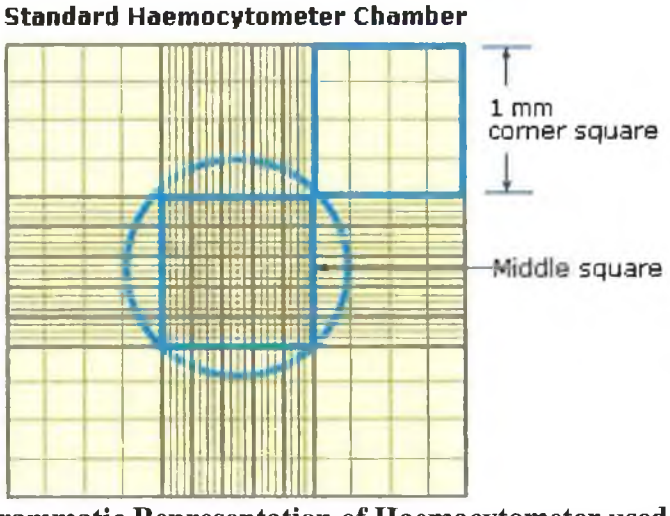

Fig. 2.0 Diagrammatic Representation of Haemocytometer used to count cells

2.3 Protein Analysis

2.3.1 Preparation of cell lysates for western blotting analysis

Preparation of lysates for western blotting from cultured cells was achieved as follows; Cells were plated after cell enumeration and viability at $2x10^6$ cells/well in 6 well plates and stimulated for the required time with Lipopolysaccharide or PAM3CSK4. Supernatants were removed using a pasteur pipette and 100µl sample buffer added. Cells were scraped using a pasteur pipette and transferred to a sterile eppindorf and kept on ice. The cell lysates (on ice) were then sonicated for 60 secs at 40mA with 2.5 pulses/sec. Lysates were finally boiled at 100°C for 5mins. The samples were then either run on a SDS-PAGE gel or stored at -20°C for use at a later date.

2.3.2 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were fractionated by SDS denaturing polyacrylamide gel electrophoresis (SDS PAGE). 10-12 % (w/v) acrylamide gels are suitable for separation of proteins of high molecular weight (20 – 100 kDa). The gel apparatus (E5889 Vertical Electrophoresis Unit, Sigma Chemical Ltd, USA) was cleaned with isopropanol and assembled. 8.3ml bis-acrylamide (30 % (w/v)) (Sigma Ireland), 6.5 ml 1.875 M Tris-HCl pH 8.8, 9.9 ml dH_2O, 250µl SDS (10 % (w/v)), 72 µl TEMED (Sigma Ireland), 250 µl ammonium persulfate (APS) (10 % (w/v)) (Sigma Ireland). The solution was mixed well, (TEMED and APS were added to mixture immediately prior to pouring the gel), and poured between the assembled vertical plates. Isopropanol was layered on top of the gel mix to exclude air and aid polymerization. The gel was allowed to set for approximately 30 mins. Once the gel had set the isopropanol was decanted and

the gel rinsed with dH_2O to remove any remaining isopropanol. A 5 % (w/v) stacking gel containing 650µl bis-acrylamide (30 % (w/v)), (Sigma Ireland), 1.25 ml 1.5 M Tris-HCl pH 6.8, 3ml dH_2O, 50 µl SDS (10 % (w/v)), 30 µl TEMED (Sigma Ireland), and 50 µl APS (Sigma Ireland), (10 % (w/v) was added to the top of the separating gel and a 12-well comb was inserted. The gel was allowed to polymerize for 20 min after which time the comb was removed carefully and the wells washed briefly with dH_2O to remove any un-polymerized material. The wells were straightened using the needle of a 1 ml insulin syringe. 10µl of samples were added to the wells of the polyacrylamide gel and fractionated at 30 mA per gel in 1 × electrode buffer (prepared from a 10 × stock: 15 g Tris, 72 g Glycine, 10 ml SDS (10 % (w/v)), made up to 100 ml with dH_2O), until the bromophenol blue dye front approached the bottom of the gel. Pre-stained protein molecular weight markers (New England Biolabs Ltd.; MA USA) ranging from 7 – 175 kD were added to one well in each gel. These proteins were: 7 kD, aprotinin; 16.5 kD, lysozyme; 25 kD, β-lactoglobulin A; 32.5 kD, triosephosphatase isomerase; 47.5 kD, aldolase; 62 kD, glutamic dehydrogenase; 83 kD, MBP paramyosin; 175 kD, MBP-β-galactosidase.

2.3.3 Protein transfer using iBlot dry blotting system

When the gel was approaching the end of its run, the iBlot dry blotting electrophoresis system was set up. Each protein transfer required a complete iBlot run pack, which contained a bottom anode stack and top cathode stack, filter paper and a disposable sponge. The bottom anode stack package consisted of a copper electrode, nitrocellulose (0.2µM) membrane and a transfer gel layer all contained in a transparent plastic tray, which served as the support for the assembled transfer packs. The top cathode stack consisted of a copper electrode and a top transfer gel layer. The bottom anode stack was removed from its package and kept in its transparent plastic

tray. The tray was then aligned with the gel barriers on the blotting surface of the iBlot. The pre-run gel was placed on the nitrocellulose membrane of the anode stack and a pre-soaked filter paper was placed on the pre-run gels. The blotting roller was then used to remove any air bubbles between the membrane and the gel. The top cathode stack was removed from its packaging and placed on top of the pre-soaked filter paper with the copper electrode side facing upwards. Any bubbles were again removed using the blotting roller. The disposable sponge was then placed on the inner side of the lid of the iBlot, ensuring its brass metal contact is in exact position for contact with the electrical contact on the lid as well as the electrode on the assembled iBlot Gel transfer stacks. Gels were then stained with coomassie blue and membranes stained with poncheau s or immuno-probed.

2.3.4 Coomassie blue staining

Fractionated proteins were visualized by staining with Coomassie Brilliant Blue dye (250 mg Coomassie Brilliant Blue, 45 % (v/v) methanol, 10 % (w/v) glacial acetic acid) to determine if electrophoresis was successful and whether proteins were intact and loaded equally. The gel was immersed in at least 5 volumes of staining solution for approximately 1hr on a rotating platform. The gel was destained (destaining solution: 45 % (v/v) methanol, 10 %(v/v) glacial acetic acid) on a slowly rotating platform for approximately 3-4 h until all protein bands were clearly visible and background staining was fully eliminated. The bands on both the stained gel and the membrane were compared to confirm even loading of protein samples.

2.3.5 Ponceau S staining

After protein transfer the nitrocellulose membrane was stained with Ponceau S (Sigma Ireland) to view quality of protein transfer. The membrane was stained with 20 ml of stain for 5 min on a rotating platform. The transferred proteins were

visualized following several washes with dH_2O to enhance clarity. The protein bands appear pink with ponceau S stain. A longer wash with dH_2O was used to totally remove the stain.

2.3.6 Immunological probing

Membranes were blocked in blocking buffer (5 % (w/v) dried milk in TBS (TBS 137mM Sodium Chloride, 10 mM Tris, pH is 7.4)), for 1 hr at RT unless otherwise stated. Membranes were incubated with the primary antibody (Table 2.1), diluted in blocking buffer to the manufactures specifications on a rotating platform (Bellco Glass model 7744: Bellco Glass Inc., NJ, USA) for 1.5 h. Membranes were washed three times for 5 min each in TBST (TBS containing 0.5 - 1.0 % (v/v) Tween 20 Sigma Ireland) on the rotating platform. The appropriate species-specific horseradish peroxidase (HRP) conjugated secondary antibody (diluted in TBS at 1/2000 unless otherwise stated), was then added for 1 hr. Membranes were washed four times for 5 min each in TBST.

2.3.7 Autoradiography and densitometry

HRP-labeled antibody complexes were visualized using Supersignal West Pico Chemiluminescent substrate kit (Pierce ILL USA) for 5 min on a rotating platform. Excess substrate was decanted; the membrane was sealed in plastic wrap without allowing it to dry out and exposed to X-ray film (Kodak Ltd., Herts, UK) immediately in a dark room under red light. The film was developed using an AGFA CP1000 film processor (Agfa-Gevaert, Morstel, Belgium). Exposure times varied depending on the concentration of protein used and the intensity of signals obtained. Membranes were stored in square petri dishes containing 10ml TBS at 4°C. The X-ray film was photographed and when appropriate the density of bands on the film was calculated

using the densitometry program on the GeneSnap gel analysis and documentation system GeneTools.

2.3.8 Stripping and re-probing membranes

To re-probe membranes antibody complexes were removed by washing membrane in 20 ml stripping solution (0.2 M glycine, 10 % (w/v) SDS, 1 % (v/v) Tween 20, dH_2O to 100 ml, pH 2.2 using 12 M HCl) for 30 min at 56°C in a, followed by six 5 min washes in TBST. The membrane was then blocked and re-probed.

Table 2.1 Immunoblotting antibodies used: antibody, concentration, detection method, and source of antibody

Antibody	Concentration	Detection Method &Concentration	Secondary Antibody	Source
pP38 (Phospho-P38)	1:1000 dilution of antibody	1:2000 dilution of HRP-labeled secondary antibody	Anti-Rabbit	Cell signaling
pERK (Phospho-ERK)	1:1000 dilution of antibody	1:2000 dilution of HRP-labeled secondary antibody	Anti-Mouse	Cell signaling
pJNK (Phospho-JNK)	1:1000 dilution of antibody	1:2000 dilution of HRP-labeled secondary antibody	Anti-Rabbit	Cell signaling
P38 (Total)	1:1000 dilution of antibody	1:2000 dilution of HRP-labeled secondary antibody	Anti-Rabbit	Cell signaling
ERK (Total)	1:1000 dilution of antibody	1:2000 dilution of HRP-labeled secondary antibody	Anti-Rabbit	Cell signaling
JNK (Total)	1:1000 dilution of antibody	1:2000 dilution of HRP-labeled secondary antibody	Anti-Rabbit	Cell signaling

2.4 Enzyme-Linked Immunosorbent Assay (ELISA)

An ELISA is a biochemical technique used to detect the presence of an antibody or antigen/protein in a sample. The ELISA method used in these experiments was the sandwich ELISA for detection of secreted proteins. Samples were generated by first plating cells at a concentration of $1x10^6$/well in a 24 well plate for 24hrs and stimulating each well for the required time with either LPS or PAM3CSK4. Supernatants were then removed to fresh 24 well plates and were either assessed by ELISA straight away or frozen for later use at -20°C. A 96 well ELISA plate was coated with 100µl of a 1:180 dilution of capture antibody in PBS. The plates were incubated overnight at RT. Plates then washed 4X in PBS/Tween. 300µl of blocking buffer (1% BSA/PBS) was added to each well and left for 1hr at RT. Plates were washed again 4X in PBS/Tween. Serial dilutions of standards for each ELISA were prepared (Table 2.3). To each well 50µl of blocking buffer (1% BSA/PBS) was added (unless stated) and 50µl of either standard or sample. Plates were incubated overnight at 4°C. Plates were then washed 4X in PBS/Tween. 100µl of a 1:180 dilution of detection antibody in blocking buffer was added to each well and left to incubate for 2hrs at RT. Plates were washed 4X times in PBS/Tween. 100µl of a 1:200 dilution of Streptavidin-HRP in blocking buffer was added to each well and incubated at RT for 20mins. Plates were washed a final time in four changes of PBS/Tween and 100µl of TMB added to each well. Plates were left at RT until color development and reaction was stopped with 1M H_2SO_4. Plates were read at 450nm in a Versa Max Microplate Reader (Molecular Devices, USA).

2.5 Flow Cytometry

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. Layout and design of cytometer as shown in fig 2.1.

Samples were generated by first plating cells at a concentration of $1x10^6$ cells/ml in a 24 well plate and stimulating for the required time with either LPS or PAM3CSK4. Cells were then scraped using a transfer pipette in the media and transferred to 15ml falcon tubes. Equal amounts of FCS were added to the cells and left at RT for 15mins, to block non-specific binding. Tubes were spun at 1200rpm for 5mins. Supernatents were removed carefully and pellets were resuspended in FACs buffer, approx 1ml per $2x10^6$ cells. 200µl of each resuspended pellet was added to a 96 well plate and spun again for 5mins at 2000rpm. Supernatant was removed from wells and 100µl of antibody was added to each well as required (see table 2.2). Pellets were fully resuspended in antibody solution and incubated at 4°C for 30mins (See table 2.2 for antibodies used in experiments). Plates were spun again at 2000rpm for 5mins, supernatants removed and pellets resuspended in 200µl of FACs buffer. Plates were again spun at 2000rpm for 5 mins. This was repeated a further 3 times. Cells were finally fixed in 200µl of 4% formaldehyde/PBS and transferred to labelled FACs tubes for analysis. Tubes were read on the flow cytometer FACS calibre and data was analysed using Cellquest software (Becton Dickinson, Oxford).

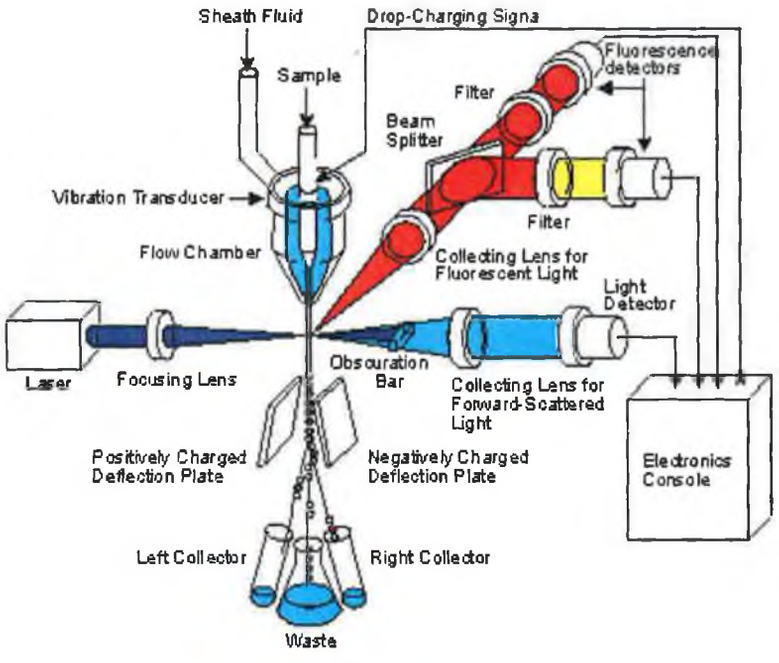

Fig 2.1 Picture taken from http://www.ueb.cas.cz

Table 2.2 Antibodies used in experiments

Antibody	Label	Catalogue Number	Supplier
TLR 2	FITC	11-9021-82	EBioscience
TLR4-MD2	PE	12-9924-82	EBioscience
Rat IgG2a	FITC	11-4321-82	EBioscience
Rat IgG2a	PE	12-4321-82	EBioscience
Rat IgG2b	FITC	11-4031-82	EBioscience
Rat IgG2b	PE	12-4031-83	EBioscience

Table 2.3 Dilutions of antibodies and source of ELISA kits.

Cytokine	Capture Antibody Dilution	Standard Dilution	Detect Antibody Dilution	Source
IL-10	1:180	1:60	1:180	R&D
IL-12p40	1:180	1:35	1:180	R&D
TNF-α	1:180	1:135	1:180	R&D

2.5 Statistical Analysis

The results of the experiment are expressed as the mean +/- the standard error of the mean (SEM). The SEM is the standard deviation of the distribution of sample mean. As sample sizes increase, the SEM. decreases. When the SEM is small, it indicates that the distribution of sample means has less error estimating the true mean. S.E.M. is calculated as the SD of the original sample divided by the square root of the sample size. The data in this study did not need to be normalised and so no transformations were necessary. Statistical significance was determined by analysis of variance (ANOVA), where the number of groups in the experiment was three or more. A probability (P) value of <0.05 was considered to represent a significant difference between the groups. A Newman-Keuls Post Hoc test for non-parametric statistical analysis was necessary after using ANOVA to determine which groups were significantly different to each other. Statistical analysis was computed using GraphPad Prism software.

Chapter 3
Results

3.1 Introduction

Inflammation is the complex biological response of vascular tissues to harmful stimuli. Inflammation, which runs unchecked, can also lead to a host of inflammatory diseases, such as atherosclerosis and rheumatoid arthritis (RA). The accumulation and activation of macrophages is the hallmark of chronic inflammation. In the case of autoimmune diseases pharmaceutical intervention is required, for example in patients suffering with ulcerative colitis, corticosteroids are used to reduce inflammation. While these treatments are quite effective, the side effects can be severe. Nutritional studies have shown the n-3 PUFA, found in fish oil, to reduce inflammation in patients with ulcerative colitis. Several studies show specific effects of these PUFA on inflammatory cytokine production, which may explain some of their beneficial effects. EPA and DHA suppress the production of inflammatory cytokines TNF-α, IL-1β and IL-6, following LPS stimulation of immune cells (Weldon et al., 2007). Another PUFA, CLA, as well as EPA have been shown to decrease IL-12 production in dendritic cells. The mechanisms by which these PUFA exert these effects are still relatively unclear.

Macrophages play an important role in innate immunity. They constantly test the surrounding area for pathogens and engulf them using pseudopods or by the binding of PAMPs present on pathogens, to PRRs on the macrophage cell surface. The most common of these PRRs are the TLRs, which are highly expressed by macrophages. In macrophages, the presence of these TLRs, serve to identify the nature of the pathogen and turn on an effector response appropriate for dealing with it. The resultant signaling cascade leads to the expression of various cytokine genes. Activation of

macrophages results in production of pro-inflammatory cytokines, which if dysregulated, can play a major role in inflammatory disease. The cytokines and factors produced as a result of activation of PPRs can serve to initiate or sustain host responses that are anti-inflammatory (eg, interleukin 10 and transforming growth factor β) or pro-inflammatory (eg, TNFα and interleukin 12).

TLR4 binds LPS as well as heat shock proteins released at site of infection and damage. TLR4 ligation results in the production of inflammatory cytokines such as IL-12 and TNFα. Expression levels of TLR4 have been shown to be enhanced in patients with IBD, suggesting that an abnormal mechanism may provide an excess of inflammatory mediators during the active phases of IBD. Polymorphisms of TLR4 have also been studied with higher levels observed in ulcerative colitis and Crohns disease patients then in normal population (Harris et al, 2006). Furthermore, TLR4 is now believed to play a role in atherosclerosis (Li et al, 2007)

Upon recognition TLR4 triggers intracellular signalling pathways including MAPK pathways ERK, JNK and p38 resulting in The MAPK pathways are one of the intracellular signalling pathways utilised by TLR4. Activation of ERK has been shown to promote IL-10 production and regulate IL-12 production (Yi et al, 2002). JNK activation is involved in IL-1β production. Indeed JNK inhibitors have shown to be mildly anti-inflammatory in a rat model of arthritis (Han et al, 2001). Finally p38 has shown to be involved also in production of IL-1 and TNFα. Inhibitor studies have revealed that inhibition of p38 can reduce expression of cytokines IL-1β and TNFα, and increased production of IL-10 (Foey et al, 1998). Interestingly p38 is increased within the inflamed intestinal mucosal of IBD patients (Waetzig GH et al, 2003).

This study examines the effects of PUFA on LPS induced cytokine production in macrophage as well as expression of TLR4. Furthermore, it investigates the role of MAPK activation in mediating these effects.

3.2 Results

3.2.1 The elucidation of the effects of PUFA concentrations on cell viability.

The concentrations of PUFA used in this study are well established in the laboratory and have been shown to exert effects on immune cell function while maintaining cell viability. To establish that the effects of PUFA we see in our studies are not a result of cell death, we examined the effect of several concentrations of PUFA on cell viability. J774 murine macrophages, were cultured for 7 days in 25μM, 50μM and 100μM concentrations of fatty acids. Supernatants were removed and cell viability assessed using Cell Titre 96 Aqueous One Solution (Promega, WI, USA) as per manufacturer's instructions. The selected concentrations of fatty acids used in all subsequent experiments have no cytotoxic effects on J774 macrophage *in vitro*. These are as follows - DMSO (25μM), CLA (50μM), EPA (25μM), DHA (25μM), LA (25μM), SA (25μM). (Fig 3.1)

3.2.2 Assessment of cytokine production by macrophages to increasing doses of TLR4 ligand.

To determine the correct dose of the TLR4 ligand for this study we cultured J774 murine macrophages in fatty acids (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM) for 7 days, plated them at $1x10^6$ cells/ml, and stimulated them for 24hrs with 10ng/ml, 100ng/ml and 1μg/ml of the TLR4 ligand, LPS. Supernatants were removed after 24hrs and assessed for levels of IL-10 and IL-12p40 using specific immunoassay. Activation of TLR4 by addition of LPS resulted

in production of IL-10 and IL-12p40. Fig 3.2 demonstrates that IL-10 production was enhanced in cells treated with by DHA and CLA but not by EPA. Given the use of 100ng/ml of LPS in a number of published studies and our observation that shows significant activation of TLR4 at this concentration, we used this concentration for the rest of the study.

3.2.3 Effect of PUFA on TLR4 expression

As we were examining the effects of PUFA on TLR4 activation, we felt it was important to look at the concomitant effects on expression of TLR4. J774 murine macrophages were cultured in fatty acids for 7 days (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM), plated at $1x10^6$ per ml and stimulated with 100ng/ml LPS for 0, 6 and 24hrs. Cells were then stained with a fluorescent antibody specific for TLR4 and expression levels were analysed by flow cytometry. Stimulation of J774 macrophages with LPS increased expression of TLR4 at 6hr and 24hr 6hr treated cells (Fig 3.3(A)). When we examined the effects of PUFA on this expression, we observed an increase in TLR4 expression in cells treated with all CLA, EPA and DHA. At 24hr post stimulation, the increase in TLR4 expression persisted for CLA-treated cells, with the other groups showing no change at this time point (Fig 3.3(B)).

3.2.4 PUFA modulate cytokine production by macrophages.

We examined the effects of PUFA on the production of cytokines in response to stimulation with the TLR4 ligand, LPS. J774 murine macrophages were cultured in presence of fatty acids for 7 days (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM), plated at $1x10^6$ per ml and stimulated for 24hrs

with 100ng/ml LPS. Supernatants were removed and assessed for levels of IL-10, IL-12p40 and TNFα using specific immunoassay (Fig 3.4). Treatment of cells with CLA and DHA resulted in a significant increase in production of IL-10 following TLR4 ligation ($p<0.001$, $p<0.01$ respectively). The levels of IL-10 were not significantly affected by the remaining fatty acids. CLA and DHA also suppressed IL-12p40 production ($p<0.001$) with SA also exerting a small but significant effect. All of the fatty acids examined significantly inhibited LPS-induced TNFα production ($p<0.01$, $p<0.001$).

3.2.5 Effects of PUFA on cytokine production by macrophages in the presence of MAPK inhibitors

In order to determine the involvement of MAPK in mediating the effects of PUFA on LPS-induced cytokine production in macrophages, cells were cultured in presence of fatty acids (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM) for 7 days, plated at $1x10^6$ per ml, then specific MAPK inhibitors (ERK U0126, JNK inhibitor 1, p38 SB20210) were added at 5μM, 1hr prior to stimulation with 100ng/ml LPS for 24hrs. Supernatants were removed and assessed for levels of IL-10, IL-12p40 and TNFα using specific immunoassay. Treatment of cells with CLA resulted in increased production of IL-10 in J774 macrophages following LPS stimulation ($p<0.001$; Fig 3.5). This increase was maintained in the presence of the specific JNK inhibitor ($p<0.001$) the levels were dramatically decreased in the presence of the ERK and p38 inhibitors, although the levels still reached statistical significance ($p<0.001$, $p<0.05$). DHA also significantly enhanced TLR4-induced IL-10 production by macrophages ($p<0.01$). This increase was maintained in the presence of specific inhibitors to JNK ($p<0.001$) and also in the presence of inhibitors

to ERK and p38, but to a much lesser extent ($p<0.001$, $p<0.05$). Treatment of cell with CLA resulted in a suppression of LPS-induced IL-12p40 ($p<0.001$; Fig 3.6). This effect was maintained in the presence of the ERK and JNK inhibitors, however inclusion of a specific inhibitor to p38 reversed this suppressive effect. DHA also inhibited LPS-induced IL-12p40 production ($p<0.001$). The inclusion of inhibitors to ERK, p38 and JNK did not reverse this effect. Similar results were obtained for the SA-treated group. All of the fatty acids examined induced a significant decrease in TLR4 induced TNFα production ($p<0.01$; Fig 3.7). This effect persisted in the presence of inhibitors to ERK and JNK, but were reversed by the p38 inhibitor.

3.2.6 Modulation of MAPK pathway by PUFA.

Given that the presence of some MAPK inhibitors were shown to reverse the effects of PUFA on cytokine production in J774 macrophages after LPS stimulation, we investigated whether PUFA enhanced or suppressed activation of these MAPK at a number of time points following activation of the cells with the TLR4 ligand, LPS. Cells were cultured in presence of fatty acids for 7 days (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM) and plated at $1x10^6$ per ml. Cells were stimulated with 100ng/ml LPS for the following time points; 0hr, 5 min, 10 min, 15 min, 30 min, 60 min, 120min, 240 min and 360 min. Cells were then scraped and collected into sample buffer, sonicated and boiled prior to loading on SDS-PAGE gels. Gels were blotted using iBLOT system. All membranes were blocked in 5% Marvel / TBST, then incubated with phosphospecific ERK, p38 or JNK 5% BSA, followed by HRP-conjugated secondary Antibody 5% Marvel / TBST unless otherwise stated. Membranes were then treated with Chemiluminescent

Substrate (Pierce) and exposed for required time and developed using FUJI processor. Blots were then stripped and re-probed for total protein to demonstrate equal loading. Representative blots are shown in the figures. We carried out densitometry using GeneSnap and GeneTool in order to graph the results using arbitary units. All DMSO groups at all time points were assigned a value of 100 and changes in density of the bands calculated accordingly.

3.2.6.1 Modulation of ERK pathway by PUFA

Exposure of J774 macrophages to CLA enhanced activation of ERK following TLR4 ligation. This was evident 5 min, 30 min, 60 min and 120 min after activation with LPS (Fig 3.8, 3.8(A)). Treatment of cells with EPA and DHA did increase ERK activation (except for one time point). Indeed a marked decrease in ERK phosphorylation was observed at most of the time points examined (Fig 3.9, 3.9(A)). In both experiments, the SA treated group inhibited ERK activation at many of the time points

3.2.6.2 Modulation of p38 pathway by PUFA

Treatment of J774 macrophages with CLA was observed to enhance phosphorylation of p38 at a number of time point post LPS stimulation – this was evident at 5 min, 10 min, 15 min and 30 min (Fig 3.10, 3.10(A)). EPA and DHA also enhanced p38 activation with increased levels observed at 5 min, 10 min, 2hr, 4hr and 6hr post LPS stimulation (Fig 3.11, 3.11(A)).

3.2.6.3 Modulation of JNK pathway by PUFA

Treatment of J774 murine macrophages with CLA showed a decrease JNK activity at 0hr, 5 min, 10 min, 15 min, 30 min and 2 hr post LPS stimulation (Fig 3.12, 3.12(A)). EPA had little if any effect on JNK activation, however DHA treated cells displayed enhanced phosphorylation of JNK 5 min, 10 min, 1hr, 2hr and 6hr post stimulation with LPS (Fig 3.13, 3.13(A)).

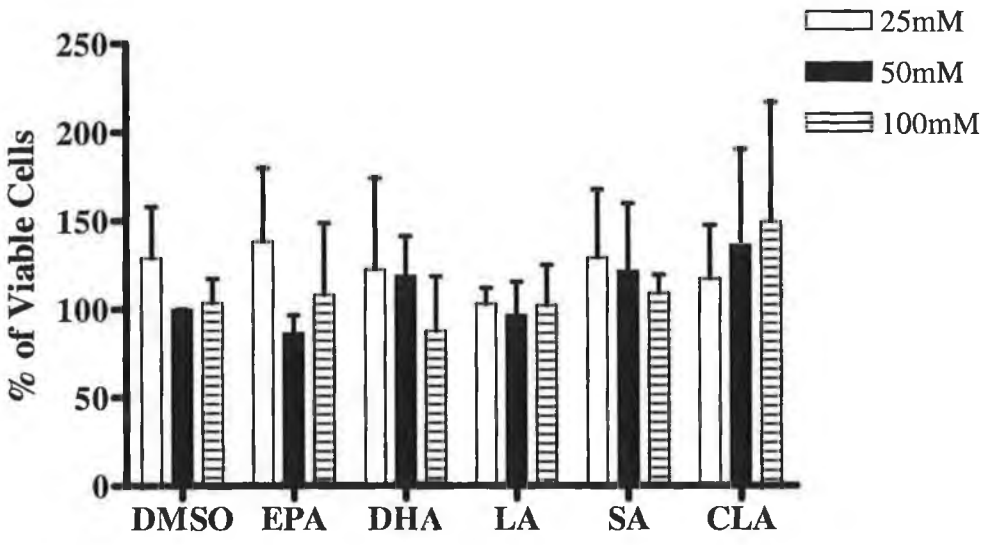

Figure 3.1 Fatty Acid concentrations do not affect viability of J774 cells *in vitro*. J774 cells were cultured in fatty acids for 7 days at either 25mM, 50mM, or 100mM. Supernatants were removed and cell viability assessed using Cell Titre 96 Aqueous One Solution (Promega, WI, USA) as per manufacturer's instructions. Results are expressed as mean +/- SEM. As this experiment was a dose response study comparing the best concentrations eliciting an effect no further statistical analysis was required.

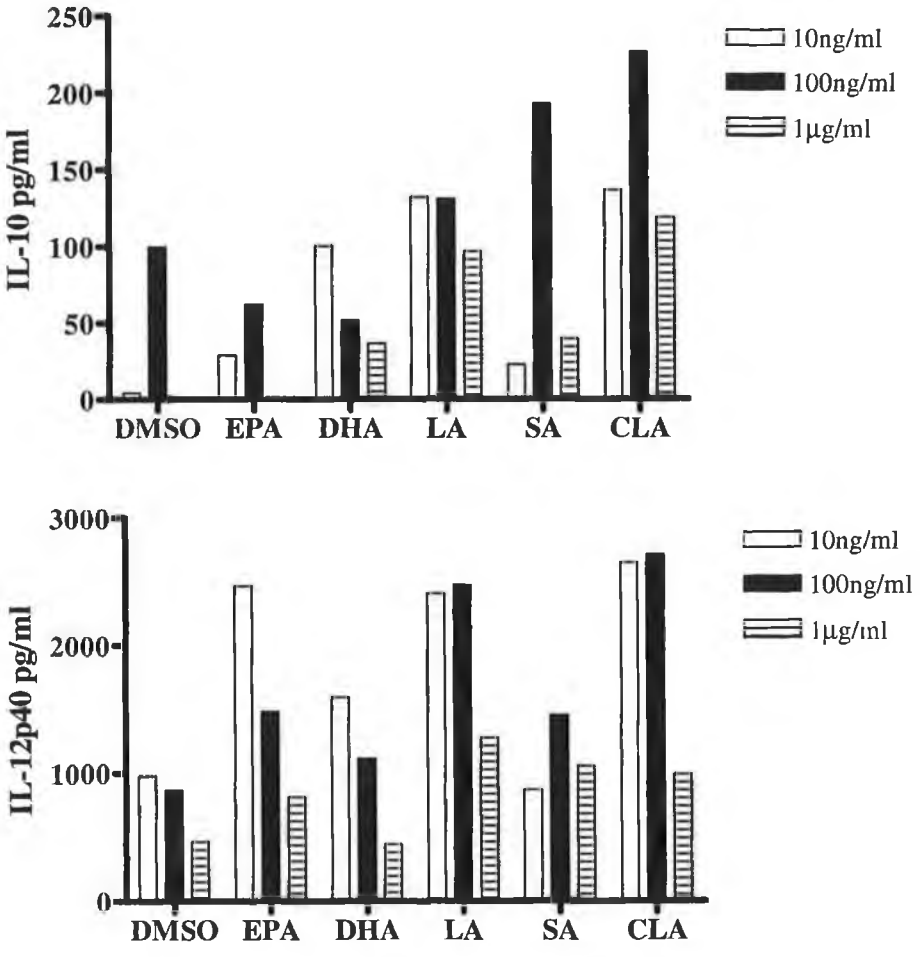

Fig 3.2 Cytokine production by J774 murine macrophages following TLR4 activation. J774 murine macrophages were cultured in fatty acids (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM) for 7 days, plated at $1x10^6$ per ml then stimulated for 24hrs with 10ng/ml, 100ng/ml and 1µg/ml LPS. Supernatants were removed and assessed for levels of IL-10 and IL-12p40 using specific Immunoassay.

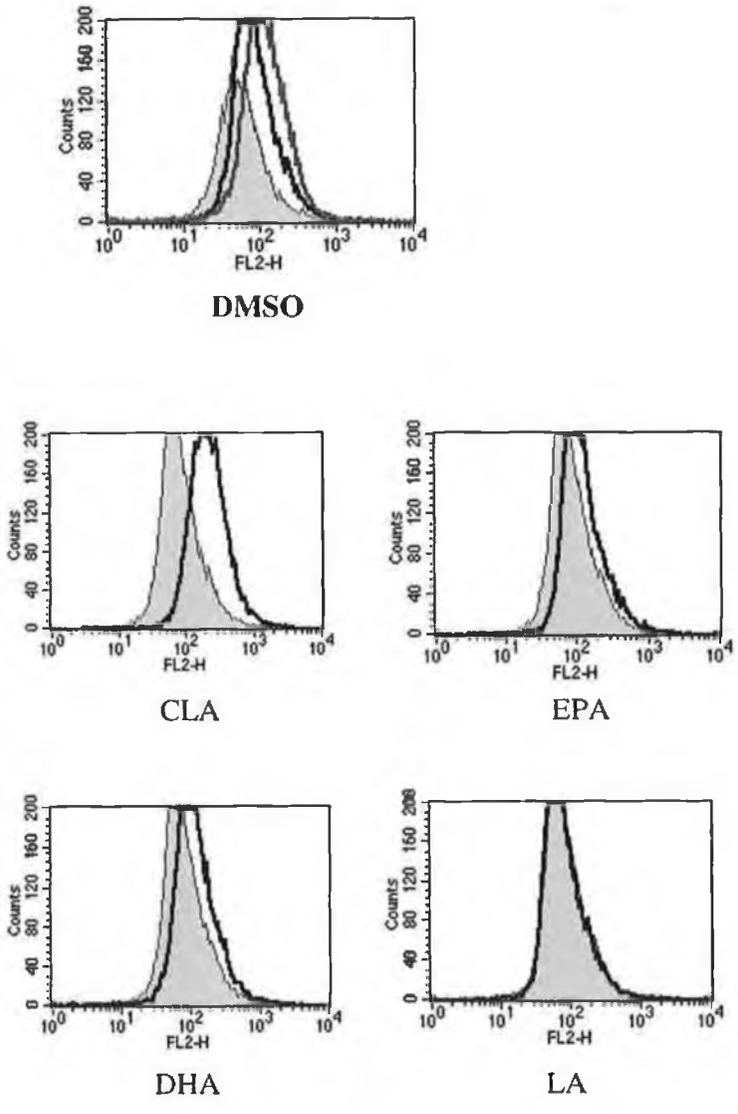

Fig 3.3(A) PUFA modulate TLR4 expression on macrophages. J774 murine macrophages were cultured in fatty acids (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM) for 7 days, plated at $1x10^6$ per ml then stimulated for 6hrs with 100ng/ml LPS. Cells were then stained with antibody specific for TLR4. In DMSO figure, grey (fill) represents DMSO treated cells (no stimulation), black line (no fill) represents DMSO 6hr stimulated cells, grey line (no fill) represents DMSO 24hr stimulated cells. In all other cases grey (Fill) represents DMSO 6hr LPS treated cells, black line (no fill) represents 6hr fatty acid treated cells.

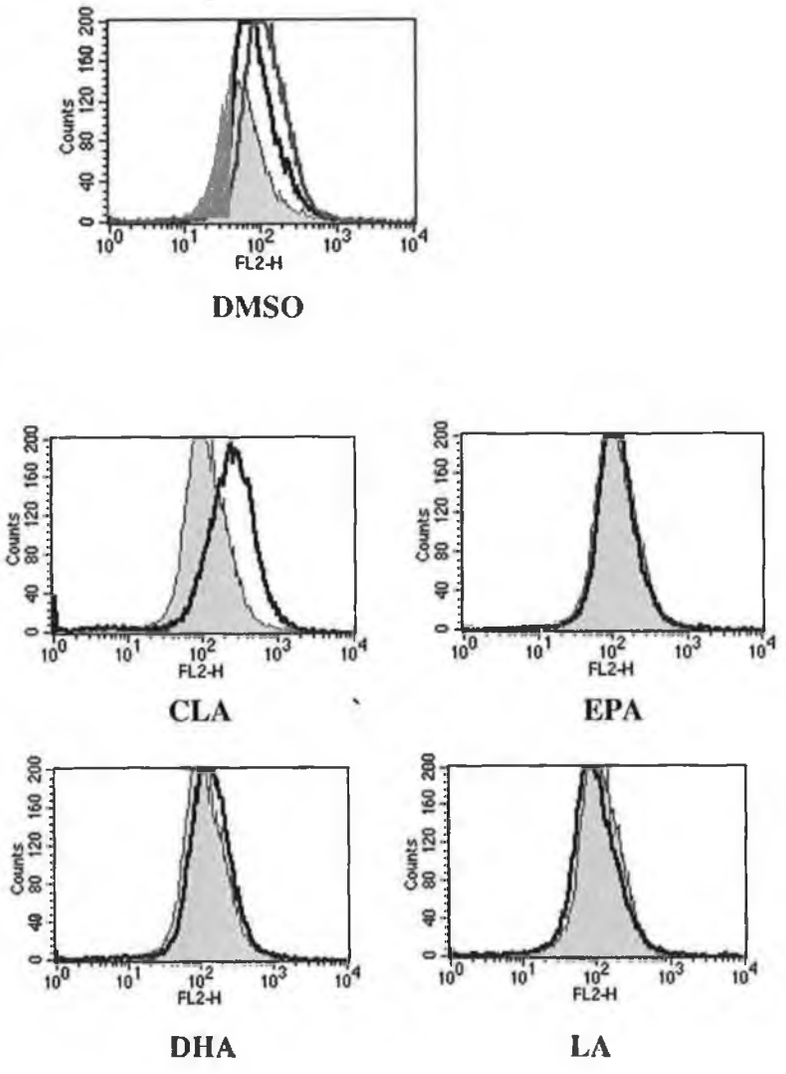

Fig 3.3(B) PUFA modulate TLR4 expression on macrophages. J774 murine macrophages were cultured in fatty acids (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM) for 7 days, plated at $1x10^6$ per ml then stimulated for 24hrs with 100ng/ml LPS. Cells were then stained with antibody specific for TLR4. In DMSO figure, grey (fill) represents DMSO treated cells (no stimulation), black line (no fill) represents DMSO 24hr stimulated cells, grey line (no fill) represents DMSO 24hr stimulated cells. In all other cases Grey (Fill) represents DMSO 24hr LPS treated cells, black line (no fill) represents 6hr fatty acid treated cells.

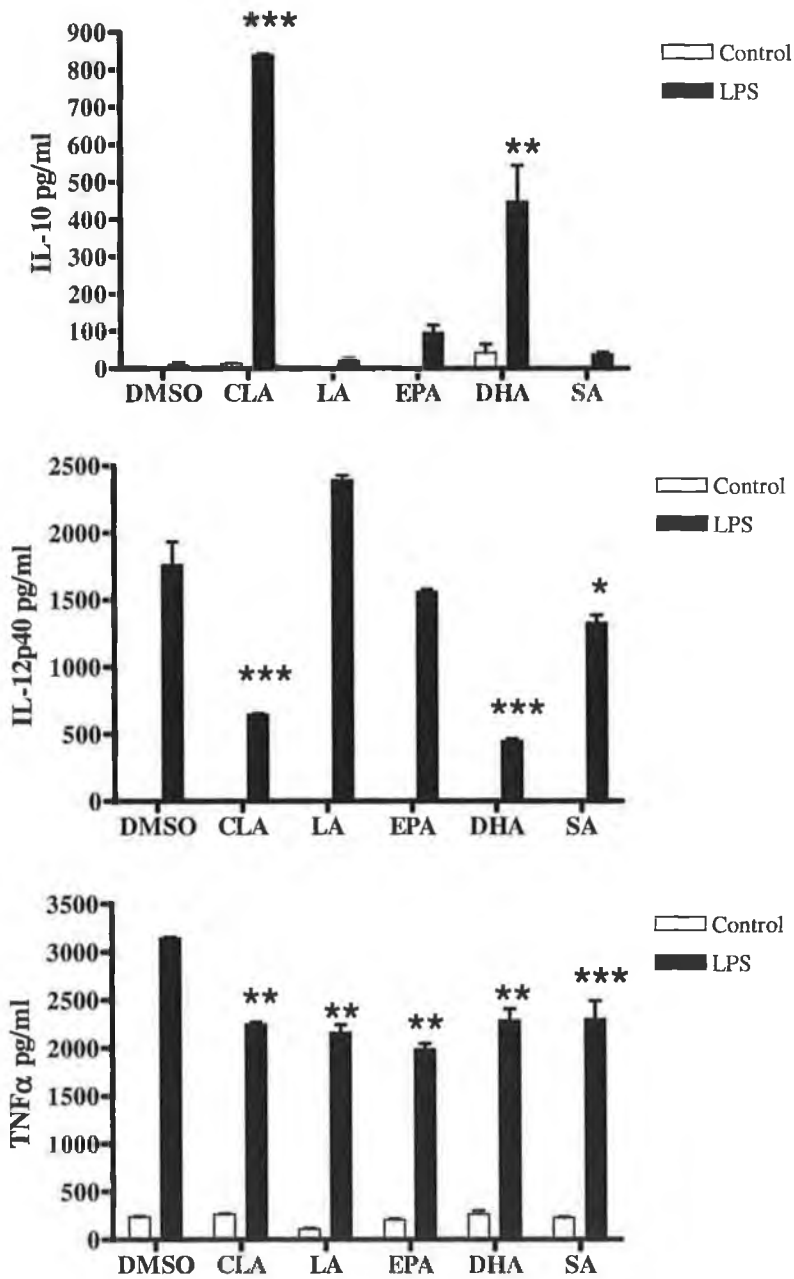

Fig 3.4 PUFA modulate cytokine production by macrophages following TLR4 activation. J774 murine macrophage were cultured in presence of fatty acids (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM) for 7 days, plated at $1x10^6$ per ml and stimulated for 24hrs with 100ng/ml LPS. Supernatants were removed and assessed for levels of IL-10, IL-12p40 and TNFα using specific immunoassay. Results are expressed as mean ±SEM. *p<0.05; **p<0.01; ***p<0.001, ANOVA, comparing DMSO and fatty acid treated groups.

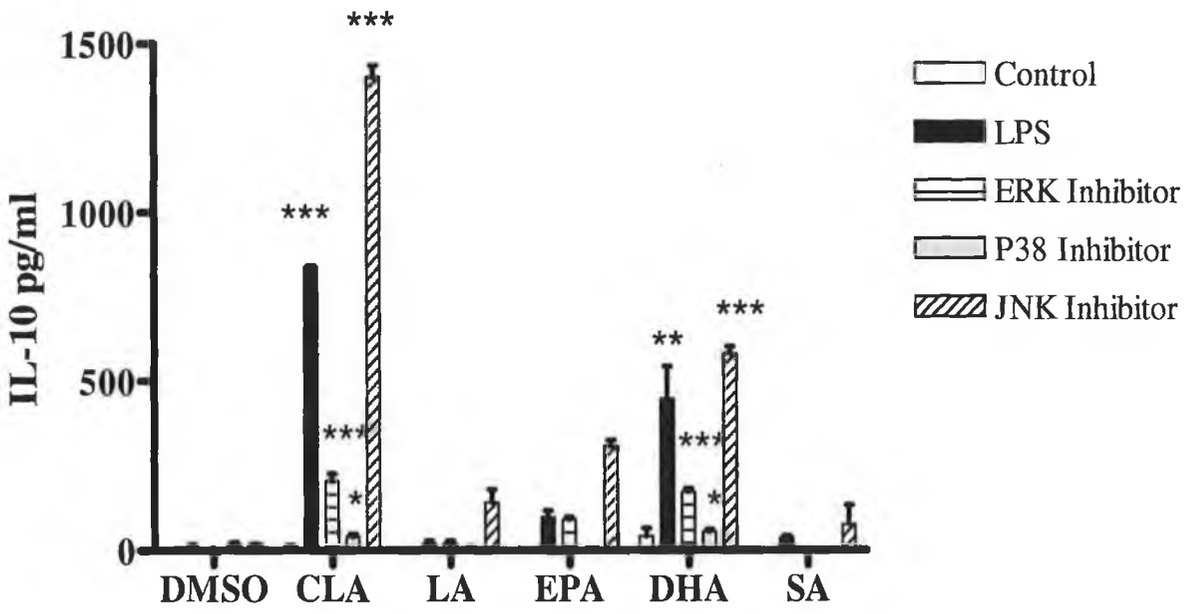

Figure 3.5 The effects of PUFA on IL-10 production by macrophages in the presence of specific MAPK inhibitors. J774 murine macrophages were cultured in the presence of fatty acids for 7 days (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM), plated at $1x10^6$ per ml +/- specific inhibitors (ERK U0126, JNK inhibitor 1, p38 SB20210) at 5μM added 1hr prior to stimulation with 100ng/ml LPS for 24hrs. Supernatants were removed and assessed for levels of IL-10 using specific immunoassay. Results are expressed as mean ±SEM. *$p<0.05$; **$p<0.01$, ***$p<0.001$, ANOVA, comparing DMSO and fatty acid treated groups in the presence and absence of specific inhibitors.

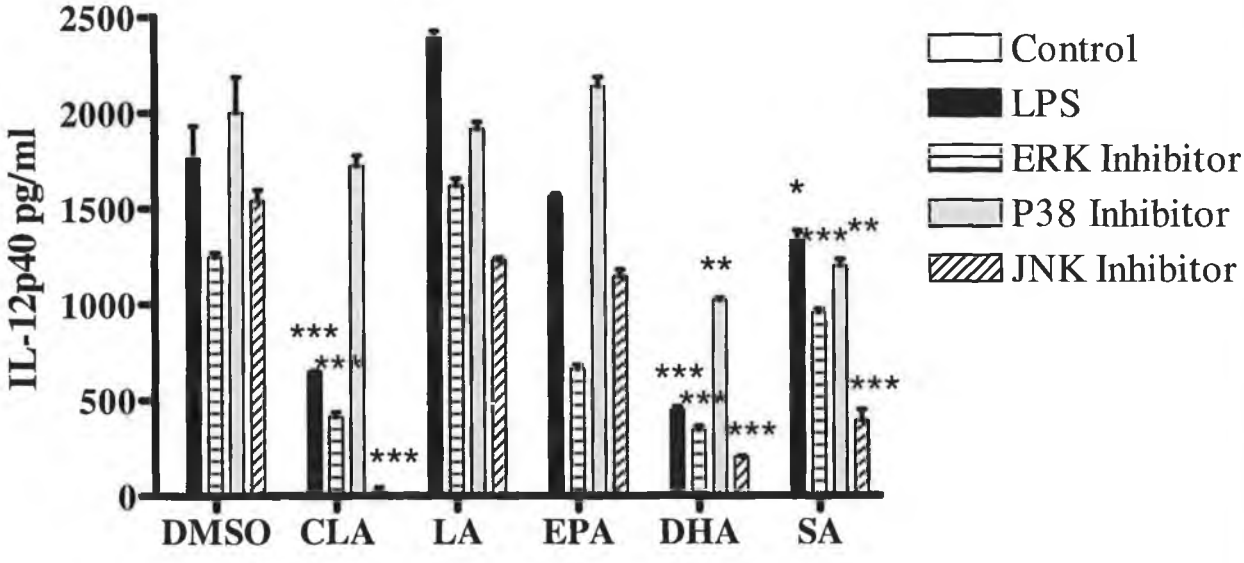

Figure 3.6 The effects of PUFA on IL-12p40 production by macrophages in the presence of specific MAPK inhibitors. J774 murine macrophages were cultured in the presence of fatty acids for 7 days (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM), plated at $1x10^6$ per ml +/- specific inhibitors (ERK U0126, JNK inhibitor 1, p38 SB20210) at 5μM added 1hr prior to stimulation with 100ng/ml LPS for 24hrs. Supernatants were removed and assessed for levels of IL-12p40 using specific immunoassay. Results are expressed as mean ±SEM. *p<0.05; **p<0.01; ***p<0.001, ANOVA, comparing DMSO and fatty acid treated groups in the presence and absence of specific inhibitors.

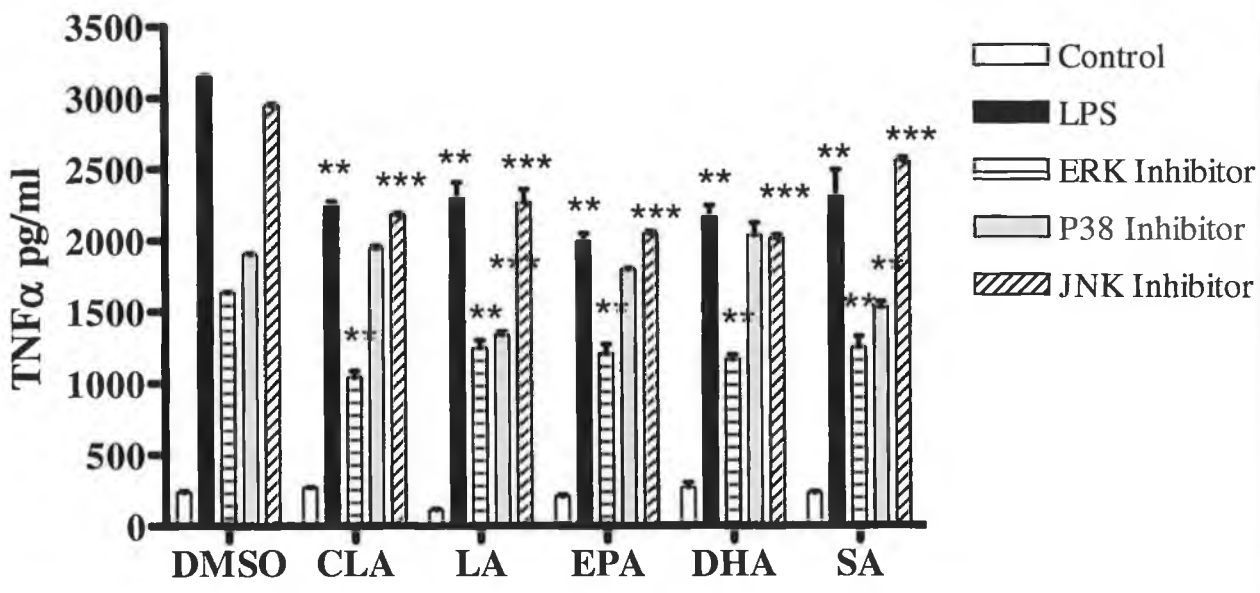

Figure 3.7 The effects of PUFA on TNFα production by macrophages in the presence of specific MAPK inhibitors. J774 murine macrophages were cultured in the presence of fatty acids for 7 days (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM), plated at $1x10^6$ per ml +/- specific inhibitors (ERK U0126, JNK inhibitor 1, p38 SB20210) at 5μM added 1hr prior to stimulation with 100ng/ml LPS for 24hrs. Supernatants were removed and assessed for levels of TNFα using specific immunoassay. Results are expressed as mean ±SEM. **p<0.01; ***p<0.001, ANOVA, comparing DMSO and fatty acid treated groups in the presence and absence of specific inhibitors.

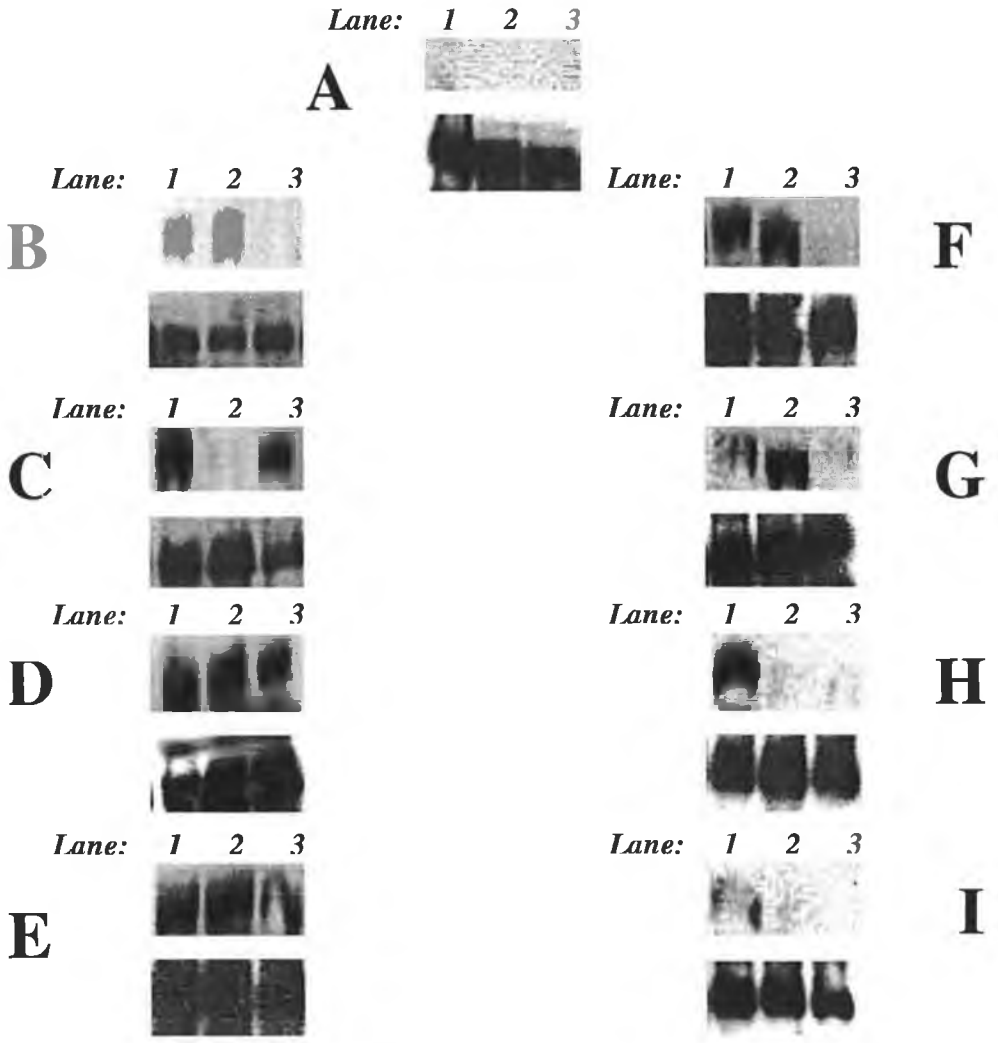

Figure 3.8: CLA modulates activation of ERK in J774 macrophages following TLR4 activation. Cells were cultured with either DMSO, c9, t11-CLA (50uM) or SA (25uM) for 7 days and then stimulated with LPS (100ng/ml) or medium alone. Cells were harvested 0-6h following stimulation and total (bottom panel) and phosphorylated ERK (top panel) was determined in whole cell lysates by western blotting. A representative immunoblot is shown. **(A)** 0 hr time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(B)** 5 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(C)** 10 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(D)** 15 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(E)** 30 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(F)** 60 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(G)** 120 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(H)** 4 hr time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA.
(I) 6hr time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA.

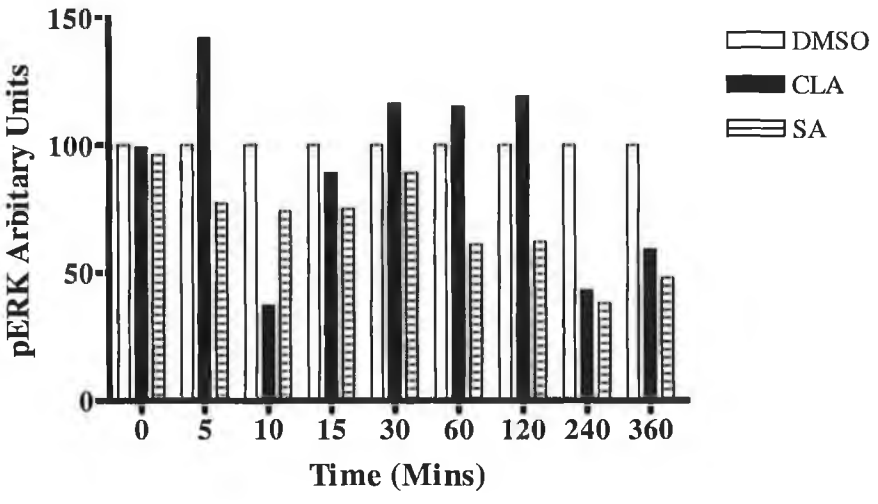

Fig 3.8(A) CLA modulates activation of ERK in J774 macrophages following TLR4 activation. Densitometric analysis was carried out on immunoblots shown in Fig 4.8. pERK is expressed as arbitary units.

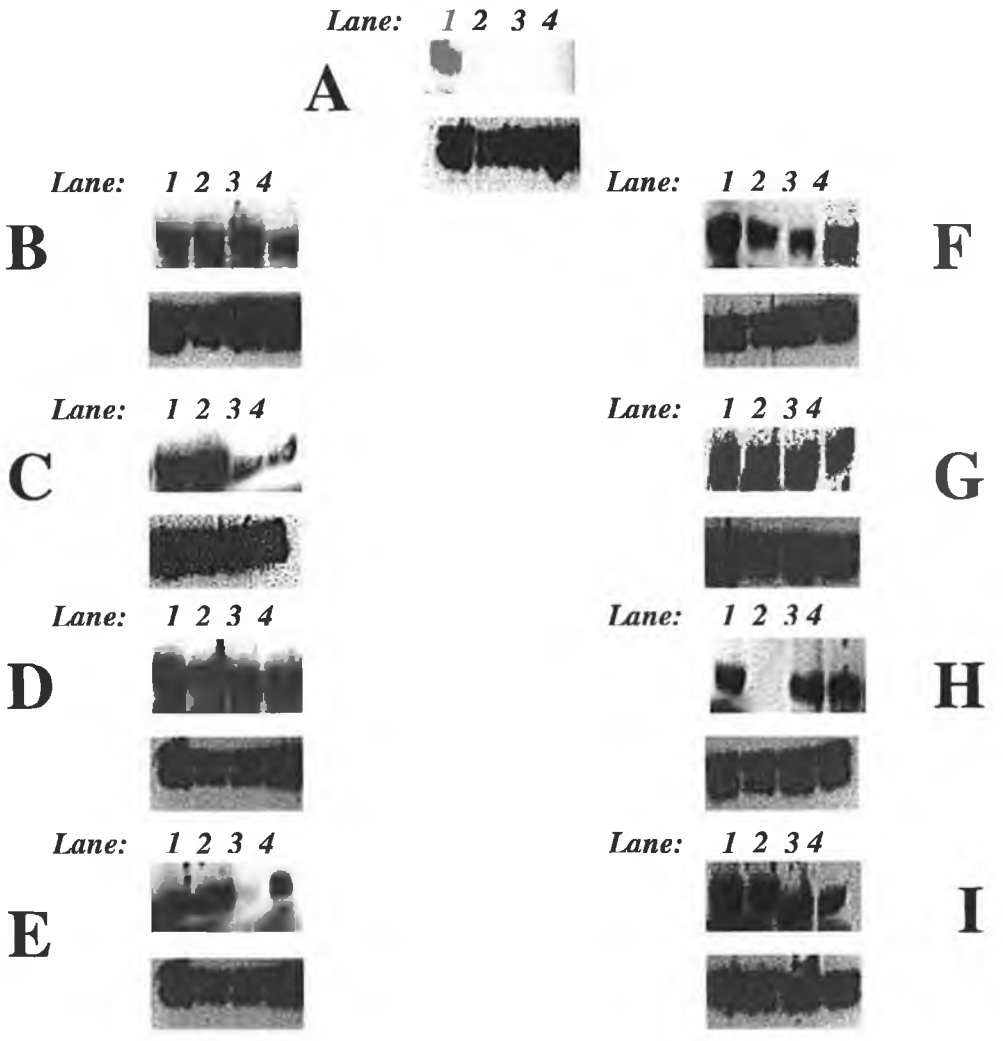

Figure 3.9: EPA and DHA modulate activation of ERK in J774 macrophages following TLR4 activation. Cells were cultured with either DMSO, EPA (25uM), DHA (25uM) or SA (25uM) for 7 days and then stimulated with LPS (100ng/ml) or medium alone. Cells were harvested 0-6h following stimulation and total (bottom panel) and phosphorylated ERK (top panel) was determined in whole cell lysates by western blotting. A representative immunoblot is shown. (**A**) 0 hr time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; (**B**) 5 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; (**C**) 10 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; (**D**) 15 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; (**E**) 30 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; (**F**) 60 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; (**G**) 120 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; (**H**) 4 hr time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; (**I**) 6hr time point: Lane 1 DMSO; lane 2 EPA; lane DHA; lane 4 SA.

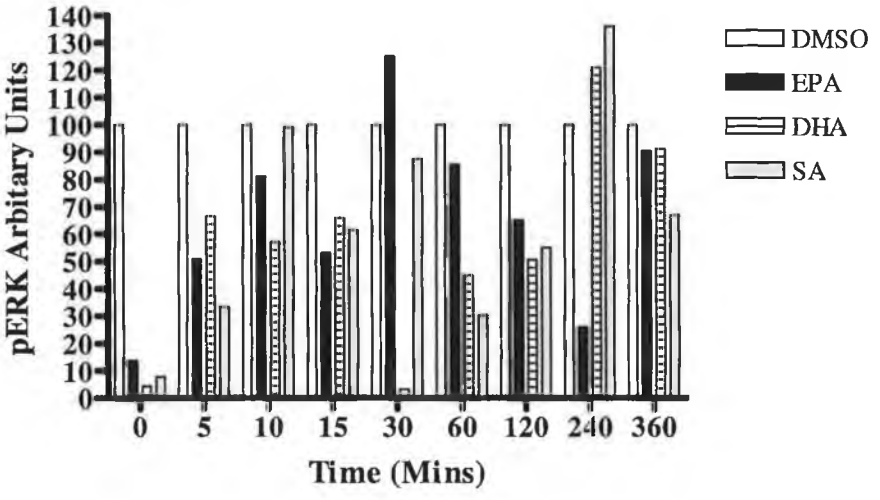

Fig 3.9(A) EPA and DHA modulate activation of ERK in J774 macrophages following TLR4 activation. Densitometric analysis was carried out on immunoblots shown in Fig 4.9. pERK is expressed as arbitary units.

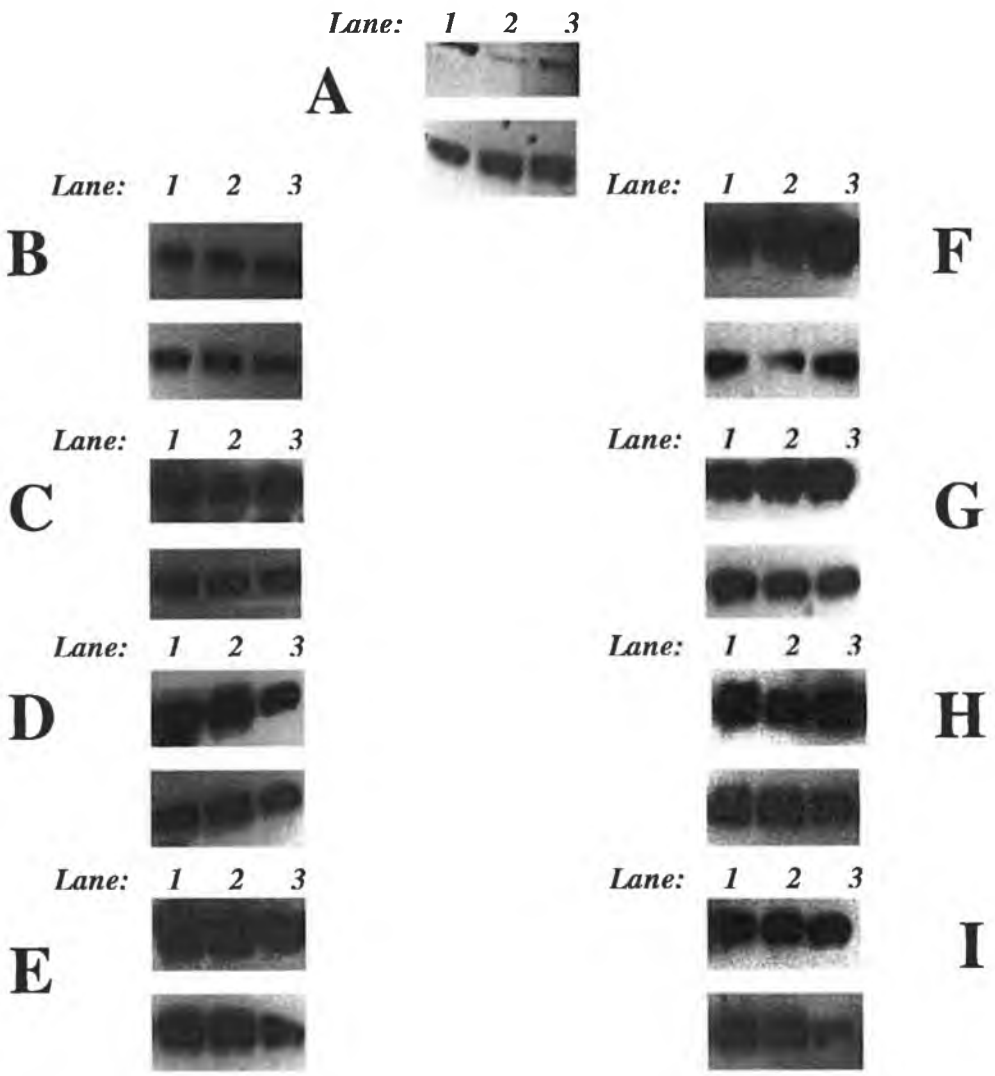

Figure 3.10: CLA modulates activation of p38 in J774 macrophages following TLR4 activation. Cells were cultured with either DMSO, c9, t11-CLA (50uM) or SA (25uM) for 7 days and then stimulated with LPS (100ng/ml) or medium alone. Cells were harvested 0-6h following stimulation and total (bottom panel) and phosphorylated p38 (top panel) was determined in whole cell lysates by western blotting. A representative immunoblot is shown. (**A**) 0 hr time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; (**B**) 5 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; (**C**) 10 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; (**D**) 15 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; (**E**) 30 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; (**F**) 60 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; (**G**) 120 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; (**H**) 4 hr time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA.
(**I**) 6hr time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA.

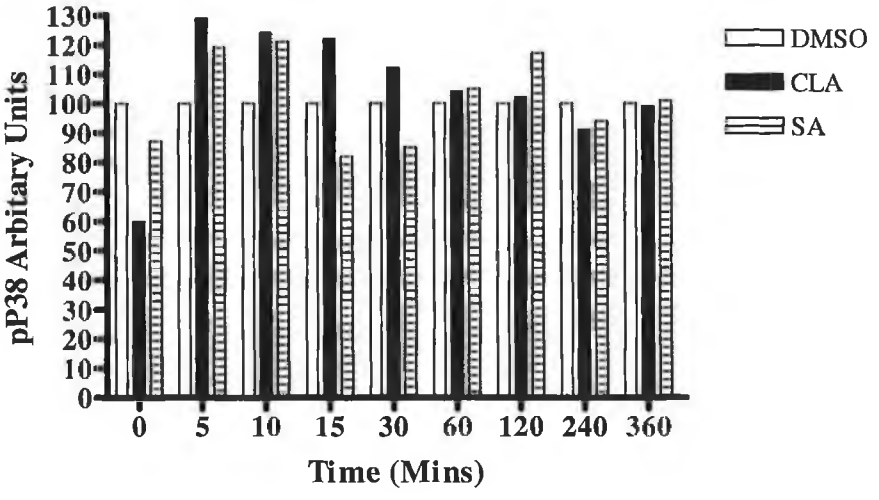

Fig 3.10(A) CLA modulates activation of p38 in J774 macrophages following TLR4 activation. Densitometric analysis was carried out on immunoblots shown in Fig 4.10. Pp38 is expressed as arbitary units.

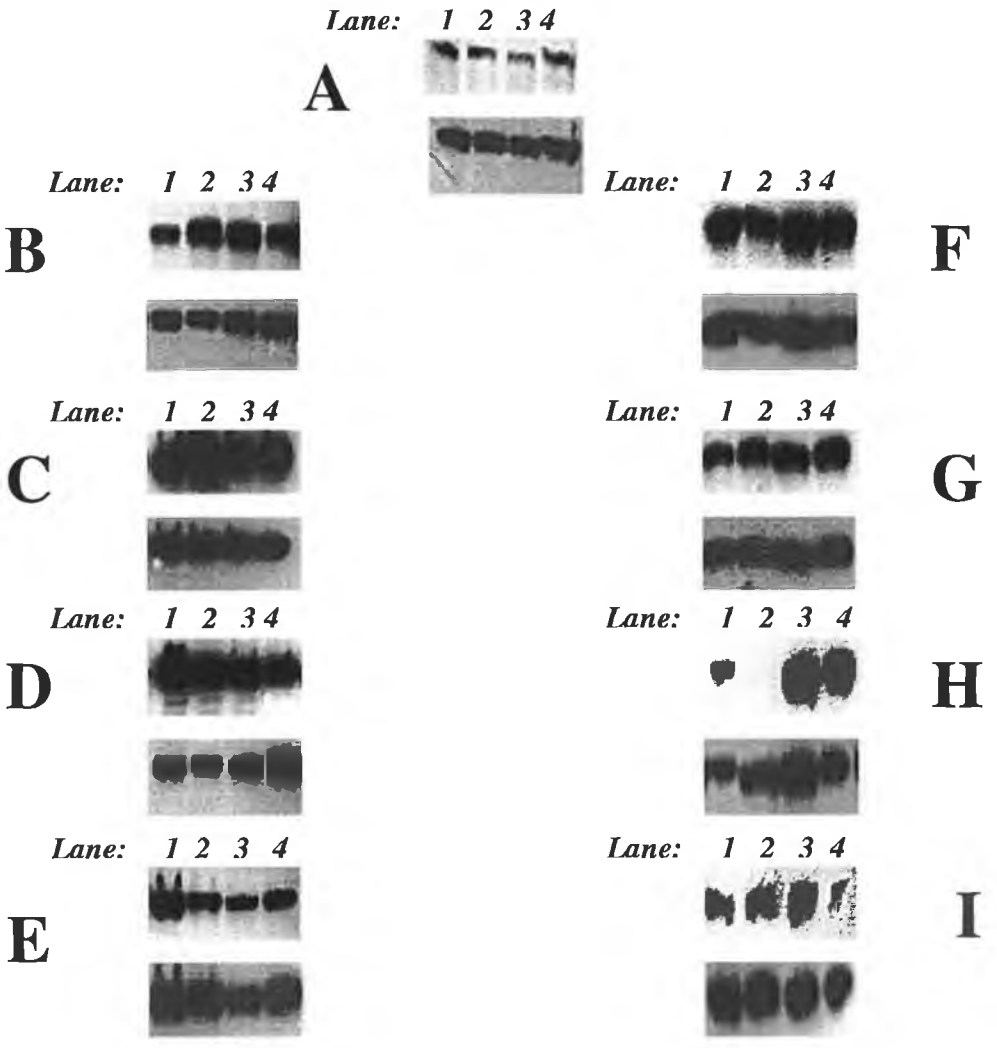

Figure 3.11: EPA and DHA modulate activation of p38 in J774 macrophages following TLR4 activation. Cells were cultured with either DMSO, EPA (25uM), DHA (25uM) or SA (25uM) for 7 days and then stimulated with LPS (100ng/ml) or medium alone. Cells were harvested 0-6h following stimulation and total (bottom panel) and phosphorylated p38 (top panel) was determined in whole cell lysates by western blotting. A representative immunoblot is shown. **(A)** 0 hr time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(B)** 5 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(C)** 10 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(D)** 15 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(E)** 30 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(F)** 60 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(G)** 120 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(H)** 4 hr time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(I)** 6hr time point: Lane 1 DMSO; lane 2 EPA; lane DHA; lane 4 SA.

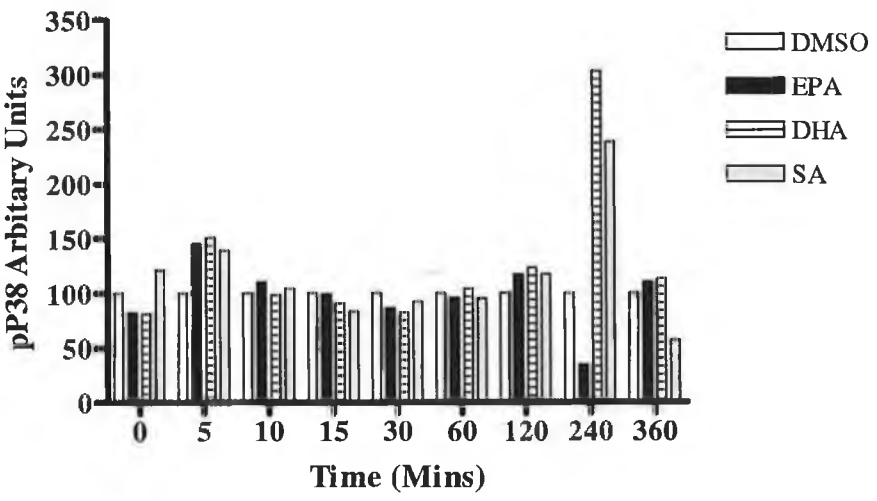

Fig 3.11(A) EPA and DHA modulate activation of p38 in J774 macrophages following TLR4 activation. Densitometric analysis was carried out on immunoblots shown in Fig 4.11. Pp38 is expressed as arbitary units.

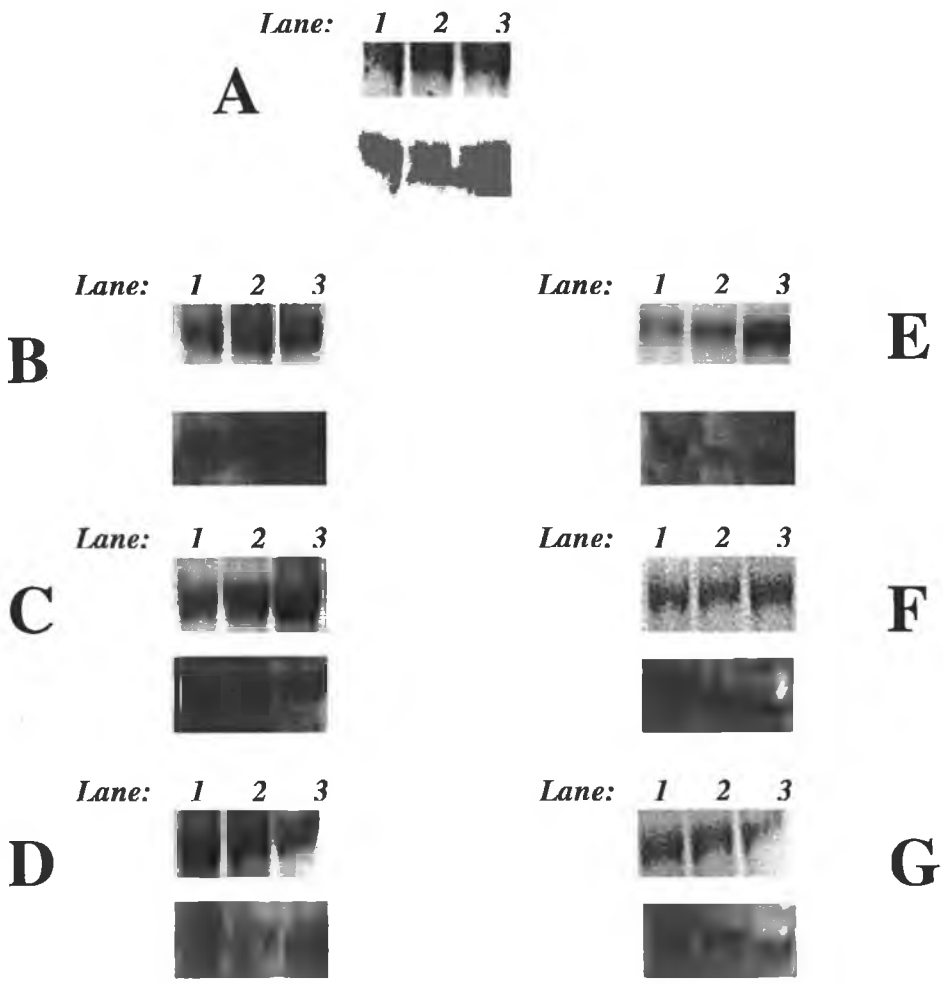

Figure 3.12: CLA modulates activation of JNK in J774 macrophages following TLR4 activation. Cells were cultured with either DMSO, c9, t11-CLA (50uM) or SA (25uM) for 7 days and then stimulated with LPS (100ng/ml) or medium alone. Cells were harvested 0-2h following stimulation and total (bottom panel) and phosphorylated JNK (top panel) was determined in whole cell lysates by western blotting. A representative immunoblot is shown. (**A**) 0 hr time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; (**B**) 5 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; (**C**) 10 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; (**D**) 15 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; (**E**) 30 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; (**F**) 60 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; (**G**) 120 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA.

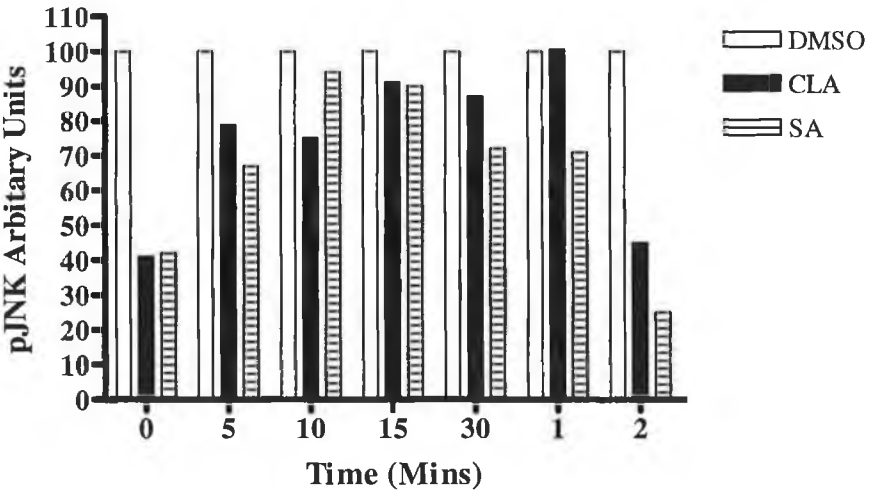

Fig 3.12(A) CLA modulates activation of JNK in J774 macrophages following TLR4 activation. Densitometric analysis was carried out on immunoblots shown in Fig 4.12. pJNK is expressed as arbitary units.

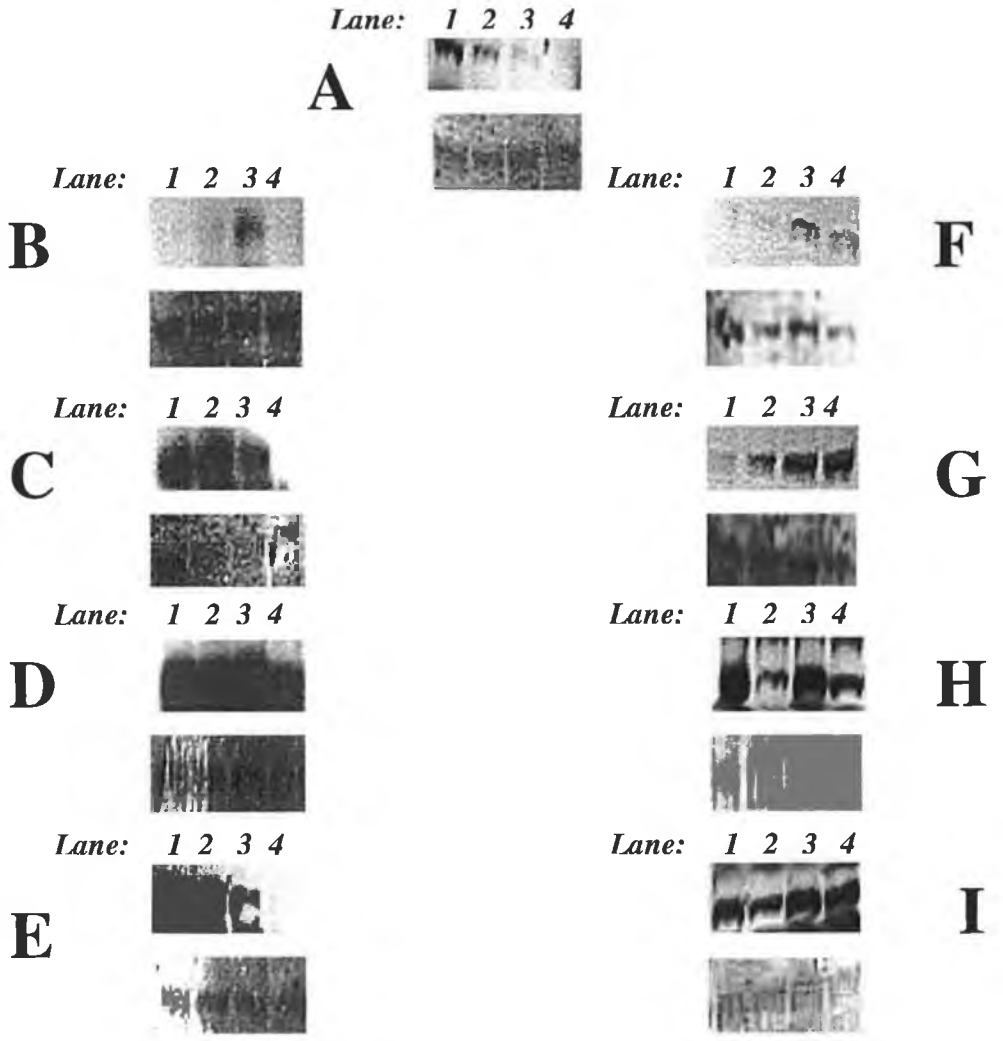

Figure 3.13: EPA and DHA modulate activation of JNK in J774 macrophages following TLR4 activation. Cells were cultured with either DMSO, EPA (25uM), DHA (25uM) or SA (25uM) for 7 days and then stimulated with LPS (100ng/ml) or medium alone. Cells were harvested 0-6h following stimulation and total (bottom panel) and phosphorylated JNK (top panel) was determined in whole cell lysates by western blotting. A representative immunoblot is shown. **(A)** 0 hr time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(B)** 5 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(C)** 10 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(D)** 15 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(E)** 30 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(F)** 60 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(G)** 120 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(H)** 4 hr time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(I)** 6hr time point: Lane 1 DMSO; lane 2 EPA; lane DHA; lane 4 SA.

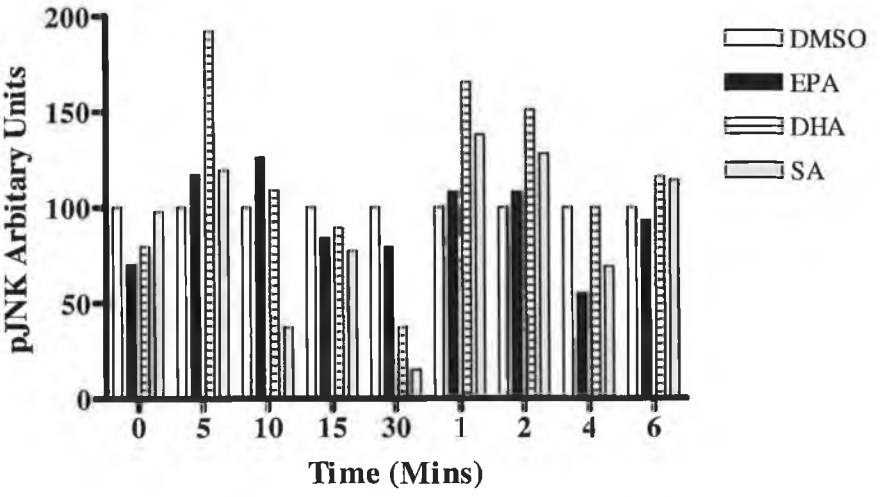

Fig 3.13(A) EPA and DHA modulate activation of JNK in J774 macrophages following TLR4 activation. Densitometric analysis was carried out on immunoblots shown in Fig 4.13. pJNK is expressed as arbitary units.

3.3 Discussion

The main findings of this study are that PUFA can modulate the response of macrophages to the TLR4 ligand, LPS. This is evidenced by changes in MAP kinase activation, cytokine production, and expression of TLR4. In addition, this study also establishes the differential effects of PUFA suggesting that these effects are mediated through a number of different pathways.

Treatment of macrophages with CLA results in a significant enhancement of IL-10 following stimulation with LPS. CLA exerted similar effects in the presence of the JNK inhibitor, however CLA's positive effect on IL-10 was reversed in the presence of ERK and P38 inhibitors. Western blotting analysis demonstrated an increase in phosphorylation of p38 and ERK but not JNK, with CLA treatment. CLA was also shown to up-regulate TLR4 expression in macrophages. Similar results were observed with DHA treatment, which enhanced LPS-induced IL-10. This effect was lessened considerably in the presence of inhibitors to ERK and p38. Further analysis showed that DHA increased p38 activity but had almost no effect on activation of ERK. DHA was also shown to induce modest up-regulation of TLR4 expression in macrophage. The effects observed with DHA were not seen with EPA. There have been very few studies that demonstrate differential actions of these two PUFA. Many studies have shown similarities but no definitive differences.

IL-10 is one of the most important anti-inflammatory cytokines within the human immune response. It is especially important as it is a potent deactivator of macrophage pro-inflammatory cytokine synthesis (Brandtzaeg et al., 1996, Clarke et al., 1998).

IL-10 also inhibits Th1 immune responses by reducing the capacity of macrophages to produce IL-12, a potent inducer of Th1 responses. IL-10 is a potent anti-inflammatory therapy in IBD as it also has a role in the regulatory network of cytokines in controlling mucosal tolerance. Indeed, IL-10 deficient mice have been shown to spontaneously develop colitis further emphasising its role as an anti-inflammatory therapy in IBD (Tamagawa et al., 2007). Few studies have examined the effects of PUFA on production of IL-10. We have previously reported that CLA enhances IL-10 production by dendritic cells (Loscher et al., 2005). Furthermore, EPA has been shown to increase IL-10 in the brain, suggesting that it exerts anti-inflammatory and neuroprotective effects in the central nervous system (Lynch et al., 2003). More recently, Sierra *et al*, showed that n-3 PUFA reduced innate and specific immune responses partially through an increase in IL-10 (Sierra et al., 2006). However, there are still a number of contradictory studies that show dietary fish oil to suppress IL-10 production (Skuladottir et al., 2007). We have reported a significant difference between the effects of EPA and DHA on IL-10 production, which may explain some of the findings of these studies.

We also report that CLA inhibited LPS-induced IL-12p40 in murine macrophage. Inhibitor studies show that this inhibition was dependent on the P38 MAPK kinase intracellular signalling cascade but not on the ERK or JNK cascades. Furthermore, western blotting analysis showed a sustained increase in phosphorylation of p38 in CLA-treated cells following LPS stimulation, with only small amounts of ERK phosphorylation, and no change in JNK. CLA was also shown to up-regulate TLR4 expression in macrophage. DHA treatment also inhibited LPS-induced IL-12p40 production, an effect that was maintained even in the presence of inhibitors to ERK,

p38 and JNK. Although cells treated with DHA did demonstrate increased p38 activity. Again there were clear differences between the effects of EPA and DHA, with EPA showing no significant effect on IL-12p40 production. All of the PUFA examined in this study resulted in suppression of TNFα production following stimulation with LPS. This effect was unaffected by the addition of inhibitors to ERK and JNK but was reversed by inhibition of p38.

IL-12 and TNFα are important pro-inflammatory cytokine that have been found to be involved in autoimmune diseases like IBD. Indeed dysregulation of their synthesis is key in the inflammatory pathogenesis of these diseases. Therefore tight control of IL-12 and TNFα are crucial to avoid inflammatory and autoimmune disease. (H Wang et al). TNFα is mainly produced by macrophages although lymphoid cells, mast cells, endothelial cells, fibroblasts and neuronal tissue can also produce it. TNFα plays a central role in chronic inflammation. High levels of pro-inflammatory cytokines, including TNFα, in the intestinal mucosa are pivotal in the development of relapses and for sustaining chronic inflammation (Mascheretti et al., 2004). Our findings that PUFA suppress pro-inflammatory cytokine production by immune cells are in agreement with numerous studies. We have previously reported that CLA suppressed IL-12 production by DC (Loscher et al., 2005), an effect also reported for EPA and DHA (Wang et al., 2007).

It is well established that MAPK activation is involved in the regulation of cytokines. Activation of ERK has been shown to promote IL-10 production and regulate IL-12 production. p38 has been shown to be involved in production of IL-1 and TNFα. While the effects of PUFA on suppression of pro-inflammatory cytokine production

have been widely reported, the involvement of MAPK in mediating these effects, are less well defined. Further complexity is added by reports that demonstrate PUFA to have different effects on MAPK activity in different cell populations. We have reported that CLA activated ERK in DC with subsequent effects on cytokine production. Furthermore, DHA has been shown to inhibit apoptosis via activation of ERK (German et al., 2006). However, both DHA and EPA have been shown to suppress ERK activity in T cells (Denys et al., 2001). These conflicting effects can also be seen when examining the literature on PUFA and p38 activation. DHA and EPA have been shown to inhibit mineralisation of vascular cells via activation of p38 (Abedin et al., 2006), however, recently these PUFA were reported to modulate activation of DC through inhibition of p38 activity (Wang et al., 2007) and were also shown to decrease activation of p38 as well as ERK and JNK in microglia (Moon et al, 2007). It is clear from these reports that the effects of PUFA on activation of MAPK, varies greatly between cell types.

Our study clearly shows that CLA enhances IL-10 and suppresses IL-12p40 production by macrophages following TLR4 activation and these effects are mediated by p38 and ERK. We also show that DHA significantly increases IL-10 production in LPS-stimulated macrophages via the same pathway but can suppress IL-12p40 production independently of MAPK activation. These effects were not seen with EPA and therefore demonstrate differential effects of these PUFA. Given that TLR4 has been shown to be involved in a number of inflammatory diseases, these data may explain the beneficial effects of PUFA in these diseases.

Chapter 4
Results

4.1: Introduction

As discussed in the previous chapter, inflammation, which runs unchecked, leads to inflammatory diseases such as atherosclerosis, rheumatoid arthritis and IBD. Dietary intervention with PUFAs have been shown to have beneficial effects in these diseases. TLR2 has now been shown to play a role in the development and progression of these diseases; however the effects of PUFA on TLR2 have not yet been explored.

TLR2, in association with TLR1 or TLR6, recognizes various bacterial components, including peptidoglycan, lipopeptide and lipoprotein of Gram-positive bacteria and mycoplasma lipopeptide. There are a variety of cytokines produced as a result of TLR ligation, including IL-10, IL-2, IL-6 and IL-12. TLR2-mediated cellular activation may play a role in protection from pathogens that contain specific TLR2 ligands. Higher expression of TLR2 was also found in both Crohns disease and ulcerative colitis suggesting that an abnormal mechanism may provide an excess of inflammatory mediators during the active phases of IBD (Canto et al., 2006). Alternatively, the increase in TLR2 expression could serve to enhance levels of the anti-inflammatory cytokine, IL-10, in an effort to restore homoeostasis to the local environment in this disease.

Activation of TLR2 leads to a signaling cascade involving the MAP kinases – ERK, p38 and JNK. These have been shown to be involved in the regulation of cytokines downstream of TLR activation. For example, ERK activation has been shown to promote IL-10 production and regulate IL-12 production. Studies using ERK inhibitors have shown that ERK activation suppresses IL-12 production in

macrophages (Feng et al., 1999) and dendritic cells (Puig-Kroger et al., 2001). ERK activation has been shown to also be essential for IL-10 production in dendritic cells, as inhibition of ERK completely abrogated IL-10 production (Loscher et al., 2005).

This study examines the effects of PUFA on TLR activation in macrophages by assessing their effects on cytokine production and TLR2 expression. Furthermore, it investigates the role of MAPK activation in mediating these effects.

4.2 Results

4.2.1 The effects of PUFA concentrations on cell viability.

The concentrations of PUFA used in this study are well established in the laboratory and have been shown to exert effects on immune cell function while maintaining cell viability. To establish that the effects of PUFA we see in our studies are not a result of cell death, we examined the effect of several concentrations of PUFA on cell viability. J774 murine macrophages were cultured for 7 days in 25μM, 50μM and 100μM concentrations of fatty acids. Supernatants were removed and cell viability assessed using Cell Titre 96 Aqueous One Solution (Promega, WI, USA) as per manufacturer's instructions. The selected concentrations of fatty acids used in all subsequent experiments have no cytotoxic effects on J774 macrophage *in vitro.* These are as follows - DMSO (25μM), CLA (50μM), EPA (25μM), DHA (25μM), LA (25μM), SA (25μM). (Fig 4.1)

4.2.2 Assessment of cytokine production by macrophages to increasing doses of TLR2 ligand.

To determine the correct dose of the TLR2 ligand for this study we cultured J774 murine macrophages in fatty acids (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM) for 7 days, plated them at $1x10^6$ cells/ml, and stimulated them for 24hrs with 10ng/ml, 100ng/ml and 1μg/ml of the TLR2 ligand, PAM3CSK4. Supernatants were removed after 24hrs and assessed for levels of IL-10 and IL-12p40 using specific immunoassay. Activation of TLR2 by addition of PAM3CSK4 resulted in production of IL-10 and IL-12p40. Fig 4.2 demonstrates that

IL-10 and IL-12p40 production was enhanced in cells treated with by EPA, CLA but not by DHA. Given the use of 1μg/ml of PAM3CSK4 in a number of published studies and our observation that shows significant activation of TLR2 at this concentration, we used this concentration for the rest of the study.

4.2.3 PUFA modulate TLR2 expression on macrophages.

When determining the effects of compounds on TLR2 activation, it is important to look at the concomitant effects on expression of TLR2. J774 murine macrophages were cultured in fatty acids for 7 days (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM), plated at $1x10^6$ per ml and stimulated with 1μg/ml PAM3CSK4 for 0, 6 and 24hrs. Cells were then stained with a fluorescent antibody specific for TLR2 and expression levels were analysed by flow cytometry. Stimulation of J774 macrophages with PAM3CSK4 increased expression of TLR2 at 6hr and 24hr 6hr treated cells (Fig 4.3(A)). When we examined the effects of PUFA on this expression, we observed an increase in TLR2 expression in cells treated with all fatty acids. At 24hr post stimulation, the increase in TLR2 expression persisted for CLA-treated cells, with EPA and LA- treated cells showing no change and indeed DHA was shown to decrease TLR2 expression at this time point (Fig 4.3(B)).

4.2.4 PUFA modulate cytokine production by macrophages.

We examined the effects of PUFA on the production of cytokines in response to stimulation with the TLR2 ligand, PAM3CSK4. J774 murine macrophages were cultured in presence of fatty acids for 7 days (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM), plated at $1x10^6$ per ml and stimulated for 24hrs with 1μg/ml PAM3CSK4. Supernatants removed and assessed

for levels of IL-10, IL-12p40 and TNFα using specific immunoassay. (Fig 4.4) Treatment of cells with CLA and EPA resulted in a significant increase in production of IL-10 following TLR ligation ($p<0.001$). The levels of IL-10 were unaffected by the remaining fatty acids. All of the fatty acids examined induced an increase in IL-12p40 production in macrophages activated with PAM3CSK4 ($P<0.001$). The levels of TNFα measured were well below the detection limit of the ELISA kit and so no statistical analysis was performed on this data.

4.2.5 Effects of PUFA on cytokine production by macrophages in the presence of MAPK inhibitors

In order to determine the involvement of MAPK in mediating the effects of PUFA on cytokine production in macrophages, cells were cultured in presence of fatty acids (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM) for 7 days, plated at $1x10^6$ per ml, then specific MAPK inhibitors (ERK U0126, JNK inhibitor 1, p38 SB20210) were added at 5μM, 1hr prior to stimulation with 1μg/ml PAM3CSK4 for 24hrs. Supernatants were removed and assessed for levels of IL-10, IL-12p40 and TNFα using specific immunoassay. Treatment of cells with CLA resulted in increased production of IL-10 in J774 macrophages following TLR ligation ($p<0.001$; Fig 4.5). This increase was not maintained in the presence of specific inhibitors to ERK, p38 or JNK. EPA also significantly enhanced TLR2-induced IL-10 production by macrophages ($p<0.001$). This increase was maintained in the presence of specific inhibitors to ERK, p38 and JNK, with significant increases still being detected in these groups ($p<0.001$, $p<0.001$, $p<0.001$ respectively). All of the fatty acids examined induced a significant increase in TLR-2 induced IL-12p40 production in J774 macrophages ($p<0.001$; Fig 4.6). This increase was maintained in

all fatty acid groups upon the inclusion of a specific ERK inhibitor ($p<0.001$) and JNK inhibitor ($p<0.001$) except for the SA group. The effects of these fatty acids on IL-12p40 were reversed in the presence of the specific p38 inhibitor. Once again the levels of TNFα detected were nominal (Fig 4.7) and so the data was not analysed statistically.

4.2.6 Modulation of MAPK pathway by PUFA.

Given that the presence of some MAPK inhibitors were shown to reverse the effects of PUFA on cytokine production in J774 macrophages, we investigated whether PUFA enhanced or suppressed activation of these MAPK at a number of time points following activation of the cells with the TLR2 ligand, PAM3CSK4. Cells were cultured in presence of fatty acids for 7 days (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM) and plated at $1x10^6$ per ml. Cells were stimulated with 1ug/ml PAM3CSK4 for the following time points; 0hr, 5 min, 10 min, 15 min, 30 min, 60 min, 120min, 240 min and 360 min. Cells were then scraped and collected into sample buffer, sonicated and boiled prior to loading on SDS-PAGE gels. Gels were blotted using iBLOT system. All membranes were blocked in 5% Marvel / TBST, then incubated with phosphospecific ERK, p38 or JNK 5% BSA, followed by HRP-conjugated secondary Antibody 5% Marvel / TBST unless otherwise stated. Membranes were then treated with Chemiluminescent Substrate (Pierce) and exposed for required time and developed using FUJI processor. Blots were then stripped and re-probed for total protein to demonstrate equal loading. Representative blots are shown in the figures. We carried out densitometry using GeneSnap and GeneTool in order to graph the results using arbitary units. All DMSO

groups at all time points were assigned a value of 100 and changes in density of the bands calculated accordingly.

4.2.6.1 Modulation of ERK pathway by PUFA

Exposure of J774 macrophages to CLA enhanced activation of ERK following TLR2 ligation. This was evident 5 min, 10 min, 15 min and 60 min after activation with PAM3CSK4 (Fig 4.8, 4.8(A)). Cells treated with EPA and DHA also showed increased phosphorylation of ERK at the following time points 0hr, 5 min, 15 min, 30 min and then again later at 2hr and 4 hr post stimulation with PAM3CSK4 (Fig 4.9, 4.9(A)). In contrast the saturated fatty acid control, SA, suppressed ERK activation at a number of time points.

4.2.6.2 Modulation of p38 pathway by PUFA

Treatment of J774 macrophages with CLA had little effect on p38 activation. There were some very small increases observed 2hr and 4 hr post stimulation (Fig 4.10, 4.10(A)). EPA had the most noted effect with increased activation of p38 at 5 min, 10 min, 15 min and later at 4hr and 6hr post TLR2 ligation (Fig 4.11, 4.11(A)). DHA treatment of macrophages also enhanced p38 activation at 5 min and to a lesser extent at 30 min post stimulation. SA had mixed effects with some increases at early time points and decreased activation at later time points.

4.2.6.3 Modulation of JNK pathway by PUFA

Treatment of J774 murine macrophages with CLA had no effect on JNK activation at any of the time points examined (Fig 4.12, 4.12 (A)). While EPA and DHA enhanced

JNK activation in resting cells, they inhibited JNK activation 5 min, 10 min, 15 min, 30 min and later at 6 hrs post stimulation with PAM3CSK4 (Fig 4.13, 4.13(A)).

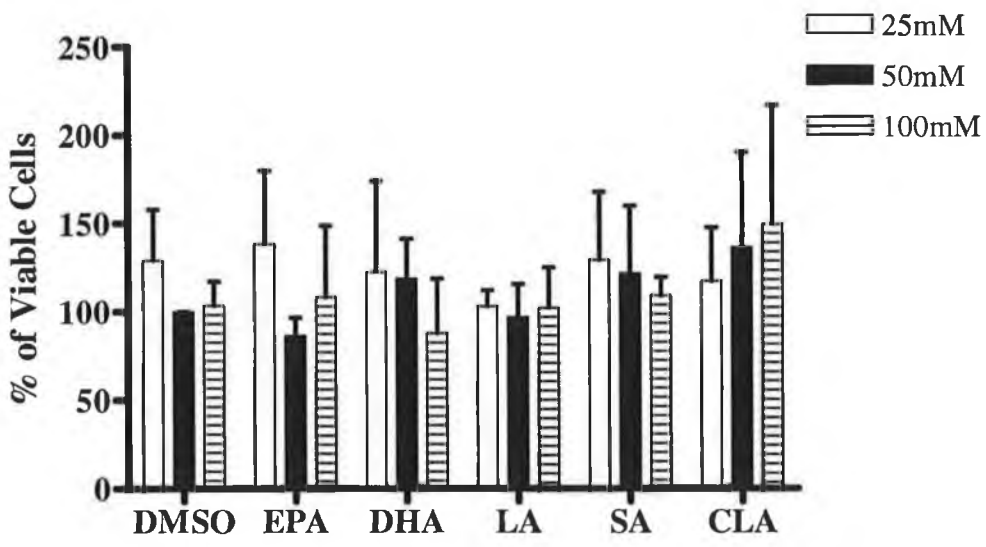

Figure 4.1 Fatty Acid concentrations do not affect viability of J774 cells *in vitro*. J774 cells were cultured in fatty acids for 7 days at either 25mM, 50mM, or 100mM. Supernatants were removed and cell viability assessed using Cell Titre 96 Aqueous One Solution (Promega, WI, USA) as per manufacturers instructions. Results are expressed as mean +/- SEM. As this experiment was a dose response study comparing the best concentrations eliciting an effect no further statistical analysis was required.

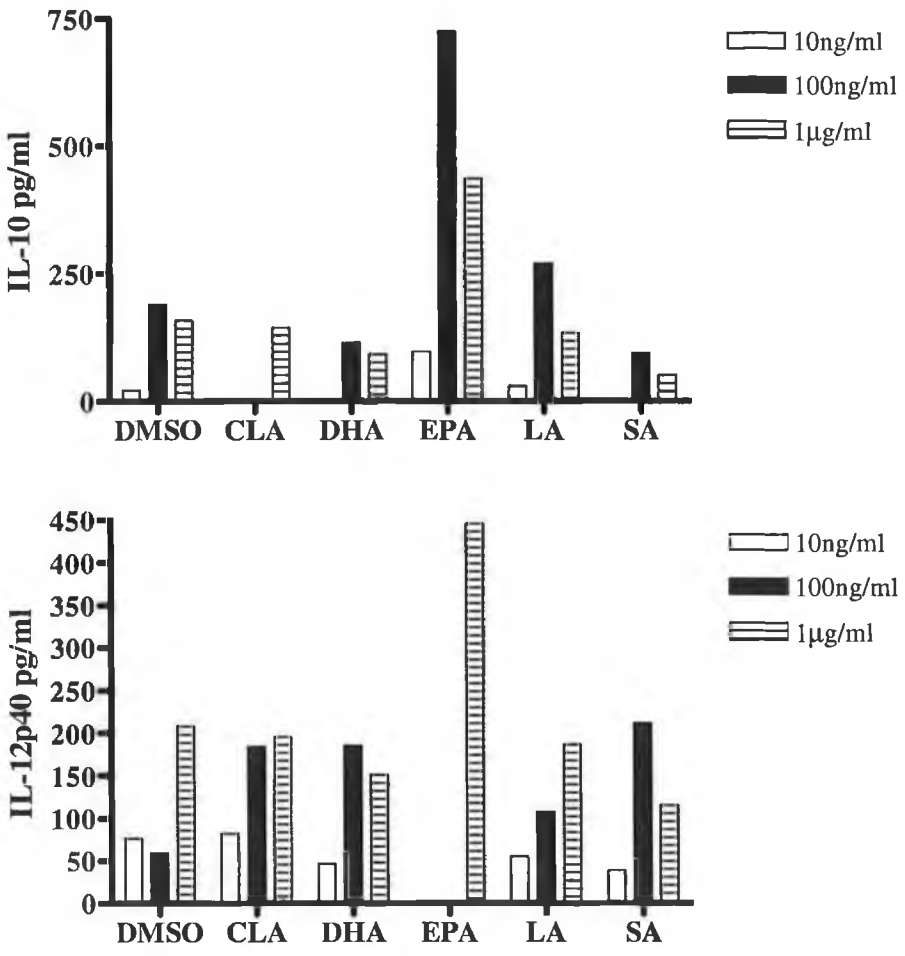

Fig 4.2 Cytokine production by J774 murine macrophages following TLR2 activation. J774 murine macrophages were cultured in fatty acids (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM) for 7 days, plated at $1x10^6$ per ml then stimulated for 24hrs with 10ng/ml, 100ng/ml and 1µg/ml PAM3CSK4. Supernatants were removed and assessed for levels of IL-10 and IL-12p40 using specific immunoassay.

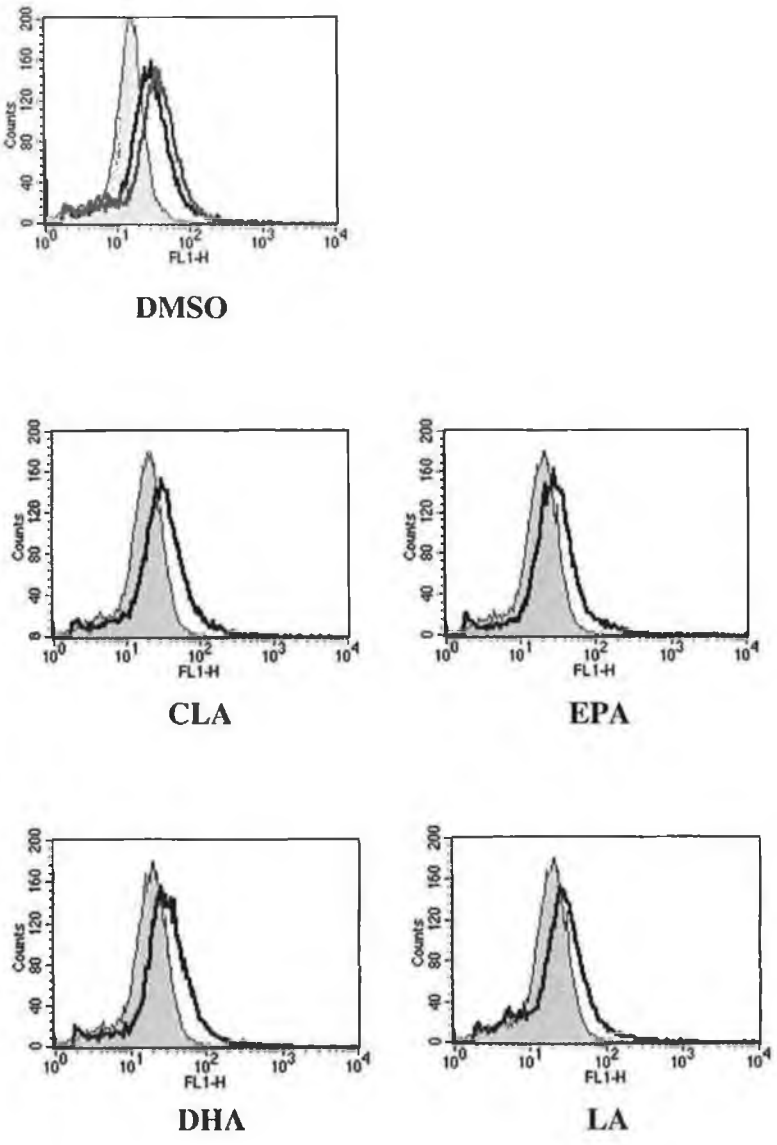

Fig 4.3(A) PUFA modulate TLR2 expression on macrophages. J774 murine macrophages were cultured in fatty acids (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM) for 7 days, plated at $1x10^6$ per ml then stimulated for 6hrs with 1μg/ml PAM3CSK4. Cells were then stained with antibody specific for TLR2. In DMSO figure, grey (fill) represents DMSO treated cells (no stimulation), black line (no fill) represents DMSO 6hr stimulated cells, grey line (no fill) represents DMSO 24hr stimulated cells. In all other cases grey (Fill) represents DMSO 6hr PAM3CSK4 treated cells, black line (no fill) represents 6hr fatty acid treated cells.

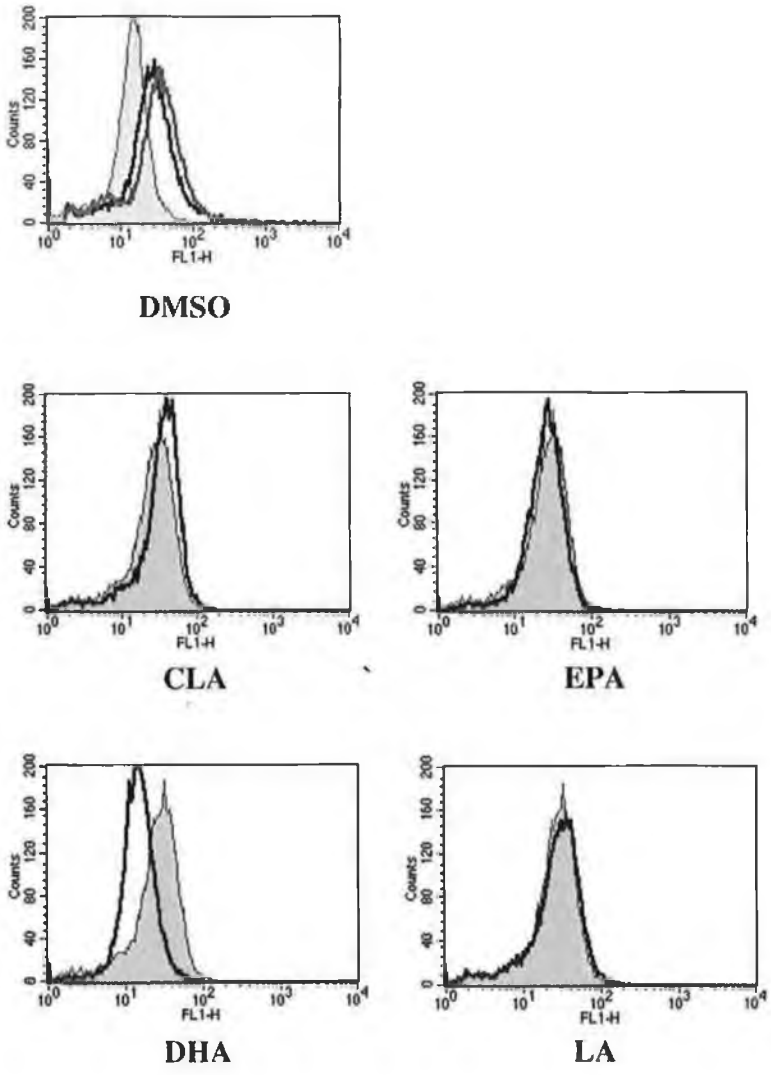

Fig 4.3(B) PUFA modulate TLR2 expression on macrophages. J774 murine macrophages were cultured in fatty acids (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM) for 7 days, plated at $1x10^6$ per ml then stimulated for 24hrs with 1μg/ml PAM3CSK4. Cells were then stained with antibody specific for TLR2. In DMSO figure, grey (fill) represents DMSO treated cells (no stimulation), black line (no fill) represents DMSO 6hr stimulated cells, grey line (no fill) represents DMSO 24hr stimulated cells. In all other cases grey (Fill) represents DMSO 24hr PAM3CSK4 treated cells, black line (no fill) represents 24hr fatty acid treated cells.

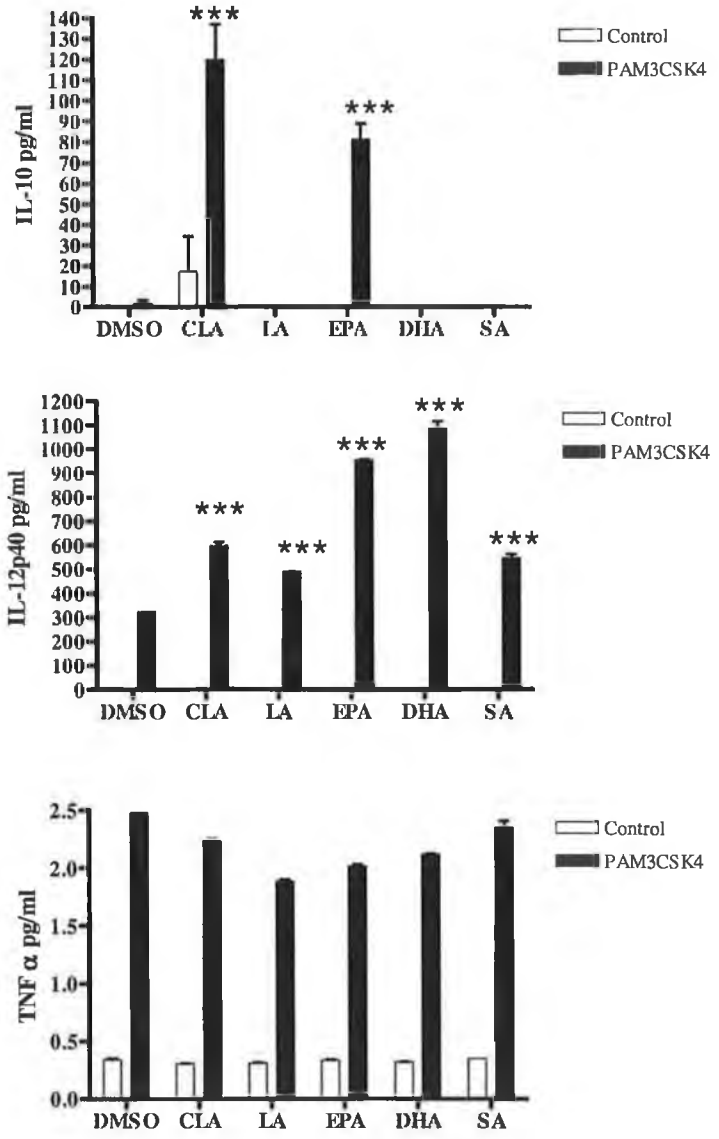

Fig 4.4 PUFA modulate cytokine production by macrophages following TLR2 activation. J774 murine macrophage were cultured in presence of fatty acids (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM) for 7 days, plated at $1x10^6$ per ml and stimulated for 24hrs with 1μg/ml PAM3CSK4. Supernatants were removed and assessed for levels of IL-10, IL-12p40 and TNFα using specific immunoassay. Results are expressed as mean ±SEM. ***$p<0.001$, ANOVA, comparing DMSO and fatty acid treated groups.

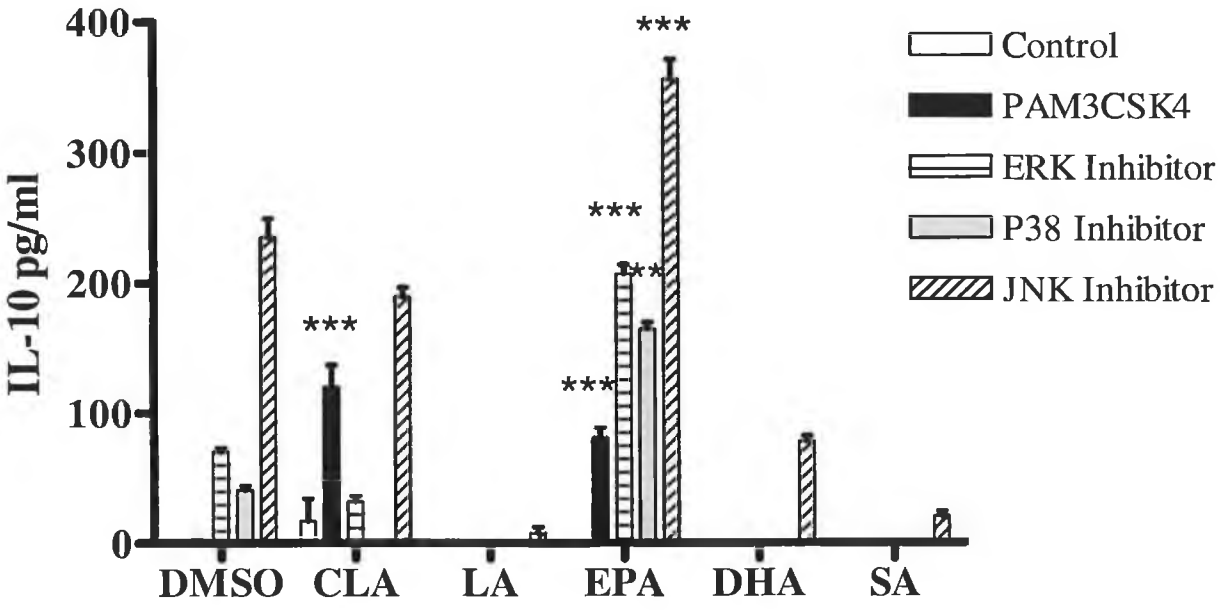

Figure 4.5 The effects of PUFA on IL-10 production by macrophages in the presence of specific MAPK inhibitors. J774 murine macrophages were cultured in the presence of fatty acids for 7 days (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM), plated at $1x10^6$ per ml +/- specific inhibitors (ERK U0126, JNK inhibitor 1, p38 SB20210) at 5μM added 1hr prior to stimulation with 1μg/ml PAM3CSK4 for 24hrs. Supernatants were removed and assessed for levels of IL-10 using specific immunoassay. Results are expressed as mean ±SEM. **p<0.01, ***p<0.001, ANOVA, comparing DMSO and fatty acid treated groups in the presence and absence of specific inhibitors.

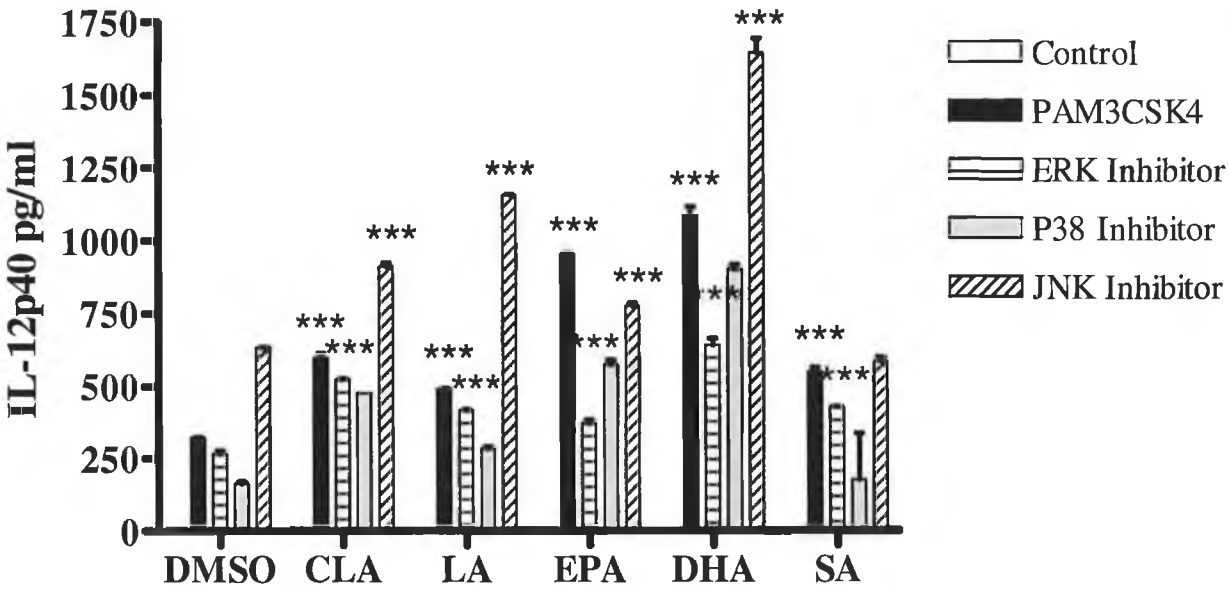

Figure 4.6 The effects of PUFA on IL-12p40 production by macrophages in the presence of specific MAPK inhibitors. J774 murine macrophages were cultured in the presence of fatty acids for 7 days (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM), plated at $1x10^6$ per ml +/- specific inhibitors (ERK U0126, JNK inhibitor 1, p38 SB20210) at 5μM added 1hr prior to stimulation with 1μg/ml PAM3CSK4 for 24hrs. Supernatants were removed and assessed for levels of IL-12p40 using specific immunoassay. Results are expressed as mean ±SEM. ***p<0.001, ANOVA, comparing DMSO and fatty acid treated groups in the presence and absence of specific inhibitors.

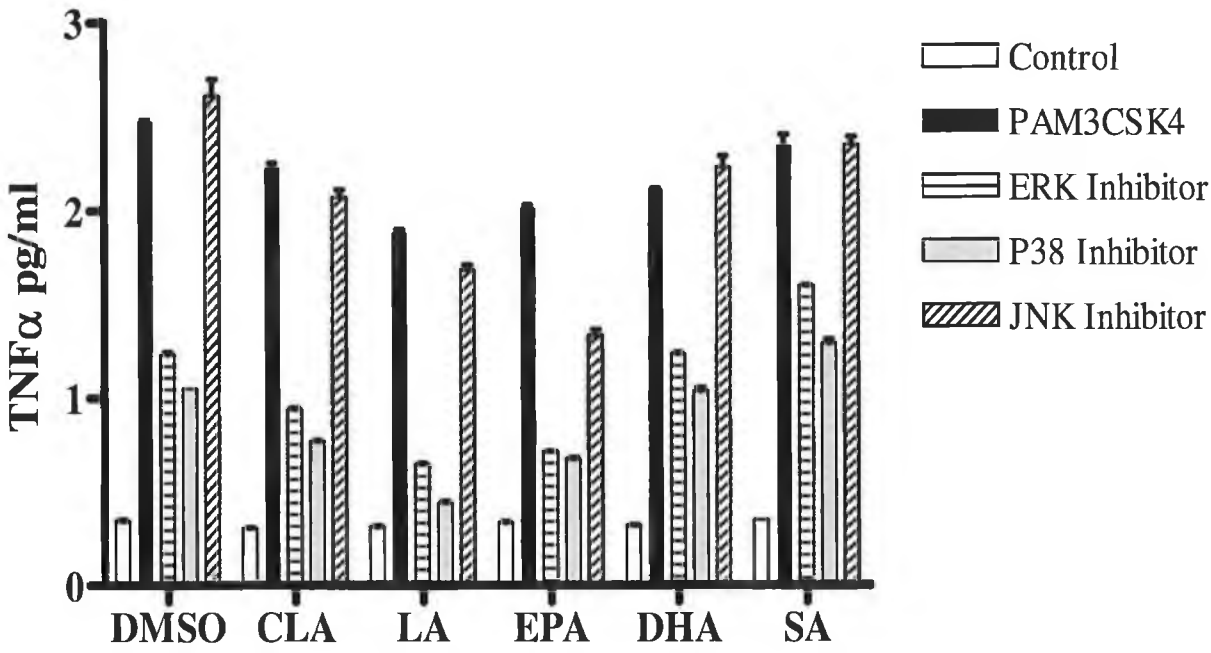

Figure 4.7 The effects of PUFA on TNFα production by macrophages in the presence of specific MAPK inhibitors. J774 murine macrophages were cultured in the presence of fatty acids for 7 days (DMSO 25mM, CLA 50mM, DHA 25mM, EPA 25mM, LA 25mM, and SA 25mM), plated at $1x10^6$ per ml +/- specific inhibitors (ERK U0126, JNK inhibitor 1, p38 SB20210) at 5μM added 1hr prior to stimulation with 1μg/ml PAM3CSK4 for 24hrs. Supernatants were removed and assessed for levels of TNFα using specific immunoassay. Results are expressed as mean ±SEM. The levels detected were below the recommended detection level of the immunoassay and so statistical analysis was not performed on this data.

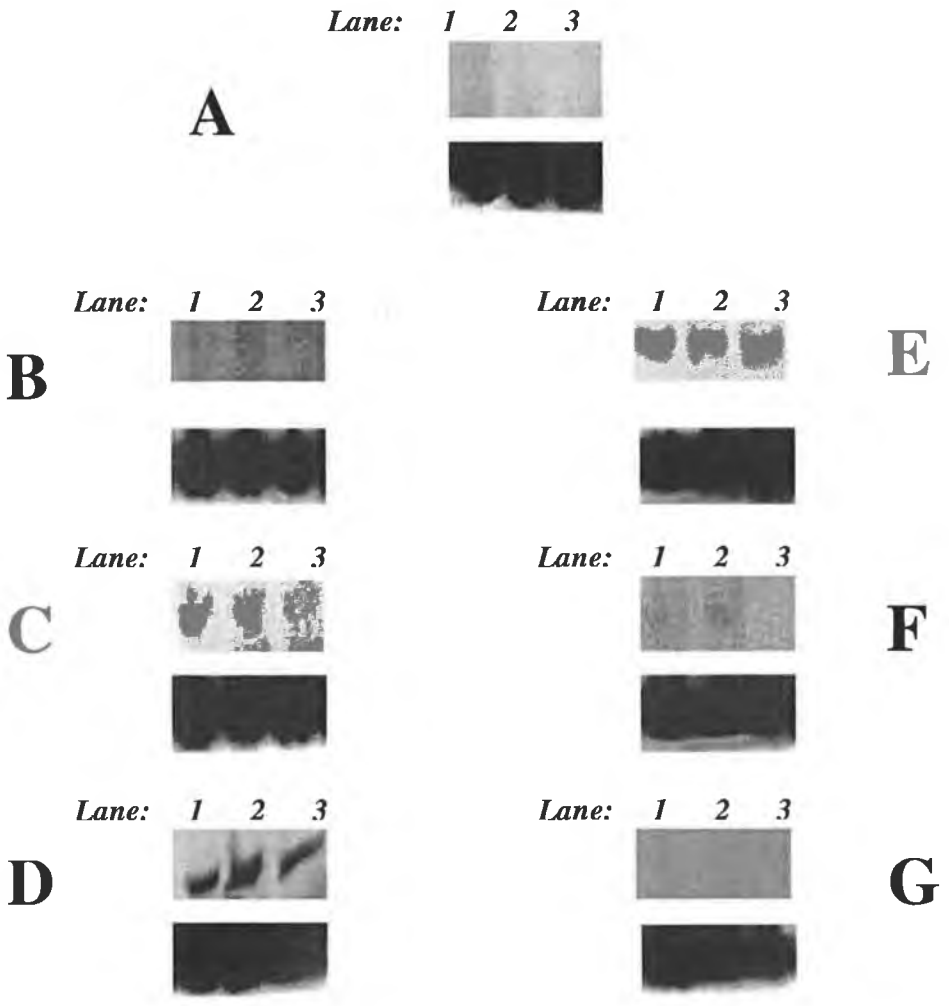

Figure 4.8: CLA modulates activation of ERK in J774 macrophages following TLR2 activation. Cells were cultured with either DMSO, c9, t11-CLA (50uM) or SA (25uM) for 7 days and then stimulated with PAM3CSK4 (1ug/ml) or medium alone. Cells were harvested 0-2h following stimulation and total (bottom panel) and phosphorylated ERK (top panel) was determined in whole cell lysates by western blotting. A representative immunoblot is shown. **(A)** 0 hr time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(B)** 5 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(C)** 10 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(D)** 15 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(E)** 30 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(F)** 60 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(G)** 120 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA.

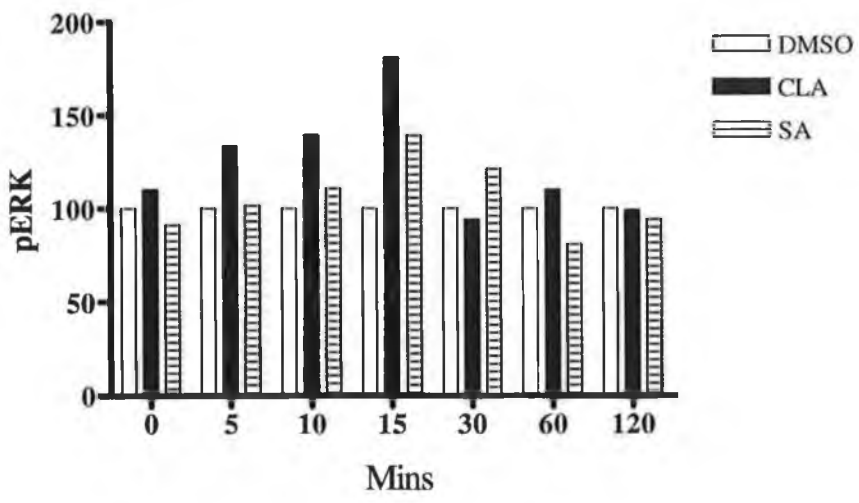

Fig 4.8(A) CLA modulates activation of ERK in J774 macrophages following TLR2 activation. Densitometric analysis was carried out on immunoblots shown in Fig 3.8. pERK is expressed as arbitary units.

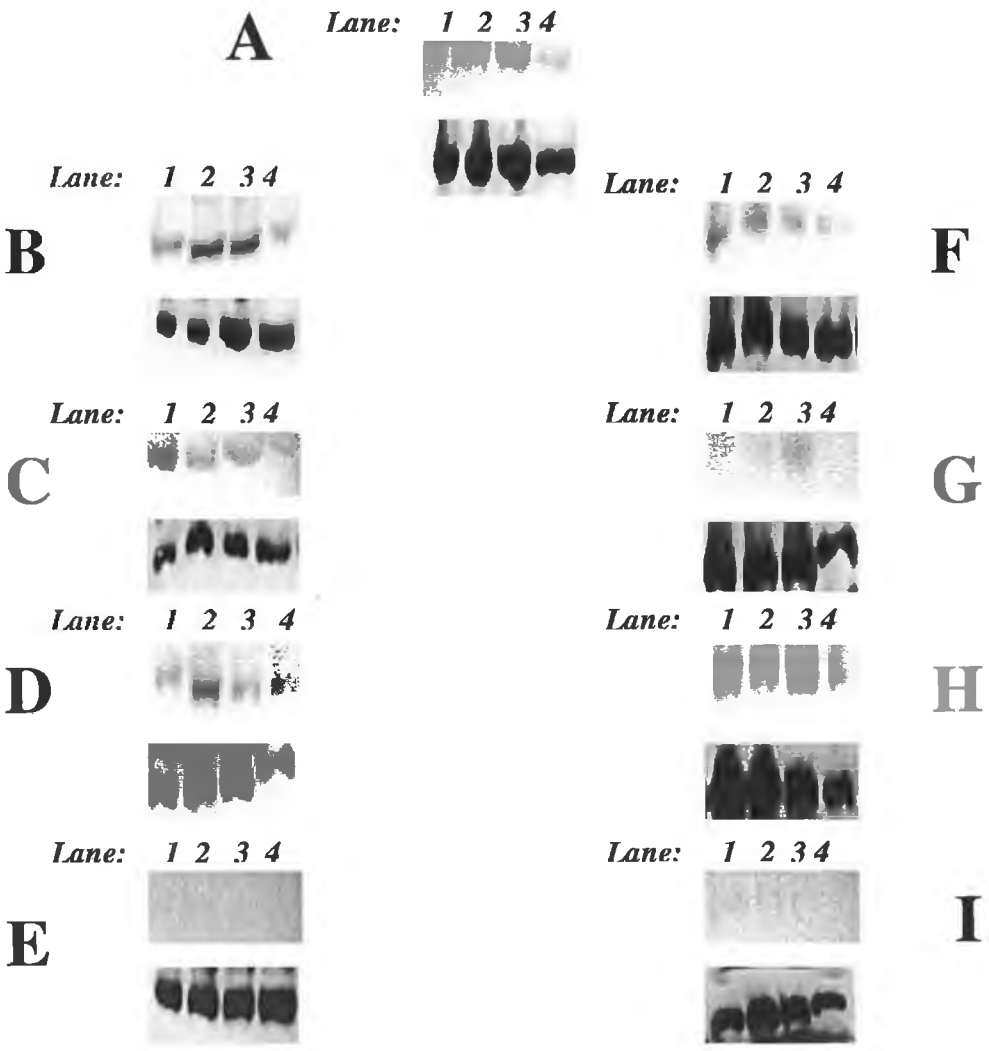

Figure 4.9: EPA and DHA modulate activation of ERK in J774 macrophages following TLR2 activation. Cells were cultured with either DMSO, EPA (25uM), DHA (25uM) or SA (25uM) for 7 days and then stimulated with PAM3CSK4 (1ug/ml) or medium alone. Cells were harvested 0-6h following stimulation and total (bottom panel) and phosphorylated ERK (top panel) was determined in whole cell lysates by western blotting. A representative immunoblot is shown. **(A)** 0 hr time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(B)** 5 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(C)** 10 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(D)** 15 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(E)** 30 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(F)** 60 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(G)** 120 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(H)** 4 hr time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(I)** 6hr time point: Lane 1 DMSO; lane 2 EPA; lane DHA; lane 4 SA.

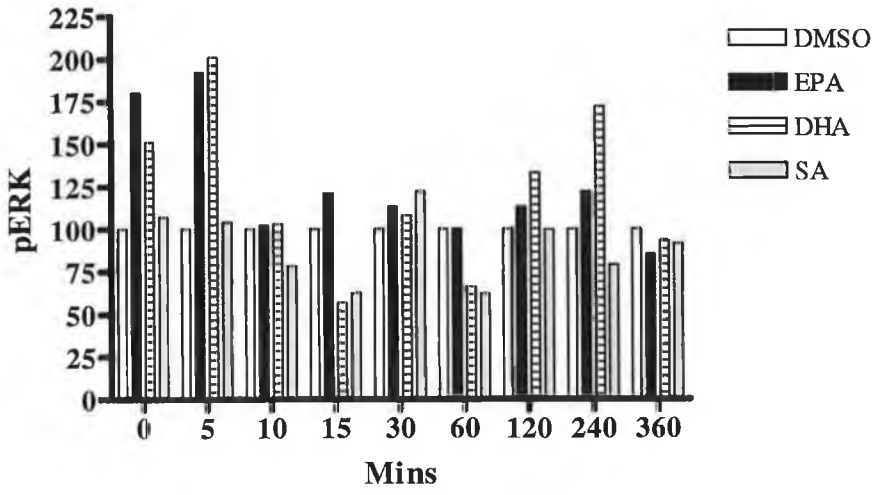

Fig 4.9 EPA and DHA modulate activation of ERK in J774 macrophages following TLR2 activation. Densitometric analysis was carried out on immunoblots shown in Fig 3.9. pERK is expressed as arbitary units.

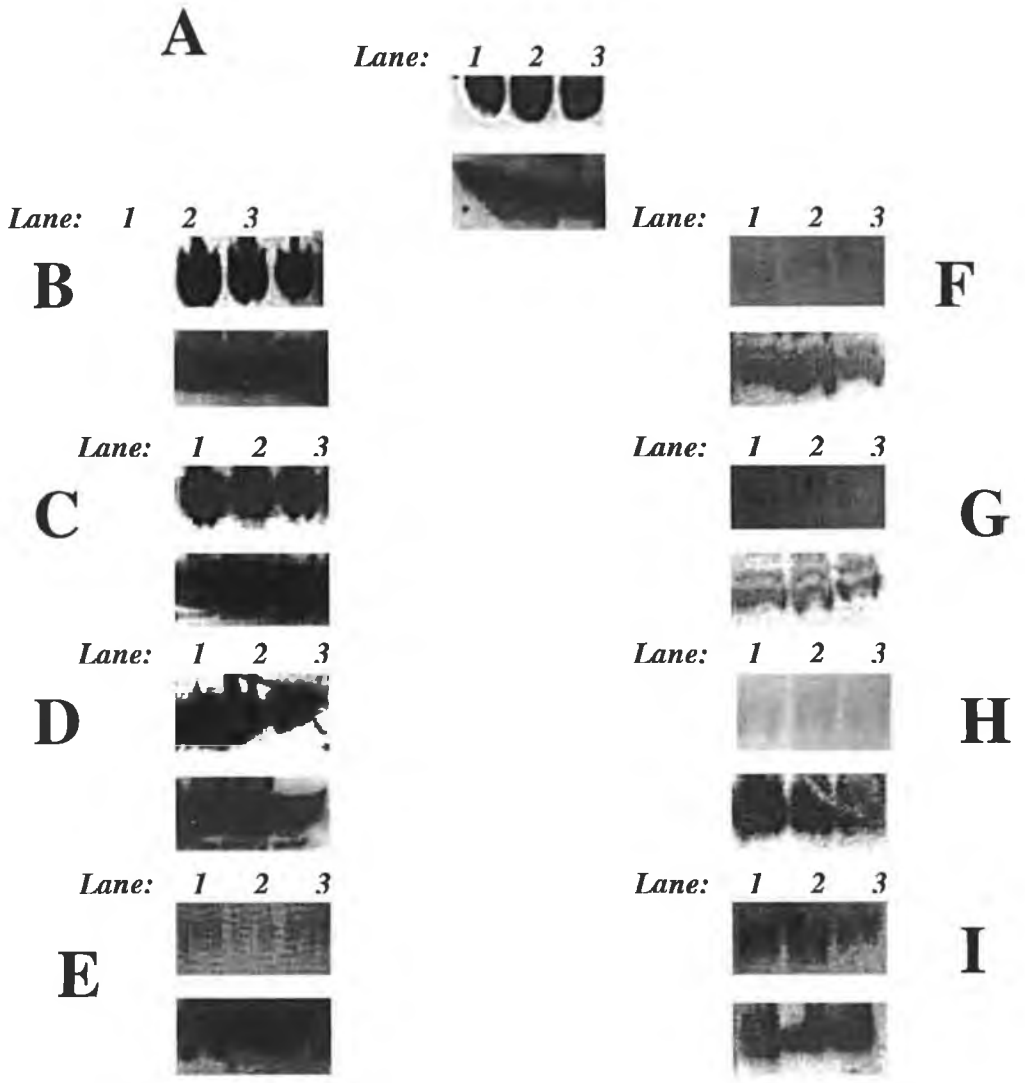

Figure 4.10: CLA modulates activation of p38 in J774 macrophages following TLR2 activation. Cells were cultured with either DMSO, c9, t11-CLA (50uM) or SA (25uM) for 7 days and then stimulated with PAM3CSK4 (1ug/ml/ml) or medium alone. Cells were harvested 0-6h following stimulation and total (bottom panel) and phosphorylated p38 (top panel) was determined in whole cell lysates by western blotting. A representative immunoblot is shown. **(A)** 0 hr time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(B)** 5 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(C)** 10 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(D)** 15 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(E)** 30 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(F)** 60 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(G)** 120 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(H)** 4 hr time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA. **(I)** 6hr time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA.

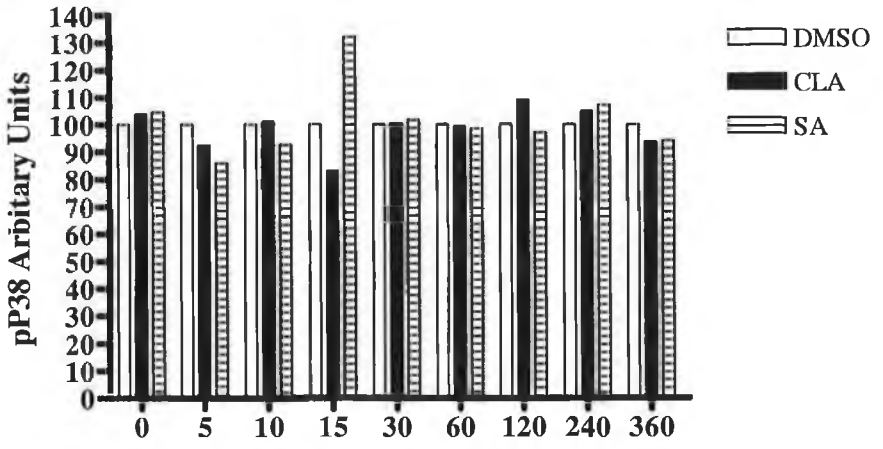

Fig 4.10 (A) CLA modulates activation of p38 in J774 macrophages following TLR2 activation. Densitometric analysis was carried out on immunoblots shown in Fig 3.10. p38 is expressed as arbitary units.

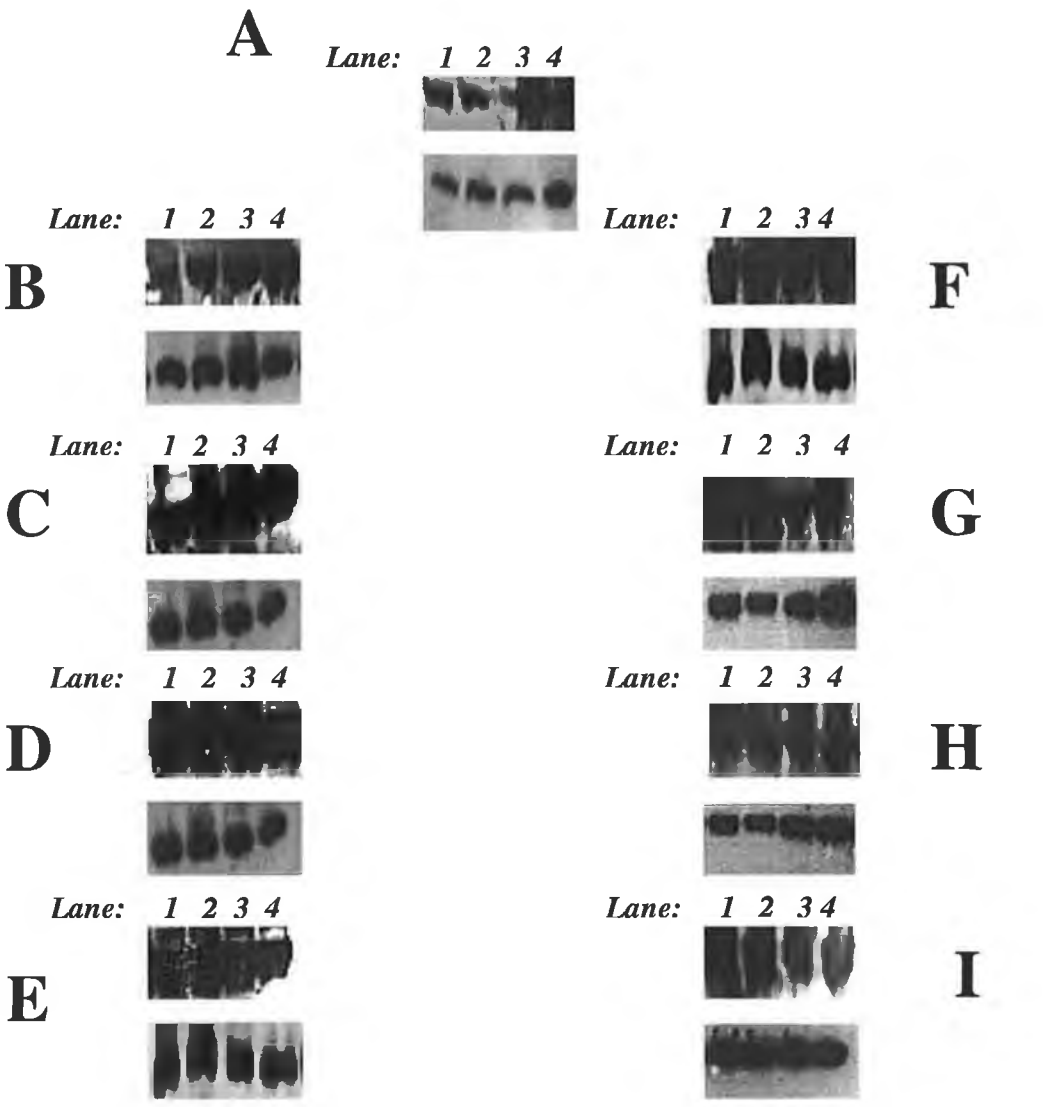

Figure 4.11: EPA and DHA modulate activation of p38 in J774 macrophages following TLR2 activation. Cells were cultured with either DMSO, EPA (25uM), DHA (25uM) or SA (25uM) for 7 days and then stimulated with PAM3CSK4 (1ug/ml) or medium alone. Cells were harvested 0-6h following stimulation and total (bottom panel) and phosphorylated p38 (top panel) was determined in whole cell lysates by western blotting. A representative immunoblot is shown. **(A)** 0 hr time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(B)** 5 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(C)** 10 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(D)** 15 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(E)** 30 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(F)** 60 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(G)** 120 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(H)** 4 hr time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(I)** 6hr time point: Lane 1 DMSO; lane 2 EPA; lane DHA; lane 4 SA.

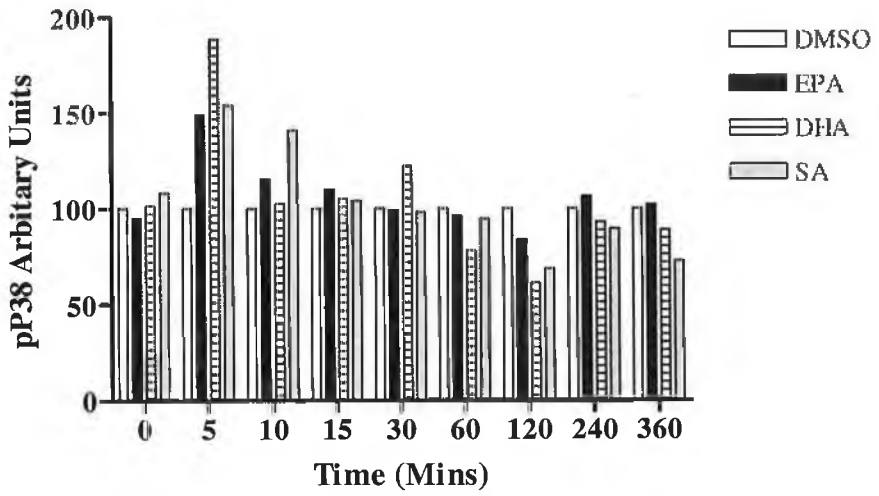

Fig 4.11 EPA and DHA modulate activation of p38 in J774 macrophages following TLR2 activation. Densitometric analysis was carried out on immunoblots shown in Fig 3.10. P38 is expressed as arbitary units.

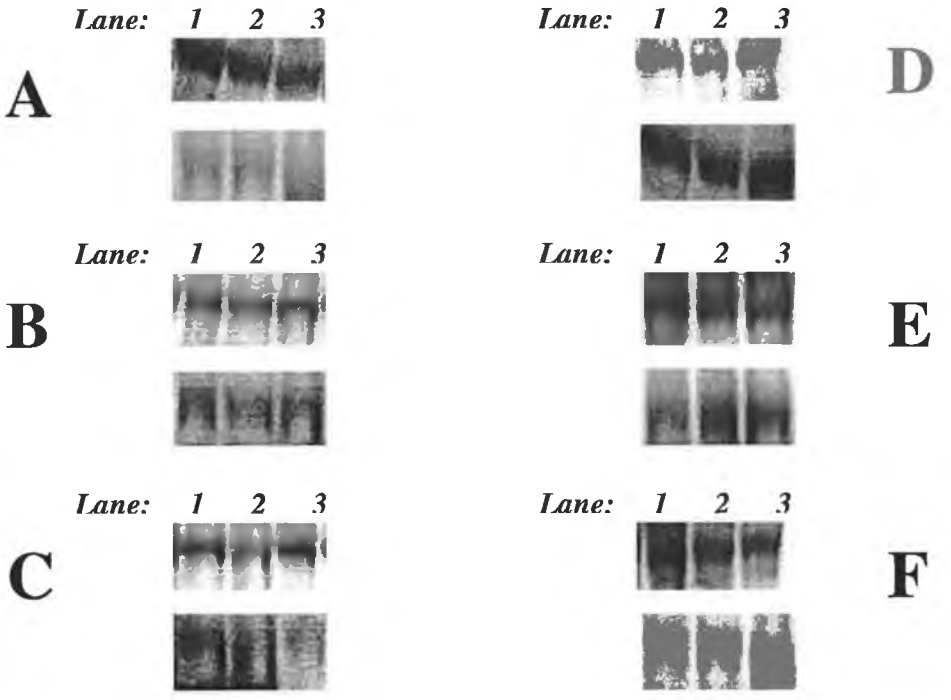

Figure 4.12: CLA modulates activation of JNK in J774 macrophages following TLR2 activation. Cells were cultured with either DMSO, c9, t11-CLA (50uM) or SA (25uM) for 7 days and then stimulated with PAM3CSK4 (1ug/ml) or medium alone. Cells were harvested 0-2h following stimulation and total (bottom panel) and phosphorylated JNK (top panel) was determined in whole cell lysates by western blotting. A representative immunoblot is shown. **(A)** 0 hr time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(B)** 30 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(C)** 60 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(D)** 120 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(E)** 240 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA; **(F)** 360 min time point: Lane 1 DMSO; lane 2 CLA; lane 3 SA.

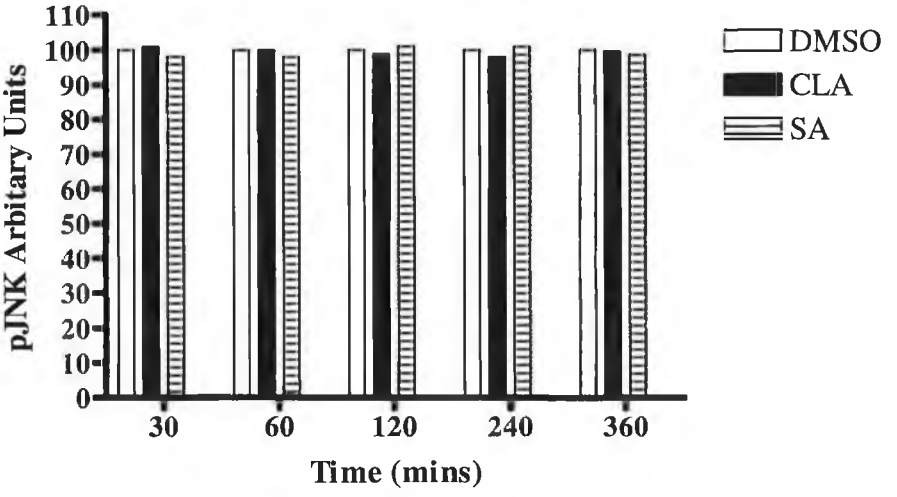

Fig 4.12(A) CLA modulates activation of JNK in J774 macrophages following TLR2 activation. Densitometric analysis was carried out on immunoblots shown in Fig 3.10. pJNK is expressed as arbitary units.

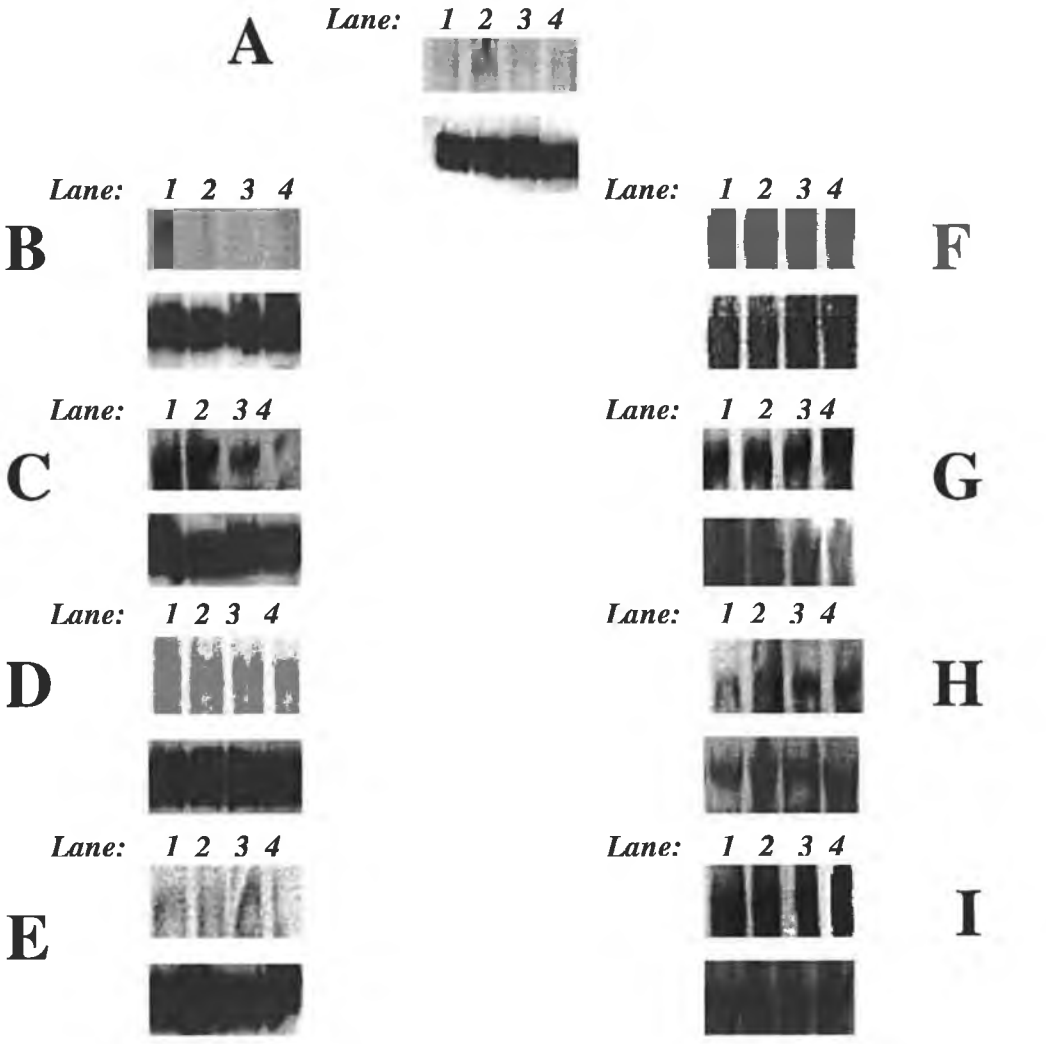

Figure 4.13: EPA and DHA modulate activation of JNK in J774 macrophages following TLR2 activation. Cells were cultured with either DMSO, EPA (25uM), DHA (25uM) or SA (25uM) for 7 days and then stimulated with PAM3CSK4 (1ug/ml) or medium alone. Cells were harvested 0-6h following stimulation and total (bottom panel) and phosphorylated JNK (top panel) was determined in whole cell lysates by western blotting. A representative immunoblot is shown. **(A)** 0 hr time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(B)** 5 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(C)** 10 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(D)** 15 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(E)** 30 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(F)** 60 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(G)** 120 min time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(H)** 4 hr time point: Lane 1 DMSO; lane 2 EPA; lane 3 DHA; lane 4 SA; **(I)** 6hr time point: Lane 1 DMSO; lane 2 EPA; lane DHA; lane 4 SA.

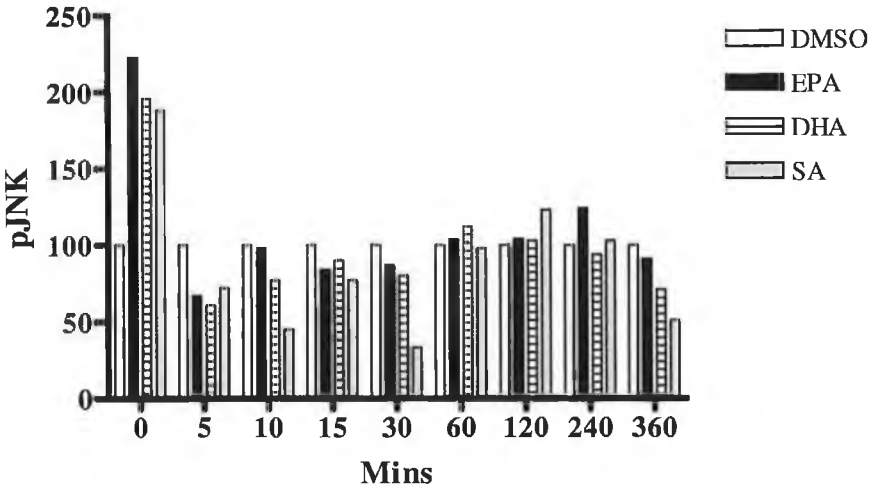

Fig 4.13(A) EPA and DHA modulate activation of JNK in J774 macrophages following TLR2 activation. Densitometric analysis was carried out on immunoblots shown in Fig 3.10. pJNK is expressed as arbitary units.

4.3 Discussion

The main findings of this study are that PUFA can modulate the response of macrophages to activation with the TLR2 ligand, PAM3CSK4. This is evidenced by the changes in MAP kinase activation, cytokine production, and even expression of TLR2 itself. Furthermore, this study demonstrates differential effects of these PUFA, suggesting that they may exert their effects through a number of different pathways.

This study shows that treatment of macrophages with CLA results in a significant enhancement of IL-10 production following TLR2 ligation. CLA exerted similar effects in the presence of a specific JNK inhibitor, however its positive effect on IL-10 was completely abrogated in the presence of inhibitors for ERK and p38. Analysis of the activation of these MAPK by western blotting revealed that CLA increased ERK activation following stimulation with PAM3CSK4, however there was little if any effect on p38 phosphorylation. These results suggest that the enhanced IL-10 production by CLA following TLR2 ligation, are mediated through activation of ERK.

EPA-treated macrophage also exhibited an increase in PAM3CSK4-induced IL-10 production, an effect that was maintained in the presence of specific inhibitors to all three MAPK examined. Even though the effect of EPA on IL-10 production was not apparently mediated by MAPK activation, we observed that EPA increased activity of ERK and to a lesser extent p38, following TLR2 ligation. DHA did not enhance IL-10 production following PAM3CSK4 stimulation of macrophages. This is surprising as it significantly enhanced IL-10 production following stimulation with the TLR4 agonist,

LPS (previous chapter). Interestingly while EPA enhances IL-10 through TLR2, it failed to have this effect following activation of TLR4 with LPS. Many studies examining the effects of the n-3 PUFA, EPA and DHA, have failed to show any individual effects. They have both been shown to suppress NFκB activation and have similar effects on production of cytokines such as IL-1β and TNFα in many cell types (Trebble et al., 2003, Weldon et al., 2007). Our data clearly show distinct effects of EPA and DHA, depending on the mode of activation of the cell.

IL-10 is the most potent deactivator of macrophage proinflammatory cytokine synthesis. IL-10 production has been shown to be enhanced in DC in the gut upon encountering gram positive commensals partially through TLR2 activation (Ferwerda et al., 2007). Peptidoglycan triggers IL-10 secretion, thereby suggesting a specific function in tolerance to commensal Gram-positive bacteria in Human Langerhans cells (Flacher et al., 2006). Induction of IL-10 by zymosan, a TLR2 agonist, has also been shown to require activation of ERK MAP kinase in murine DC (Slack, 2007). These studies demonstrate the importance of ERK and IL-10 following TLR2 ligation. Furthermore a role for p38 in TLR2 signaling has also been described. TLR agonists, particularly PAM3CSK4, have also shown to stimulate human neutrophil migration via the activation of ERK and p38 MAPK (Yoshioka and Asai, 2007). Furthermore, Horie et al (2007) show that the p38 MAPK pathway is functionally linked to IL-10 gene expression and is a step in the upregulation of IL-10 gene expression (Horie et al., 2007). Our data suggest that PUFA can enhance IL-10 production by increasing activation of p38 and ERK, which are downstream of TLR2 and have already been shown to be important in the production of IL-10.

Treatment of macrophages with all of the PUFA resulted in an increase in IL-12p40 production following stimulation with PAM3CSK4. This effect was maintained in the presence of the ERK and JNK inhibitor but was somewhat reversed with the addition of an inhibitor to p38. EPA perhaps demonstrates the strongest effect on p38 activity, as measured by western blot, while the other PUFA have little or no effect. The observed effect on IL-12p40 does not seem to be dependent on MAPK activation and appears non-specific as all of the PUFA, regardless whether they are saturated or unsaturated, exert the same effect. IL-12p40 is an important pro-inflammatory cytokine found to be involved in autoimmune disease such as IBD. It was surprising to see an increase in this cytokine, given that PUFA suppressed production of this cytokine following TLR4 activation. Furthermore, CLA and EPA exert such a dramatic effect on IL-10 that the increase in IL-12p40 may not be very significant overall.

Activation of TLR2 in macrophages did not result in significant production of TNFα, therefore we were unable to assess the impact of the PUFA on this cytokine. In the previous chapter, the PUFA had clear effects on TNFα following LPS stimulation and the consequences of this have already been discussed.

Our study clearly shows that PUFA can significantly enhance IL-10 and this is predominantly associated with activation of ERK. Given that TLR2 had now been implicated in a number of diseases such as atherosclerosis and IBD (Harris et al., 2006, Mullick et al., 2006), and the evidence that PUFA have beneficial effects in these disease (Bassaganya-Riera et al., 2002), it is now important to assess the effects of PUFA on TLR2 expression and activation as it may provide an insight into how

PUFA mediate their positive effect in these diseases. At present, ours is the first study to demonstrate that PUFA can modulate cytokine production following TLR2 ligation. Our findings with regard to IL-10 are particularly important. Enhanced production of the anti-inflammatory cytokine, IL-10 by PUFA may explain their benefits in diseases where increased expression of TLR2 has been shown. The ability of PUFA to enhance IL-10 through this receptor may result in significant production of IL-10 into the local environment, which inhibits some of the inflammation at the site.

Chapter 5
General Discussion

5.1 General Discussion

PUFA are nutritionally essential PUFA, essentiality referring to man's inability to synthesise these PUFA leading to complete dependency on the requirement for dietary supply. The dietary intake of PUFA results in their incorporation into every cell membrane of the body, where they bind to fatty-acid binding proteins and undergo metabolic conversions. There appear to be a number of targets for the anti-inflammatory effects of PUFA, none of which are mutually exclusive. Their effects on changes in cytokine production and mode of activation reported in this study will be discussed here.

EPA and DHA have been shown to decrease production of pro-inflammatory and T-helper 1 cytokines by monocytes and lymphocytes. Sierra et al., 2007, showed that n-3 PUFA down-regulate the inflammatory response by enhancing IL-10. Sierra et al., 2006, have also previously demonstrated that PUFA reduce innate and specific Th1 and Th2 immune responses increase immunomodulatory cytokines such as IL-10 and regulate gene expression. Fish oil rich diets (n-3 PUFA) are associated with higher TGF-β1 expression in T cells, a growth factor implicated in immune response modulation (Sierra et al, 2007). Studies involving THP-1 monocyte-derived macrophages have revealed that DHA suppresses the production of inflammatory cytokines TNF-α, IL-1β and IL-6, during LPS stimulation to a greater degree than EPA (Weldon et al, 2007). These studies show that PUFA can exert an anti-inflammatory effect. This response is important in the dampening of inflammatory disease through IL-10's ability to inhibit IL-12 production. Our data aggress with these studies and show that PUFA can suppress pro-inflammatory cytokine

production. Furthermore we provide evidence that these effects are mediated by activation of MAPK. Many studies using EPA and DHA have shown similarities between the two PUFA, however publications showing clear differences in the actions of EPA and DHA are lacking and the mechanisms of action of PUFA are still relatively unclear. This study has shown clear differences in their actions.

So far little data is available on the effects of PUFA on TLR2 activation. We however have shown that treatment of macrophage with PUFA resulted in a significant enhancement of IL-10 in both TLR2 and TLR4 activation. This data suggests that the PUFA enhancement of IL-10 is independent of specific TLR activation and may show that the relative potent anti-inflammatory nature of IL-10 is through increased production of IL-10 by several TLRs. Enhancement of IL-10 by all PUFA was also shown to be critically dependent on ERK MAPK activation and to a lesser extent by p38. This involvement of ERK was observed in both TLR2 and TLR4 activation. Mechanisms determining how PUFA modulate IL-10 are unclear but our data suggests that ERK MAPK is of critical importance in inducing increased expression of IL-10 by PUFA. CLA enhanced IL-10 production in both TLR2 and TLR4 activated cells. EPA enhanced IL-10 in TLR2 activated cells only, but no effect was observed with TLR4. DHA enhanced IL-10 production in TLR4 activated cells only, no effect was observed with TLR2. IL-10 production in all PUFA of this study was found to rely on an ERK dependent mechanism. These data suggest differences in the modes of activation of IL-10 by PUFA, in particular EPA and DHA. Many studies examining the n-3 PUFA, DHA and EPA have failed to show individual effects. Consequently, our data is the first to show that differing effects of EPA and DHA on IL-10 production is dependent on mode of activation of cell. Furthermore, it clearly

shows that the mode of activation of cells is critically important in regulation of cytokine production.

Our studies show that all PUFAs examined enhanced IL-12p40 production following TLR2 activation, however CLA and DHA had an inhibitory effect, while EPA had no effect on IL-12p40 following TLR4 activation. Again our data provides evidence that DHA and EPA have clear differences in modulation of cytokine production. The inhibition of IL-12p40 in TLR4 activated cells shows an anti-inflammatory mode of action by the PUFA, as IL-12p40 is a potent pro-inflammatory cytokine. TLR4 is known to be involved in many inflammatory diseases and this suppression of inflammatory mediator IL-12p40 and enhancement of IL-10 lends weight to growing evidence that PUFA exert anti-inflammatory properties. The enhancement of IL-12p40 by TLR2 is unclear as there have been no studies to date on the role of PUFA on TLR2. Further investigation is required to determine the significance and mechanism of action of this increase.

In nutritional studies the n-3 PUFA in fish oil have been shown to reduce inflammation in patients with ulcerative colitis. For these reasons the interest in inflammation and nutritional therapies has become a major area of research. TLR2 and TLR4 have been shown in previous studies to be potent producers of pro-inflammatory and anti-inflammatory cytokines. Furthermore, TLR4 is now believed to play a role in atherosclerosis (Li and Sun, 2007). Higher expression of TLR2 and TLR4 were found in both Crohns disease and ulcerative colitis suggesting that an abnormal mechanism may provide an excess of inflammatory mediators during the active phases of IBD (Canto et al., 2006). Zhao et al., 2007, have showed that

activation of TLR4 and TLR2, leucine-rich repeat-containing pattern recognition receptors, were differentially modulated by saturated and n-3 PUFA in macrophages and dendritic cells (Zhao et al., 2007). Naturally occurring PUFA and their metabolites are endogenous PPAR γ ligands. Bassaganya-Riera et al., 2004, showed dietary CLA induced colonic PPAR γ expression providing protection against disease in a pig model of bacterial-induced colitis (Bassaganya-Riera et al., 2002). Studies in animal models and humans show that CLA suppresses inflammation while enhancing antigen-specific responses against viral and bacterial pathogens. The decreased production of proinflammatory cytokines such as TNF-α in both human and animal models by CLA Song et al., 2003, suggests that the immune status is channelled into an anti-inflammatory profile. Evidence from diverse sources has suggested that TLRs can affect atherosclerosis in multiple ways (Song et al., 2003). Interestingly, CLA has been shown to suppress the development of atherosclerosis in experimental models. CLAs were shown to lower the release of vasoactive prostanoids from vascular smooth muscle cells (SMCs) which play a central role in atherosclerosis

Together our data suggests that PUFA may exert their beneficial effects in these diseases, by modulating cytokine production following ligation of multiple TLRs. Indeed the effects of PUFA on other TLRs should be examined.

Clearly there is a role for dietary fat modification in altering the inflammatory status of cells. Elucidating the exact molecular mechanisms involved in the beneficial effects of dietary PUFA will be important in designing novel nutritional-based therapies for the prevention and treatment of these diseases.

Chapter 6
Bibliography

6.0 Bibliography

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