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The Effects of Conjugated Linoleic Acid on the Human Immune System.

By

Anne Nugent

A Thesis for the degree of Doctor of Philosophy (Ph.D.)

Submitted to the University of Dublin, Trinity College

April 2003

Department of Clinical Medicine
University of Dublin
Trinity College
This thesis is dedicated to my parents, Margaret and James.
Declaration

I, the undersigned hereby declare that this thesis is my own work and it has not been previously submitted for any other degree at this or any other University except where otherwise acknowledged. This thesis is submitted to the University of Dublin Trinity College for examination for the degree of Ph.D., and may be made available from the library for consultation or copying.

Anne Nugent

Anne Nugent.


Date.
Summary

The cells of the immune system are sensitive to their exogenous environment. Indeed the amount and type of nutrients, including fatty acids, which this environment contains all influence subsequent immune cell functioning. Dietary fatty acids, in particular polyunsaturated fatty acids, modulate immune function. However, less is known about the effects of the dietary fatty acid conjugated linoleic acid (CLA) on immune function. CLA is a mix of positional and geometric isomers of the fatty acid linoleic acid. The predominant isomers are a cis-9, trans-11 CLA and trans-10, cis-12 CLA. Although it can be synthesised industrially, natural dietary sources include dairy products and certain meats (e.g. lamb). This thesis aims to investigate the influence of these two CLA isomers on human immune function ex vivo and in vitro and to investigate the molecular mechanisms underlying their effects.

The effects of supplementing the human diet with two isomeric blends of CLA were investigated in chapter three. Linoleic acid was included as a control. The CLA blends contained either an 80:20 or 50:50 mix of the cis-9, trans-11 CLA: trans-10, cis-12 CLA isomers. The 80:20 CLA blend enhanced mitogen stimulated lymphocyte proliferation whereas the 50:50 CLA blend decreased mitogen stimulated lymphocyte proliferation. Both isomeric blends enhanced mitogen stimulated TNFα and IL-2 secretion, as did linoleic acid. None of the fatty acids affected mitogen stimulated IL-4, PGE\(_2\) and LTB\(_4\) production or the circulating levels of sICAM-1.

Chapter four investigated the influence of the individual CLA isomers, and of the fatty acids (linoleic, stearic, oleic and palmitic acids), on mitogen stimulated cytokine (IL-4, IL-2 and TNFα) mRNA expression and production in vitro. The cis-9, trans-11 CLA isomer was the most influential of all the fatty acids tested. It consistently decreased IL-4 and IL-2 mRNA expression and IL-2 production. Trans-10, cis-12 CLA, linoleic and oleic acids all significantly decreased IL-2 production, whereas stearic acid reduced IL-2 mRNA expression. In addition, treatment with stearic and oleic acids decreased IL-4 mRNA expression. Palmitic acid significantly increased IL-2 mRNA expression, IL-4 production and TNFα mRNA expression and production. Gas-liquid chromatography confirmed that all the fatty acids were incorporated into the total cell lipids. Stearic acid alone reduced mitogen stimulated lymphocyte proliferation while none of the fatty acids affected PGE\(_2\).

The effects of fatty acids on the transcription factor NF-κB are presented in chapter five. The cis-9, trans-11 CLA isomer decreased the activity of this transcription factor in resting PBMC’s and in resting and activated monocytes of the THP-1 cell lineage. Linoleic acid and the trans-10, cis-12 CLA isomer decreased NF-κB activity to a lesser
extent, whereas palmitic acid was without effect. Both the cis-9, trans-11 CLA isomer and linoleic acid increased IkBα nuclear expression in LPS-activated THP-1 monocytes. However, none of the fatty acids tested affected cytoplasmic IkBα, nuclear p65 or cytoplasmic p65 expression.
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Firstly, I would like to thank Dr. Helen Roche for providing me with the opportunity to carry out this research, and for her valued encouragement and input throughout. I would also like to thank Professor Michael Gibney for his constant support and guidance.

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Abbreviations

AA  Arachidonic Acid
AP-1  Activating Protein 1
APC  Antigen Presenting Cell
CAM  Cell Adhesion Molecule
cAMP  Cyclic Adenosine Monophosphate
CD  Cluster of Differentiation
CD40L  CD40 Ligand
CLA  Conjugated Linoleic Acid
CO  Coconut Oil
Con A  Concanavalin A
COX  Cyclooxygenase
cPLA2  Cytoplasmic Phospholipase A2
CRM-1  Chromosome Maintenance Region 1
DAG  Diacylglycerol
DD  Death Domain
DEPC  Diethyl Pyrocarbonate
DHA  Docosahexaenoic Acid
DHGLA  Dihomogama Linoleic Acid
DMSO  Dimethyl Sulfoxide
DNA  Deoxyribonucleic Acid
DTH  Delayed Type Hypersensitivity
EC  Endothelial Cell
EMSA  Electrophoretic mobility shift assay
EPA  Eicosapentaenoic Acid
EPO  Evening Primrose Oil
ERK  Extracellular Signal Regulated Kinase
FADD  Fas-associated Death Domain
FBS  Foetal Bovine Serum
FCS  Foetal Calf Serum
FO  Fish Oil
GLA  Gamma Linoleic Acid
GPI  Glycosyl Phosphatidylinositol
GTP  Guanosine Triphosphate
HBSS  Hanks Balanced Salt Solution
HCO  Hydrogenated Coconut Oil
HETE  Hydroxy fatty acids
HLA  Human Leukocyte Antigen
HPETE  Hydroperoxy eicosantrienoic acids
HSVEC  Human Saphenous Venous Endothelial Cell
HUVEC  Human Umbilical Venous Endothelial Cell
IBTS  Irish Blood Transfusion Service
ICAM  Intercellular Adhesion Molecule
ICOS  Inducible Costimulatory Molecules
IFN  Interferon
Ig  Immunoglobulin
IKK  IKB Kinase
IL  Interleukin
IP  Inositol Phosphate
IP3  Inositol Triphosphate
IRS  Insulin Receptor Substrate
ITAM  Immunoreceptor Tyrosine Based Activation Motif
IkB  I kappa B
JAK  Janus Kinase
JNK  c-Jun Amino Terminal Kinases
LA  Linoleic Acid
LFA  Lymphocyte function associated molecule-1
LNA  Linolenic Acid
LOX  Lipoxigenase
LPS  Lipopolysaccharide
LT  Leukotriene
MA  Mead Acid
MAPK  Mitogen-Activated protein Kinase
MEK  MAPK Kinase
MEKK  MEK Kinase
MHC  Major Histocompatibility Complex
MO  Mendahen Oil
mRNA  Messenger Ribonucleic Acid
MUFA  Monounsaturated Fatty Acid
NES  Nuclear Export Sequence
NFAT  Nuclear Factor of Activated T-cells
NF-κB  Nuclear Factor κappa B
NIK  NF-κB Inducing Kinase
NKC  Natural Killer Cell
NLS  Nuclear Localisation Sequence
NO  Nitric Oxide
OA  Oleic Acid
Oct  Octamer Protein
OO  Olive Oil
PA  Phosphatidate
PA  Palmitic Acid
PBL  Peripheral Blood Lymphocyte
PBMC  Peripheral Blood Mononuclear Cell
PBS  Phosphate Buffered Saline
PCR  Polymerase Chain Reaction
PG  Prostaglandin
PHA  Phytohemaglutinin
PI-3K  Phosphoinositol-3-Kinase
PKA, PKB & PKC  Protein Kinases A, B & C
PLC & PLD  Phospholipases C & D
PMA  Phorbol-12-myristate-13-acetate
PPAR  Peroxisome Proliferator Activated Receptor
PTK  Protein Tyrosine Kinase
PUFA  Polyunsaturated Fatty Acid
PWM  Pokeweed Mitogen
RHD  Rel Homology Domain
RIP  Receptor Interacting Protein 1
RNA  Ribonucleic Acid
Rpm  Revolutions per minute
RPMI  Roswell Park Memorial Institute
RT  Reverse Transcribed
RT-PCR  Reverse Transcribed-Polymerase Chain Reaction
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>RXR</td>
<td>Retinoid X Receptor</td>
</tr>
<tr>
<td>SAF</td>
<td>Safflower Oil</td>
</tr>
<tr>
<td>SBO</td>
<td>Soybean Oil</td>
</tr>
<tr>
<td>sCAM</td>
<td>Soluble Cell Adhesion Molecule</td>
</tr>
<tr>
<td>SCF</td>
<td>SKp1-cullin-F-box type E3 ubiquitin ligase</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<td>SFA</td>
<td>Saturated Fatty Acid</td>
</tr>
<tr>
<td>SH2</td>
<td>Src Homology 2 Domain</td>
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<tr>
<td>SO</td>
<td>Sunflower Oil</td>
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<tr>
<td>SOCS</td>
<td>Suppressors of Cytokine Signalling</td>
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<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
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<td>TAD</td>
<td>Transactivation Domain</td>
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<tr>
<td>TCR</td>
<td>T-cell receptor</td>
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<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
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<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>Thp</td>
<td>T helper precursor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumour Necrosis Factor Receptor</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR1-associated Death Domain</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF Receptor Associated Factor</td>
</tr>
<tr>
<td>Tregs</td>
<td>T regulatory T-cells</td>
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<tr>
<td>TX</td>
<td>Thromboxane</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular Cell Adhesion Molecule</td>
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<td>VLA</td>
<td>Very Late Activating Antigen</td>
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Chapter One.

General Introduction.
1.0 The Immune System – Innate and Acquired immunity.

The Immune System represents a complex array of cells and secreted molecules whose main function is to defend the body (self) against a wide variety of infectious agents (including bacteria, viruses, fungi, parasitic worms, protozoa) and foreign (non-self) molecules (e.g. tumours) (Janeway & Travers, 1997). The immune system can be divided into two distinct parts termed innate and adaptive (acquired) immunity. Innate immunity represents the body’s (host’s) initial response to a pathogen or invading agent. Generally, the innate immune system involves physical, chemical and non-specific cellular protection against infection. It includes tears, mucous membranes and skin along with cell types such as neutrophils, basophils and mast cells (Lydyard et al, 2000). Adaptive immunity involves cells that possess the ability to recognize pathogens specifically and can mount a faster and stronger response on re-exposure to that pathogen, i.e. exhibit immunological memory (Todd, 2001). Principle cell types of the innate and adaptive immune systems including B- and T-lymphocytes are listed in Table 1.1. The research presented in this thesis is concerned with T- and B-lymphocytes and their interactions with lymphoid and non-lymphoid cells. Such interactions are mediated by communication molecules of the immune system e.g. cytokines, eicosanoids and adhesion molecules.

1.1.1 B- and T-lymphocytes.

Two major types of lymphocytes exist in the circulation. They both originate in the bone marrow from common progenitors but are classified according to their area of differentiation – B-cells in the bone marrow, T-cells in the thymus (Alberts et al, 1994). Both types of lymphocytes are distinguishable by their large round single nucleus surrounded by a thin rim of cytoplasm (Alberts et al, 1994). Two other types of lymphocytes can be identified – natural killer cells (NKC, also called large granular lymphocytes) and lymphokine activated T-cells (NKC’s activated with Interleukin-2). These lymphocytes have a larger cytoplasm, have distinct cytoplasmic granules and play a smaller (though vital) role in immunological responses (Lydyard et al, 2000, see Table 1.1). B- and T-lymphocytes express surface receptors, which bind specifically to antigens (i.e. non-self molecules). A process called clonal selection and expansion allows for millions of different antigen receptors to be expressed by lymphocytes hence yielding lymphocytes with a powerful sensitivity and ability to recognise, bind and react to a vast array of foreign molecules (Todd, 2001).

B-lymphocytes constitute 5-15% of human blood lymphocytes. Their main function is to secrete antibodies (Immunoglobulins, Ig) or soluble binding molecules that bind antigens
<table>
<thead>
<tr>
<th>Cell Type of Immunity:</th>
<th>Function</th>
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<tr>
<td>Granulocytes:</td>
<td></td>
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<tr>
<td>• Neutrophils</td>
<td>Innate</td>
</tr>
<tr>
<td>• Eosinophils</td>
<td>Innate</td>
</tr>
<tr>
<td>• Basophils</td>
<td>Innate</td>
</tr>
<tr>
<td>• Mast cells</td>
<td>Innate</td>
</tr>
<tr>
<td>• Monocytes &amp; macrophages</td>
<td>Innate</td>
</tr>
<tr>
<td>Lymphocytes:</td>
<td></td>
</tr>
<tr>
<td>• T-cells</td>
<td>Adaptive</td>
</tr>
<tr>
<td>• B-cells</td>
<td>Adaptive</td>
</tr>
<tr>
<td>• Natural Killer cells (LGL)</td>
<td>Innate</td>
</tr>
<tr>
<td>• LAK</td>
<td>Innate</td>
</tr>
<tr>
<td>Other:</td>
<td></td>
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<tr>
<td>• Dendritic cells</td>
<td>Innate</td>
</tr>
<tr>
<td>• Platelets</td>
<td>Innate</td>
</tr>
<tr>
<td>• Erythrocytes</td>
<td>Innate</td>
</tr>
</tbody>
</table>

LAK, lymphokine activated killer cells; LGL, large granular lymphocytes;

recognised specifically by that cell (Janeway & Travers, 1997). Five main classes of Ig exist – IgG, IgM, IgA, IgE and IgD. All are crucial in antigen recognition and have different biological functions (Lydyard et al, 2000). By somatic mutation (changing their antigen-binding properties), switching from one class of antibody to another, turning into antibody secreting plasma cells and forming a bridge whereby other cells of the immune system, particularly the innate immune system, recognise and bind to the B-cell receptor (Fc chain), B-cells play a vital role in propagating an immune response. They bind antigen rendering it inactive and retain this information as part of immunological memory (Todd 2001).

T-lymphocytes comprise 70% of all blood lymphocytes hence exert a dominant influence on all immunological reactions (Janeway & Travers, 1997). T-lymphocytes are mainly responsible for regulating other cells of the immune system and for the killing and elimination of infected or malignant cells (Janeway & Travers, 1997). T-cells express a critical surface receptor (T-cell receptor, TCR) and indeed are classified according to the polypeptides included in this receptor. Most TCR contain polypeptides called α and β chain. These αβ T-cells are concentrated in particular areas/organs e.g. lymph nodes and the spleen but are also found in the circulation (De La Hera et al, 1991). However, a smaller subset of T-cells (approx. 10-15% all T-cells) contains γδ polypeptide chains in their TCR. These γδ T-cells are believed important in protecting the host against epithelial pathogens, as they are mainly concentrated in the gut, lung and skin epithelium (Carding & Egan, 2002). However, their exact immuno-biology remains unknown (Carding & Egan, 2002). αβ T-cells are more abundant and the focus of this thesis. Their structure is represented in Fig 1.1.

T-lymphocytes are unique insofar as they only recognise processed antigen or antigen that is presented to them by other cells of the immune system (antigen presenting cells, APC), including B-lymphocytes, monocytes, macrophages and dendritic cells (Todd, 2001). Antigen presented by these APC has been recognised, processed and are presented on their external surface in conjunction with molecules called major histocompatibility complex (MHC). This antigen-MHC complex is then recognised by the αβ TCR, binds to and initiates a T-cell response (Janeway & Travers, 1997) (see Fig 1.1). Two distinct class of MHC exist (MHC class I and class II), their difference lying in the type of antigenic peptide they present to one of two subclasses of αβ T-cells expressing either a CD8 or CD4 receptor on their surface. APC expressing MHC class I molecules generally process and present peptides produced by intracellular microbes or virally infected cells to CD8 T-cells (Lydyard et al, 2000). These CD8 T-cells (cytotoxic T-cells) then kill the infected or
Fig 1.1 Outline of the TCR: MHC complex.
malignant cells by secreting proteins called perforins and granzymes which puncture target cell membranes, and subsequently activate capase enzymes which initiate apoptosis (Todd, 2001). Cytotoxic T-cells also express ligands called Fas ligand and can secrete cytokines (e.g. TNFα), which are also involved in apoptosis.

APC expressing MHC class II molecules present bacterial peptides to a group of CD4αβ T-cells called T-helper cells (Alberts et al, 1994). Two types of T-helper cells exist: Th1 and Th2 cells. These T-helper cells provide help to cells of the innate and adaptive immune systems and originate from a common antigen naïve T-cell precursor or Thp cell (Seder & Paul, 1994). Upon exposure to antigen and in the context of an APC, this Thp cell can undergo differentiation into an uncommitted cell called a Th0 cell (Bucy et al, 1995, Zhai et al, 1999). The exact nature of this Th0 cell is unclear. However, it is proposed as a common precursor that can differentiate into either a Th1 or Th2 cell (Sad & Mossman, 1994, Nakamura et al, 1997). This differentiation process is dependent upon the nature of the antigenic stimulation and the surrounding cytokine profile (O’Gara, 1998). Thp cells are capable of producing Interleukin 2 (IL-2) (Sad & Mossman, 1994), while Th0 cells are described as producing IL-2, Interleukin-4 (IL-4) and Interferon-γ (IFN-γ) (Seder & Paul, 1994). Th0 cells presented with antigen in the presence of IL-4 result in B-cell activation and antibody production (humoral immunity). They are also implicated in allergic reactions (Swain, 1990) and immune responses to helminths and extracellular parasites (Nelms et al, 1999). The source of IL-4 initiating this process is debated (Swain, 1990), however Th2 cells are characterised by the secretion of IL-4, Interleukin-5 (IL-5) and Interleukin-10 (IL-10) (Swain, 1990, Farrar et al, 2002). On the other hand, exposure of Th0 cells to Interleukin-12 (IL-12) from activated macrophages and dendritic cells, promotes Th-1 development. Th1 cells secrete IFN-γ and IL-2 (Hsieh et al 1993, Farrar et al, 2002). They are important in activating cells involved in cell-mediated immunity (CMI), inflammation, graft rejection, and delayed-type hypersensitivity (DTH) (Hsieh et al, 1993). Negative feedback exists between the different Th1 and Th2 derived cytokines. A schematic representation of the development of Th1 and Th2 cells is outlined in Fig. 1.2.

Antigen dose and affinity have been suggested to influence Th1/Th2 development. Very low and very high antigen doses are suggested to promote a Th2 response, whereas moderate antigen levels prejudice naïve cells to develop a Th1 phenotype (Murray, 1998). However, when antigen dose and affinity are considered concurrently, the exact opposite occurs. Low and high doses of high affinity antigen yield Th1 development, while moderate doses of high affinity antigen lead Th2 cell formation (Rogers & Croft, 1999).
**Fig 1.2.** Overview of the immune system focusing on lymphokine (Th1/Th2 cytokine profile) and monokine production.
The secretion of cytokines and subsequent development of Th1/Th2 subsets is also affected by the frequency of cell division. Initial IL-2 secretion is cell cycle independent as Thp/Th0 cells secrete it (Sad & Mossman, 1994). IFN-γ expression increases thereafter, whereas expression of IL-4 requires at least three cell divisions (Bird et al., 1998, Gett & Hodgin, 1998). Therefore development of Th1 cells appears to have the initial edge. However IL-4 is dominant acting over IL-2 or IFN-γ and although produced in later cell divisions, it can drive cells into a Th2 phenotype (O’Gara, 1998).

Recently it was reported that the balance of calcium and protein kinase C (PKC) could direct human T-cells towards a Th1 or Th2 phenotype: stimulation of calcium signalling or inhibition of PKC favoured Th1 type differentiation, whereas stimulation of PKC or inhibition of calcineurin resulted in the development of Th2 cells. Further research is needed in this area (Noble et al. 2000).

Finally a third class of Th lymphocyte has been identified called regulatory T-cells (Treg). Tregs are CD4+CD25+ Th cells and include distinct populations of T-cells called Th3 cells, Tr1 cells, and anergic cells (reviewed in Read & Powrie, 2001). These T-cells specialise in the suppression of immune responses e.g. Th3 subset produces immunosuppressive cytokines e.g. transforming growth factor β (TGFβ), which are inhibitory to Th1 and Th2 cells (Read & Powrie, 2001).

### 1.1.2 Cytokines.

Cytokines are small soluble polypeptides that function as intercellular signal mediators acting in an autocrine, paracrine and even endocrine manner (Oppenheim & Saklatvala, 1993). Cytokines act as communicators between other cells of the immune system as well as non-lymphoid tissues. Cytokines range in size from 6,000 to 60,000 kilodaltons (kDa) but are extremely potent, active at concentrations as low as 1 pg/ml and bind to complimentary surface receptors with high affinity (kDa $10^{-9}$ to $10^{-12}$ M). Cytokine-receptor occupancy of even <10% can activate a number of intracellular signalling pathways and initiate gene transcription (Oppenheim & Saklatvala, 1993). Cytokines can be classified into a number of families depending on their function and origin, e.g. T and B cells produce lymphokines, monocytes secrete monokines and antigen-stimulated lymphocytes secrete antiviral cytokines called interferons (IFN) (Lydyard et al., 2000). Functional properties of cytokines include redundancy (whereby different cytokines can act on the same cell type to mediate similar effects) and pleiotrophy (cytokines exhibit a range of biological effects on tissues and cells) (Lydyard et al., 2001). Finally, a given cell type can produce a range of cytokines once stimulated, however
normal cells do not produce cytokines unless stimulated. Excessive cytokine production has the potential for tissue damage, hence cytokine production is strictly controlled and the cytokine profile of healthy populations and individuals mounting an immune response will therefore be different (Kishimoto et al, 1994).

1.1.3 Interleukin-2.

Interleukin-2 (IL-2) is a T-cell derived lymphokine (15.5 kDa) pivotal in T-cell, B-cell and NKC proliferation and maturation. These cell types (particularly Th1 cells) are the primary producers of IL-2. Some of the biological effects of IL-2 are listed in Table 1.2. The human IL-2 gene has been mapped to chromosome 4q bands 26-28 (Siegel et al, 1984). Inducible expression of this gene is controlled at a transcriptional level by a 5’ enhancer element containing cis-acting regulatory sequences including binding sites for the transcription factors NFAT, NF-κB, AP-1 and octamer proteins (Durand et al, 1988, Shaw et al, 1988, Hoyos et al, 1989, Muegge & Durum, 1989, Serfling et al, 1989). Central to the functioning of IL-2 is the binding to its receptor IL-2R. The IL-2R is composed of three distinct, membrane-associated subunits: a 55kDa α chain (IL-2Ra, Tac, p55), a 70-75 kDa β chain (IL-2Rβ, p70/75), and a 64 kDa γ chain (IL-2Rγ, p64). Each individual subunit can bind IL-2 with low affinity, however subunit heterodimerisation (generally αβ or αγ complexes) or heterotrimerisation (αβγ) permits binding with intermediate and high affinity respectively (Goldsmith & Greene, 1996). Detailed information in relation to intracellular IL-2 signalling is dealt with in Section 1.2.6 (i).

1.1.4 Interleukin 4.

Interleukin-4 (IL-4) is also a member of the lymphokine family of cytokines. It is a pleiotrophic cytokine that plays a critical role in the regulation of immune responses (Nelms, 1999). IL-4 is produced chiefly by Th2 cells, basophils and mast cells (Nelms et al, 1999) and to a lesser extent by γδ T-cells and eosinophils (Zuany-Amorim et al, 1998). Some of the biological functions of IL-4 are listed in Table 1.2. IL-4 is mapped to chromosome 5q31 (Arai et al 1989). Transcriptionally, the IL-4 gene has known binding sites for STAT-6 (Fenghao et al, 1995) and NFAT (Schmidt-Weber et al, 2000). Like IL-2R, IL-4R consists of subunits, namely an IL-4Rα chain (140 kDa, high affinity receptor) and the common IL-4γc chain. The IL-4γc receptor is also a component of the IL-2R (Russell et al, 1993). Although IL-4Rγc only slightly enhances the affinity of IL-4 for the IL-4R heterodimer, it is critical for activation of the subsequent signalling pathway and exerting physiological effects (Russell et al, 1993). Recently it has also been shown that
IL-4Rα also functions as a part of the IL-13 receptor (reviewed in Nelms et al., 1999). A secreted from of the receptor resulting from alternatively spliced mRNA also exists – this may function as a competitive inhibitor for IL-4 (Idzerda et al., 1990). IL-4 signalling is presented in Section 1.2.6 (ii).

**Table 1.2. Immunological functions of IL-2 and IL-4.**

**IL-2**
- Autocrine & paracrine stimulation of IL-2R expressing T-cells
- Progression of T-cell cycle from G1 to S phase (i.e. activation)
- Augment IFN-γ & IL-4 production (differentiation factor Th1 & Th2 cells)
- Activation, tumoricidal activity and growth of NKC & LAK.
- Enhance B-cell growth and Ig production
- Secretion of monocytic IL-6 & macrophage differentiation
- Modulate histamine release from stimulated basophils
- Modulate expression of IL-2R
- Thymic T-cell development, signalling and TCR-rearrangement.
- Anti-apoptotic signals.

**IL-4**
- Differentiation Ag-stimulated naïve T-cells into Th2 cells
- Induces secretion IL-5, IL-10 & IL-13 from Th2 cells
- Suppresses Th1 cells (downregulates IL-2Ra)
- Co-mitogen for B-cell growth
- Ig class switching to IgE and IgG4.
- Increases MHCII expression on B-cells
- Enhances expression of CD23
- Upregulates IL-4R expression
- Upregulates VCAM-1 with TNFα
- Downregulates E-selectin
- Involved in allergic reactions and protection from helminths & parasites
- Ameliorates the tissue damaging effects of autoimmunity

1 Table adapted from Nelms et al. (1999) & Goldsmith & Greene (1996).

1.1.5 Tumor Necrosis Factor alpha (TNFα)

TNFα is often described as a pro-inflammatory cytokine with an important role in infection, injury, cachexia and sepsis (See Table 1.3 for functions). There are two forms of TNF: TNFα (cachectin) and TNFβ (lymphotoxin), however this thesis will focus on TNFα. TNFα is principally synthesised by macrophages, but also by lymphocytes, polymorphonuclear leukocytes and eosinophils in response to viruses (e.g. HIV, influenza), mycobacteria, fungi, parasites, products of complement activation, antigen-antibody complexes, other cytokines and bacterial toxins (e.g. lipopolysaccharide, LPS) (Tracey & Cerami, 1994). However unlike IL-4 and IL-2, TNFα can exert systemic effects
on adipocytes and myocytes, as well as cells of the immune system (Tracey & Cerami, 1994, Tracey, 1995).

The gene for TNFα is located on chromosome 6 and encodes for a prohormone of 26kDa (Wang et al, 1985, Muller et al, 1987). This prohormone is believed to anchor the precursor to the extracellular cell membrane surface, is bioactive and implicated in the paracrine activities of TNFα in tissues (Tracey & Cerami, 1994). On activation, this prohormone is cleaved yielding a 17kDa hormone. Three of these TNF monomers associate noncovalently to form the biologically active TNF trimer (Jones et al, 1989). Two forms of TNFα receptor exist: Type 1 (TNFR-55) and Type 2 (TNFR75) and are present on virtually every cell type except erythrocytes (Hohmann et al, 1989). They have similar structural homology and ligand affinity but induce separate cytoplasmic signalling pathways following receptor-ligand binding (Smith et al, 1990, Thoma et al, 1990)(See Section 1.2.6). Cleaved fragments of both receptor types called TNF-binding proteins are detectable in the urine and serum of patients with cancer, aids and sepsis (Aderka et al, 1991, Kalinkovich et al, 1992). Their exact role is unclear but is thought to involve inhibition TNFα activity by preventing receptor-ligand interaction, or prolonging the activity of circulating TNFα by stabilising the TNFα trimeric structure and preventing clearance (Aderka et al, 1992). TNFα signalling is outlined in Section 1.2.6 (iii).

Table 1.3. Immunological & physiological functions of TNFα

<table>
<thead>
<tr>
<th>TNFα</th>
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<tbody>
<tr>
<td>Inflammatory</td>
<td>• activation of cell cytotoxicity &amp; enhanced NKC function,</td>
</tr>
<tr>
<td></td>
<td>• mediation of IL-2 tumour toxicity,</td>
</tr>
<tr>
<td></td>
<td>• cytokine induction including IL-1, IL-6, IL-8, IFNγ</td>
</tr>
<tr>
<td>Central Nervous System</td>
<td>• fever &amp; anorexia</td>
</tr>
<tr>
<td></td>
<td>• altered pituitary hormone secretion</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>• shock &amp; capillary leakage syndrome</td>
</tr>
<tr>
<td>Renal</td>
<td>• acute tubular necrosis &amp; nephritis</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>• ischaemia,</td>
</tr>
<tr>
<td></td>
<td>• colitis &amp; hepatic necrosis</td>
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<tr>
<td>Metabolic</td>
<td>• lipoprotein lipase suppression,</td>
</tr>
<tr>
<td></td>
<td>• net protein and lipid catabolism,</td>
</tr>
<tr>
<td></td>
<td>• insulin resistance &amp; stress hormone release.</td>
</tr>
</tbody>
</table>

1Table adapted from Tracey & Cerami (1994) & Tracey (1996).
1.1.6 Eicosanoids.

Eicosanoids have been described as autacoid lipid mediators (Fitzpatrick & Soberman, 2001) affecting the cellular function and microenvironment of many cell types, including those of the immune system (Sperling, 1998). Eicosanoids are derived from 20-carbon polyunsaturated fatty acids (PUFA) e.g., arachidonic acid (AA; C20:4n-6), eicosapententaenoic acid (EPA; C20:5n-3) and dihomo-γ-linolenic acid (DHGLA; C20:3n-6). It is possible for eicosanoids to be formed from mead acid (MA; C20:3n-9), however only in essential fatty acid deficient states. The fatty acids or their precursors are derived from dietary fat intake and are readily incorporated into the cell membrane phospholipids at the sn-2 position (Sardesai, 1992). Western diets rich in n-6 fatty acids, particularly AA, result in a predominance of AA-derived eicosanoids in the circulation. N-3 PUFA can also function as substrates for eicosanoid synthesis. N-3 PUFA displace AA from the phospholipid membrane, and result in the production of an independent set of less biologically active eicosanoids (Calder et al., 2002).

The cell membrane bound AA (n-6 PUFA) is mobilised by the phospholipid enzyme phospholipase A2 (PLA2) yielding 2-lyso-phospholipids and non-esterified PUFA (Anggard & Samuelsson, 1965). Non-esterified PUFA are the principal substrates for the two major families of eicosanoids – cyclooxygenase (COX) and lipoxygenase (LOX) derived eicosanoids (See Fig 1.3). Two isoenzymes of COX exist; COX-1 and -2. Different genes encode these two isoenzymes but they show a high degree of homology (Hla & Neilson, 1992). COX-1 is constitutively found in most cell types and involved in initial responses to immunological challenge, whereas COX-2 is undetectable, expression only increases following stimulation e.g. increased expression is rapidly detected in monocytes following lipopolysaccharide (LPS) exposure (McAdams et al., 2000). COX-2 metabolises most of the AA when it is present at a low concentration (Tilley et al., 2001). The main derivatives of the COX pathway include the prostaglandin (PG), the prostacyclin (PC) and the thromboxane (TX) families. There are three LOX isoenzymes (5-, 12- and 15- LOX). Derivatives of the LOX pathway include hydroperoxy eicosantrienoic (HPETE) acids, hydroxy fatty acids (HETE), lipoxins and leukotrienes (LT). Many different compounds exist belonging to each class of eicosanoid but they are formed in a cell-specific manner. Mast cells produce PGD2, neutrophils PGE2 while macrophages and monocytes secrete large quantities of PGE2 and PGF2 (Tilley et al., 2001, Yaqoob & Calder, 2002). The three LOX enzymes have different tissue distributions. 5-LOX chiefly found in mast cells, monocytes, granulocytes and macrophages whilst 12- and 15- LOX predominate in epithelial cells (Tilley et al., 2001).
Fig 1.3. Synthesis of eicosanoids from arachidonic and eicosapentaenoic acids.\(^1\)

Control of eicosanoid biosynthesis is thought to involve co-ordinated action of specific phospholipases, restricted expression of eicosanoid biosynthetic enzymes (e.g. COX-2) and the “suicide” inactivation of these biosynthetic enzymes (Fitzpatrick & Soberman, 2001). For both COX and LOX derived eicosanoids, critical to their physiological effect is the type of cell acquiring the eicosanoid, eicosanoid concentration, the timing of their release, target sensitivity, level of immune cell activation, presence of other mediators and the physiological state of the organism (Tilley et al., 2001, Calder et al., 2002). PGE\(_2\) and LTB\(_4\) are two potent eicosanoids affecting the immune system and were the focus of investigation of this thesis.

1.1.7 Prostaglandin \(E_2\) (PGE\(_2\))

PGE\(_2\), a 20-carbon carboxylic acid with a cyclopentane ring, two aliphatic chains and terminal carboxyl group, is a primary product of AA metabolism in many cells. PGE\(_2\) does not exist in any cellular reservoir but is synthesised \textit{de novo} by prostaglandin endoperoxides (e.g. PGG\(_2\) and PGH\(_2\)) and released into the extracellular space following cell activation or substrate availability (Hamberg & Samuelsson, 1971). PGE\(_2\) is rapidly converted to 13, 14-dihydro-15-keto PGF\(_2\) by the prostaglandin 15-dehydrogenase pathway, having a half-life of just 30 seconds (Fitzpatrick et al., 1980). Although present in very low concentrations, normal plasma levels range between 3-12 pg/ml (Fitzpatrick et al., 1980), PGE\(_2\) determines the intensity and duration of immune responses. PGE\(_2\) exerts both pro- and anti-inflammatory effects. PGE\(_2\) is credited with inducing fever and redness, increasing vascular permeability and enhancing pain and oedema caused by other agents e.g. histamine (Ferreria, 1974, Richardson et al., 1976, Tilley et al., 2001). However PGE\(_2\) has also been shown to inhibit lymphocyte proliferation (Goodwin et al., 1974, Webb et al., 1980), cytokine production (e.g. IL-2, IL-1, TNF\(_{\alpha}\), IL-6 & IFN-\(\gamma\)) (Gordon et al., 1976, Knudsen et al., 1986), the generation of cytotoxic cells (Plaunt, 1979) and NKC activity (Roder & Klein, 1979). The EP2 receptor is postulated as the receptor that mediates actions of PGE\(_2\) to inhibit T-cell proliferation (Tilley et al., 2001).

1.1.7 Leukotriene \(B_4\) (LTB\(_4\))

LTB\(_4\), derived by the action of 5-LOX on AA, has a structure consisting of four double bonds and is a chief mediator of inflammation (Sardesi et al., 1992). Leukocyte stimulation causes plasma levels of LTB\(_4\) to rise from below 100pg/ml to more than 100ng/ml (Doyle et al., 1990). LTB\(_4\) is metabolised in leukocytes and hepatocytes first by cytochrome P450 enzymes, followed by \(\beta\)-oxidation to less active LTB\(_4\)-derivatives.
LTB₄ is a potent mediator of inflammation, in particular responses initiated by the innate immune system. It is a potent chemotactic agent for leukocytes, induces release of lysosomal enzymes, enhances the generation of reactive oxygen species, increases vascular permeability and local blood flow, promotes NKC activity, inhibits lymphocyte proliferation and enhances the production of inflammatory cytokines (Ford-Hutchinson, 1990, Mayatepek & Hoffmann, 1995). LTB₄ function is concentration dependent: at subnanomolar ranges (3.9 x 10⁻⁶ M) LTB₄ causes chemotaxis and chemokinesis in human polymorphonuclear leukocytes. At higher concentrations (1.0 x 10⁻⁷ M) LTB₄ causes neutrophil aggregation and degranulation along with superoxide anion production (Ford-Hutchinson, 1990).

1.1.9 Adhesion molecules.

Central to any immunological response is the process of physical cell-cell contact amongst lymphoid cells and also between lymphoid and non-lymphoid cells. Cellular adhesion molecules (CAM) orchestrate this physical 'link' between the different cells involved in any immune response. They result in intercellular adherence and attachment to extracellular matrices thus allowing for host defence against infections, wound healing and normal immune functioning (Wayne Smith, 1996). Three main families of adhesion molecule exist according to their structural architecture: selectins, integrins and the Ig superfamily.

Three members of the selectin superfamily exist called L-, P- and E-selectins. Selectin molecules are characterised by a lectin-like domain attached to an epidermal growth like factor domain and a number of complement regulatory protein repeat sequences comprising the extracellular motif (Kansas, 1996). Selectins are important calcium dependent carbohydrate binding proteins (Wayne-Smith, 1996). L-selectin is constitutively expressed on leukocytes and rapidly shed upon activation. P-selectin is stored pre-formed in platelet α-granules, endothelial cells and Weibel-Palade bodies, released to the cell surface following activation and subsequently either shed or internalised. E-selectin is only expressed following cytokine (e.g. TNFα, IL-1) or endotoxin stimulation (Bevilacqua et al, 1989, McEver, 1991, Kansas, 1996, Meager, 1999). Selectins are important for the localisation of granulocytes and monocytes to sites of inflammation. L-selectin is also necessary for lymphocyte homing into peripheral lymph nodes (Meager, 1999). Collectively they are primarily involved in leukocyte trafficking and the initiation of leukocyte rolling (Bevilacqua & Nelson, 1993).
Integrins are composed of non-covalently linked α and β heterodimers containing a large N-terminal extracellular domain, many N-linked glycosylation sites, a transmembrane domain and a C-terminal cytoplasmic domain (Meager, 1999). At least 14 α subunits and 8 β subunits exist hence many combinations are possible. Integrins are found on leukocytes, monocytes, granulocytes, basophils and eosinophils (Meager, 1999). These CAM are important in the firm adhesion of immune cells to endothelial extracellular matrix proteins (e.g. fibronectin, laminin), counter receptors on other cells (e.g. Intercellular adhesion molecule, ICAM) or soluble, multivalent molecules (e.g. fibronectin or von Willebrand factor) (Price & Loscalzo, 1999). Some integrin subfamilies include very late appearing antigen (VLA), leukocyte integrins (LFA) and the cytoadhesion integrins. Integrin expression varies with state of cell or tissue differentiation: cellular stimulation (e.g. by cytokines) can increase the expression and functional characteristics of integrins (Wayne Smith, 1996).

The Ig superfamily are primarily expressed on endothelial cells and function as ligands for the integrins expressed on leukocytes and platelets (Price & Loscalzo, 1999, Meager, 1999). The Ig Superfamily consists of a series of immunoglobulin-like domains of 90 to 100 amino acids, a transmembrane region and a short cytoplasmic tail. They partake in cellular firm adhesion and extravasation, signal transduction resulting in neutrophil and lymphocyte activation and in phagocytosis (Kevil & Bullard, 1999). Members include intercellular adhesion molecules -1 -2 and -3 (ICAM -1 -2 -3), vascular cell adhesion molecule-1 (VCAM-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1). They are primarily expressed on endothelial cells, polymorphonuclear leukocytes, fibroblasts, lymphocytes and monocytes (Gearing & Newman, 1993). With respect to the adhesion of mononuclear cells to activated endothelial cells, Abe et al. (1996) reported the interactions between VCAM-1 and α4 integrin to be the most significant. E-selectin and ICAM-1 played a lesser, more supporting roles in their in vitro system.

### 1.1.10 The interactions between cytokines and cell adhesion molecules.

Cytokines are important regulators of CAM expression. Indeed, cytokine-induced enhanced adhesiveness of the endothelial cells depends on de novo protein synthesis and increased CAM expression (Springer, 1994). As mentioned earlier, cytokines such as TNF-α and-β, IL-1-α and-β strongly upregulate E-selectin, ICAM and VCAM expression (Meager, 1999). IL-4 and IL-13 can upregulate VCAM-1, particularly in concert with TNFα but inhibit late expression of E-selectin (Palmer Crocker & Pober, 1995, Ying et al.,
IL-10 inhibits TNFα induced ICAM-1 expression and TGFβ inhibits E-selectin (Chang et al, 1994, Lastres et al, 1994.). Finally IFNγ alone is a weak inducer of ICAM-1 but strong inducer in the presence of either TNF or IL-1 (Romer et al, 1995).

1.1.2 Soluble cell adhesion molecules.

Many cell adhesion molecules are shed from the cell surface into the serum by activated endothelial cells and macrophages. These soluble CAM (sCAM) are generated by a combination of enzymatic cleavage and/or alternative splicing of mRNA yielding only the extracellular part of the molecule (Ghaisas et al, 1997, Kevil & Bullard, 1999). As sE-selectin is expressed solely on activated endothelial cells, sE-selectin is thought to reflect the activation state of endothelial cells. ICAM-1 and VCAM-1 are expressed on a variety of cells and changes in the levels of these CAM are though to be related to the ‘activity’ of many cell types in atheroscleortic lesions (Abe et al, 1998). Elevated levels of sCAM have been demonstrated in a variety of pro-inflammatory conditions including ischemia-reperfusion injury, acute lung injury, rheumatoid arthritis and graft rejection (Bevilacqua et al, 1994). Plasma concentrations of sCAM are also elevated in patients with atherosclerosis (Blann & McCollum, 1994), unstable angina (Ghaisas et al, 1997) and hypertriglycerideridemia (Hackman et al, 1996). A prospective study showed that sICAM-1 predicted the risk of future myocardial infarction (Ridker et al, 1998). This was shown to be independent of cigarette smoking (Blann et al, 1997) and aspirin intake (Ridker et al, 1997). However, it still remains to be confirmed whether a particular adhesion molecule can be indicative of active vascular disease (Kevil & Bullard, 1999).

1.2 Signalling pathways of the immune system.

When an antigen / mitogen / stimulus / ligand encounters an immune cell and is recognised by the appropriate receptor, some form of communication is required to transmit this signal to the nucleus, initiate gene transcription and the appropriate immunological response. Such signalling pathways generally follow the following pathway: the receptor can by itself or in concert with effectors (e.g. adenylate cyclase and phospholipid) alter the amount or distribution of intracellular mediators or second messengers (e.g. cyclic AMP (cAMP), diaeylglycerol (DAG), inositol phosphate (IP) and calcium). These second messengers act upon target proteins (typically protein kinases / phosphatases), which can directly (receptor tyrosine kinases) or indirectly (nonreceptor tyrosine kinases) activate a cascade (involving further kinases, adapter proteins etc.) and
lead to the modulation of transcription factor activity, gene expression and protein translation. Fig 1.4 outlines a brief schematic of the above pathway. It now appears that fatty acids not only function as structural molecules and eicosanoid precursors, but also act as second messengers or regulators of signal-transducing molecules (Hwang & Rhee, 1999).

Along with second messenger activation, kinase-induced phosphorylation and phosphatase-induced dephosphorylation play a central role in all of the signalling pathways. Phosphorylation of protein serine /threonine residues results in altered functional activity, whereas tyrosine residue phosphorylation is important for the recruiting, localisation and interaction of molecules. Both types of phosphorylation play a key role in the transmission, amplification and specificity of these signalling cascades (Hwang, 2000). Typically, specific ligand-receptor binding results in the dimerization or oligomerization of the receptors. For receptors with intrinsic tyrosine kinase activity, this results in the autophosphorylation of different tyrosine residues in the cytoplasmic domain (Ulrich & Schlessinger, 1990). Such enhanced kinase activity, leads to signal amplification via the phosphorylation of kinases outside the receptor kinase domain and on other signalling molecules, including adapter molecules. Adapter molecules lack enzymatic and transcriptional activities, but influence signalling pathways by mediating inducible or constitutive protein-protein (or protein-lipid) interactions via modular interaction domains (Leo et al, 2002). An example of a modular domain involved in adapter molecule signalling is the SH2 domain, so called as it contains a tyrosine specific Src homology 2 (SH2) domain (Ulrich & Schlessinger, 1990).

However, most cytokine and antigen receptors lack intrinsic kinase activity in their cytoplasmic domain (e.g. IL-2, IL-4 etc.) (Hwang & Rhee, 1999). These receptors require the association of non-receptor tyrosine kinases (e.g. the Src and janus kinase (JAK) families) for signal propagation (Takeda & Akira, 2000). The JAK family is constitutively associated with the intracellular domains of cytokine families (Takeda & Akira, 2000). Activation of JAKs phosphorylates downstream signal transducers, e.g. signal transducers and activators of transcription (STAT). STAT proteins are transcription factors present in a latent form in the cytosol (Horvath & Darnell, 1997, Takeda & Akira, 2000). Once tyrosine-phosphorylated, STATs then dimerise via juxtaposed SH2 domains, translocate to the nucleus and modulate target gene expression (including cytokine-inducible genes) (Karin & Hunter, 1995, Horvath & Darnell, 1997, Takeda & Akira, 2000). In addition, JAKs phosphorylate tyrosine residues on cytokine receptors, thereby creating docking sites
Fig 1.4. Overview of the signal transduction pathway involved in many immune responses.

- **Ligand** (e.g. Cytokines, mitogens, eicosanoid, specific ligand, antigen presented in the appropriate form etc.)
- **Receptor** (e.g. TCR, BCR, cytokine receptor etc.)
- **Transducer** (G-Proteins)
- **Effector** (adenyl cyclase, phospholipids etc.)
- **Second Messenger** (cAMP, DAG, IP₃, Calcium etc.)
- **Target A** (Protein Kinases A, C, PI-3K, phosphatases, phospholipases)
- **Target B** (e.g. kinases such as MAPK, p38, Raf etc. adapter proteins eg. Grb2, Sos etc.)
- **Target C** (NF-κB, PPAR, AP-1, NFAT, STAT)
- **Gene** (e.g. IL-2, IL-4, TNFα etc.)
for other signalling molecules with SH2 domains (Horvath & Darnell, 1997, Takeda & Akira, 2000).

The major signalling pathways involved in mounting an immune response can be grouped according to the different second messenger (phospholipid) pathways and protein kinase cascades activated and will be described briefly below.

1.2.1 Second messengers and enzymes: G-proteins, calcium and phospholipases.

G-proteins act as a link between agonists and enzyme systems, while calcium is an important second messenger which plays a critical role in initiating and terminating cellular responses (Alberts et al, 1994). Both G-proteins and calcium are important components in the activation of phospholipases. All of the phospholipases (A, C and D) have been implicated in cellular activation by cytokine and antigen receptor ligands (Wakelam & Harnett, 1998).

1.2.1 (i) Phospholipase A2 (PLA2).

Activation of cytosolic Phospholipase A2 (cPLA2) is catalysed by mitogen-activated protein kinases (Section 1.2.2) and an increase in intracellular calcium (Wakelam, 1994). Polyunsaturated fatty acids at the sn-2 position are the preferential substrates for cPLA2 (Wakelam, 1994). The activation of cPLA2 results in the hydrolysis of the membrane phospholipid phosphatidylcholine, yielding lyso-phosphatidylcholine and AA. AA is an important precursor of eicosanoid synthesis (Section 1.1.6), is thought to affect some GTP-binding proteins (Wakelam & Harnett, 1998), and as will be described later, is a co-activator of PKC and can directly affect transcription factor activity (Sections 1.2.2(vii) & 1.2.3). However, cPLA2 is thought to play a role in mature T-cells primed for activation-induced cell death, hence cPLA2 may be detrimental for lymphocyte survival, selection and proliferation (Voelkel Johnson et al, 1996, Wakelam & Harnett, 1998).

1.2.1 (ii) Phospholipase C (PLC)

G-protein-induced phospholipase C (PLC) activation results in the hydrolysis of phosphatidylinositols and the release of two important secondary messenger molecules: inositol 1,4,5 triphosphate (IP3) and diacylglycerol (DAG) (Wakelam & Harnett, 1998). IP3 promotes the release of intracellular calcium from the endoplasmic reticulum and subsequent signal transduction (Berridge, 1993). Calcium-induced responses terminate with the dephosphorylation of IP3 and the pumping of calcium out of the cell (Berridge, 1993). Slower sustained release of calcium is stimulated by the production of IP3 from IP4.
The protein calmodulin can function as an intracellular calcium receptor, targeting calcium to various enzymes and transport proteins (Head, 1992). DAG is involved in eicosanoid synthesis as it can be cleaved to form AA or can act as a second messenger itself activating PKC (See Section 1.2.2(vii)) (Nishizuka, 1992).

1.2.1 (iii) Phospholipid D (PLD)

Phospholipid D (PLD) hydrolys phosphatidylcholine to yield free choline and phosphatidate (PA) (Wakelam, 1994). PA acts as a direct messenger molecule, or it can be dephosphorylated to generate DAG and thereby result in sustained PKC activation (Wakelam & Harnett, 1998). DAG with a greater polyunsaturated content and PA with a richer saturated or monounsaturated content are reported to be more physiologically relevant signalling molecules (Pettit et al., 1997). Interestingly PLD has been found coupled to the TCR-CD3 complex in the Jurkat cell line (Stewart, et al. 1993) downstream of PLC and PKC activation (Reid et al., 1997). Conversely, PLD is reported, via PA, to transduce negative immuno-modulatory signals in mature B-lymphocytes (Gilbert et al., 1993).

1.2.2 Protein Kinases.

The major protein kinase families will be described below, emphasis will be placed on the PKC family and any interactions with fatty acids highlighted.

1.2.2 (i) Protein Kinase A (PKA).

Phosphorylation of G-proteins increases adenylate cyclase activity, causes the release of cAMP from the plasma membrane and the activation of the enzyme protein kinase A (PKA, also known as cyclic AMP-dependent protein kinase). PKA catalyses the transfer of a phosphate group from ATP to specific protein serine / threonine residues. This phosphate transfer ultimately results in enhanced kinase activity, initiation of gene transcription and enhanced cell proliferation. These effects are mediated through a cAMP-responsive element in promoter regions of target genes (Daniel et al., 1998). Protein phosphatases I, IIA, IIB (calcineurin) and IIC, remove the phosphate group and inactivate PKA (Cohen, 1989).

1.2.2 (ii) Mitogen-activated protein kinase (MAPK) signal-transduction pathways.

To date, three major MAPK pathways that play an important role in transmitting a range of receptor-mediated signals to intracellular targets: Extracellular signal regulated...
kinases (ERKs), c-Jun amino terminal kinases (JNK) and p38 (Su & Karin, 1996, Hwang & Rhee, 1999). ERK 1/2 is activated by TCR and growth factor receptor occupancy (Su et al., 1994). JNK and p38 pathways are activated by stress conditions (e.g. UV, oxidant and osmotic stress), endotoxins (e.g. LPS) and cytokines (e.g. IL-1 and TNFa) (Su & Karin, 1996, Hwang & Rhee, 1999). 

**Fig 1.5** outlines the main steps involved in these three pathways. However in general three kinases families are required: MAPK, MEK (MAPK kinase) and MEKK (MEK activator). The transcription factors affected by the three pathways include: TCF/ELK-1 and NF-IL6 by ERK (Karin & Hunter, 1995), c-Jun by JNK (Davis, 1994) and ATF2 by p38 (Gupta et al., 1995). The response elements involved include a serum response element (TCF/ELK-1), TPA response element (c-Jun) and cAMP response element (ATF2) respectively (Su & Karin, 1996, Hwang & Rhee, 1999). As will be outlined in Section 1.2.3 (i, ii), c-Jun and ATF2 in particular play an important role in the activities of the transcription factors AP-1 and NFAT and in the transcription of IL-2 and IL-4 in particular. It should also be noted that ERKs can phosphorylate cPLA2, which as mentioned in Section 1.2.1(i), catalyses the release of membrane-bound AA (Lin et al., 1993).

1.2.2 (iii) JAK-STAT pathways.

As mentioned earlier, the JAK-STAT pathway plays a critical role in cytokine signal transduction, including IL-2, IL-3, IL-4, IL-6, IFN-α, IFN-γ (Hwang & Rhee, 1999).

1.2.2 (iv) Phosphoinositol-3-kinase (PI-3K) pathway.

The PI-3K pathway is intrinsically linked to phospholipids, phospholipid hydrolysis and phosphoinositide formation (Kapellaret al., 1994, Hwang & Rhee, 1999). Phosphoinositides are substrates for PI-3Ks, and PI-3Ks are involved in cell functions e.g. cell adhesion, chemotaxis, apoptosis, secretory responses, platelet activation and cytoskeletal reorganisation (Hwang & Rhee, 1999, Nelms et al., 1999). However, with respect to the immune system, cytokines are known PI-3K ligands, and the SH2 domain of PI-3Ks can directly interact with phosphorylated tyrosine residues of other tyrosine kinases, e.g. PKC (See Section1.2.2(vii)).

The primary form of PI-3K is a complex of two subunits: an 85-kDa regulatory (p85) and a 110-kDa catalytic (Kapellar et al., 1994). The p85 SH2 domain mediates interaction with and activation of the p110 catalytic subunit (Dhand et al., 1994). The activated p110 phosphorylates membrane lipids and serine/threonine residues of proteins.
Fig 1.5. A representation of the three mitogen-activated protein kinase (MAPK) signalling pathways.

Extracellular-signal-regulated kinase (ERK)-1 & -2, c-Jun amino terminal kinase-stress-activated protein kinase (JNK-SAPK), and p38. For growth factors, receptor dimerisation or oligomerisation induces autophosphorylation of tyrosine residues in the cytoplasmic domain. This provides a docking site for the adapter molecule, Grb2 (via a SH2 domain). Grb2 recruits the guanine nucleotide exchange factor, SOS, through its SH3 domain. SOS activates Ras which in turn activates downstream MAPK cascades, leads to the translocation of phosphorylated MAPKs to the nucleus where they activate (phosphorylate) transcription factors. The proximal steps in the stress-induced activation of MAPK are less characterised.

CRE, cAMP response element; MAPK, mitogen activated protein kinase; MEK or MKK, MAPK kinase; MEKK, MEK kinase; SEK, SAPK-ERK kinase; TRE, TPA response element; UV, ultraviolet.
1.2.2 (v) Sphingomyelin pathways.

Cell surface receptors known to activate the hydrolysis of sphingomyelin and release of ceramide include TNFα and the LPS receptor, CD14 (Hannun & Obeid, 1995). Ceramide is a key membrane phospholipid derived second messenger and can activate MAPK, NF-κB and cPLA2 (Hannun & Obeid, 1995, Hwang & Rhee, 1999).

1.2.2 (vi) IκB kinase pathways.

The protein kinase IκB is involved in the activation of the ubiquitously expressed transcription factor NF-κB and will be dealt with in Section 1.2.3 (iv).

1.2.2 (vii) Protein Kinase C (PKC)

Protein kinase C (PKC) is a family of heterogeneous lipid-regulated serine-threonine kinases that phosphorylate a variety of cellular proteins, and play a pivotal role in intracellular signalling events (Jaken, 1996, Newton, 1997, Ron & Kazanietz, 1999, Buchner, 2000). Activation of G-protein coupled receptors, receptor and non-receptor tyrosine kinases all result in PKC activation (Newton 1997, Ron & Kazanietz, 1999). This activation is mediated either by stimulation of PLC to yield DAG, or PLD to yield PA and subsequently DAG. The secondary messenger calcium, generated from the hydrolysis of phosphatidylinositol bisphosphate (PIP$_2$) and IP$_3$, and the phorbol ester tumour promoters are also known PKC activators (Jaken, 1996, Newton, 1997, Ron & Kazanietz, 1999).

PKC consists of at least 12 structurally related phospholipid-dependent protein kinases (Newton, 1997, Mellor & Parker, 1998, Ron & Kazanietz, 1999). PKC isoenzymes can be divided into three main subclasses depending on their cofactor requirements (Nishizuka, 1992, Ron & Kazanietz, 1999). The ‘classical’ or ‘conventional’ PKCs (cPKCs) include PKC α, βI, βII and γ, and are activated by calcium, DAG, phosphatidlyserine, cis-unsaturated fatty acids, lysophosphatidylcholine and phorbol esters (Nishizuka, 1992, Jaken, 1996, Keenan et al. 1997, Newton, 1997, Ron & Kazanietz, 1999). The ‘novel’ PKCs (nPKCs) are PKC δ, ε, η and θ, and are regulated by DAG and phosphatidlyserine (i.e. are calcium independent). PKCε is activated by cis-unsaturated fatty acids, whereas PKCδ is not (Keenan et al, 1997). The third class of PKC isozyme are called ‘atypical’ PKC (PKC ζ, ι and λ, αPKC) and are unresponsive to calcium, DAG or
phorbol esters. Atypical PKC are stimulated by phosphatidlyserine, IP\textsubscript{3} and ceramide (Keenan \textit{et al.} 1997, Newton, 1997, Ron & Kazanietz, 1999).

Structurally, PKC isozymes consist of a single polypeptide chain that contains an amino-terminal regulatory region and a carboxy-terminal kinase or catalytic domain (Jaken, 1995, Newton, 1997). Like most protein kinases, phosphorylation is essential for PKC activation. PKC is phosphorylated at three key positions in the kinase core and the pattern of phosphorylation is similar for all PKC isozymes (Newton, 1997).

Cofactors are essential for PKC activation and include lipid derivatives and calcium (Jaken, 1996, Ron & Kazanietz, 1999). Lipid derivatives, e.g. phosphatidylserine, are potent regulators of PKC activity (Khan \textit{et al.} 1995, Newton, 1995, Keenan \textit{et al.} 1997). However, their activity is enhanced by the presence of DAG (Newton, 1995, 1997). Other lipids and lipid derivatives, which can stimulate and synergise with DAG-dependent activation, include free fatty acids (e.g. AA, Oleic Acid (OA)), IP\textsubscript{3}, short-chain phosphatidylcholine derivatives and lysophosphatidic acid (Shinomura \textit{et al.}, 1991, Khan \textit{et al.}, 1995, Sando & Chertihin, 1996). The influence of calcium as a cofactor is twofold; at low concentrations, calcium is thought to promotes membrane interactions, while at higher concentrations, calcium produces a conformational change in PKC that causes PKC activation (Bazzi & Nelsestuen, 1990, Keranen & Newton, 1997).

With respect to the immune system specifically, T-lymphocytes are known to express the PKC isoforms \(\alpha, \beta\text{I}, \delta, \epsilon, \zeta, \eta\) and \(\theta\) (Long \textit{et al.} 2001). The control of each of the cPKC to T-cell function is reportedly dependent on the stage of lymphocyte development, and on the interactions with other PKC isoforms activated by specific stimuli (Keenan \textit{et al.} 1997). However, PKC\(\varepsilon\) (and to a lesser extent PKC\(\alpha\)) have been reported to increase T-cell AP-1 and NFAT-1 expression (Genot \textit{et al.} 1995). PKC\(\theta\) is implicated as an upstream regulator of the IL-2 promoter (Ghaffari-Tabrizi \textit{et al.} 1999) and in the activation of T-cell NF-\(\kappa\)B (Dienz \textit{et al.} 2000). More recently, PKC\(\beta\) was found necessary for the release of the IL-2 molecule from T-cells but not for the transcription and translation of IL-2 within these T-cells (Long \textit{et al.} 2001). PKC is also thought to play a role in the development of Th1 and Th2 lymphocytes. However its exact involvement is unclear. Kawakami & Parker (1992) and Casolaro \textit{et al.} (1996) reported PKC important in Th1 rather than Th2 T-lymphocyte signalling. However, Noble \textit{et al.} (2000) showed that inhibition of PKC favoured Th1 development in human T-cells and PKC activation resulted in the development of Th2 response. Further research is needed in this area.
1.2.3 Transcription factors.

This thesis is mainly concerned with, and will focus on, the transcription factor NF-κB. However the transcription factors AP-1, NFAT and PPAR are intimately linked to NF-κB activity and are expressed in various immune cells. Furthermore, it is believed that the effects of fatty acids on immune function are mediated in part through these four transcription factors. Hence this subsection is concerned with a short review of AP-1, NFAT and PPAR, but will focus mainly on NF-κB.

1.2.3 (i) Activating protein-1 (AP-1)

AP-1 is a family of transcription factors consisting of Jun, Fos or ATF (activating transcription factor) subunits that dimerise and bind the AP-1 DNA binding site (5′TGA (C/G)TCA-3′) in the promoter and enhancer regions of a wide range of mammalian genes including IL-2 (Karin et al. 1997, Mechta-Grigoriou, 2001). AP-1 participates in the control of cellular responses such as proliferation, differentiation, apoptosis and oncogenesis (Chinenov & Kerppola, 2001). AP-1 is activated by environmental stimuli such as stress, serum, mitogens, pro-inflammatory cytokines, radiation and growth factors (Mechta-Grigoriou et al, 2001, Shaulian & Karin, 2001). The Jun family (c-Jun, JunB and JunD) can form homo- or hetero- dimers with the Fos (v-Fos, c-Fos, Fos B, Fra1, Fra2) or the ATF (ATF2, ATF3/LRF1, B-ATF) families of basic region-leucine zipper proteins (Chinenov & Kerppola, 2001, Vogt, 2001). Fos proteins require heterodimerisation to bind DNA (Chinenov & Kerppola, 2001). c-Jun: c-Fos dimers are most stable and have the highest affinity for the DNA target sequence (Halazonetis et al. 1988). Phosphorylation of AP-1 family members is required for transactivation activity. With respect to cytokine-induced AP-1 activity, the PKC and Ras activated MAPK cascades, JNK and p38 are thought most influential (Macian et al, 2001 Karin, 1995, Shaulian & Karin, 2001). c-Jun is phosphorylated by JNK at serines 63 and 73, thereby enhancing its transcriptional activity and stability (Smeal et al, 1991). Cis- elements mediate c-fos induction including sis-inducible enhancer elements, serum-response element and ternary complex factors stimulated by cAMP and calcium-dependent, STAT and ERK MAPK signalling pathways respectively (Gille et al, 1992, Treisman, 1992, Darnell et al, 1994, Karin et al, 1997). Proinflammatory cytokine mediated activation of fos is particularly dependent on p38 MAPK and JNK induced phosphorylation (Treisman, 1992, Raingeaud et al, 1995).

With respect to precursor T-cells, it is reported that AP-1: DNA binding requires TCR-mediated and costimulatory (CD28: B7.1 / B7.2) signalling (Rincon & Flavell, 1994). However in effector T-cells, TCR-mediated activation of AP-1 is sufficient
(Rincon & Flavell, 1999). Rincon & Flavell (1999) also reported that although large amounts of AP-1 are present in effector Th1 and Th2 cells, the nature of the complexes formed is different. Th2 effector T-cells have a higher proportion of JunB complexes and respond more strongly to antigen-induced AP-1 transcriptional activity (Rincon & Flavell, 1994). During T-cell activation and in the presence of DNA, AP-1 generally acts in concert with nuclear factor of activated T-cells (NFAT) rather than alone (Macian et al, 2001). This fact is underlined by the close proximity of the specific sequences for AP-1 and NFAT in the IL-2 gene (Hughes & Pober, 1996).

1.2.3(ii) Nuclear Factor of Activated T-cells (NFAT).

Five different members belonging to the NFAT family have been identified thus far (NFAT1-5) (Macian et al, 2001). The first four members (NFAT 1-4) act synergistically with the AP-1 complex on composite DNA elements expressing NFAT and AP-1 binding sites (the binding site for NFAT is GGAAAA) (Macian et al, 2001). This interaction results in the formation of stable ternary complexes, which can regulate the expression of a wide range of inducible genes in a stimulus- and cell-specific manner (Rincon & Flavell, 1999, Macian et al, 2001). NFAT -1 and -4 are constitutively expressed in T-cells, while NFAT-2 is strongly induced upon T-cell activation (Serfling et al, 2000). NFAT-5 is involved in the cellular response to osmotic stress (Lopez-Rodriguez et al, 1999) and will not be dealt with here.

NFAT binds to DNA as a monomer (Chinenov & Kerppola, 2001). Its structure consists of a highly phosphorylated N-terminal region controlling cellular distribution and transcriptional activation (Kiani et al, 2000), a DNA binding domain that shows a 15-17% homology to the Rel region of NF-kB (see Section 1.2.3d, Chen et al, 1998) and a C-terminal region (Avots et al, 1999). Transcriptional activation of NFAT involves intracellular free calcium release, calcineurin activation and the stimulation of protein kinases (e.g. p56lck, p21ras) and GTP binding proteins (Serfling et al, 2000). Calcineurin induces NFAT dephosphorylation, nuclear translocation and DNA binding, while the protein kinase cascades (Ras/Raf/ERK) control transcriptional activation and induction of AP-1 (Okamura et al, 2000, Serfling et al, 2000). Kinases thought to inactivate NFAT include casein kinase 1 (Zhu et al, 1998) and glycogen synthase kinase-3 (Beals et al, 1997). TCR and BCR engagement typically trigger calcium release and calcineurin activation (Kiani et al, 2000). Dolmetsch et al, (1997) reported optimal NFAT activation to occur following low sustained increases of intracellular calcium. Conversely, JNK and
NF-κB activation occur as a result of large transient peaks of calcium entry (Dometsch et al., 1997)

NFAT -1, -2 and -4 are the main forms expressed in the immune system. Mice deficient in NFAT -1 and -4 have an allergic phenotype while T-cells deficient in NFAT1 and NFAT2 are incapable of cytokine production, including IL-2 (Ranger et al., 1998, Peng et al., 2001). NFAT proteins induce a variety of genes during T-cell activation but typically as part of composite AP-1/NFAT regulatory elements (Chinenov & Kerppola, 2001). NFAT and AP-1 only interact in the presence of DNA and indeed such interactions induce a bend in the DNA that brings the NFAT and Fos-Jun dimmers closer together (Chen et al., 1998). At least five composite NFAT: AP-1 sites are found on the IL-2 promoter separated by two base pairs, where Jun binds the AP-1 half-site closer to NFAT (Chen et al., 1998, Serfling et al., 2000, Kerppola et al., 2001). With respect to human IL-2, two high affinity and three low affinity binding sites have been identified (Serfling et al., 2000). The best-studied ('proto-typical') site is called the distal antigen receptor response element (ARRE-2) (Chen et al., 1998). It has been reported that that NFAT 1 and 2 bind with higher affinity to the IL-2 promoter (Ho et al., 1995), but also that NFAT-1, -2 and -4 have overlapping functions in the control of IL-2 promoter activity (Yoshida et al., 1998).

Other cytokines that involve NFAT during gene activation include IL-4, IL-5, IL-13, IFNγ, COX-2 and TNFα. IL-4 is reported to have at least five different NFAT sites in its promoter, three of which are composite NFAT: AP-1 binding sites (reviewed in Avni & Rao, 2000). However, the interactions between NFAT and another family of transcription factors (GATA-3) proteins are thought more important than AP-1 (Avni & Rao, 2000). The T-cell COX-2 promoter has two NFAT sites, one of which resembles an NFAT: AP-1 site and appears to regulate COX-2 gene expression (Iniguez et al., 2000). Although TNFα contains NFAT: AP-1 composite elements in its promoter region, it is also thought that NF-κB induced activation is more significant. Hence TNFα is classified as a gene independent of NFAT: AP-1 cooperation (Macian et al., 2001).

Finally signalling pathways known to repress NFAT: AP-1 cooperation include a cAMP-inducible transcriptional repressor, a leucine zipper protein p21SNF which prevents IL-2 activation and the a serine/threonine kinase HPK1 (reviewed in Macian et al., 2001)

1.2.3 (iii) Peroxisome Proliferator Activated Receptors.

Peroxisome proliferator activated receptors (PPARs) are members of a family of ligand activated transcription factors that have been implicated in a variety of biological processes including inflammation (Issemann and Green 1990, Kelly, 2001). PPARs upon
heterodimerisation with retinoid X receptors (RXRs), bind to common DNA response elements called PPAR response elements (PPRE's) in the 5' flanking regions of target genes thereby regulating gene transcription. Co-activators and co-repressors modulate PPAR activity and gene transcription by mediating contact between the PPAR-RXR dimer, chromatin and other transcriptional machinery involved in the activation and/or repression of target genes (Escher & Wahli, 2000, Lehman et al., 2000, Kelly, 2001). After activating gene expression, the nuclear complexes are ubiquitinized and degraded by the proteasome (Bassaganya-Riera et al., 2002b). In addition, ERK-MAPK, PKC, PKA and p38 MAPK are all reported to affect PPAR transcriptional activity (Hu et al., 1996, Lazennec et al., 2000, Barger et al., 2001, Yaacob et al., 2001).

Three different PPAR subtypes have been identified to date: PPARα, PPARγ and PPARβ (also called NUC-1 or PPARδ). PPARα regulates genes involved in β- and ω-oxidation of fatty acids, and is highly expressed in the liver, kidney, skeletal and cardiac muscle and adrenal glands of both humans and rodents (Schoonjans et al., 1996). However, it is also present in aortic smooth muscle cells (Staels et al., 1998), macrophages (Chinetti et al., 1998) and T- and B-lymphocytes (Jones et al., 2002). Alternative promoter usage and differential splicing result in the formation of three human isoforms of PPARγ: PPARγ-1, -2 and -3 (Elbrecht et al., 1996). PPARγ2 is mainly expressed in adipose tissue and is implicated in adipocyte differentiation (Berger & Moller, 2002, Cunard et al., 2002). However, PPARγ1 is also expressed in vascular smooth muscle cells (Staels et al., 1998), monocytes (Patel et al., 2001), macrophages (Nagy et al., 1998, Ricote et al., 1998) and T-lymphocytes (Clark et al., 2000). Interestingly, PPARα expression is greater in resting T-cells, but PPARγ expression is more pronounced in activated T-cells (Jones et al., 2002). PPARδ, also called PPARβ or NUC-1, is ubiquitously expressed in all the tissues of adult mammals. Less is known about the biology of PPARδ.

Pharmacological and synthetic ligands for PPARα include the hypolipidaemic drugs fibrates, and the synthetic ligand WY14643. The anti-diabetic drugs thiazolidinediones are specific PPARγ ligands. Non-steroidal anti-inflammatory drugs are reported to act directly and indirectly on PPARα and PPARγ activity. (Lehmann et al., 1997). Of greater interest to this thesis, fatty acid derivatives, including LTB4 and HETE's, have been identified as PPARα agonists (Devchand et al., 1996), while the eicosanoid prostaglandin G2 metabolite (15d-PGJ2) has been identified as a non-specific PPARγ ligand (Kliwer et al., 1997). More significantly, fatty acids have been discovered to bind directly to all three PPAR isotypes suggesting that fatty acids serve as endogenous ligands for PPARs (Forman et al., 1997, Kliwer et al., 1997). All three PPAR isotypes
demonstrate a preference for long chain unsaturated fatty acids e.g. OA, AA, DHA, EPA, linoleic acid (LA) and linolenic acid (LNA) in the μM range (Gottlicher et al., 1992, Kliwer et al., 1997, Forman et al., 1997, Michalik & Wahli, 1999). PPARα is also activated by the saturated fatty acid, PA (Gottlicher et al., 1992). It is possible that fatty acids may influence PPAR function either directly or indirectly through the formation of eicosanoid metabolites (Forman et al., 1997, Kliwer et al., 1997).

PPARs have been credited as regulators of inflammation processes (Michalki & Wahli, 1999). PPARα null mice show prolonged inflammatory responses (Delerive et al., 1999) and PPARα ligands were reported to decrease IL-6 and COX-2 expression (Staels et al., 1998). PPARγ ligands appear to affect the immune response more profoundly. They have been reported to decrease the secretion of IL-1β, IL-6 and TNFα in human monocytes (Jiang et al., 1998) and IL-2 secretion in T-cell clones and splenocytes (Yang et al., 1999, Clark et al., 2000). More recently, Cunard et al. (2002) reported PPARγ ligands to decrease IL-2 and IFNγ production and lymphocyte proliferation in murine mitogen activated splenocytes. The PPARα ligand, WY14643, enhanced IL-4 production in a PPARα independent mechanism (Cunard et al., 2002). Although it has been reported that PPARγ ligands have anti-inflammatory effects that are independent of PPARγ (Chawla et al., 2001, Boyault et al., 2001, Berger & Moller, 2002), it has been demonstrated that PPAR ligands affect the immune system by antagonising the transcriptional activities of NF-κB, NFAT, AP-1 and the JAK-STAT transcription factors (Delerive et al., 1999, Zhou & Waxman 1999, Yang et al., 2000). PPARα has also been reported to interact directly with p65 subunit of NFκB and c-jun and to induce IkBa gene expression (Delerive et al., 1999, 2000). The effects of PPAR ligands, particularly fatty acids, merits further study to ascertain whether the immuno-modulatory effects observed are PPAR dependent or independent.

1.2.3 (iv) Nuclear Factor kappa B (NF-κB)

Nuclear factor kappa B (NF-κB) is a transcription factor involved in the expression of many genes, particularly of the inflammatory and immune responses (May & Ghosh, 1998, Jobin & Sartor, 2000, Yamamoto & Gaynor, 2001). NF-κB is ubiquitously expressed in the cytoplasm as homo-or hetero-dimers of a family of structurally related proteins (May & Ghosh, 1998). Five mammalian proteins have been identified to date as members of the NF-κB family. Each of these members has a conserved N-terminal region (~ 300aa) called the Rel-homology domain (RHD). In this RHD lie the DNA-binding and dimerization domains, the nuclear localization signal (NLS) and the IkBa interacting domain (Baeuerle
The members of this family found in mammalian cells include: p65, c-Rel, RelB, p50/p105 and p52/p100. p65, c-Rel and RelB are produced as transcriptionally active proteins. p50 and p52 are produced as longer precursor molecules of p105 and p100 respectively and processed to yield the smaller transcriptionally active forms (Jobin & Sartor, 2000). NK-κB subunits can form homo- or hetero- dimers. p65, RelB and c-Rel contain additional C-terminal transactivation domains. These domains, which are thought to strongly activate NF-κB transcription are absent in p50 and p52 (Lienhard Schmitz et al., 1995). The predominant dimer is composed of the p65/p50 subunits and this variant is the most potent gene transactivator among the NF-κB family (Ballard et al., 1992). The secondary structure of p65/p50 dimer bound to target DNA is likened to a butterfly. Briefly, each dimer subunit has two sets of β-sheet Ig folds in the N-terminal domain which interacts with DNA, base and backbone specifically and a C-terminal domain that mediates dimerization and nonspecific DNA contacts (Chen & Ghosh, 1999).

NF-κB is activated by a wide variety of agents including other cytokines and growth factors, phorbol esters, LPS, bacteria and viral transactivators (May & Ghosh, 1998, Bowie & O’Neill, 2000, Jobin & Sartor, 2000). Table 1.4 lists some inducers of NF-κB. The importance of NF-κB is underlined not only by its ubiquitous expression, but also by its influence in the expression of a large number of inducible genes (May & Ghosh, 1998, Jobin & Sartor, 2000). Table 1.5 details some of the NF-κB –inducible genes involved in immune responses, including IL-2 and TNFα.

**Table 1.4. Inducers of NF-κB.**

<table>
<thead>
<tr>
<th>Class of inducers:</th>
<th>Class member:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines &amp; Growth Factors:</td>
<td>IL-1β, IL-2, IL-17, IL-18 &amp; Lymphotoxin</td>
</tr>
<tr>
<td></td>
<td>LTB4, TNFα, MCSF, PDGF.</td>
</tr>
<tr>
<td>T-cell Mitogens:</td>
<td>Antigen &amp; Anti- CD2, -CD3, CD28,</td>
</tr>
<tr>
<td></td>
<td>Calcium ionophores,</td>
</tr>
<tr>
<td></td>
<td>Lectins (PHA, Con A).</td>
</tr>
<tr>
<td>Oxidative Stress:</td>
<td>Hydrogen peroxide,</td>
</tr>
<tr>
<td></td>
<td>Ozone &amp; Reactive oxygen species.</td>
</tr>
<tr>
<td>Bacteria &amp; Bacterial Products:</td>
<td>LPS &amp; Peptidoglycan-polysaccharide,</td>
</tr>
<tr>
<td></td>
<td>H. Pylori &amp; Lactobacilli,</td>
</tr>
<tr>
<td></td>
<td>Salmonella, Shigella &amp; Enteropathogenic E. coli</td>
</tr>
<tr>
<td></td>
<td>Mycoplasma fermentans &amp; Listeria monocytogenes</td>
</tr>
<tr>
<td></td>
<td>Toxic shock syndrome toxin 1.</td>
</tr>
<tr>
<td>Viruses &amp; Viral Products:</td>
<td>Adenovirus &amp; Epstein-Barr virus,</td>
</tr>
<tr>
<td></td>
<td>HIV type I &amp; Human T-cell leukaemia virus type I,</td>
</tr>
<tr>
<td></td>
<td>Hepatitis B virus B &amp; Herpes simplex virus type I,</td>
</tr>
</tbody>
</table>

### Table 1.5. NF-κB inducible genes involved in the immune responses.¹

<table>
<thead>
<tr>
<th>Class of inducers</th>
<th>Class member</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines, Cytokine Receptors &amp; Growth Factors</td>
<td>IL-1α, IL-1β, IL-2, IL-3, IL-6, IL-8 &amp; IL-12, TNFα &amp; Lymphotoxin, IFN-β, macrophage inflammatory protein-2, IL-2Ra, CD95/APO-1 (Fas), RANTES, G-CSF, M-CSF &amp; GM-CSF.</td>
</tr>
<tr>
<td>Stress Proteins</td>
<td>Serum amyloid A protein, Complement factors B, C3 &amp; C4, α1-acid glycoprotein</td>
</tr>
<tr>
<td>Leukocyte adhesion molecules</td>
<td>ICAM-1 &amp; VCAM-1, MAdCAM-1, E-selectin</td>
</tr>
<tr>
<td>Inflammatory enzymes</td>
<td>Inducible nitric oxide synthase, COX-2</td>
</tr>
<tr>
<td>Apoptotic genes</td>
<td>c-IAP1, c-IAP2, TRAF1 &amp; TRAF2, A1/Bfl-1</td>
</tr>
<tr>
<td>Immunoregulatory molecules</td>
<td>MHC class I &amp; II, Igκ, TCRα &amp; TCRβ, β2-microglobulin, II (invariant chain), Transporter associated with antigen processing</td>
</tr>
</tbody>
</table>


The multi-functional and potent consequences of NF-κB activation dictate that NF-κB is not active in healthy tissue (Baeuerle, 1998). A class of cytoplasmic inhibitors called IκBs ensure tight control over NF-κB transcription (Jobin & Sartor, 2000). IκBs complex with, and sequester NF-κB in the cytoplasm of inactive cells rendering it transcriptionally inactive (Zandi et al., 1997). Seven IκB molecules have been identified to date: IκBα, IκBβ, IκBe, IκBγ (p100), IκBδ (p105) and Bcl-3. IκB’s act as direct inhibitors by binding to the RHD in NF-κB proteins via a series of amino acids called ankyrin repeats (Beg & Baldwin, 1993, Gilmore and Morin, 1993, Baldwin, 1996). The pathway of NF-κB activation is best understood for IκBα, a 37 kDa protein, which is known to bind to the p65 subunit (Huxford et al., 1998). IκBα consists of six ankyrin repeats and a C-terminal PEST sequence that interacts directly with p65/p50 (Karin & Ben-Neriah, 2000). In this manner, the IκBα molecules mask the NLS of NF-κB and prevent NF-κB nuclear translocation and activation (Huxford et al., 1998). IκBα also plays a critical role in the termination of NF-κB dependent transcription (Arenzana-Seisdedos et al., 1995). This pathway of NF-κB activation and extinction is outlined in *Fig* 1.6 and described below.

During NFκB activation, stimuli (e.g. IL-1, LPS etc.) activate upstream kinases (e.g. PKC and NIK etc.), which in turn activate a complex of IκB kinases (IKK). The IKK complex consists of a high molecular weight signalsome complex of two kinases, IKK- α
and -β, and a non-catalytic regulatory subunit, IKKγ (Li et al., 2002). Each protein contains an N-terminal kinase domain, a leucine zipper region and a C-terminal helix loop helix domain (Zandi et al., 1997). The leucine zipper domain is essential for homo- or hetero-dimerization, while the helix loop helix region is crucial for efficient kinase activity (Mercurio et al., 1997, Woronicz et al., 1997, Zandi et al., 1997). The exact function of the IKK-α, -β and -γ subunits is still controversial, but all are critical for the activation of NF-κB-dependent TNFα and IL-1 responsive genes (Li et al., 2002.) The IKK complex phosphorylates IkBα on the amino terminus at serine residues 32 and 36 (Brockman et al., 1995, Chen et al., 1995, Zandi et al., 1997). This phosphorylation marks the IkBα for polyubiquination by a specific ubiquitin ligase (β-Trcp) belonging to the SKP1-Cullin-F-box (SCF) type E3 ubiquitin ligase family. This SCF E3 ligase results in the polyubiquitination of IkBα at lysines 21 and 22, and its subsequent rapid degradation via a nonlysosomal, ATP-dependent 26S proteolytic complex composed of a 700-kDa proteasome (Palombella, 1994, Chen et al., 1995, Li et al., 1995, Scherer et al., 1995, Yaron et al., 1998, Karin & Ben Neriah, 2000). IkBα degradation exposes the NLS of NF-κB, and the NF-κB heterodimer is then free to bind to DNA containing the κB-responsive sequence, 5’-GGGPuNNPyPyCC-3’, and initiate gene transcription (Blackwell & Christman, 1997).

Traditionally, it was thought that the NF-κB: IkBα complex was ubiquinated and degraded in the cytoplasm. However recent research has revealed that the NF-κB: IkBα complex shuttles dynamically between the cytoplasm and nucleus, even in resting cells (Johnson et al., 1999, Rodriguez et al., 1999, Huang et al., 2000, Ghosh & Karin, 2002). This process is dependent upon the presence of NLS and nuclear export sequence (NES) on the IkBα and p65/p50 structures. The NLS mediate nuclear import, and crystallographic studies have shown that IkBα completely masks the NLS on the p65, but not the p50 subunit (Huxford et al., 1998, Jacobs & Harrison, 1998). This ‘exposed’ NLS on p50 would therefore allow the NF-κB: IkBα complex to enter the nucleus (Ghosh & Karin, 2002). However, upon nuclear entry and in resting cells, NES work in partnership with a nuclear export protein called chromosome maintenance region 1 (CRM-1), resulting in the immediate removal of the NF-κB: IkBα complex from the nucleus (Ossarch-Nazari et al., 1997, Renard et al., 2000, Ghosh & Karin, 2002). The combined presence of a NES on IkBα, and of an additional NES on the p65 subunit, ensure the immediate nuclear export of the NF-κB: IkBα complex and prevent NF-κB activation in resting cells (Harhaj & 1999, Ghosh & Karin, 2002). Conversely in activated cells, IkBα previously
Fig 1.6. A schematic model of NF-κB activation.\(^1\)

LPS, dsRNA, Ionizing Radiation, ROS, Okadic acid etc

Cytokine receptors e.g. TNFα & IL-1

LPS, dsRNA, Ionizing Radiation, ROS, Okadic acid etc

UV Others?

IKKγ

IKKα

IKKβ

IKK

P65

IκBα

P65

P50

IκBα

P65

P50

Proteasome degradation

NF-κB responsive genes e.g. IL-1, TNFα, IL-2, chemokines, immunoreceptors, Adhesion molecules, Acute phase proteins, COX

Transcription

NF-κB responsive gene

E3RSIKb, E3 ubiquitin ligase; IκBα, Inhibitor of kappa B; IKK, Inhibitor of kappa B kinase; IL-1, -2, Interleukin-1 and -2; NF-κB, Nuclear factor kappa B; p50, NF-κB p50 subunit; p65: NF-κB p65 subunit; ROS, Reactive oxygen species; TNFα, tumour necrosis factor alpha; Ub, ubiquitin.

\(^1\) Adapted from Ghosh & Karin (2002).
phosphorylated at serines 32 and 36 by the IKK family, is primed for further polyubiquination and degradation (Brown et al., 1995, DiDonato et al., 1996). Finally, it has been postulated that IkBα ubiquination also occurs in the nucleus upon recognition by the nuclear receptor β-Trcp. This β-Trcp receptor targets the phosphorylated IkBα for ubiquination, proteasome-mediated degradation and NF-κB activation (Davis et al., 2002)

Following NF-κB activation, IkBα also participates in the inhibition of NF-κB dependent transcription as part of a negative feedback loop (Baeuerle, 1998, Turpin et al., 1999). Degradation of IkBα occurs within 10 min of NF-κB activation, but it is followed by the induction of IkBα mRNA by a mechanism whereby NF-κB binds to the κB sequence in the promoter region of IkBα (Velasco et al., 1997). Hence IkBα is resynthesised within 60 min in an NF-κB dependent manner (Sun et al., 1993). Newly synthesised IkBα enters the nucleus and binds to NF-κB (Turpin et al., 1999). As the affinity of NF-κB is greater for IkBα than for DNA, the NF-κB re-associates with the newly formed IkBα, and is subject to a retrograde transport from the nucleus to the cytoplasm via the CRM1-dependent export pathway (Arenzana-Seisdedos et al., 1997, Ossareh-Nazari et al., 1997).

The other predominant forms of mammalian IkB include IkBβ and IkBγ (both 45 kDa) (Baldwin, 2001). Differences exist between the IkB proteins with respect to the affinity for NF-κB and their mechanisms of action (Baeuerle 1998, Simeonidis et al. 1998, Thompson et al. 1995, Whiteside et al. 1997). IkBβ is not regulated by NF-κB, binds to p65 and c-Rel but not to p50, and is slowly degraded over 2 h (Thompson et al. 1995, Blackwell & Christman, 1997, Weil et al. 1997, Jobin & Sartor, 2000). Following IL-1 and LPS stimulation, IkBβ dependent-NF-κB activation can persist for over 20 h (Johnson et al., 1996). It is thought that the newly synthesised unphosphorylated IkBβ may escort the NF-κB into the nucleus and prevent IkBα binding, i.e. act as a ‘chaperone’ (Suyang et al., 1996). Less is known about IkBe, however it is thought to exert its inhibitory effect in the cytoplasm, to bind to the p65 and c-Rel complexes and to have a slower degradation rate than IkBα (Blackwell & Christman, 1997, Whiteside et al. 1997). Bcl-3 is unusual in that it can be present in the nucleus and can bind the p50 and p52 homodimers and act as a transactivator (Baldwin, 1996, 2001). p100 and p105 can bind to p65 (RelA) and thereby function as NF-κB inhibitors (Blackwell & Christman, 1997). The C-terminal portions of p105 and p100 have been called IkBγ and IkBδ respectively. These units contain ankyrin repeat domains, which allow interaction with NF-κB in a configuration that masks the NLS and prevents nuclear transport (Siebenlist et al., 1994, Miyamot & Verma, 1995).
There are several other points of regulation along the NF-κB: IκB cascade, including upstream kinase activity, subunit post-translational modifications, variations in κB binding sites and interactions with co-activators and co-repressors. Cytokine and bacterial products are known to signal through the NF-κB: IκB pathway. As the cytokine and microbial product receptors lack intrinsic kinase activity, they rely on scaffolding and adaptor proteins to transmit their extracellular signal inside the cells (Jobin & Sartor, 2000). Key points at which these signal pathways coalesce with, and modulate the NF-κB: IκB pathway, include NF-κB-inducing kinase (NIK) (Malinin et al., 1997), a scaffold protein called IKK complex-associated protein (IKAP) (Scheidereit, 1998) and mitogen-activated protein kinase kinase-1 (MEKK-1) (Baumann et al., 2000). In particular, NIK is known to bind to and phosphorylate IKKα, thereby initiating the NF-κB activation cascade (Malinin et al., 1997). Similarly NF-κB subunit activity, particularly p65, is regulated by post-translational modification. The p65 subunit is modified post-translationally by acetylation and the phosphorylation of a ‘transactivation domain’ (TAD) (Lienhard Schmitz et al., 1995, Vermeulen et al., 2002). These processes enhance p65 activity. Kinases which phosphorylate this TAD and increase p65 activity include casein kinase II, PKCζ, PI-3K, ERK, p38 and the catalytic domain of PKA. (Vermeulen et al., 2002). Acetylation of p65 modulates the efficiency of interaction with IκBα. Acetylation is probably mediated by histone acetyl transferases and the co-activator CBP/p300, increases the nuclear retention of p65 and enhances NF-κB transcriptional activity (Vermeulen et al., 2002). Deacetylation, mediated by histone deacetylases, enhances p65 binding to newly synthesised IκBα and subsequent nuclear export (Vermeulen et al., 2002). Other points of regulation that can affect NF-κB activity include differences in κB binding sites and binding affinities. Not all κB binding sites are identical and variations in both protein and DNA sequence result in varying affinities, crystal structures and protein:DNA interactions (Chen & Ghosh, 1999). Finally NF-κB can interact with other transcription factors, transactivators and repressors in the context of individual promoters to coordinate transcription (Ray & Prefontaine, 1994, Blackwell & Christman, 1997). For example, cooperative binding of NF-κB and NF-IL-6 is necessary for IL-6 gene transcription (Akira & Kishimoto, 1992).

1.2.4 Membrane-associated events: Lipid Rafts.

Recently, interest has focused on the study of sphingolipid/cholesterol enriched-lipid domains in the plasma membrane, known as membrane rafts (Brown & London, 1998, Langlet et al., 2000). These rafts are thought to correspond to a liquid-ordered phase of the
plasma membrane that has fluidity intermediate to that of the liquid-disordered and gel phases of the lipid bilayer (Langlet et al., 2000). The different phases are thought to allow a high degree of protein lateral mobility within the plasma membrane and undergo dynamic rearrangement ('patching') during immune cell activation (Langlet et al., 2000). The sphingolipids contain large saturated acyl chains that are tightly packed together (Brown & London, 1998). However, critically these lipid rafts are known to contain glycosyl phosphatidylinositol (GPI)-anchored, myristoylated or palmitoylated proteins (Brown & London, 1998), the G-protein α subunits and proteins involved in T-cell signalling, e.g. kinases, receptors and integrins (Mitchell et al., 2002). Lipid rafts are known to include T-cell associated signalling proteins (e.g. Ras, Lck, Grb-2, PI-3K, LAT and Fyn) (Xavier et al., 1998, Langlet et al., 2000) and receptors (e.g. TCR and CD59) (Mitchell et al., 2002). CD45, β1 integrin, CD43 and LFA-3 are not localised in lipid rafts in resting cells (Mitchell et al., 2002). (See Section 1.2.5) Functionally, cholesterol depleted micro-domains resulted in decreased CD3-induced TCR phosphorylation (Moran & Miceli, 1998) and lipid raft ‘patching’ is known to induce calcium flux (Janes et al., 1999). With respect to fatty acids, it has been reported that biosynthetic incorporation of PUFA in T-cells caused a displacement of Lck from membrane rafts and inhibited TCR signalling (Horejsi et al., 1999).

1.2.5 T-cell signalling pathways.

Different pathways are involved in both T-cell and B-cell signalling, however as this thesis is principally concerned with T-cells and T-cell induced responses, focus will be placed on T-cell signalling pathways. T-cell signalling can be divided into two main steps: i) initiation of activation and ii) subsequent intracellular signalling. There are three major steps involved in the initiation of T-cell activation: a) antigen-specific TCR engagement, b) costimulation and c) inhibition of costimulation (Frauwirth & Thompson, 2002). Once a T-cell is activated, a signal is generated which activates a cascade of intracellular signalling pathways and results in transcription, translation, cellular proliferation, cytokine production and an immunological response.

1.2.5 (i) Initiation of Activation.

Central to T-cell activation is the recognition by the TCR of antigen-MHC complexes on the membranes of antigen presenting cells (Hudrisier & Bongrand, 2002) (See Section 1.1.1). The TCR (α and β chains) are associated to a complex of proteins called CD3 (γ, δ, ε and ξ). CD3 complexes are known as the signal transducing subunits due to
the presence of immunoreceptor tyrosine-based activation motifs (ITAMs) on their cytoplasmic tails (Acuto & Cantrell, 2000). This association is necessary as the TCR has a very small cytoplasmic domain that is unable to signal efficient MHC-TCR engagement to the nucleus (Sharfe & Roifman, 1997). CD3 γ, δ and ε also have small cytoplasmic domains and help stabilise the TCR/CD3/antigen complex, although CD3ε has been implicated in tyrosine kinase signalling (Letourner & Klausner, 1991). The disulphide linked homodimer CD3 ξ, with its small extracellular domain and large cytoplasmic domain, (containing repeated SH2 domains), is chiefly responsible for effective signal transduction (Clevers et al., 1988).

TCR/CD3 engagement alone is not sufficient to induce cytokine production and lymphocyte proliferation (Frauwirth & Thompson, 2002). Cytokines (e.g. IL-2, IL-4, IL-12, etc.) and chemokines (e.g. CCR7, MIP3α etc.) act themselves as co-stimulatory molecules (Campbell & Butcher, 2000, Frauwirth & Thompson, 2002). However, interactions between receptor-ligand pairs on the APC and lymphocyte surfaces are essential for enhanced signalling, cross-talk and specific regulation of lymphocyte activation (Frauwirth & Thompson, 2002). CD4 and CD8 are co-receptors that play an important role in the differential recognition of MHC type molecules (MHC II and MHC I respectively) (Janeway & Travers, 1997). However, other critical co-stimulatory molecules include CD28, inducible costimulatory molecules (ICOS), CD40 and CD2 (Frauwirth & Thompson, 2002). CD28 is expressed on the T-cell and interacts with B7.1 and / or B7.2 on the APC surface (Mueller, 2000). Low constitutive levels of B7.1 and / B7.2 on the APC activate CD28 which in turn upregulates CD40 Ligand (CD40L) expression on T-cells (Mueller, 2000). CD40L interacts with CD40 expressed on the APC, which in turn upregulates B7.1/B7.2 expression as part of a positive feed back loop (Frauwirth & Thompson, 2002). CD28 also induces expression of ICOS on T-cells, which interacts with ICOSL on the APC surface (Mueller, 2000). Naive and resting T-cells are reported to use the CD28-mediated pathway, while activated and effectors use the ICOS pathway (Frauwirth & Thompson, 2002). CD4+ Th cells appear more dependent than CD8+ cytotoxic T-cells on CD28 costimulation (Frauwirth & Thompson, 2002), and CD28 is involved in the activation of GTPases (e.g. Rac and CDC42), MAPK cascades and IL-2 secretion (Krummel & Allison, 1995). Finally PI-3K can associate with the CD28 and ICOS cytoplasmic tails (Frauwirth & Thompson, 2002). Adhesion molecule expression acts in concert with these co-stimulatory molecules to stabilise cell/cell contact and send positive or negative signals to the T-cell pathway (Clevers et al., 1988). Typical examples
include CD2, which complexes with LFA-3 on the APC surface and LFA-1, which interacts with ICAM-1 thereby enhancing adhesion (Clevers et al., 1988).

Finally, some sort of negative feedback loop or regulation of co-stimulatory molecules is required. CTLA-4 has been identified as a CD28 receptor (Oosterwegel et al., 1999). Binding of CD28 to CTLA-4 rather than the B7.1/B7.2 family results in T-cell anergy and poor IL-2 production (Wells et al., 2001). Another negative regulator of CD28 activity is the ligand PD-1, which is expressed on activated T and B cells and inhibits T-cell proliferation and cytokine production (Agata et al., 1996).

In conclusion, a complex series of interactions occur at the T-cell/ APC interface (the immunological synapse) (Dustin, & Cooper, 2000). These strength of these interactions determine whether signal amplification (T-cell activation) or T-cell anergy transpire. It is now becoming evident that these interactions involve molecule recruitment either through lipid raft (Brown & London, 1988) and/or cytoskeletal (Hudrisier & Bongrand, 2002) rearrangement. It is also known that small amounts of antigen can rapidly and efficiently trigger TCR signalling (Harding & Unanue, 1990). Less is known about the fate of the TCR and the actual process of antigen transfer. It is thought that the engaged TCR is internalised and degraded, thereby preventing excessive T-cell activation (Valitutti et al., 1995). With respect to antigen transfer, three mechanisms have been hypothesised: transfer of cleavage fragments of membrane molecules, shedding of membrane material in vesicles or direct transfer of intact molecules between apposed membranes (Hudrisier & Bongrand, 2002). Recently the presence of membrane bridges at the synapse of CTL and targets cells have been reported, however this area merits further study (Stinchcombe et al., 2001).

1.2.5 (ii) T-cell signalling.

TCR engagement initiates a cascade of signalling events involving various protein kinases, including the Src, Syk and Tec families of protein tyrosine kinases (PTKs) (Aucto & Cantrell, 2000). Fig 1.7 outlines a general overview of the major signalling molecules involved in T-cell signalling. In T-cells Lck is found associated to CD4 and CD8, while Fyn is found associated to the TCR itself (Sharfe & Roifman, 1997). The transmembrane phosphatase CD45 is thought responsible for the activation of Lck and Fyn kinases (Herminston et al., 2002). Initially, the Src family PTKs, Lck and / or Fyn phosphorylate ITAM tyrosine residues on the CD3 subunits and TCR ξ (Hermiston et al., 2002). This creates a binding site for the SH2 domain of the PTK, ZAP-70, which upon binding becomes phosphorylated and activated (Zhang et al., 1999). Activation of this ZAP-70
Figure 1.7. A model of TCR activation.

Representation of the various intracellular signalling cascades involved during T-cell signaling (MAPK, PKC and calcium). Together these result in the activation of the nuclear transcription factors: NFAT, NF-κB and AP-1.

AP-1, activating protein-1; MAPK, mitogen activated protein kinases; NFAT, nuclear factor of activated T-cells; NF-κB, nuclear factor kappa B.
See Section 1.2.5 for a more detailed description of activation pathways.

Adapted from Pahlavani (1998)
molecule results in the phosphorylation of two categories of proteins: adapter (linker molecules) or enzymes that are regulated by tyrosine phosphorylation. (Zhang et al., 1999). Linker for activated T-cells (LAT) is an integral membrane protein, is phosphorylated on multiple tyrosine sites and activated by ZAP-70 (Zhang et al., 1999). Phosphorylation of LAT results in the recruitment of the adapter molecule Grb2 (which binds to LAT via its SH2 domain) to the plasma membrane (Zhang et al., 1999). Bound to the SH3 domain of Grb2 are other linkers and enzymes including activators of the GTP binding proteins Ras (activated by Sos) and Rac (activated by a complex of SLP-76, SLAP and Vav) (Janeway & Travers, 1997, Zhang et al., 1999, Herminston et al., 2002). Activation of Ras leads to stimulation of the MAPK pathways, and ultimately AP-1 transcription (Janeway & Travers, 1997). LAT also binds to, and activates the enzymes PLC and PI-3K (Leo et al., 2002). As mentioned in Sections 1.2.1(ii) and 1.2.2(iv), these enzymes result in the generation of DAG and calcium that activate PKC and calcineurin, which in turn stimulate NF-κB and NFAT activity respectively (Taylor-Fishwick et al., 1997).

1.2.6 Cytokine signalling pathways.

The principal cytokines of interest in this thesis are IL-2, IL-4 and TNFα: their respective signalling pathways and regulation of gene expression are described below.

1.2.6 (i) Interleukin-2

IL-2 signalling pathways.

IL-2 signalling pathways can be divided into two categories depending on effector functions: induction of cellular proliferation or anti-apoptotic mechanisms (Ellery & Nicholls, 2002). These pathways also appear linked to whether the IL-2 βγ or αβγ subunits and JAK -dependent and -independent signalling pathways respectively are involved (Ellery & Nicholls, 2002).

Classical IL-2R activation following TCR engagement induces expression of the IL2-Rα, the formation of the potent IL-2αβγ heterotrimer and the activation of genes critical in cell proliferation and differentiation (Lin & Leonard, 1997). As outlined in Section 1.2.5, various (and in many cases overlapping) signalling pathways are activated upon T-cell stimulation including the Src family kinases (typically lck), the PTK Syk, the Shc-Ras-Raf-MAP kinase pathway, PI-3K, JAK-STATs and other signal transducing adaptor molecules (Lin & Leonard, 1997, Ellery & Nicholls, 2002). Lck, is implicated in IL-2R signalling, in particular, anti-apoptotic mechanisms. The role of the PTK Syk in IL-
2R mediated signalling is dubious. Syk, a member of the same family as ZAP-70, is activated by IL-2 in peripheral blood lymphocytes (PBL) and interacts with the IL-2Rβ (Minami et al., 1995). However, Syk activation is not thought critical for IL-2 signalling (Xiu & Leonard, 1997). Within one minute of IL-2-induced T-cell stimulation, there is an increase in the phosphorylation of the adaptor proteins Shc, Grb2 and the guanine nucleotide exchange factor Sos (Zhu et al., 1994). These molecules are thought to provide a key link between IL-2R engagement and the activation of Ras, the serine/threonine kinase Raf1, MEK, and MAP kinase (Perkins et al., 1993). PI-3K activity (and that of its downstream products, the serine/threonine kinases p70 S6 kinase and protein kinase B (also called Akt)) is detectable following IL-2Rβ stimulation (Franke et al., 1997).

With respect to IL-2R mediated cell proliferation, JAKs particularly JAK1 and JAK3 are important contributors. Both JAK-1 and-3 interact with the IL-2R (Ellery & Nicholls, 2002). In T-cells, upon IL-2 stimulation or TCR engagement and induction of the high affinity IL-2Rαβγ subunit, JAK3 complexes with the γc subunit and becomes phosphorylated (Boussiotis et al., 1994). Phosphorylated JAK3 is thought to mediate the activation and phosphorylation of JAK1, which is associated with the β subunit (Miyazaki et al., 1994). These receptor-associated JAKs result in the activation of the cytosolic transcription factors STATs. In lymphocytes STAT3, STAT5a and STAT5b are activated in response to IL-2 (Lin & Leonard, 1997). JAK1 phosphorylates STAT -1, -3, -5a and 5b in a JAK3 dependent manner (Lin & Leonard, 1997). In freshly isolated PBL, IL-2 activates only STAT5 proteins, however in PBL pre-activated with PHA, STAT -5a, -5b and STAT3 are activated (Lin et al., 1995). These STATs upon dimerization, travel to the nucleus and modulate target gene activity by binding to specific response elements (Section 1.2(iii), Lin & Leonard, 1997, Ellery & Nicholls, 2002). A signal transducing adaptor molecule is also phosphorylated by JAK3 following IL-2 stimulation (Xiu & Leonard, 1997). This adapter molecule is thought important in the amplification of IL-2 mediated signals.

The above pathways are implicated in the activation of genes important in cellular proliferation and cell cycle progression including c-myc, c-fos, c-jun, c-myb, bcl-2, bcl-X, cyclin family proteins and a family of kinases called cdc2 kinases (Xiu & Leonard, 1997). The activation of these genes confirms the role of IL-2 as a proliferative and differentiation signal (Goldsmith & Greene, 1996, Xiu & Leonard, 1997). However, IL-2 is also thought to play a role in anti-apoptotic mechanisms. The IL-2Rβγ receptor, Ick and PI-3K are considered important contributors to this anti-apoptotic role (Ellery & Nicholls, 2002).
Following IL-2Rβγ (intermediate affinity receptor) engagement and activation, the src family kinase lck, phosphorylates tyrosine kinases in the IL-2Rβ subunit (Hatakeyama et al., 1991). Lck must be dephosphorylated before activation, indicating that some other factors (e.g. phosphatases) are involved (Ellery & Nicholls, 2002). Recently it has been suggested that a protein phosphatase associated with the proximal region of the γc subunit may promote lck kinase activity, independent of JAK1 and JAK3 (Ellery & Nicholls, 2002). Lck is known to phosphorylate PI-3K associated with the β subunit. It is now believed that this phosphorylation of PI-3K, by lck, on βγ receptors is critical for inducing anti-apoptotic (i.e. 'survival') mechanisms (Gonzalez-Garcia et al., 1997). Resting and anergised T-cells express the IL-2Rβγ (intermediate affinity) receptor but not JAK3 (Ellery & Nicholls, 2002). It is postulated that this lck-dependent signalling via the βγ receptor and PI-3K is involved in the long-term survival of immune cells in the circulation and provides a source of memory upon antigenic re-challenge (Ellery & Nicholls, 2002).

Regulation of IL-2 gene expression.

Due to the positive effect of IL-2 on cell proliferation and cell cycle progression, its secretion must be tightly controlled (Hughes & Pober, 1996, Burlinson et al., 1997). The IL-2 promoter region contains a number of functional regions necessary for maximal gene induction (Burlinson et al., 1997). Activated T-cells require full occupancy of these DNA-binding sites within this IL-2 promoter (Rooney et al., 1995). The functional regions include proximal and distal binding sites for NFAT and AP-1 (AARE -2 and -1 respectively), binding sites for NF-κB, CD28-activated factors (CD28RE), octamer factors and NF-IL-2B (a non-classical NF-κB binding site) (Rooney et al., 1995, Goldsmith & Greene, 1996, Hughes & Pober, 1996). Another AP-1 binding site (designated NFAP-1) has been located downstream of (and appears to be functionally part of) the distal NFAT binding site (Boise et al., 1993). The CD28RE is the binding site for the co-stimulatory molecule CD28, and appears to work in tandem with the NF-κB and NFAT binding sites during CD28-mediated IL-2 promoter activation (Zhou et al., 2002). Octamer (Oct) proteins are a family of transcription factors that are found tightly coupled to the AP-1 binding site in the IL-2 promoter (Taylor-Fishwick et al., 1997). Oct proteins namely Oct-1, which is ubiquitously expressed and Oct-2, which is expressed in T- and B- Cells (Taylor-Fishwich et al., 1997), are thought to act in concert with an octamer associated binding protein (OAP-40) in antigen mediated stimulation of IL-2 gene activity in T-cells (Burlinson, 1997). Fig 1.8 highlights the binding sites of the major transcription factor binding sites in the IL-2 promoter.
Fig 1.8. Regulatory Binding sites on the IL-2 promoter.


CD28RE, CD28 response element; dAP-1, distal activating protein 1; dOct, distal octamer protein; dNFAT, distal nuclear factor of activated T-cells; NFAP-1, AP-1 binding protein distal of NFAT; NF-κB, nuclear factor kappa B; pAP-1, proximal activating protein-1; pNFAT, proximal nuclear factor of activated T-cells; pOct, proximal octamer protein.
A recent study investigating the transcriptional regulation of IL-2 in human PBL and Jurkat T-cells reported differences in the transcriptional regulation of IL-2 between these two cell types (Hughes & Pober, 1996). In human PBL, mutation studies revealed NF-κB, proximal AP-1 and proximal NFAT binding sites important for PHA-stimulated IL-2 promoter activity, proximal AP-1 being the single most important site. However, in Jurkats the distal NFAT site and NFAP-1 were more important (Hughes & Pober, 1996). Clearly, NFAT appears less important for IL-2 promoter activity in PBL than Jurkats (Hughes & Pober, 1996). Finally this study also concluded that co-stimulation provided by different co-stimulatory molecules (e.g. CD2, CD28, CD3) function through the same cis-acting elements on the IL-2 promoter (chiefly NF-κB, proximal AP-1 and to a lesser extent NFAT, NFAP-1 and proximal Oct sites) (Hughes & Pober, 1996). These results underline the differences transcriptional activity between cell types and have important implications when conducting inter-study comparisons.

1.2.6 (ii) IL-4 signalling pathways.

The IL-4R (as mentioned in Section 1.1.4) consists of α and γc subunits. The IL-4Rα chain is a member of the hematopoietin receptor superfamily (Nelms et al. 1999). IL-4 binding to the IL-4Rα initiates cross-linkage, subsequent IL-4Rα: IL-4Rγc heterodimerisation and activation of IL-4R signalling pathway (Kammer et al., 1996). Both IL-4R subunits lack intrinsic tyrosine kinase activity. However, JAK-1 (and sometimes JAK-2) have been shown to associate with the IL-4Rα (Russell et al., 1994, Murata et al., 1996), and JAK-3 to associate with the γc chain (Takeda & Akira, 2000). Following IL-4: IL-4Rα engagement, JAK-1 and JAK-3 become tyrosine phosphorylated, resulting in the rapid phosphorylation of IL-4Rα (Murata et al., 1996, Nelms et al., 1999). The cytoplasmic domain of the IL-4Rα domain contains five highly conserved functional tyrosines and the resulting activity of IL-4 may be subdivided according to these tyrosine residues. Y497 is proximal to the membrane and a critical proliferative signal. The next three tyrosines (Y575, Y603 & Y631) are involved in the induction of IL-4 responsive genes (via JAK- STAT activation), while the final C-terminal tyrosine (Y713) is within a motif that appears to serve as a docking site for different phosphatases (Nelms et al., 1999).

A 170kDa phosphoprotein, uniquely phosphorylated in response to IL-4, was identified (Sun et al., 1995). Initially called IL-4 phosphorylation substrate, this phosphoprotein was subsequently renamed insulin receptor substrate (IRS) -2 due to its high homology to IRS-1 (Sun et al., 1995). IRS-1/2 physically interact with the IL-4Rα.
chain, via Y497, which is contained in a region called the insulin IL-4 receptor (I4R) motif (Paul, 1997, Nelms et al, 1999). 14R: IRS-1/-2 interaction results in the phosphorylation of IRS-1/-2 molecules by IL-4R associated kinases (e.g. JAK-1, -2, -3) (Sun et al, 1995, Nelms et al, 1999) and in response to stimulation by cytokines including IL-2, IL-4, IL-7, IL-9 and IL-15 (Johnston et al, 1995). Phosphorylated IRS-1/-2 is reported to interact with the p38 PI-3K subunit and the Grb-2 adapter molecule (Sun et al, 1995). This results in the downstream activation of PI-3K and Ras/MAPK pathways respectively and is thought to explain the role of IL-4 as a proliferation signal (Sun et al, 1995, Nelms et al, 1999).

The IL-4Rα tyrosine residues Y575, Y603 and Y631 are critical residues in IL-4-induced gene activation (Nelms et al, 1999). Upon IL-4: IL-4R engagement, JAK1 and JAK3 become activated and phosphorylate Y575, Y603 and Y631 on the IL-4Rα (reviewed in Nelms et al, 1999, Takeda & Akira, 2000). STAT6 can then associate via a conserved SH2 domain and becomes phosphorylated at a C-terminal residue (Darnell, 1997). Upon phosphorylation, STAT6 disengages from the IL-4Rα, dimerizes and translocates to the nucleus (Schmidt-Weber et al, 2000, Takeda & Akira, 2000). The exact mechanisms of how STAT6 activates transcription are still unclear. However, they are thought to involve interactions with other transcription factors (e.g. C/EBP, NF-κB) or require phosphorylation by kinases activated by the Ras/MAPK cascades (e.g. ERK/1/2) (Delphin et al, 1995, Nelms et al, 1999).

The importance of STAT6 in IL-4 signalling is emphasised in STAT6 knockout mice where IL-4-mediated increases in cellular proliferation, Th2-cell development, surface expression of MHC class II and IL-4Rα, and IgE class switching are ablated (Shimoda et al, 1996 Takeda & Akira, 2000). Furthermore STAT6 knockout mice displayed impaired responses to models of Th2-type infection (e.g. Leishmania mexicana) (Stamm et al, 1999).

IL-4 is also subject to negative regulation by various phosphotyrosine phosphatases. General control of IL-4R signalling is mediated by the phosphotyrosine phosphatases, SH2-containing phosphatases SHP-1 and SHP-2 and the SH2-containing inositol-5-phosphatases (SHIP) (Nelms et al, 1999). STAT proteins are subject to negative regulation by a family of SH2 containing proteins called suppressor of cytokine signalling (SOCS) proteins (Takeda & Akira, 2000). SOCS protein expression is enhanced by cytokine stimulation and SOCS1 and SOCS3 can bind directly to JAK proteins, thereby reducing their activity (Takeda & Akira, 2000). Finally, another family called protein inhibitors of activated STAT (PIAS) are known to bind to and inactivate STAT protein activity directly (Takeda & Akira, 2000).
Recently, attention has focused on the role of IL-4 in Th2 subset development and the interactions between TCR- and STAT- signalling pathways. Most peripheral T-cells have a naïve phenotype and do not secrete IL-4 (Kubo et al., 1997). Recently the functional IL-4 promoter was found in B- and Th1- cells (cells which do not normally secrete IL-4) as well as Th2- cells (Kubo et al., 1997). Kubo and co-workers (1997) also noted the presence of an IL-4 silencer element in the 3’ untranslated region of Th1 cells alone. This region contained a STAT-6 consensus site, which was found to be essential for STAT6 function in Th2 cells (Kubo et al., 1997). Further research is needed to elucidate the exact role of this STAT-6 sequence/silencer region in the development of Th1/Th2 phenotype from Th0 cells.

TCR cross-linking is reported as insufficient for Th2 development. Following IL-4 stimulation, TCR cross-linking increased the expression and binding properties of the IL-4R, however CD28 costimulation was necessary to enhance IL-4R sensitivity (as demonstrated by the phosphorylation of JAK 3, IL-4Rα and STAT-6) (Kubo et al., 1999). TCR-mediated signal transduction in Th2 cell differentiation is also reported to exhibit a preferential requirement for the activation of p56 lck (Yamashita et al., 1998), while IL-4 was reported to indirectly suppress IL-2 production by human T lymphocytes via PPARγ (Yang et al., 2002). Finally, Yamashita et al. (1999) found that Th2 cell differentiation was dependent on TCR-mediated activation of Ras/MAPK pathway. They established that activation of the Ras/MAPK pathway altered IL-4R function directly and enhanced STAT6 tyrosine phosphorylation, (probably by up-regulating JAK1 kinase activity) (Yamashita et al., 1999). However, it still remains unclear how the TCR-IL-4 signalling pathways interact during Th-cell differentiation.

1.2.6 (iii) TNFα signalling pathways.

TNFα receptor occupancy and cross-linking elicits a variety of cell death mechanisms and cell signalling pathways, the true understanding of which remains incomplete (Vanden Berghe et al., 2000, Baud & Karin 2001). It has been shown that clustering of the intracellular domains of the TNF receptors can lead to TNFα activation (Vanden Berghe et al., 2000). Although at least four receptors are known to interact with TNFα and lymphotoxin ligands, this thesis will focus mainly on the TNFR1 (p55) and TNFR2 (p75) receptors. p55 (also called CD120a) is implicated in both positive and negative regulation of both apoptosis and inflammation. However, p75 initiates an anti-apoptotic and pro-inflammatory response (Baud & Karin, 2001). TNFα-induced effects can be classified into two signalling networks defined by the presence of either a death
domain (DD) or a TNF receptor associated factor (TRAF) domain (Wajant et al., 1999, Wallach et al., 1999).

The DD was originally identified as the C-terminal intracellular portion of p55, and a related molecule called Fas, which played a critical role in the cytotoxic effects of these receptors (Tartaglia et al., 1993). The DD acts as a protein-docking site and transducer of conformational change (Tartaglia et al., 1993). Other DD-containing molecules can therefore interact with the TNFα receptors via this DD-domain, e.g. TNFR1-associated death domain protein (TRADD), receptor-interacting protein 1 (RIP1), Fas-associated death domain protein (FADD). Receptor signalling causes the recruitment and activation of the caspase family of apoptotic proteins and ultimately results in cell death (apoptosis) (Nicholson & Thornberry, 1997, Wallach et al., 1999, Wajant et al., 1999, Baud & Karin, 2001).

The TRAF family are also involved in protein-protein interactions during the TNFα-induced cell signalling cascade (Wajant et al., 1999). TRAF family members are distinguishable by the presence of a conserved C-terminal TRAF domain, a variable N-terminal ring finger, zinc finger motifs and a central coiled-coil region (Wajant et al., 1999, Wallach et al., 1999). The C-terminal region is required for receptor binding while both the C- and N-terminal regions are necessary for signal transduction (Rothe et al., 1994). p55 upon activation firstly recruits TRADD, which results in the recruitment of RIP1, FADD and critically TRAF2 (Baud & Karin, 2001). p75 is known to bind TRAF2 directly and subsequently recruit TRAF1 (Rothe et al., 1994, Baud & Karin, 2001). TRAF2 plays a critical role in the activation of IKK (NF-κB) and MAPK (AP-1) pathways (Baud et al., 1999, Baud & Karin, 2001). Indeed, TRAF2 null cells do not produce TNFα (Song et al., 1997, Vanden Berghe et al., 2000). Over-expression of TRAF proteins 2, 5, and 6 has been reported to activate NF-κB (Rothe et al., 1994, reviewed in Wajant et al., 1999) and AP-1 signalling pathways (Song et al., 1997). RIP1 was found to be a key effector in the activation of NF-κB by p55, however its precise role has not yet been established (Ting et al., 1996). Apart from TRAF4, all mammalian TRAF can interact with NIK thereby leading to the activation of NF-κB (Malinin et al., 1997), however it appears that other cooperative mechanisms may be involved during this activation (Wajant et al., 1999).

NF-κB and AP-1 cross talk and cooperation is thought to play an important role in the in the induction of TNFα target gene and TNFα-stimulated cell proliferation. This cooperation is underlined by the presence of both NF-κB and AP-1 binding sites in the TNFα promoter, which are subject to autoregulation (Karin et al., 1997). Other molecules implicated in this cross talk include lipid mediators e.g. sphingomyelinase (Wiegmann et
phosphatidylcholine-specific phospholipase C (van den Bosch et al., 1992), phospholipase A₂ (van den Bosch et al., 1992), phospholipase D (De Valck et al., 1993) and PI-3K (Madrid et al., 2001). Sphingomyelinase-induced ceramide is known to contribute to the JNK activation, however its contribution to NF-κB signalling in this system is unclear (Wallach et al., 1999). TNFα induced signalling is also known to activated PKC (particularly epsilon and zeta), probably via phsophatidylcholine-PLC activated DAG (Berra et al., 1995).

Control of TNFα signalling is mediated by the availability and interactions of ligands and receptors, various docking proteins, signalling enzymes, other signalling pathways etc (Wallach et al., 1999). The seemingly parallel functions of TNFα (i.e. cell growth on one hand and apoptosis on the other) make the task of elucidating TNFα signalling (and control of) more difficult. Two groups of proteins called inhibitors of apoptosis (IAP) and A20 have been identified (reviewed in Wajant et al., 1999). Both of these proteins are anti-apoptotic. The IAP family have been found to interact with TRAF-1 and-2 and to inhibit caspase family members, while A20 interacts with TRAF2 and NF-κB to prevent apoptosis (Wajant et al., 1999). Further research is needed to determine the roles and interactions of the various TNFα-related proteins and their signalling pathways.

1.3 Nutrients & The Immune System.

The development, maintenance and optimal performance of the immune system depends upon balanced and adequate nutrition. Immune cell functioning is influenced by both dietary excess and deficiency. Indeed the magnitude and efficiency of an immune response, in both acute and chronic illness, is affected by nutritive and non-nutritive dietary constituents (Kim, 1999). Chronic energy deficiency (e.g. in protein-energy malnutrition) results in impaired immune functioning and can potentiate the effects of bacterial and viral infections (Chandra, 1997, ILSI, 1999). Particular nutrients / molecules that have been reported to exert immuno-modulatory influences include glutamine (Andrews & Griffiths, 2002), vitamins A, B6, B12, C and E (Chandra, 1997), nucleotides (Sanchez-Pozo & Gill, 2002) and minerals such as zinc, copper, selenium and iron (Cunningham-Rundle et al., 2002). Dietary fatty acids in particular are also known to modulate the immune and inflammatory responses. This section of the review will focus on the immuno-modulatory effects of fatty acids.
1.3.1 Fatty acids.

Fatty acids not only provide a source of energy to the body but by their incorporation into plasma membranes contribute to cell growth, maintenance, structure, fluidity and function. Biological membranes are composed of a phospholipid bilayer with the hydrophilic phospholipid heads oriented outwards and the hydrophobic tails facing inwards towards each other (fluid mosaic model, Singer & Nicolson, 1972). Proteins are embedded in this lipid bilayer integrally and peripherally (Zubay, 1993), and have specific functions (e.g. signal transduction). The lipid bilayer provides structural integrity and a permeability barrier. However it also plays an important role in hydrophobic interactions between the membrane proteins (Zubay, 1993).

Dietary fatty acids are known to influence lipid bilayer composition and mobility. Structurally fatty acids are composed of a glycerol backbone with fatty acids attached. Fatty acids can be divided into three categories; saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). This classification is dependent upon the presence or absence of double bonds; SFA have none, MUFA have one, and PUFA have more than one double bond in their carbon chain. PUFA can be further subdivided into \(\omega\)-9, \(\omega\)-6 and \(\omega\)-3 series, depending on the position of the first double bond from the methyl end (between carbon (C) 9 and C10, C6 and C7, and C3 and C4, respectively). \(\omega\)-9 PUFA are normally only present in essential fatty acid deficiency. Common \(\omega\)-9 fatty acids include the MUFA oleic acid (OA; 18:1), \(\omega\)-6 PUFA include linoleic acid (LA; 18:2), arachidonic acid (AA; 20:4), \(\gamma\)-linoleic acid (GLA; 18:3) and conjugated linoleic acid (CLA; 18:2). \(\omega\)-3 PUFA comprise \(\alpha\)-linolenic acid (ALA; 18:3), and the fish oils (FO) eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6). The three \(\omega\)-classes of fatty acid are not inter-convertible but are metabolised by a common series of elongase and desaturase enzymes (See Fig 1.9). In this manner, ALA can be elongated and desaturated to EPA and DHA, LA can be elongated and desaturated to GLA, dihomogamma linolenic acid (DHGLA), AA and finally docosapentaenoic acid (DPA). OA can be elongated and desaturated to mead acid (MA; C22:3n-9), in states of essential fatty acid deficiency. Competitive interaction exists between the fatty acid families whereby \(\omega\)-3 fatty acids suppress \(\omega\)-6 fatty acid metabolism and vice versa though not as potently. Both \(\omega\)-3 and \(\omega\)-6 fatty acids suppress \(\omega\)-9 fatty acid metabolism (Miles & Calder, 1998).

Sources of some of the common fatty acids (SFA, MUFA & PUFA) are listed in Table 1.6). It is worth noting that the \(\omega\)-3 PUFA can only be sourced from marine plants.
**Fig 1.9.** Elongation and desaturation of ω-3, ω-6 and ω-9 PUFA.

<table>
<thead>
<tr>
<th>n-3 PUFA</th>
<th>n-6 PUFA</th>
<th>n-9 PUFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:3n-3 (ALA)</td>
<td>18:2n-6 (LA)</td>
<td>18:1n-9 (OA)</td>
</tr>
<tr>
<td>↓ Δ-6 desaturase</td>
<td>↓ Δ-6 desaturase</td>
<td>↓ Δ-6 desaturase</td>
</tr>
<tr>
<td>18:4n-3</td>
<td>18:3n-6 (GLA)</td>
<td>18:2n-9</td>
</tr>
<tr>
<td>↓ Elongase</td>
<td>↓ Elongase</td>
<td>↓ Elongase*</td>
</tr>
<tr>
<td>20:4n-3</td>
<td>20:3n-6 (DHGLA)</td>
<td>20:2n-9</td>
</tr>
<tr>
<td>↓ Δ-5 desaturase</td>
<td>↓ Δ-5 desaturase</td>
<td>↓ Δ-5 desaturase*</td>
</tr>
<tr>
<td>20:5n-3 (EPA)</td>
<td>20:4n-6 (AA)</td>
<td>20:3n-9</td>
</tr>
<tr>
<td>↓ Elongase</td>
<td>↓ Elongase</td>
<td>↓ Elongase*</td>
</tr>
<tr>
<td>22:5n-3 (DPA)</td>
<td>22:4n-6</td>
<td>22:3n-9</td>
</tr>
<tr>
<td>↓ Elongase</td>
<td>↓ Elongase</td>
<td></td>
</tr>
<tr>
<td>24:5n-3</td>
<td>24:4n-6</td>
<td></td>
</tr>
<tr>
<td>↓ Δ-6 desaturase</td>
<td>↓ Δ-6 desaturase</td>
<td></td>
</tr>
<tr>
<td>24:6n-3</td>
<td>24:5n-6</td>
<td></td>
</tr>
<tr>
<td>↓ Δ-5 desaturase</td>
<td>↓ Δ-6 desaturase</td>
<td></td>
</tr>
<tr>
<td>22:6n-3 (DHA)</td>
<td>22:5n-6 (DPA)</td>
<td></td>
</tr>
</tbody>
</table>

*Only in essential fatty acid deficiency.

**Table 1.6.** Sources of common dietary fatty acids.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lauric acid (12:0)</td>
<td>*De Novo synthesis &amp; coconut oil</td>
</tr>
<tr>
<td>Myristic acid (14:0)</td>
<td>*De Novo synthesis &amp; milk</td>
</tr>
<tr>
<td>Palmitic acid (16:0)</td>
<td>*De Novo synthesis, milk, eggs, animal fats, meat, palm oils, FOs, cocoa butter</td>
</tr>
<tr>
<td>Stearic acid (18:0)</td>
<td>*De Novo synthesis, milk, eggs, animal fats, meat cocoa butter</td>
</tr>
<tr>
<td>Oleic acid (18:1n-9)</td>
<td>Desaturation of stearic acid, olive oil, milk, eggs, animal fats, meat, cocoa butter</td>
</tr>
<tr>
<td>Linoleic acid* (18:2n-6)</td>
<td>Some milks, eggs, animal fats, meat, green leaves, maize, sunflower, safflower and soybean oils.</td>
</tr>
<tr>
<td>α-Linoleic acid* (18:3n-3)</td>
<td>Green leaves, rapeseed, soyabean and linseed oils.</td>
</tr>
<tr>
<td>γ-Linolenic acid* (18:3n-6)</td>
<td>Synthesised from linoleic acid, borage and evening primrose oils.</td>
</tr>
<tr>
<td>Dihomo-γ-linolenic acid (20:3n-6)</td>
<td>Synthesised from γ-linolenic acid.</td>
</tr>
<tr>
<td>Arachidonic acid (20:4n-6)</td>
<td>Synthesised from linoleic acid via γ-Linolenic acid &amp; DHGLA.</td>
</tr>
<tr>
<td>Eicosapentaenoic acid (20:5n-3)</td>
<td>Synthesised from α-linolenic acid, FOs</td>
</tr>
<tr>
<td>Docosahexaenoic acid (22:6n-3)</td>
<td>Synthesised from α-linolenic acid via eicosapentaenoic acid, FOs</td>
</tr>
</tbody>
</table>

*Signifies cannot be synthesised in mammals. Table adapted from Calder (1998).
and seafood or by synthesised from ALA. In addition, dietary fatty acid composition is known to determine tissue fatty acid composition (Gibney & Hunter, 1993). The ratios between the different classes of dietary fatty acids has been found to be more important in determining effects on tissue fatty acid composition than absolute amounts (Kelley, 2001). Dietary fatty acids can modulate the immune response and indeed immune cell activation leads to the production and secretion of free fatty acids from these cells (Kelley & Rudolph, 2000).

1.3.2 Fatty acids and lymphocyte proliferation.

The effects of fatty acids on lymphocyte function has been investigated in vitro and ex vivo using whole blood, splenic, thymic and lymph node lymphocytes, lung and gut – associated lymphoid tissue, cell lines and isolated peripheral blood mononuclear cells (PBMC’s) which are composed of approx 85-90% lymphocytes and 10-15% monocytes (Calder et al, 2002). In vivo measures generally involve tissue staining and organ weight, measuring the numbers, types and percentages of circulating lymphocytes and their surface molecule expression and lastly skin prick testing or antigen intradermal challenge, which is used as a measure of delayed type hypersensitivity (DTH). Mitogen or antibody induced lymphocyte proliferation is used as an indicator of lymphocyte function. Common mitogens used to stimulate polyclonal proliferation include concanavalin A (Con A) and phytohaemagglutinin (PHA) which stimulate T-cells, pokeweed mitogen (PWM) which stimulates T-cell dependent B-cells and bacterial lipopolysaccharides (LPS), which stimulates monocytes / macrophages. Monoclonal antibodies e.g. anti-CD3 (OKT3) and intracellular signal stimulators (e.g. Phorbol 12-myristate 13 acetate, PMA) are also used.

Level and type of fat in the diet has been highlighted as influential in lymphocyte functioning. In vitro studies reported low concentrations of fatty acid (<5µM) to enhance mitogen-stimulated proliferation of lymphocytes. However once a ‘threshold’ range of concentrations is reached (approx. 10-15µM), fatty acids were reported to inhibit lymphocyte proliferation in the order:

Lauric = myristic < palmitic < stearic = oleic < linoleic < α-linolenic < γ-linolenic <
dihomo-γ-linolenic = docosahexaenoic < arachidonic ≤ eicosapentaenoic acids.

(See Table 1.7 for a summary of these in vitro studies). Hence, degree of unsaturation modulates lymphocyte proliferation. However, fatty acid concentration, the time during culture when the oils were added and period of time to which the cells are exposed to the fatty acid can also influence blastogenesis (Calder, 1996, 1998). Time of addition of fatty acid appears critical; Khalfoun et al (1996a) reported human peripheral blood lymphocytes
(PBL) pre-incubated with PUFA for 48h, washed and subsequently stimulated with PHA
exhibited reduced proliferation compared to untreated, washed and stimulated cells. However, PUFA added to the PBL's 48 h post stimulation did not affect lymphocyte proliferation. Inhibitors of cPLA$_2$, COX or LOX did not prevent this fatty acid-induced inhibition, suggesting that effects were eicosanoid independent (Santoli et al, 1990, Calder et al, 1992c, Kumar et al, 1992, Soyland et al, 1993, Rotondo et al, 1994, Khalfoun et al, 1996a). Similarly, anti-oxidants e.g. α-tocopherol did not prevent this fatty acid-induced proliferation, hence it seems unlikely that the effects are due to lipid peroxidation (Calder & Newsholme, 1993, Soyland et al, 1993, Khalfoun et al, 1996a).

Ex vivo measures of lymphocyte proliferation following supplementation in animals, confirm that high-fat diets reduce blastogenesis relative to low-fat diets. However this is influenced by fat type and source: high-fat diets inhibit proliferation in the order SFA < n-6 PUFA < olive oil ≤ linseed oil ≤ FO (Calder, 1996). (See Table 1.7 for a summary of in vitro, animal and human trials of fatty acid and lymphocyte function). There exists the possibility of interactions between the different fatty acids, including palmitic acid (PA), oleic acid (OA) and linoleic acid (LA) and lymphocyte proliferation. Jeffrey et al, (1997a) supplemented rats with diets containing 178 g fat/kg, a constant ratio of n-6 : n-3 PUFA but with different levels of PA, OA and LA. She found that there was a significant inverse relationship between dietary OA: LA and lymphocyte proliferation, suggesting an interaction between the different fatty acids (Jeffrey et al 1997a). However, feeding rats 178 g fat/kg at two levels of PUFA (17.5 or 35 g/100g fatty acids) and a range of n-6: n-3 ratios (100, 20, 10, 5, 1) resulted in reduced lymphocyte proliferation with decreasing n-6: n-3 PUFA content in the low PUFA diet, but had no effect in the high PUFA diet (Jeffrey et al, 1997c). It has also been demonstrated that the position of the fatty acid in the triacylglycerol moiety determines immune response; rats fed PA at the sn-2 position had an increased proliferative response relative to rats fed PA at the sn-1 position, medium-chain triglycerides, lauric (12:0, LA), PA or stearic acids (18:0, SA) (Jeffrey et al, 1997c). Finally, dietary n-3 PUFA modulated murine T-cell subset activation. Arrington et al, (2001) fed C57BL/6 mice FO at 2% diet for 14 days. She reported that dietary n-3 PUFA down regulated IL-2 driven CD4 and CD8 activation while up-regulating the activation of the Th2 CD4 subset (i.e. IL-4 driven) (Arrington et al, 2001). Therefore the authors concluded that the anti-inflammatory effects of dietary n-3 PUFA were a combination of a directly depressed Th1 type response and the suppressive effect of a Th2 type response on Th1 cells and cell mediated immunity.
There are many discrepancies between study results. This may be explained by confounding factors such as a) dietary factors, b) animal models used, c) cell types and d) cell culture conditions. With respect to dietary intakes differences in the amount of fat in the diet, duration of feeding and type of comparison can all influence the immune function. Examples of different types of dietary comparisons include high and low-fat diets using the same fatty acid, or a low- or high-fat diet but studying the effects of different fatty acids. Strain of animal is important in the context of their ability to mount a Th1 or Th2 dominant responses and their differences in cytokine production, e.g. the C57Bl/6 murine strain has a lower IL-2 secretion, higher IFN-γ and lower IL-4 and IL-10 than Balb/C mice (Calder, 2002). Clearly this difference in cytokine production will result in potentially different outcomes following fatty acid supplementation in these two strains. With respect to cell types and culture conditions used, anatomical source of lymphocytes (PBMC, splenocytes, thymus etc), mitogen used and its concentration, may all influence the immuno-modulatory effects of fatty acids. It has also been reported that for dietary studies, the use of autologous serum in the culture media is preferable to the traditionally used foetal calf serum. Foetal calf serum contains amongst its ingredients lipids (e.g. triglycerides, cholesterol and lipoproteins), which can reverse the changes in fatty acid composition brought about by dietary lipid manipulation (Yaqoob et al. 1995a) and counteract any effects of dietary lipid manipulation on cell function. (Meydani et al. 1985, Fritsche et al. 1991, Yaqoob et al. 1995a).

Decreasing dietary fat intake from 40 to 25 % total energy intake enhanced mitogen stimulated proliferation of human PBMC’s, indicating that high-fat diets may blunt lymphocyte proliferation in humans (Kelley et al. 1989, 1992). A low-fat diet with different levels of n-6 PUFA (12.9 and 3.5% energy) had no effect on the proliferation of human PBMC’s (Kelley et al. 1989, 1992a), however a low-fat, low-cholesterol diet with 1.23 g/day FO decreased Con A and PHA induced PBMC proliferation (Meydani et al. 1993). Clearly type of fat and amount of fat is important in dietary intervention studies. However the effects of dietary fatty acids on the human immune response appear less profound relative to animal and in vitro studies. GLA (770 mg/d) and FO (720 mg evening primrose oil (EP0) & 280 mg DHA) for 12 weeks caused a significant decrease in lymphocyte proliferation in healthy volunteers ages 55-75 y (Thies et al. 2001). Supplementation with ALA, AA, DHA alone or a palm oil (PO): Sunflower oil (SO) mix did not affect proliferation (Thies et al. 2001). The effects of olive oil on human immune function have been investigated. Yaqoob et al. (1998) supplemented the diets of healthy middle-aged men with olive oil (OO) (18.4% energy) at the expense of SFA (11.3%
energy) for 8 weeks. Although a trend towards reduced lymphocyte proliferation was observed, this was not significant (Yaqoob et al., 1998). Similarly, supplementation with AA (1.5 g/day for 50 d) had no effect on lymphocyte proliferation (Kelley et al., 1997, 1998a). The observed lack of effect may be due in part, to confounding dietary, lifestyle and environmental factors as well as experimental designs. Age may also affect the immuno-modulatory effects of fatty acids; postmenopausal women supplemented with n-3 PUFA (2.4 g/day) for 24 weeks displayed reduced PHA induced lymphocyte proliferation post-supplementation, however supplementation had no effect on pre-menopausal women (Meydani et al., 1991). Similarly, Calder et al. (2002) reported a decrease in thymidine incorporation and lymphocyte proliferation in healthy males with increasing age.

Other influences on lymphocyte proliferation include genetic polymorphisms (single nucleotide polymorphisms) of MHCII (Human Leukocyte Antigen, HLA), cytokines, their receptors, adhesion molecules etc. Increasing emphasis is now being placed on the investigation of the importance and extent of these polymorphisms. TNF in particular is known to have at least ten single nucleotide polymorphisms in the TNF promoter region (Allen, 1999). With respect to one of these polymorphisms (NcoI polymorphism), it has been found that PHA-stimulated PBMC's from TNFβ1 homozygotes have increased TNFβ production, while PHA and endotoxin stimulated PBMC's from TNFβ2 homozygotes have enhanced IL-1β and TNFα production (Poicot et al., 1993, Majetschak et al., 1999). However, de Jong et al. (2002) reported that alleles of TNFα microsatellite and carriehship of TNF polymorphisms were not related to TNF production. The authors concluded that the genes determining the differences in endotoxin-induced TNF production have not yet been identified (de Jong et al., 2002). Recently, Grimble et al. (2002), showed that the ability of dietary FO supplementation to decrease TNFα production ex vivo is influenced by polymorphisms in the TNFα gene. Therefore, single nucleotide polymorphisms can influence the capacity of an individual to mount an immune response – they may render that individual more / less susceptible to the immuno-modulatory effects of fatty acids. Clearly, this area merits further investigation.

It has been reported that lifestyle and environmental factors that may also contribute to variations in lymphocyte responses in healthy humans (e.g. sex, smoking factors, exercise, alcohol consumption and dietary habits, history of infections and vaccinations). These factors may influence the propensity of an individual to respond to fatty acids intervention (Kelley & Rudolph, 2000, Calder et al., 2002). Vitamin E status influences the immuno-modulatory effects of fatty acids. Supplementation with n-3 rich FOs, while maintaining adequate vitamin E levels has been reported to enhance PHA or
Con A-induced lymphocyte proliferation in monkeys (Wu et al., 1996). However, no immuno-modulatory benefits were conferred after supplementing healthy humans with CO or safflower oil (SAF), OA, EPO or FO enriched in α-tocopherol (Yaqoob et al., 2000). Indeed vitamin E has been reported to directly affect immune function (Van Tits et al., 2000). A more comprehensive review of the effects of fatty acids on human proliferation is reviewed in Table 1.7.

1.3.3 Fatty acids and other parameters of lymphocyte function.

Investigations concerning the effects of dietary fatty acid on cell mediated immunity generally examine other parameters of lymphocyte function including cytotoxic T lymphocyte activity (CTL), NKC activity, the expression of various cell surface and co-stimulatory molecules (including HLA), DTH, organ transplantation, graft/host rejection models and antibody production.

Animal studies indicate that feeding high-fat diets can reduce CTL activity in the order: SFA < n-6 PUFA rich oils < linseed oil < FO (Fritsche & Johnstone, 1990, Fritsche & Cassidy, 1992, Calder, 1998). NKC activity also appears reduced in a similar manner in animal studies in the order: SFA < n-6 PUFA rich oils < OO < linseed oil < FO (Jeffrey et al., 1996, 1997abc). Two intervention trials found no significant effect of dietary supplementation with AA and OO on human NKC activity (Kelley et al., 1997, Yaqoob et al., 1998). One study reported dietary EPA & DHA but not ALA, GLA, AA or DHA alone to decrease NKC activity in healthy subjects greater than 55 years (Thies et al., 2001). Similarly, fatty acids appear to affect DTH more profoundly in animal models than in human supplementation trials. DTH has been described as the consequence of a cell-mediated response to an antigenic test, the individual having already been exposed (primed) to that antigen (Calder, 1998). Studies in rats, guinea-pigs, mice, sheep and beagle dogs found that fatty acids diminished the DTH response in the magnitude: SFA < n-6 PUFA < n-3 PUFA (Friend et al., 1980, Yoshino & Ellis, 1987, Crevel et al., 1992, Taki et al., 1992, Wander et al., 1997). However, human investigations have found no effect of reducing total fat intake (Kelley et al., 1992a), altering the PUFA level (Kelley et al., 1992a), eating FO rich (Kelley et al., 1992b), linseed oil-rich (Kelley et al., 1991) or AA-rich diets (Kelley et al., 1997) on the DTH response to various recall antigens. Only one study supplementing a low-fat low-cholesterol diet with 1.25 g n-3 PUFA/d observed a reduction in the DTH response to seven recall antigens (Meydani et al., 1993).

Animal studies also reveal that high-fat diets can reduce the graft v host response (B-cell predominant response) and the host v graft response (CMI response) activated by
the infection of allogenic cells into the footpad of the host (popliteal lymph node assay) relative to low-fat diets. Again the order of potency in animals is: low-fat = high SFA < high n-6 PUFA < high linseed oil < high FO (Hinds & Sanders, 1993, Sanderson et al., 1995, Jeffrey et al., 1996a, Peterson et al., 1998, Calder 1998). Indeed fatty acid supplementation appears to reduce organ / allograft rejection, as well as the symptoms of rheumatoid arthritis in animals and humans, FO appearing most beneficial (Berthoux et al., 1992, Bennett et al., 1995, Grimm et al., 2002).

With respect to antibody production and / or circulating antibody titres, dietary fatty acids by virtue of their immuno-suppressive effects on the Th-1 type response (lymphocyte proliferation etc) would be expected to enhance the Th-2 type response. This was found to be true in animal studies showing raised IgM, IgG and even IgE production (Friend et al, 1980, Prickett et al, 1982). However, no difference in antibody response was reported in two monkey species following a CO or maize oil rich diet (Meydani et al, 1985). Kelley et al (1989) reported no effect of reducing total fat intake or varying PUFA content on human circulating IgG, IgM, IgA or IgE levels. Likewise, there was no effect of dietary supplementing with linseed oil or salmon (Kelley et al, 1991, 1992). Sugano et al, (2000) reported a decrease in the histamine content of peritoneal exudate cells incubated with n-3 and n-6 PUFA in vitro. However, the exudate cells tended to increase the release of histamine irrespective of activation, this release being more profound with n-3 PUFA than n-6 PUFA (Sugano et al., 2000).

### 1.3.4 Fatty acids and cytokine production.

With respect to the effects of fatty acids on cytokine production, emphasis has traditionally rested upon the Th1 type lymphokines (e.g. IL-2, IFN-γ) and the pro-inflammatory cytokines (e.g. TNFα, IL-1β, IL-6) rather than the Th2 type lymphokines (IL-4, IL-10, IL-5). In vitro studies in rat lymph node and human PBL suggest that OA, LA, ALA, AA, EPA and DHA acids result in lower IL-2 concentrations compared to fatty-acid free cultures or cell incubated with SFA (Calder & Newsholme, 1992ab). DHA, EPA & ALA have been found to inhibit and low concentrations of GLA to stimulate IL-2 production in human PBMC’s in vitro (Das, 1994, Devi & Das, 1994, Purasiri et al, 1997). With respect to IFN-γ, low levels of DHA alone were found to enhance production (Purasiri et al, 1997).

Animal ex vivo cytokine production following dietary supplementation with various fatty acids has produced conflicting results, whereby fatty acids decreased (Turek et al, 1994, Jolly et al, 1997), increased (Lokesh et al, 1990, Hardardottir & Kinsella, 1991,
### Table 1.7. The effects of fatty acids on lymphocyte proliferation from a sample of *in vitro* and *ex vivo* studies.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Fatty acid</th>
<th>Stimulus</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In Vitro:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human blood</td>
<td>ALA, EPA</td>
<td>IL-2</td>
<td>↓</td>
<td>Santoli <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>Human blood</td>
<td>EPA</td>
<td>Anti-CD3</td>
<td>↓</td>
<td>Virella <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>Human blood</td>
<td>EPA, DHA</td>
<td>PHA</td>
<td>↓</td>
<td>Brouard &amp; Pascaud, 1993</td>
</tr>
<tr>
<td>Human LAK</td>
<td>DHA, EPA, GLA</td>
<td>PHA, PWM</td>
<td>↓</td>
<td>Purasiri <em>et al.</em>, 1997</td>
</tr>
<tr>
<td>Human PBL</td>
<td>ALA, EPA, DHA, AA, SA, OA, PA, MA</td>
<td>Con A</td>
<td>↓</td>
<td>Calder &amp; Newsholme, 1992</td>
</tr>
<tr>
<td>Monocytic cell line</td>
<td>FO</td>
<td>PHA</td>
<td>↓</td>
<td>Watson <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>Rat lymph node</td>
<td>MA, PA, LA, AA, EPA, LNA, OA, SA, DHA</td>
<td>Con A</td>
<td>↓</td>
<td>Khalfoun <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>Rat lymph node</td>
<td>n-3 PUFA rich Emulsion</td>
<td>Con A</td>
<td>↓</td>
<td>Calder <em>et al.</em>, 1994</td>
</tr>
<tr>
<td><strong>Ex Vivo:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human blood</td>
<td>2.4 g/d n-3 PUFA, 12 wks</td>
<td>Con A, DHA</td>
<td>↓</td>
<td>Meydani <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>Human blood</td>
<td>8 g/d FO, 6 wks</td>
<td>Anti-CD3</td>
<td>↓</td>
<td>Virella <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>Human blood</td>
<td>LF, LC &amp; 1.23 g/d FO, 24 wks</td>
<td>Con A, PHA</td>
<td>↓</td>
<td>Meydani <em>et al.</em>, 1993</td>
</tr>
<tr>
<td>Human blood</td>
<td>FO 18 g/d, 6 wks</td>
<td>PHA</td>
<td>↓</td>
<td>Endres <em>et al.</em>, 1993</td>
</tr>
<tr>
<td>Human blood</td>
<td>AA rich diet, 1.5 g/d, 50d</td>
<td>Con A, PHA, PWM, flu virus</td>
<td>NSD</td>
<td>Kelley <em>et al.</em>, 1997</td>
</tr>
<tr>
<td>Human blood</td>
<td>MUFA rich diet (18.4 % energy v 11.3% energy)</td>
<td>Con A</td>
<td>NSD</td>
<td>Yaqoob <em>et al.</em>, 1998</td>
</tr>
<tr>
<td>Human blood</td>
<td>2.4 g/d GLA</td>
<td>PHA</td>
<td>↓</td>
<td>Rossetti <em>et al.</em>, 1997</td>
</tr>
<tr>
<td>Human blood</td>
<td>LF diet &amp; 1.5 g/d AA, 8 wk</td>
<td>Con A, PHA</td>
<td>NSD</td>
<td>Kelley <em>et al.</em>, 1998</td>
</tr>
<tr>
<td>Human blood</td>
<td>LF diet &amp; 1.5 g/d ALA, 6 wks</td>
<td>Con A, PHA</td>
<td>↓</td>
<td>Kelley <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>Mouse spleen</td>
<td>30 g/kg SO v 20 g/kg SO &amp; 10 g/kg AA or EPA or DHA</td>
<td>Con A</td>
<td>↓ EPA / DHA only</td>
<td>Jolly <em>et al.</em>, 1997</td>
</tr>
<tr>
<td>Mouse spleen</td>
<td>2% FO, SAF or EPA/DHA ester mix</td>
<td>α-CD3/α-CD28 PMA/mononycin α-CD3/PMA</td>
<td>↑ LP</td>
<td>Arrington <em>et al.</em>, 2001</td>
</tr>
<tr>
<td>Mouse spleen</td>
<td>200 g/kg CO, SAF or FO 6 wks</td>
<td>Con A</td>
<td>↑ CO only</td>
<td>Wallace <em>et al.</em>, 2001</td>
</tr>
<tr>
<td>Rabbit spleen &amp; blood</td>
<td>76g FO/kg / 20 wks</td>
<td>Con A, PHA</td>
<td>↓</td>
<td>Kelley <em>et al.</em>, 1988</td>
</tr>
<tr>
<td>Rat spleen</td>
<td>150 g RO/ kg / 8 wks</td>
<td>Con A</td>
<td>↓</td>
<td>Calder <em>et al.</em>, 1995</td>
</tr>
<tr>
<td>Rat spleen</td>
<td>200g LO/kg/10 wks</td>
<td>Con A</td>
<td>↓</td>
<td>Jeffery <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>Rat spleen</td>
<td>200 g/kg SO v LO v SOLO</td>
<td>Con A</td>
<td>↓ LO</td>
<td>Jeffery <em>et al.</em>, 1996a</td>
</tr>
<tr>
<td>Rat spleen</td>
<td>200 g/kg SO v OOv HOSO</td>
<td>Con A</td>
<td>↓ OO &amp; HOSO</td>
<td>Jeffery <em>et al.</em>, 1996b</td>
</tr>
<tr>
<td>Rat spleen</td>
<td>178 g/kg, varying proportions PA, OA, LA, ALA, 6 wks</td>
<td>Con A</td>
<td>↓ as OA:LA</td>
<td>Jeffery <em>et al.</em>, 1997</td>
</tr>
<tr>
<td>Rat spleen, thymus &amp; lymph node</td>
<td>200 FO/kg / 10 wks</td>
<td>Con A, (PHA - spleen &amp; lymph node only)</td>
<td>↓</td>
<td>Yaqoob <em>et al.</em>, 1994</td>
</tr>
</tbody>
</table>

AA, Arachidonic acid; ALA, α-linolenic acid; CO, coconut oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, FO; GLA, γ-linolenic acid; HOSO, high oleic sunflower oil; LA, linoleic acid; LF, Low-fat; LO, linseed oil; MA, myristic acid; MUFA, monounsaturated fatty acid; OO, olive oil; PA, palmitic acid; RO, rapeseed oil; SAF, safflower oil; SO, sunflower oil; SOLO, sunflower oil & linseed oil.
Somers & Erickson, 1994) or had no effect on cytokine production (Hubbard et al, 1994, Yaqoob & Calder, 1995bc). This may depend not only on cytokine under investigation and the variables outlined in Section 1.3.2, but also the state of activation of the cell, particularly if macrophages were used as the cytokine source. Macrophages from animal studies are generally derived from the liver, lung and peritoneal cavity and can be in a resident, inflammatory or activated state. Wallace et al, (1999) reported dietary fat to modulate murine peritoneal macrophage cytokine production. However these effects were influenced by the activation state of the macrophage (resident or inflammatory). Inflammatory macrophages from FO fed mice exhibited significantly decreased production of TNFα relative to low-fat, CO and SAF diets. On the other hand, resident macrophages from FO fed mice had increased TNFα production compared to CO fed mice. Clearly, the activation state of a cell will dictate the cytokine profile it secretes (Calder, 1996).

Most studies tended to focus on the macrophage-derived pro-inflammatory cytokines (e.g.IL-1β, TNFα), rather than the lymphokines IL-2, IL-4 etc. Indeed it appears that dietary fatty acids affect pro-inflammatory cytokines and Th-1 derived lymphokines rather than the Th-2 derived cytokines including IL-4, IL-10 (Yaqoob & Calder, 1995, Wallace et al, 2001). These effects appear to be mediated at the mRNA level. Recently Lo et al. (1999a) reported that the murine macrophage cell line RAW264.7, grown in an EPA-rich media had decreased TNFα mRNA expression and TNFα production. In an animal feeding study, Wallace et al, (2001) fed C57B16 mice for 6 wks with a low-fat diet (50 g/kg diet) or on high-fat diets containing CO, SO or FO (225g/kg diet). After the feeding-period, IL-2, IFNγ and IL-4 mRNA were lowest in the FO-fed animals (Wallace et al, 2001). A FO diet has also been reported to decrease splenic IL-1β mRNA relative to a beef tallow diet in female BALB/c mice (Robinson et al, 1996). This reduction was due to more rapid shutting down of gene transcription rather than changes in the initial rate of gene transcription (Robinson et al, 1996). Diabetes prone BB mice fed a FO rich diet demonstrated a shift in cytokine production from a Th1 type response to a Th2 type response (IFNγ/IL-10) by a factor of 15 when compared at mRNA level (Kleeman et al, 1998). However, decreases in IL-1β, IL-6 and TNFα mRNA (Renier et al, 1993, Chandrasekar & Fernandes, 1994) but increases in splenic IL-2, IL-4 and TGF-β mRNA have all been reported following FO supplementation (Fernandes et al, 1994). Clearly fatty acids affect cytokine production at a transcriptional level in animals, the precise effects of which merit further investigation. Table 1.8 provides a review of the in vitro and animal studies investigating the effects of fatty acids on cytokine production.
The effect of dietary supplementation with fatty acids on the production of human cytokines has been investigated both in healthy populations and in individuals suffering from immunological related disorders. Supplementation with the n-6 PUFA AA at 1.5 g/d for 50 days did not have any effect on the ex vivo production of IL-1, IL-2 and TNFα (Kelley et al. 1997, 1998). GLA (770 mg/d), ALA (2 g/d), AA (680 mg/d), DHA (720 mg/d) or EPA & DHA (720 mg EPA & 280 mg DHA) for 12 wk did not affect IL-2 or IFN-γ production in healthy 55-75 y volunteers (Thies et al. 2001). However, most studies have focused on the immuno-modulatory effects of n-3 PUFA. A 25-75% decrease in ex vivo secretion of the monokines IL-1β and TNFα has been reported when EPA and DHA are supplemented in the diets of young adults at a level of 2.4 g/d or greater for a minimum of 4 weeks (Endres et al. 1989, Caughey et al. 1996, Kelley et al. 1998, 1999). Indeed as little as 1.2 g/d was found to reduce cytokine production in elderly volunteers after 24 weeks (Meydani et al. 1993). N-3 PUFA have also been reported to reduce IL-2 and IFN-γ production (Meydani et al. 1991, Virella et al. 1991, Gallai et al. 1995). Such decreases in cytokine production have also been found in patients with diseases such as multiple sclerosis (Gallai et al. 1995), psoriasis (Allen et al. 1985) and advanced colorectal cancer (Purasiri et al. 1995).

However, the results of these studies is dependent on the intake (and level) of vitamin E during the study period; a reduction in cytokines has been observed in studies that provided less than 10 mg/d of vitamin E level. Higher doses of vitamin E supplementation (200 mg/d for 8 weeks) reversed the effects of FO supplementation (15 g/d for 10 weeks) (Kramer et al. 1991). Similarly Yaqoob et al. (2000), found no effect of fatty acid supplementation (9 g/d CO: soybean oil (SBO) mix, OO, EPA, SAF or FO for 12 wks) on the production of a range of cytokines (IL-1α, IL-1β, IL-2, IL-10, TNFα and IFN-γ). A relatively high dose of vitamin E (205 mg/d) was given to all volunteers throughout the study period.

Finally, most studies have investigated the effects of short-term fatty acid supplementation on cytokine production. Blok et al. (1997) supplemented a cohort of monks with various doses of FOs for a year. They found no effect of long-term supplementation of FO on ex vivo cytokine production. (Blok et al. 1997). A more comprehensive list of studies investigating the effects of dietary fatty acids on human cytokine production is outlined in Table 1.9.
Table 1.8. The effects of fatty acids on cytokine production from a sample of human and animal *in vitro* and animal *ex vivo* studies.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Fatty acid</th>
<th>Stimulus</th>
<th>Cytokine</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In Vitro:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human blood</td>
<td>EPA, ALA, DHA</td>
<td>PHA</td>
<td>↓ IL-2</td>
<td>Das, 1994</td>
</tr>
<tr>
<td>Human blood</td>
<td>EPA, DHA, ALA</td>
<td>PHA</td>
<td>↓ IL-2</td>
<td>Devi &amp; Das, 1994</td>
</tr>
<tr>
<td>Human LAK</td>
<td>DHA, EPA, GLA</td>
<td>PHA, PWM, Con-A</td>
<td>↓ IL-1β, DHA↓ IFN-γ</td>
<td>Purasiri <em>et al</em>, 1997</td>
</tr>
<tr>
<td>Human PBL</td>
<td>ALA, EPA, DHA, AA,</td>
<td>Con A</td>
<td>↓ IL-2</td>
<td>Calder &amp; Newsholme, 1992a</td>
</tr>
<tr>
<td></td>
<td>SA, OA, PA, MA</td>
<td></td>
<td></td>
<td>Santoli &amp; Zurier, 1989</td>
</tr>
<tr>
<td>Human blood</td>
<td>DHGLA, EPA, AA, OA,</td>
<td>PHA</td>
<td>↓ IL-2, DHGLA,</td>
<td>Baldie <em>et al</em>, 1993</td>
</tr>
<tr>
<td></td>
<td>SA, PA</td>
<td></td>
<td>EPA &amp; AA only</td>
<td></td>
</tr>
<tr>
<td>U937 cells</td>
<td>AA, DHGLA, EPA, OA,</td>
<td>LPS</td>
<td>↑ IL-1: AA&gt;EP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LA, PA</td>
<td></td>
<td>AA&gt;DHG, LA&gt;LA&gt;</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>OA.</td>
<td></td>
</tr>
<tr>
<td>Rat lymph node</td>
<td>Phosphatidylycholine</td>
<td>Con A</td>
<td>↓ IL-2 &amp; IFN-γ</td>
<td>Nishiyama <em>et al</em>, 2000</td>
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<tr>
<td></td>
<td>containing AA</td>
<td></td>
<td>NSD IL-4 &amp; IL-10</td>
<td></td>
</tr>
<tr>
<td><strong>Ex Vivo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse spleen</td>
<td>30 g/kg SO v 20 g/kg</td>
<td>Con A</td>
<td>↓ IL-2</td>
<td>Jolly <em>et al</em>, 1997</td>
</tr>
<tr>
<td></td>
<td>SO &amp; 10 g/kg AA or</td>
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<tr>
<td></td>
<td>EPA or DHA</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Autoimmune prone mice</td>
<td>HF (200g/kg diet) v</td>
<td>Con A</td>
<td>↑ IL-6 &amp; TNFα</td>
<td>Fong Lin <em>et al</em>, 1996</td>
</tr>
<tr>
<td>Mouse spleen</td>
<td>LF (50g/kg diet)</td>
<td></td>
<td>NF grp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(equal amts lard &amp; SBO)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mouse spleen</td>
<td>10% wt fat – high &amp;</td>
<td>LPS</td>
<td>↑ TNFα HF n-3</td>
<td>Hardardottir &amp; Kinsella, 1991</td>
</tr>
<tr>
<td>Mouse spleen</td>
<td>low n-6 &amp; n-3.</td>
<td></td>
<td>diet</td>
<td></td>
</tr>
<tr>
<td>Mouse spleen</td>
<td>LF (2% wt) or HF</td>
<td>Con A</td>
<td>↑ IL-2 v LF/ HCO,</td>
<td>Yaqoob &amp; Calder, 1995</td>
</tr>
<tr>
<td></td>
<td>(20% wt) HCO, OO, SO,</td>
<td></td>
<td>NSD IL-10, IL-4,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MO, 8 wks</td>
<td></td>
<td>IFN-γ</td>
<td></td>
</tr>
<tr>
<td>Rat spleen</td>
<td>100g LO v FO v CO v</td>
<td>LPS</td>
<td>↓ TNFα</td>
<td>Tappia &amp; Grimble, 1994</td>
</tr>
<tr>
<td>Monkey blood</td>
<td>1.3% or 3.3% en EPA &amp;</td>
<td>Con A</td>
<td>↓ IL-1 &amp; IL-6</td>
<td>Wu <em>et al</em>, 1996</td>
</tr>
<tr>
<td></td>
<td>DHA or 3.5% or 5.3%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PHA</td>
<td></td>
<td>↑ IL-2 EPA &amp; DHA only</td>
<td></td>
</tr>
</tbody>
</table>

AA, Arachidonic acid; ALA, α-linolenic acid; CO, coconut oil; DHA, docosahexaenoic acid; DHGLA, dihomogammalinoleic acid; EPA, eicosapentaenoic acid; FO, FO; GLA, γ-linolenic acid; HOSO, high oleic sunflower oil; LA, linoleic acid; L.F, Low-fat; LO, linseed oil; MA, myristic acid; MUFA, monounsaturated fatty acid; OO, olive oil; PA, palmitic acid; RO, rapeseed oil; SAF, safflower oil; SO, sunflower oil; SOLO, sunflower oil & linseed oil.
Table 1.9. The effects of fatty acids on human *ex vivo* cytokine production.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Fatty acid</th>
<th>Stimulus</th>
<th>Cytokine</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human blood</td>
<td>2.4 g/d n-3 PUFA, 12 wks</td>
<td>Con A,</td>
<td>↓ IL-1β &amp; TNFα</td>
<td>Meydani <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>Human blood</td>
<td>8 g/d FO, 6 wks</td>
<td>Anti-CD3</td>
<td>↓ IL-2</td>
<td>Virella <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>Human blood</td>
<td>2.4 g/d FO, 3 mths, old &amp; young women</td>
<td>Con A, PHA</td>
<td>↓ IL-2, IL-1β, IL-6 &amp; TNFα</td>
<td>Meydani <em>et al.</em>, 1993</td>
</tr>
<tr>
<td>Human blood</td>
<td>18 g/d FO, 6 wks</td>
<td>LPS</td>
<td>↓ IL-1β &amp; TNFα</td>
<td>Endres <em>et al.</em>, 1989</td>
</tr>
<tr>
<td>Human blood</td>
<td>FO 2.4 g/d, 3 mths young healthy women (YW) and old healthy women (OW)</td>
<td>LPS, PHA</td>
<td>↓ IL-1β, IL-6 YW &amp; OW</td>
<td>Endres <em>et al.</em>, 1993</td>
</tr>
<tr>
<td>Human blood</td>
<td>FAS oil 4 wk &amp; then FO 4 wk</td>
<td>LPS</td>
<td>↓ IL-2 OW</td>
<td>Caughey <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>Human blood</td>
<td>9 g/d CO: SBO (3:1) or OO, SAF, EPO or FO, 12 wk &amp; 205 mg/d α-toc</td>
<td>Con A</td>
<td>NSD IL-1α, β, IL-2, IL-10, TNFα, IFN-γ</td>
<td>Yaqoob <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>Human blood</td>
<td>4 g/d PO &amp; SO (placebo) or ALNA, GLA, AA, DHA, FO</td>
<td>LPS</td>
<td>NSD IL-2 &amp; IFN-γ</td>
<td>Thies <em>et al.</em>, 2001</td>
</tr>
<tr>
<td>Human blood</td>
<td>6 g/d DHA &amp; 20 mg α-toc, 13 wks</td>
<td>LPS</td>
<td>↓ TNFα &amp; IL-1 β</td>
<td>Kelley <em>et al.</em>, 1999</td>
</tr>
<tr>
<td>Human blood</td>
<td>LF diet &amp; 1.5 g/d AA, 7 wks</td>
<td>LPS &amp; PHA</td>
<td>NSD IL-1, IL-2, TNFα</td>
<td>Kelley <em>et al.</em>, 1997 &amp; 98</td>
</tr>
<tr>
<td>Human blood</td>
<td>ALA rich diet (6.3% en) v ALA poor diet (0.3% en) 66 mg α-toc / wk, 18 wks</td>
<td>PHA</td>
<td>NSD IL-2</td>
<td>Kelley <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>Human blood</td>
<td>4 g/d n-3 Ethyl esters 18 wks (85% EPA &amp; DHA)</td>
<td>LPS</td>
<td>↓</td>
<td>Abbate <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>Human blood</td>
<td>LF diet &amp; 1.5 g/d ALA, 6 wks</td>
<td>PHA</td>
<td>NSD IL-2 &amp; IL-2R, ALA ↓ IL-1β &amp; TNFα</td>
<td>Kelley <em>et al.</em>, 1991</td>
</tr>
</tbody>
</table>

A, Arachidonic acid; ALA, α-linolenic acid; CO, coconut oil; DHA, docosahexaenoic acid; DHGLA, dihomogammalinoleic acid; EPA, eicosapentaenoic acid; FO, FO; GLA, γ-linolenic acid; HOSO, high oleic sunflower oil; LA, linoleic acid; LF, Low-fat; LO, linseed oil; MA, myristic acid; MUFA, monounsaturated fatty acid; OO, olive oil; OW, old women; PA, palmitic acid; PO, palm oil; RO, rapeseed oil; SAF, safflower oil; SO, sunflower oil; SOLO, sunflower oil & linseed oil; YW, young women.
1.3.4 Fatty acids, surface molecule and adhesion molecule expression.

Cluster of definition (CD) molecules comprise the group of molecules that provide an integral link between adhesion and cellular activation – they include cytokine receptors, co-stimulatory molecules (e.g. CD28, MHC (HLA) complexes) and classical cellular adhesion molecules. These CD molecules (including CAM) operate by a ligand receptor mechanism – e.g. the integrin LFA-1 (CD11a/CD18) associates with ICAM-1 (CD54), LFA-1: CD102, CD2:CD58 and CD49d/CD29 (VLA-4): CD106 (VCAM-1).

Dietary fatty acids have also been reported to modulate the expression of these surface molecules.

Several studies have investigated the effects of fatty acids on cytokine receptor expression e.g. IL-2R, IFN-γ. Calder & Newsholme (1992a) and Yaqoob et al. (1994) reported no effect of incubating male rat lymph nodes with various fatty acids (all at a concentration of 100µM) or feeding rats a high-fat diet (20% weight) of either OO, SO, EPO, hydrogenated coconut oil (HCO) or mendehen oil (MO) for 10 wks on IL-2R expression. However, unsaturated fatty acids decreased transferrin receptor expression in Con A-stimulated rat lymphocytes (Calder & Newsholme, 1992a). Fatty acids also appear to affect the expression of MHC complexes (or species equivalent). Khair -El-Din et al, (1995) investigated the effects of fatty acids on la-bearing macrophages, the murine equivalent of human MHC class II molecules. DHA added in vitro reduced the expression of IFN-γ-induced la expression to a greater extent than EPA and AA. This inhibition occurred at the mRNA level and was eicosanoid independent, i.e. was not inhibited by indomethacin. Jenski et al, (1995) investigated whether DHA, CO and HCO affected the expression of the murine surface proteins Thy-1.2 and CD8. To study the in vivo / ex vivo effects of fatty acids, mice were fed FO at 10% w/w of each of the fatty acids or a mixture of all three fatty acids for 3 weeks, splenocytes were fused with DHA-containing phosphatidylcholine vesicles and splenocytes were cultured with DHA. As DHA incorporation into the lymphocytes increased there was a decrease in the expression of one Thy-1.2 epitope and one CD8 epitope while the expression of two CD8 epitopes increased. The decrease in Thy-1.2 was maintained for more than a week after removal of n-3 fatty acids from the diet. Sherrington et al, (1995) demonstrated a decreased expression of MHC II and CD18 on macrophages from rats fed 200g/kg diet MO compared to HCO and OO fed diets.

Animal studies also reveal an effect of fatty acids on other surface molecules (e.g. CAM and co-stimulatory molecules) and to directly affect the adhesion of fatty acid treated immune cells to activated endothelial cells. Feeding male Lewis rats either a low-fat diet
(25 g/kg diet) or one of five high-fat diets (200g/kg of either HCO, OO, SO, EPO or FO) for 8 weeks. The FO reduced the level of expression of CD2, CD11a, CD18 and CD44 on the surface of freshly isolated lymphocytes and CD2, CD11a, CD18, ICAM-1 and L-selectin on the surface of Con- A stimulated lymphocytes from FO fed mice only (Sanderson & Calder, 1998a). The OO diet reduced expression of CD11a, CD2 and CD18 on fresh lymphocytes only. As part of the same study, the high-fat diets diminished the ability of Con-A stimulated lymphocytes to adhere to untreated endothelial cells, this decrease in adherence being even more pronounced in TNFα stimulated endothelial cells (Sanderson & Calder, 1998a). Miles et al., (2000) fed mice a low fat (25g/kg corn oil) or a high-fat (200g/kg) diet of either CO, SO or FO. FO significantly decreased the expression of ICAM-1 and macrophage scavenger receptor-A at both the level of mRNA and cell surface expression. More recently Arrington et al., (2001) reported a decrease in IL-2 secretion following FO supplementation in mice that was CD28 dependent. Conversely Barbeau et al., (1995) fed rats an atherogenic diet (4% cholesterol, 1% cholic acid, 0.5% thiouracil) for two weeks with a commercial FO (MaxEPA, 5% w/w) or antioxidant (Probucol, 1% w/w). FO had no significant effect on mononuclear cell adhesion to the aortic endothelium.

Scientific attention has also focused on the effects of fatty acids on human CAM expression and endothelial adhesion. In one of the first studies in this area, Khalifou et al., (1996a) reported EPA, DHA and AA (all at 100μM) g/mL had no effect on PHA-induced lymphocyte expression of CD25, CD71 and HLA-DR. Likewise, Kelley et al., (1998) showed no effect of supplementing healthy humans with arachidonic acid at 1.5g/d for 49 d on IL-2R expression. Hughes et al., (1996a) reported a decrease in human monocytic expression of HLA-DR and ICAM-1 following in vitro incubation with EPA (66 μmol/L), whereas DHA (61 μmol/L) resulted in a greater intensity of expression of HLA-DR and HLA-DP. If these monocytes were stimulated with IFN-γ both EPA and DHA decreased the intensity of expression of HLA-DR, -DP and ICAM-1. Following on from these in vitro studies Hughes et al., (1996b) supplemented six healthy volunteers with 3/g day FO mix (1.5: 1 mix EPA: DHA) for 21 d. The FO mix decreased the intensity of expression of monocytic HLA-DP, -DR –DQ, ICAM-1 and LFA-1 (with and without IFN-γ. HLA-DQ being affected in un-stimulated monocytes only) (Hughes et al., 1996b). Subsequently, Hughes & Pinder (2000) reported a decrease in the ability of activated monocytes to present antigen to autologous lymphocytes, following culture of healthy human monocytes with an EPA/DHA 3:2 mix for 48 h. This 3: 2 EPA: DHA mix (reflective of EPA: DHA intakes from commercial FO supplements) reduced the expression and intensity of ICAM-
1 positive and LFA-3 positive unstimulated monocytes but not MHC class II molecules. However if the monocytes were stimulated with IFN-γ, there was a significant decrease in the % monocytic expression of HLA-DR, -DP and ICAM-1 and in the intensity of HLA-DR, -DP, ICAM-1 and LFA-3. Yaqoob et al. (2000) supplemented healthy volunteers with diets consisting of either a 3:1 mix of CO: SBO (placebo), OO, SO, EPO, FO (2.1 g EPA: 1.1g DHA) plus 205 mg α-tocopherol per day for 12 weeks. She found no effect of supplementation on PBMC CD2, CD11b and CD54 expression. Clearly the effects of fatty acids on surface molecule expression appear to be affected by the state of the cell (quiescent v activated), cell type (monocytes appear more affected than PBL) and the inclusion or exclusion of vitamin E in study design.

A large proportion of research has focused on the effects of fatty acid on adhesion molecule expression in vitro using endothelial cell (EC) lines such as human umbilical venous endothelial cells (HUVEC's) or human saphoneous venous endothelial cells (HSVEC's). HUVECs incubated with DHA or EPA (<100μg/ml), prior to exposure to PBL, demonstrated decreased adhesion of the PBL to the unstimulated and stimulated (i.e. TNFα-, IL-4- and LPS-treated) HUVECs (Khalfoun, 1996b). Equally, decreased adhesion was observed if the PBL were pre-incubated with the fatty acids, washed and then allowed adhere to the EC. Khalfoun and co-workers concluded that this was due to the incorporation of fatty acids into the plasma membrane. Finally the n-3 PUFA decreased the EC-induced expression of VCAM-1 but not ICAM-1 or E-selectin and moderately reduced the expression of L-selectin and LFA-1 but not VLA-4. AA did not affect any of the aforementioned experiments (Khalfoun et al. 1996b).

DeCaterina & Libby (1996) reported decreased expression of VCAM-1 following incubation of EC with EPA, DHA and OA; this DHA-induced decrease in VCAM-1 was also observed at mRNA level. DHA dose- and time-dependently decreased the expression of E-selectin and ICAM-1 in response to various stimuli (IL-1, IL-4, TNFα and LPS) and reduced the adhesion of human monocytes and monocytic U937 cells to cytokine-stimulated EC. LA and AA did not affect any of the above parameters (DeCaterina & Libby 1996). DeCaterina et al. (1998) concluded that this inhibition was due to an increase in the number of double bonds of a fatty acid per se rather than carbon chain length, double bond position (i.e. cis/trans configuration), configuration (double bond position relative to the methyl end) or accessory substitution (e.g. inclusion of hydroxy groups). Likewise OO, the fatty acid implicated in conferring the anti-atherogenic benefits attributed to a Mediterranean diet, appears to modulate endothelial activation. OO decreased HUVEC VCAM-1 mRNA and cell surface expression following a 72 h
incubation period. Adhesion of monocytic U937 cells to OO treated and LPS-stimulated EC was reduced concomitantly (Carluccio et al, 1999). Finally, fatty acid derivatives also appear to affect CAM expression and cellular adhesion. 15-HPETE and 15-HETE were more effective in decreasing TNFα-induced neutrophil and monocyte adhesion to HUVEC’s than AA. These AA-derivatives also decreased TNFα-induced ICAM-1, VCAM-1 and E-selectin mRNA and surface expression in HUVEC’s (Huang et al., 1997).

There is less information with respect to the effects of fatty acids on sCAM expression. Seljeflot et al., (1998) reported increased levels of sE-Selectin and sVCAM-1 following FO supplementation (4.8g daily for 8 wks) in male smokers with hyperlipidaemia. Supplementation did not affect sP-selectin expression (Seljeflot et al., 1998). Abe et al., (1998) supplemented healthy controls and hypertriglyceridemic patients with 4 g/d of highly purified ethyl esters of n-3 fatty acids (Omacor). Short-term supplementation (6 weeks) with Omacor increased sE-selectin, while long-term supplementation (7 months) reduced sICAM-1 (9%) and sE-selectin (16%) levels. Omacor had no effect on sVCAM-1 expression. A subgroup of the study population was diabetic and a greater reduction in sICAM-1 (27%) and sE-selectin (32%) were reported in this diabetic group. Johansen and colleagues (1999) supplemented subjects from a larger trial (Coronary Angioplasty Restenosis Trail, CART) who had advanced and angioplasty-treated CHD, with either three FO or placebo (corn oil) capsules per day for 6 months. Each FO capsule contained 0.45 g EPA, 0.39 g DHA and 4 mg vitamin E. After the six months period, both groups were further supplemented with FO for another 4 wks. Johansen et al., (1999) found that the serum levels of sVCAM-1 and sE-selectin, but not sP-selectin were higher in the FO supplemented patients. This sCAM increase was reflected by a decrease in serum vitamin E levels and an increase in indicators of oxidative stress (thiobarbituric acid reactive substances, TBARS). Finally, Miles et al., (2001) supplemented two groups of healthy subjects (<40 y and >55 y respectively) with FO (1.2 g/d EPA & DHA) in a randomised placebo controlled trial for twelve weeks. The younger group (all males) had a significantly lower plasma concentration of sICAM-1 and sVCAM-1 than the older group as expected. FO supplementation did not affect plasma sICAM-1 levels in either group. sE-selectin concentrations were significantly increased in young males while both sE-selectin and sVCAM-1 concentrations decreased in the elderly subjects (Miles et al., 2001).

Dietary fatty acids, therefore appear to have differential effects on sCAM expression, depending on age, length of supplementation, presence or absence of hyperlipidaemia and also the CAM under investigation. Further investigations are needed
into the effects of other fatty acids on immune cell surface markers/ receptors and cell adhesion molecules.

1.3.5 Fatty acids and eicosanoids.

The influence of fatty acids on eicosanoid production has focused on the effects of n-3 PUFA. As mentioned in Section 1.1.6, n-3 PUFA, particularly EPA competitively inhibit the metabolism of AA for eicosanoid synthesis and generate a distinct set of eicosanoids of lesser biological activity (Lee et al., 1984, Calder et al., 2002). This has been proven in many in vitro, animal and human studies investigating the effects of FOs on eicosanoid production. Central to all of the following studies is the increase in the amount of EPA and DHA in immune cell content post-supplementation. This increase in EPA / DHA content is at the expense of AA (Lee et al., 1985, Endres et al., 1989, Sperling et al., 1993, Caughey et al., 1996). Table 1.10 shows a representative sample of studies investigating the effects of fatty acids on animal and human eicosanoid production. Generally, a decrease in PGE$_2$ and LTB$_4$ production was observed following n-3 PUFA supplementation with approximately 3-4 g EPA daily for at least 4 weeks (Sperling, 1998). In an in vitro study Urquhart et al. (2001) incubated both resting and stimulated HSEC's with EPA or DHA (50µM). The authors reported a decrease in basal and stimulated prostanoid production (PGF$_2$α, PGI$_2$, PGE$_2$, PGF$_2$ & TXB$_2$) following incubation with EPA. DHA however resulted in a decrease in PGF$_{2α}$ in resting cells, but an increase in the production of all other prostanoids upon HSEC stimulation.

FO supplementation also decreased neutrophil chemotactic responsiveness to LTB$_4$ and the chemotactic peptide N-formly-methionyl-leucyl-phenylalanine (FMLP), in vitro (Hoover et al., 1984) and in vivo (Lee et al., 1985, Terano et al., 1987). N-3 PUFA are thought to inhibit the eicosanoid signal transduction cascade between the receptor and phospholipase C. This conclusion is based on the observation that FO decreased chemotaxin-stimulated neutrophil inositol monophosphate, diphosphate and IP$_3$ formation, without affecting the number or affinity of the respective chemotaxin receptors (Sperling et al., 1993, 1998). The formation of IP$_3$ correlated strongly and negatively with EPA content of the phosphoinositol pools however, dietary n-3 PUFA did not affect neutrophil diacylglycerol formation (Sperling et al., 1993). In a recent study, Lo et al. (1999b) incubated RAW 264.7 macrophages with EPA (114µM) for 24 h. They reported an increase in basal PGE$_2$ levels but a decrease in PGE$_2$ production in LPS-stimulated macrophages. More interestingly, there was no effect of EPA on COX-1 mRNA expression, but an increase in COX-2 mRNA and protein expression. Exogenous PGE$_2$
Table 1.10. The effects of fatty acids on eicosanoid secretion (animal and human studies).

<table>
<thead>
<tr>
<th>Study group</th>
<th>Diet (fatty acid studied)</th>
<th>Eicosanoid affected</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Animal studies:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wistar rat lymph notes</td>
<td><em>In vitro</em> incubation with 100μM MA, PA, SAF, OA, LA, LnA, AA, EPO or DHA for 6-10 wks</td>
<td>PGE₂ ↓ the most by SA, ↑ the most by AA</td>
<td>Calder <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>BALB/c mice</td>
<td>10% wt CO or ALA (linseed oil diet) for 6-10 wks</td>
<td>ALA ↓ splenocyte &amp; PEC &amp; ↓ PEC LTC</td>
<td>Fritsche <em>et al.</em>, 1992</td>
</tr>
<tr>
<td>SD rats</td>
<td>FO (17% by wt &amp; 3% CO) or CO (20% by wt) + 30, 300, 900 mg/kg α-toc for 8/9 wks</td>
<td>FO diet ↓ PM &amp; splenocyte PGE₂ by 70-80% v CO diet</td>
<td>Fritsche <em>et al.</em>, 1992</td>
</tr>
<tr>
<td>Monkey PBMC</td>
<td>1.3% or 3.3% en EPA &amp; DHA or 3.5% or 5.3% en ALA for 14 wks following 14 wk baseline period</td>
<td>Both EPA &amp; DHA diets ↓ Con-A &amp; PHA induced PGE₂ only 5.3% ALA ↓ PGE₂</td>
<td>Wu <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>Autoimmune prone mouse splenic &amp; PEC</td>
<td>Both ALA, GLA, AA, EPA or DHA/100g total fatty acid) n-6:n-3 = 7:1, 35g PUFA/100g FA</td>
<td>HF diet ↑ PGE₂ v LF diet</td>
<td>Lin <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>Weanling rats</td>
<td>HF diet (178g/kg diet with 4.4 g ALA, GLA, AA, EPA or DHA/100g total fatty acid)</td>
<td>EPA &amp; DHA ↓ PGE₂</td>
<td>Peterson <em>et al.</em>, 1998</td>
</tr>
<tr>
<td>Murine resident &amp; inflamm mφ</td>
<td>LF or HF (CO, OO, SO or FO) for 6 wks</td>
<td>FO &amp; LF diet ↓ LTB₄ in resident mφ</td>
<td>Wallace <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>SD rat PEC</td>
<td>EPA or DHA at 2% f or 3 wks</td>
<td>Both ↓ LTB₄ &amp; ↑ LTB₅, EPA more effective than DHA</td>
<td>Hung <em>et al.</em>, 2000</td>
</tr>
<tr>
<td><strong>Human studies:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human neutrophils</td>
<td>Dietary supplementation GLA rich borage oil</td>
<td>↓ LTB₄ in Ca-ionophore stimulated neutrophils</td>
<td>Ziboh &amp; Fletcher, 1992</td>
</tr>
<tr>
<td>Human neutrophils &amp; monocytes</td>
<td>20g Super-EPA/d (9g EPA &amp; 5g DHA) for 10 wks, n=8</td>
<td>No effect LTB₄ despite ↑ EPA in all PL subclasses</td>
<td>Sperling <em>et al.</em>, 1993</td>
</tr>
<tr>
<td>Whole blood</td>
<td>30ml/d FO (18% EPA &amp; 12% DHA) + vit E at 1.5 IU/g or 4.5 IU/g, 4wks</td>
<td>Both diets↓ TXB₂ &amp; LTB₄, No benefit extra vit E</td>
<td>Engstrom <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>Human PBMC</td>
<td>Basal diet 15d, then 1.5 g AA 7 wks.</td>
<td>AA ↑ LPS stimulated LTB₄ &amp; PGE₂</td>
<td>Kelley <em>et al.</em>, 1998</td>
</tr>
<tr>
<td>Human PBMC</td>
<td>Basal diet 30d, then 6g DHA /d for 13 wks, 20mg α-toc/day</td>
<td>↓ LPS stimulated PGE₂ &amp; LTB₄ by 60-75%</td>
<td>Kelley <em>et al.</em>, 1999</td>
</tr>
<tr>
<td>Human neutrophils &amp; monocytes</td>
<td>3.2g EPA &amp; 2.2g DHA for 6 wks</td>
<td>↓ 5-HETE &amp; 6-trans-LTB₅ generation &amp; ↓ LTB₃ small quantities 5-HEPE &amp; LTB₃ generated</td>
<td>Lee <em>et al.</em>, 1985</td>
</tr>
<tr>
<td>Human blood</td>
<td>12 RA patients, 20 g MaxEPA /d for 6 wks.</td>
<td>↓ LTB₄ &amp; small quantity LTB₅ observed</td>
<td>Sperling <em>et al.</em>, 1987</td>
</tr>
</tbody>
</table>

α-toc, α-tocopherol; AA, Arachidonic acid; ALA, α-linolenic acid; CO, coconut oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, FO; GLA, γ-linolenic acid; 5-HETE, 5-S-hydroxy-trans-8,11,14-cis-eicosatetraenoic acid; 5-HEPE, 5-S-hydroxy-trans-8,11,14-cis-eicosapentaenoic acid; HF, high-fat; LA, linoleic acid; LF, Low-fat; LPS, lipopolysaccharide; LTB₄, leukotriene B₄; LTB₅, leukotriene B₅; MA, myristic acid; OO, olive oil; PA, palmitic acid; PBMC, peripheral blood mononuclear cells; PEC, peritoneal exudates cells; PL, phospholipid; PM, peritoneal macrophage; RA, rheumatoid arthritis; SAF, safflower oil; SD, Sprague dawley (rats); SO, sunflower oil; TXA₂, thromboxane A₂.
diminished this increase in COX-2 mRNA (Lo et al., 1999b). Two possible modes of action were proposed. Firstly, that EPA by replacing AA in the macrophage membrane lipids made more AA available initially for PGE\textsubscript{2} synthesis. Secondly, a reduction in PGE\textsubscript{2} production induces COX-2 mRNA and COX-2 activity through a COX-2 product-dependent feedback mechanism (Lo et al., 1999b).

It is important to note that in all of the above studies EPA was more active than DHA. This may be due to the stronger hydrolytic affinity of PLA\textsubscript{2} for EPA-containing phospholipids than DHA-containing phospholipids (Sperling, 1998). Similarly, DHA is oxidatively metabolised by 5-LOX to a much lesser extent than EPA or even AA (Sperling, 1998). A small number of studies have investigated the effects of GLA acid and LTB\textsubscript{4}; GLA was found to reduce LTB\textsubscript{4} generation in calcium ionophore activated neutrophils (Ziboh & Fletcher, 1992), monocytes (Pullman-Mooar et al., 1990) and lymphocytes (Rossetti et al., 1997). However, there is a paucity of information with respect to the effects of n-3, n-6 and n-9 fatty acids, other than EPA, DHA and GLA on eicosanoid production.

Rheumatoid arthritis patients represent a patient cohort with biologically significant concentrations of LTB\textsubscript{4} in the synovial fluid. Supplementation with n-3 PUFA replaced cellular AA content with EPA, decreased LTB\textsubscript{4} generation and improved depressed neutrophil chemotaxis to FMLP and LTB\textsubscript{4}. In addition a modest improvement in disease activity was reported. Hence, supplementation with n-3 PUFA may have clinical relevance in the management of this chronic inflammatory disease (Cleland et al., 1986, Kremer et al., 1985, Sperling, 1998).

1.3.6 Fatty acids and transcription machinery

In an attempt to elucidate how fatty acids interact with, and affect immunological responses, various studies have focused on fatty acid-induced changes in membrane fluidity, lipid peroxidation, eicosanoid production, calcium and PKC signalling, and transcription factor activity.

Fatty acids are thought to function by incorporating into the plasma membrane phospholipid bilayer and thereby altering intercellular interactions, receptor expression (orientation, number, and density), nutrient transport and signal transduction (Kelley, 2001). The lipid composition of the membrane bilayer is susceptible to changes in fluidity due to the degree of unsaturation of the fatty acyl chain, the ratio of phospholipids to cholesterol, and the interactions between phospholipids and proteins (Gurr, 1983). A higher degree of fluidity is ascribed to plasma membranes containing highly unsaturated
fatty acids and with a lower ratio of cholesterol. These variables will influence subsequent protein-phospholipid interactions (Gurr, 1983). Proteins embedded in the phospholipid bilayer (including protein kinases and phosphatases), are therefore particularly vulnerable to modifications in plasma membrane lipid composition (Gurr, 1983, Hwang, 2000). The fatty acid composition of cell membranes is modified when cells are incubated with fatty acids in vitro and in vivo (Gill & Clark, 1980, Gibney & Connolly, 1988, Calder et al, 1994). Animal supplementation studies have shown that changing dietary fatty acid composition alters the phospholipid and DAG molecular species of lymphocytes (Huang & Fritsche, 1992) and macrophages (Marignani & Sebalt, 1995). Indeed the fatty acid composition of cell membranes is altered by cellular activation (e.g. with a mitogen). Anel et al, (1990) stimulated human PBMC's with PHA-M and reported an increase in the phospholipid: cholesterol ratio, a decrease in the quantities of phospholipid LA and AA and an increase in the amounts of phospholipid OA, DPA & DHA relative to quiescent cells (Anel et al, 1990ab). Calder et al, (1994) reported similar alterations in phospholipid and neutral lipid fatty acid compositions following stimulation of rat lymph node lymphocytes with Con A. Lymphocyte stimulation also results in the release of free fatty acids; this release is reportedly decreased following FO feeding in rats (Sanderson et al, 2000).

One key biochemical reaction by which fatty acids may influence membrane proteins is acylation (Hwang & Rhee, 1999). Acylation involves the covalent attachment of long chain fatty acids to proteins and can influence protein translocation and function (Hwang & Rhee, 1999). Many proteins involved in transmitting extracellular signals are known to be acylated (Towler et al, 1988, Schmidt, 1989). Myristoylation and palmitoylation are two forms of acylation. Myristoylation occurs cotranslationally at the amino terminal glycine residues through covalent bond formation (Johnson et al, 1994). It increases the hydrophobicity of proteins and enhances membrane anchorage (Hwang & Rhee, 1999). GTP-binding proteins (α-subunit), PKA and Src family PTK are myristoylated (Towler et al, 1988, Schmidt 1989). However, myristoylation is cotranslational and irreversible (Hwang, 2000). It is therefore unlikely that dietary lipid modulation would affect protein myristoylation (Hwang & Rhee, 1999). Palmitoylation is post-translational and reversible (Peitzsch & McLaughlin, 1993). It involves the attachment of a palmitate molecule to cysteine residues via a labile thioester linkage (Peitzsch & McLaughlin, 1993). Palmitoylation is also important for enhancing protein membrane anchorage, stabilising protein-protein interactions and regulating mitochondrial enzyme activities (Berthiaume et al, 1994). Families of proteins which are palmitoylated
include transmembrane proteins, the Ras family proteins, Src family PTK's and the α-subunits of GTP-binding proteins (Schmidt, 1989). Although the enzymology of palmitoylation is still unclear (Hwang & Rhee, 1999), it is possible by the reversible nature of palmitoylation, and the types of proteins affected by this form of acylation, that the activities of these signal-transducing proteins could be targeted by dietary fatty acid modulation (Hwang & Rhee, 1999).

PUFA's are particularly susceptible to attack by free radicals and oxidation into lipid peroxides (Halliwell & Chirico, 1993). This oxidation results in the formation of a variety of products including aldehydes, ketones and cyclic peroxides (Esterbauer, 1993). Such reactions are thought to alter (modify) proteins and lipids, including those associated with cell membranes and lipoproteins (Halliwell & Chirico, 1993). Increasing PUFA intake without adequate antioxidant protection could cause such lipid peroxidation (Kelley, 2001). It is possible that some of the immuno-modulatory effects observed after dietary fatty acid intervention (in vitro and in vivo) may be due to such oxidant stress. Evidence of this arises from the lower number of immunologically significant effects reported in studies that included a high dose of vitamin E (or suitable antioxidant) in their study design (Wu et al. 1996, Wander et al. 1997, Yaqoob et al. 2000). In humans, levels of DNA adducts of malondialdehyde and of etheno-DNA adducts (a proposed biomarker of DNA damage in vivo) are increased following consumption of a 13% PUFA diet (Fang et al. 1996, Nair et al. 1997). Similarly, a 4 wk 15% PUFA diet resulted in increased endogenous and H2O2 induced DNA damage at vitamin E levels of 5-7 mg/d (Jenkinson et al. 1999). However, these changes were abolished by adding and extra 80 mg α-tocopherol / day (Jenkinson et al. 1999). Vitamin E has been reported to prevent DNA damage (Fablani et al. 2001) and to inhibit the activation of NF-κB and PKC (Azzi et al. 1993). However, products of lipid peroxidation can be measured and quantitated in vitro and ex vivo following dietary fatty acid supplementation. Wander & Du (2000) supplemented healthy postmenopausal women with either EPA (2.5 g/d), or DHA (1.8 g/d), and (0, 100, 200 or 400 mg/d) vitamin E for 5 wk. An increase in TBARS production was reported following fatty acid supplementation at low dose α-tocopherol. There was no change in oxidatively modified protein, as indicated by carbonyl content (Wander & Du, 2000). The authors concluded that on the basis of this study there is no basis for vitamin E supplementation after consumption of EPA and DHA (Wander & Du, 2000). Another indicator of oxidative stress is the production of nitric oxide (NO) and NO gene expression. Various studies have reported FOs to diminish (Somers et al. 1989, Boutard et al. 1994, Joe & Lokesh, 1994), to have no effect (Hubbard et al. 1994) or to increase
(Renier et al, 1993, Turek et al, 1994, Harris et al, 1997) macrophage NO production. DHA (100μM) added to murine thioglycollate-elicited peritoneal macrophages increased NO mRNA and NO production in response to LPS (or TNFα) plus IFN-γ (Khair-El-Din et al, 1996). Hence, lipid peroxidation and oxidative stress may play a role in fatty acid-induced immunomodulation. However they constitute only one part of a multifactorial process.

It is tempting to speculate that the immunomodulatory benefits of fatty acid are due to alterations in eicosanoid production. In particular PGE₂ is known to inhibit lymphocyte proliferation and therefore reduce the ability of cells to produce IL-2, transferrin receptor, ICAM and PGE₂ itself (Goodwin & Cueppens, 1983). However, a number of in vitro studies have concluded that the inhibitory effects of PUFA (particularly n-3 PUFA) are eicosanoid independent (Santoli et al, 1990, Calder et al, 1992c, Kumar et al, 1992, Soyland et al, 1993, Rotondo et al, 1994, Khalfoun et al, 1996a). In addition many in vivo studies have reported a decrease in PGE₂ production in paraller with a decrease in lymphocyte proliferation (Meydani et al, 1991, 1993). The authors concluded that the effect on lymphocyte proliferation was eicosanoid independent (Meydani et al, 1991, 1993).

Lymphocytes play a central role in any immune response. With respect to lymphocyte activation, an elevation in intracellular calcium concentration is fundamental for subsequent proliferation, cytokine production and cell-mediated cytolysis (Weiss & Littman). Unsaturated fatty acids (AA, OA, LA, ALA, DHA & EPA) in the absence of albumin were found to reversibly block calcium influx into Jurkat T-cells (Chow et al, 1990, Stulnig et al, 2000) and human PBL (Vassilopoulos et al, 1997, GLA and DHGLA only). These effects were reversed by the inclusion of albumin in culture systems, were independent of the CD3 receptor and PKC-dependent phosphorylation (Chow et al, 1990, Vassilopoulos et al, 1997). Hence, UFA’s were presumed to act directly on the receptor-operated calcium channels (Chow et al, 1990). Stulnig et al, (2000) reported the effects on calcium flux were mediated by cis-unsaturated fatty acids only; trans-unsaturated fatty acids were without effect. Lymphocyte IP₃ production was decreased followin in vitro incubation with GLA and DHGLA (Vassilopoulos et al, 1997), dietary FO and OO (Sanderson & Calder, 1998b). Dietary FO were also found to reduce agonist simulated DAG (Marignani & Sebaldt, 1995, Jolly et al, 1997), ceramide production (Joly et al, 1997), PLCγ₁ activation (Sanderson & Calder, 1998b) and PA generation (Bechoua et al, 1998) in lymphocytes. Fatty acids increased PLD and PA-phosphohydrolase activities in human PBMC’s in vitro (Bechoua et al, 1998).
It is possible that fatty acids could mediate their immuno-modulatory effects by altering protein kinase and phosphatase activities. Again, the majority of studies have focused on n-3 PUFA, where they have been found to decrease PKC activity (Seung Kim et al., 2001, Moore et al., 2001). Although free fatty acids have been reported to directly activate PKC (Khan et al., 1995), these effects are modulated by the presence / absence of phospholipids and a stimulant (e.g. PMA). In the absence of exogenous phospholipid, EPA & DHA were reported to increase (Speizer et al., 1991) or to have no effect (Holain & Nelson, 1992) on PKC activity. However, in the presence of phosphatidylserine, the authors reported a decrease in PKC catalytic activity (Speizer et al., 1991, Holain & Nelson, 1992). Similarly May et al., (1993) showed that OA, AA, EPA and DHA inhibit lymphocyte PKC activity in the presence of calcium, phospholipid and PMA. LA and ALA did not affect PKC activity in this way and none of the fatty acids affected PKA activity (May et al., 1993). OA has been reported to preferentially activate soluble and calcium-independent PKC’s over membrane-bound PKC (Khan et al., 1995) while DHA inhibited the PMA induced translocation of PKCa and ε from the cytosol to the plasma membrane (Denys et al., 2001a). DHA also significantly inhibited the anti-CD3 and PMA stimulated phosphorylation of the MAPK kinases ERK-1 & -2 (Denys et al., 2001ab), and at higher concentrations induced apoptosis in Jurkat cells via proteolysis of caspase-3 and activation of protein phosphatase -1 and -2B (Siddiqui et al., 2001). Finally AA has been reported to enhance the activity of PKC-α and -δ more so than EPA or DHA; there was no difference in the efficacy of fatty acids in activating PKCγ (Manani et al., 2001).

Fatty acids have been shown to affect transcription factor activity and DNA binding directly. The majority of studies have focused on endothelial and monocyte/macrophage cell lines. The saturate SA and the polyunsaturate LA (90μM) activated NF-κB and enhanced NF-κB dependent transcription in porcine endothelial cells. The monounsaturate OO was without effect (Hennig et al., 1996, 2000). This fatty acid-induced transcriptional activity was linked to an increase in intracellular calcium concentrations (Hennig et al., 1996), decreased glutathione levels (i.e. increased oxidative stress) and was blocked by adding vitamin E to culture systems (Hennig et al., 1996, 2000). Similarly Toborek et al., (2002) incubated HUVEC’s with various C18 fatty acids (60-180 μM, complexed to BSA). They reported LA to be the most potent activator of NF-κB and AP-1 transcriptional activity. LA also increased TNFα-, Monocyte chemoattractant protein-1-, VCAM-1- and ICAM-1 -mRNA and strongly induced phospholipid hydroperoxide glutathione peroxidase gene expression (Toborek et al., 2002). In this culture system, ALA stimulated only a moderate induction of NF-κB activity and
inflammatory gene mRNA expression, whereas OA had no effect (Toborek et al., 2002). However, the anti-inflammatory properties of OA were confirmed in a separate study whereby OA (at 50 and 100 µM) decreased NF-κB activity in HUVEC’s (Carluccio et al., 1999). Fatty acids also modulate transcription factor activity in monocytes/macrophages. AA, but not EPA (45µM), up-regulated NF-κB transcriptional activity in U937 monocytic cells and J774 macrophages (Camandola et al., 1996). Supershift assays indicated that this enhanced activity was due to an up-regulation of the NF-κB p50 and p65 subunits; the c-Rel subunit was unaffected (Camandola et al., 1996). PGE₂ enhanced, and COX and LOX inhibitors depressed, NF-κB activity in this cell system, indicating that AA metabolites may have contributed to the observed increases in NF-κB activity following AA treatment (Camandoula et al., 1996). In contrast, Lo and colleagues (1999a) reported that Raw 246.7 macrophages with EPA for 48 h and subsequently stimulated with bacterial endotoxin exhibited decreased NF-κB activity. Novak et al., (2003) subsequently demonstrated that the decrease in NF-κB activity observed following incubation of Raw 246.7 macrophages with an n-3 fatty acid emulsion was secondary to an inhibition of the phosphorylation of IκBα. However, further studies are needed to elucidate how fatty acids affect NF-κB and AP-1 transcriptional activity.

Another family of transcription factors that may be influenced by fatty acids are PPARs. Recently reviews by Jump et al., (2002) and Schoonjans et al., (1996) highlighted the effects of long chain fatty acids on PPAR activation and action. Fatty acids and their derivates have been identified as ligands for both PPAR-α and -γ (Devchand et al., 1996, Forman et al., 1997, Kliewer et al., 1997). In particular, EPA has been proposed as an endogenous ligand for PPARα and PPARγ (Jump et al., 2002). OA and EPA have been found to bind to PPARα with equal affinity in vitro (Xu et al., 1999), however this has yet to be confirmed in vivo (Ren et al., 1997). PPARs have been shown to mediate anti-inflammatory effects (Ricote et al., 1998, Devchand et al., 1996, Jiang et al., 1998, Clark et al., 2000, Cunard et al., 2001). Conversely, Chawla et al., (2001) reported PPARγ ligands to have anti-inflammatory effects that are independent of PPARγ. However all of the aforementioned studies have been preformed with pharmacological ligands rather than fatty acids. Thoennes et al., (2000) investigated the effects of a range of fatty acids on PPARγ transcriptional activity in MCF-7 and MDA-MB-231 breast cancer cells. The authors reported n-3 fatty acids (EPA, DHA, ALA) to inhibit PPARγ activation and subsequent cell proliferation to below control levels. EPA was the most effective fatty acid. N-6 PUFA, MUFA and SFA stimulated PPARγ transcriptional activity in this reporter assay. Unfortunately the authors did not compare the fatty acids at equimolar
concentrations; therefore it is difficult to draw any conclusions from this study (Thoennes et al., 2000). Further studies are required to elucidate the precise effects of fatty acids on PPAR activity in vitro and in vivo.

Less attention has focused on the early response genes (e.g. c-myc, c-fos). Sellmayer et al. (1996) reported AA to increase mRNA levels of the early response genes c-fos and Egr-1 in Swiss 3T3 fibroblasts. EPA and DHA were without effect and COX- and PKC-inhibitors abolished these fatty acid-induced effects (Sellmayer et al., 1996). In the Jurkat T-cell line, DHGLA prevented the fall in c-myc and the rise in c-fos steady state mRNA, which normally accompanies T-lymphocyte proliferation (Williams et al., 1996). AA, EPA and OA exhibited more modest effects on these proto-oncogenes (Williams et al., 1996). Roche et al. (1999) showed that PA and OA caused a pronounced accumulation of c-fos mRNA in a pancreatic β-cell line. This accumulation was reported to involve PKC and calcium signalling pathways and was coupled with an increase in AP-1 transcriptional activity (Roche et al., 1999). In an animal feeding study, autoimmune-disease-prone mice supplemented with dietary FO exhibited reduced splenic c-myc and c-ras mRNA expression (Fernandes et al., 1994).

Other mechanisms by which fatty acids can affect immune cell function include the Rac family of GTPases which are involved in PLA2 activation and AA mediated signalling (Kim & Kim, 1997), and the toll-like receptor family of receptors (Tlr), many of which are involved in LPS-mediated cell signalling (Hwang, 2001). Kim & Kim (1997) showed that exogenous AA stimulated Rac signalling to the nucleus. In addition, USFA have been found to inhibit and SFA to enhance NF-κB mediated COX-2 expression via Tlr4 (Hwang, 2001). Tlr are highly conserved amongst species and are present in cells of the innate immune system (O’Neill & Greene, 1998). They are responsible for the release of a plethora of cytokines, which interact directly and indirectly with the adaptive immune system (Aderem & Ulevitch, 2000). Tlr also contain acylated SFA (Lipid A) in their structures (Hwang, 2000). It is tempting to speculate that by modulating the fatty acid composition of the Tlr, it may be possible to alter Tlr-signalling, resultant transcription factor activity (e.g. NF-κB) and cytokine production (Hwang, 2001).

1.4 Conjugated linoleic acid (CLA).

CLA refers to a derivative of LA, which can be produced commercially but is found naturally in meat and dairy products (Belury, 2002). Interest in CLA has arisen in the last ten years following discoveries that CLA can influence lipoprotein metabolism, body composition, carcinogenesis and immune function (Roche et al., 2001, Belury, 2002).
More specifically, CLA refers to the name given to a mix of geometric and positional isomers of LA with conjugated double bonds (i.e. are contiguous and are not separated by a methylene group as in LA, see fig 1.10) (Mac Donald et al, 2000, Roche et al, 2001). Structurally, the double bonds may be present on carbons 8 and 10, 9 and 11, 10 and 12, 11 and 13 in either a cis or trans configuration (Belury, 2002). The principal isomers include cis-9, trans-11 CLA (rumenic acid), trans-10, cis-12 CLA, trans-7, cis-9 CLA and trans-9, trans-11 CLA (Griinari et al, 1998, Kramer et al, 1997, Corl et al, 2002). Industrially, CLA is produced as a result of alkaline-catalysed isomerisation of LA. Typically this yields a CLA oil of 90% purity composed of approximately 42 % cis-9, trans-11 CLA, 43% trans-10, cis-12 CLA with minor amounts of other CLA isomers (including cis-9, cis-11 CLA and trans-9 and trans-11 CLA), residual linoleate (0.5%), oleate (5.5%) and 4% unidentified fatty acid (Belury, 2002). Dietary sources of CLA are listed in Table 1.1. Dietary intakes are reported to range from approx 95 to 200 mg/day (Ens et al, 2001, Ritzenthaler et al, 2001). CLA levels have been reported to vary in foods from 0.2 mg/g fat in vegetable oils to 30 mg/g fat in milk fat, where the predominant isomer is the cis-9, trans-11 isomer (approx 85-95% total CLA content)(Sebedio et al, 1999). Previously it was thought that the cis-9, trans-11 isomer is primarily produced during the microbial biohydrogenation (Butyrivibrio fibrisolvens) of LA and linolenic acids in the rumen of cattle and other ruminant animals (Kepler et al, 1966). However, the major source of CLA in ruminant milk and tissues is endogenous synthesis via the action of Δ9 desaturase on vaccenic acid (Griinari et al, 2000). In ruminants the cis-9, trans-11 isomer is absorbed (biohydrogenated) into trans-11 octadecenoic acid (vaccenic acid), where after absorption it can be re-converted to cis-9, trans-11 CLA by the enzyme Δ9 desaturase (Grinairi et al, 1998, 2000). The second most common naturally occurring CLA isomer is trans-7, cis-9 CLA, which is derived from endogenous synthesis involving the action of Δ9 desaturase on ruminally derived trans-7 C18:1 (Corl et al, 2002). It is noteworthy that the presence of the trans-10, cis-12 CLA isomer, the second most common isomer formed during commercial production of CLA, was found to inhibit desaturase activity in vitro (Park et al, 2000b). The implications of this inhibition on desaturase activity are unknown in vivo.

CLA levels in ruminant and dairy products are influenced by rumen microbial population, are higher in animals grazed in pasture rather than feeding on forage and grain and are influenced by frequency of lactation (McDonald, 2000). Consequently, CLA levels are higher in milk from cows fed on pasture in the summer (McDonald, 2000). CLA
Fig 1.10. Structures of cis-9, trans-11 CLA, trans-10, cis-12 CLA and linoleic acid.

Table 1.11. Principal dietary sources of conjugated linoleic acid (CLA).\(^1\)

<table>
<thead>
<tr>
<th>Food Source</th>
<th>Amount of CLA (g/100g fat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole milk</td>
<td>0.55</td>
</tr>
<tr>
<td>Low-fat milk (2%)</td>
<td>0.41</td>
</tr>
<tr>
<td>Condensed milk</td>
<td>0.70</td>
</tr>
<tr>
<td>Butter</td>
<td>0.47</td>
</tr>
<tr>
<td>Plain Yoghurt</td>
<td>0.48</td>
</tr>
<tr>
<td>Low-fat yoghurt</td>
<td>0.44</td>
</tr>
<tr>
<td>Cheddar cheese</td>
<td>0.41</td>
</tr>
<tr>
<td>Processed cheese</td>
<td>0.50</td>
</tr>
<tr>
<td>Ice-cream</td>
<td>0.36</td>
</tr>
<tr>
<td>Beef</td>
<td>0.43</td>
</tr>
<tr>
<td>Lamb</td>
<td>0.58</td>
</tr>
<tr>
<td>Veal</td>
<td>0.27</td>
</tr>
<tr>
<td>Fresh ground turkey</td>
<td>0.26</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>0.07</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>0.04</td>
</tr>
</tbody>
</table>

\(^1\) Data from Chin et al. (1992) and Lin et al. (1995). Adapted from Roche et al. (2002).
content of foods is also affected by processing. Aged cheeses have lower amounts of CLA, while higher temperatures, the addition of iron, whey powder or non-fat dry milk result in higher concentrations of CLA in processed cheeses. McDonald (2000) completed a comprehensive review outlining the factors that determine CLA levels in foods. *Butyrivibrio fibrisolvens* has been identified in the digestive tract of humans, however supplementation of humans with 16g/d LA for 6 weeks did not increase plasma CLA concentrations (Herbel *et al.*, 1998). Hence it appears that man depends on dietary sources of CLA (Roche *et al.*, 2002). CLA has been identified in human plasma, serum and adipose tissue (Salminen *et al.*, 1998, Jiang *et al.*, 1999). Supplementation with either CLA supplements (Salminen *et al.*, 1998) or cheddar cheese (Huang *et al.*, 1994) increases plasma and breast milk CLA content (Park *et al.*, 1999). Adipose tissue levels of cis-9, trans-11 CLA correlate with milk fat intake (Jiang *et al.*, 1999).

CLA is credited with anti-carcinogenic, anti-atherosclerotic, anti-diabetic, anti-obesity and immuno-modulatory properties. CLA is reported to decrease the initiation, promotion and progression of carcinogenesis in *in vitro* and *in vivo* models of skin, mammary, forestomach, prostate and colon cancer (Ip *et al.*, 1991, Liew *et al.*, 1995, Belury *et al.*, 1996). Although some studies reported no effect of CLA on carcinogenesis (Petrik *et al.*, 2000), no studies have reported an increase in tumorgenesis following CLA intervention (Belury, 2002). The anti-carcinogenic effects of CLA appear particularly effective with respect to mammary cancer, where CLA experts a dose-dependent anti-cancer effect independent of level or type of other fats in the diet (Ip *et al.*, 1991, 1996). Both isomers of CLA appear to be equally effective, and CLA is active in both free fatty acid and triglyceride forms (Ip *et al.*, 1995, Belury, 2002). In addition, CLA is reported to decrease secondary tumour growth in breast cancer cell lines (Hubbard *et al.*, 2000). CLA was found to influence carcinogenesis by decreasing cellular proliferation, increasing cell differentiation and apoptosis and preventing cell cycle dysregulation involving the cyclin family (Ip *et al.*, 1991, 2001).

CLA has been found to exert anti-atherosclerotic effects in animals. It is reported to improve indicators of cardiovascular risk in animals (e.g. lower serum triglycerides, low density lipoprotein cholesterol levels) and decrease atherosclerotic plaque formation (Lee *et al.*, 1994, Nicolosi *et al.*, 1997). However, other studies have found no effect (Gavino *et al.*, 2000) or induced formation of aortic fatty streaks (Munday *et al.*, 1999). Park *et al.*, (2000) attributed these *in vitro* anti-cholesterolemic benefits to the trans-10, cis-12 isomer. However, ob/ob mice fed a diet rich in the purified cis-9, trans-11 isomer demonstrated significant decreases in triglyceride and non-esterified fatty acid
concentrations, relative to animals fed diets rich in the trans-10, cis-12 CLA isomer or LA (Roche et al., 2002). In humans, CLA is reported to have no effect (Blankson et al., 2000, Benito et al., 2000) or to decrease fasting plasma triglyceride and very low density cholesterol concentrations (Noone et al., 2002). Finally, CLA is reported to both improve insulin sensitivity and glucose tolerance (Houseknecht et al., 1998). Conversely, two other studies showed that CLA induced insulin resistance, hyperglycemia and marked lipodystrophy in C57/B16 and ob/ob mice (Tsuboyama-Kasaoka et al., 2000, Roche et al., 2002).

CLA has been found to reduce body fat accumulation and increase lean body mass in growing mice, rats, pigs and steers (Roche et al., 2002). CLA is also reported to decrease adipocyte cell size and filling, increase metabolic rate and fat oxidation, decrease white adipose tissue mass and induce apoptosis in animals (Roche et al., 2002). The trans-10, cis-12 isomer is credited with these anti-obesity effects (Houseknecht et al., 1998, Clement et al., 2002, Roche et al., 2002). Although some studies have reported small decreases in body fat mass, CLA appears to have minimal anti-obesity effects with respect to humans (Zambell et al., 2000, Smedman et al., 2002).

### 1.3.9 CLA and the immune system.

Like other fatty acids, CLA is reported to possess immuno-modulatory effects. In an in vitro dose response study CLA (46g cis-9, trans-11 CLA and 50g trans-10, cis-12 CLA / 100g fatty acids) increased porcine PHA-stimulated lymphocyte proliferation, inhibited Con A-induced IL-2 production and murine macrophage phagocytic activity (Chew et al., 1997). Recently, Yang & Cook (2003) investigated the isomer-specific effects of cis-9, trans-11 CLA and trans-10, cis-12 CLA on LPS-stimulated TNFα production in Raw264.7 macrophages. A CLA isomeric blend typical of commercial mixes was also included. The cis-9, trans-11 CLA isomer was associated with a dose-dependent decrease in LPS-induced TNFα production (Yang & Cook, 2003). A reduction in TNFα secretion was also evident with the CLA blend and the trans-10, cis-12 CLA isomer, however the decreases associated with these fatty acids were secondary to those observed with the cis-9, trans-11 CLA isomer (Yang & Cook, 2003).

Animal studies are detailed in Table 1.12. Cook et al. (1993) were the first to establish the immuno-modulatory benefits of CLA, when they noticed that chicks fed CLA (0.5% diet) lost less weight when exposed to endotoxic shock and demonstrated enhanced PHA-induced blastogenesis. Similarly Miller et al. (1994) reported increased PHA-stimulated blastogenesis after feeding mice a CLA rich diet (5g CLA /kg diet) for two
weeks. In a murine dose response study, feeding CLA for three (but not six weeks) significantly enhanced PHA-induced lymphocyte proliferation, decreased Con A-stimulated IL-2 production but had no significant effect on lymphocyte cytotoxicity, mammary tumour incidence or latency (Wong et al., 1997). Sprague-Dawley rats fed a CLA enriched diet demonstrated significantly reduced basal macrophage TNFα and LPS-stimulated IL-6 production, but had no effect on IL-1 secretion (Turek et al., 1998). Feeding a CLA-enriched diet to young and old C57BL/6NCrI BR mice significantly enhanced PHA- and Con A-stimulated splenocyte proliferation and Con A-induced IL-2 production, but had no effect on NKC activity or splenocyte IL-1 (Hayek et al., 1999). Kelley and colleagues (2002) supplemented C57BL/6N mice with diets enriched with the purified cis-9, trans-11 or trans-10, cis-12 CLA isomers at 0.5% diet for 8 weeks. The two isomers did not affect the number of immune cells in the circulation, spleen or bone marrow, ex vivo LPS- and Con A-induced splenocyte proliferation and Con A-stimulated IL-2 secretion. However both of the isomers increased LPS-stimulated TNFα and IL-6 and decreased Con A-induced IL-4 relative to control cells. The authors concluded that there was no difference in efficacy between the isomers in this animal study (Kelley et al., 2002). Finally, Yang & Cook, (2003) fed two cohorts of Balb/C mice 0.5% CLA or corn oil for six weeks. In one cohort, dietary CLA attenuated LPS-induced weight loss and anorexia, and was associated with a decrease in plasma TNFα. However in the second cohort, CLA did not alter ex vivo IFN-γ- or LPS-stimulated TNFα production in resident peritoneal macrophages (Yang & Cook, 2003). Splenocytes were also collected from these animals for cytokine production assays. The CLA fed mice exhibited decreased Con A-induced IL-4 production, increased IL-2 and an increase in the IL-2: IL-4 ratio relative to the corn oil fed mice.

CLA has also been reported to affect CD8 lymphocyte function. Bassaganya-Riera et al., (2000, 2001, 2002) recently reported that feeding pigs CLA enhanced the number and effector function of porcine CD8+ PBMC’s (e.g. granzyme activity, proliferation, cytotoxic potential). Furthermore, the effects of CLA on CD8+ effector functions persisted for 25 days after withdrawal of CLA from the diet (Bassaganya-Riera et al., 2002a). As CD8+-mediated down-regulation of CD4+ T-cell effector functions is suggested as important for the maintenance of mucosal immune and inflammatory balance (Bassaganya-Riera et al., 2002b), the authors hypothesised that the maintenance of CD8+ function following CLA supplementation, may enhance host defence in animal models of Inflammatory Bowel Disease. Subsequently, Hontecillas et al., (2002) showed that CLA fed preventively before the onset of bacterial-induced colitis in pigs decreased mucosal
Table 1.12. The effect of CLA enriched diets on animal indices of the cell-mediated immune response*.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Model</th>
<th>Intervention details</th>
<th>CLA dose (g/kg diet)</th>
<th>Study period (d)</th>
<th>Lymphocyte proliferation</th>
<th>Cytokine response</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cook et al, 1993</td>
<td>Chicken &amp; rat (spleenocyte)</td>
<td>Control diet: 2.5g fat/kg diet (LA only)</td>
<td>5</td>
<td>28</td>
<td>↑</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CLA diet (2.5% LA &amp; 0.5% CLA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miller et al, 1994</td>
<td>Mice (spleenocytes)</td>
<td>Control diet: 2.5g fat/kg diet (LA only)</td>
<td>5</td>
<td>15</td>
<td>↑</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Wong et al, 1997</td>
<td>Balb/c mice (spleenocytes)</td>
<td>Control diet: 5g fat/kg diet (SFO)</td>
<td>1</td>
<td>21 &amp; 42</td>
<td>↑ (d 21 only)</td>
<td>↑ Con-A IL-2</td>
<td>NSD: LC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low-CLA</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Medium-CLA</td>
<td>3</td>
<td></td>
<td>↑ (d 21 only)</td>
<td>↑ Con-A IL-2</td>
<td>NSD: LC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High-CLA (CLA: 35 g c-9, t-11, 9, c-11, 39 g t-10, c-12/100g fa)</td>
<td>5</td>
<td></td>
<td>↑ (d 21 only)</td>
<td>↑ Con-A IL-2</td>
<td>NSD: LC</td>
</tr>
<tr>
<td>Turek et al, 1998</td>
<td>Sprague-Dawley rats (peritoneal macrophages)</td>
<td>Control diet: 70g fat/kg diet (SBO)</td>
<td>10</td>
<td>42</td>
<td>NA</td>
<td>↓ TNFα &amp; IL-6</td>
<td>NSD: PGE&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Hayek et al, 1999</td>
<td>C57BL/6NCrIIR (spleenocyte)</td>
<td>Control diet: 50g fat/kg diet (SBO)</td>
<td>10</td>
<td>56</td>
<td>↑</td>
<td>↑ Con A IL-2</td>
<td>NSD: KNC</td>
</tr>
<tr>
<td>Bassaganya-Riera et al, 2001, 2002</td>
<td>(PBMC's)</td>
<td>Control diet: 2.21 g (SBO) /100g diet</td>
<td>1.33</td>
<td>37</td>
<td>↑</td>
<td>↑ C8&lt;sup&gt;+&lt;/sup&gt; LC &amp; granzyme activity</td>
<td></td>
</tr>
<tr>
<td>Kelley et al, 2002</td>
<td>C57BL/6N mice</td>
<td>Control diet: 5.5% corn oil</td>
<td>5</td>
<td>56</td>
<td>NSD</td>
<td>↑ TNFα &amp; IL-6</td>
<td>NSD: PGE&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Hontecillas et al, 2002</td>
<td>Pigs</td>
<td>Control diet: 2.21 g (SBO) /100g diet</td>
<td>1.33</td>
<td>49</td>
<td>N/A</td>
<td>Maintain IL-10 &amp; IFN-γ</td>
<td>Maintain CD4+ &amp; CD8+ profiles</td>
</tr>
<tr>
<td>Yang &amp; Cook, 2003</td>
<td>Balb/C mice</td>
<td>Control diet: 5.5% corn oil</td>
<td>5</td>
<td>42</td>
<td>N/A</td>
<td>↓ plasma TNFα</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CLA diet 5% corn oil &amp; 0.5% c-9, t-11, 13g t-10, c-12/100g fa)</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>CLA or t-10, c-12 CLA</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>CLAdiet(12g e-9, t-11,13gt-10,c-12,13g t-9,t-11, 13g other isomers/100g fa)</td>
<td>1.33</td>
<td>49</td>
<td>N/A</td>
<td>Maintain IL-10 &amp; IFN-γ</td>
<td>Maintain CD4+ &amp; CD8+ profiles</td>
</tr>
<tr>
<td>Roche et al, 2001</td>
<td>Pigs</td>
<td>Control diet: 2.21 g (SBO) /100g diet</td>
<td>1.33</td>
<td>49</td>
<td>N/A</td>
<td>Maintain IL-10 &amp; IFN-γ</td>
<td>Maintain CD4+ &amp; CD8+ profiles</td>
</tr>
</tbody>
</table>

↑,↓: significantly increased or decreased relative to control, c: cis, CLA: conjugated linoleic acid, Con A: concanavalin A, DTH: delayed type hypersensitivity, IL: interleukin, LC: lymphocyte cytotoxicity, NSD: not significantly different, N/A: not measured, NKC: natural killer cell, PBMC: peripheral blood mononuclear cells, PG: prostaglandin, SBO, soybean oil, SFO, safflower oil, t: trans, TNF: tumour necrosis factor.*Adapted from Roche et al, (2001).

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damage, maintained IFN-γ and IL-10 profiles and lymphocyte subset distributions resembling those of non-infected pigs.

The effects of CLA on Ig production have been investigated in Sprague-Dawley rats. Feeding CLA to Sprague-Dawley rats at a dietary level of 0.5 and 1% increased plasma IgA and IgG while decreasing IgE secretion (Sugano et al., 1998). In a dose response study, feeding CLA (0, 0.05, 0.1, 0.25 and 0.5% diet) for 3 weeks resulted in a dose dependent increase in splenocyte IgA, IgG and IgM production (Yamasaki et al. 2000). Effects reached a plateau at 0.25% (Yamasaki et al. 2000). However in vitro addition of individual CLA isomers, furan derivatives and a CLA isomeric mix (100μM), significantly suppressed Ig production in rat splenocytes (Yamasaki et al, 2000). CLA is reported to modulate eicosanoid production. In in vitro models, CLA decreased arachidonic acid content and PGE2 production in cultured keratinocytes (16μg/mL) (Liu & Belury, 1997, 1998). HSVEC’s incubated with a CLA isomeric mix (50:50 cis-9, trans-11: trans-10, cis-12, 100μM) or the individual isomers (50μM) were found to inhibit the resting production of PGF2α and calcium ionophore stimulated production of PGI2, PGF2α, PGE2, PGD2 and TXB2. Interestingly at 100μM, the cis-9, trans-11 isomer inhibited eicosanoid production, while the trans-10, cis-12 isomer caused stimulation (Urquhart et al. 2002). Animal in vivo studies reported a decrease in PGE2 production in rat bone organ cultures following a 1% CLA and n-3 PUFA rich diet for 6 weeks (Li & Watkins, 1998). Sugano et al. (1998) found that feeding Sprague-Dawley rats CLA (0.5 & 1% diet for 3 weeks) resulted in decreased spleen LTβ4, lung LTC4 and serum PGE2. Harris and co-workers (2001) fed pregnant rats a diet with and n-6: n-3 ratio of 7:1 and 34:1 with and without CLA (1.1% by weight). Addition of CLA to the diets resulted in a dose dependent depression in PGF2α synthesis in the placenta, uterus and liver (Harris et al. 2002). Furthermore, in a guinea-pig model for type I hypersensitivity, CLA decreased antigen-induced tracheal histamine and PGE2 release (Whigham et al., 2001). However, three animal feeding studies reported no effect of CLA on PGE2 production. C57BL/6NcrlBR mice and Sprague Dawley rats supplemented with a CLA isomeric mix or C57Bl/6N mice fed purified cis-9, trans-11 CLA or trans-10, cis-12 CLA exhibited no difference in ex vivo mitogen stimulated PGE2 production at the end of the study period (Turek et al. 1998, Hayek et al., 1999, Kelley et al. 2002).

There has been one human intervention study to date investigating the effect of dietary supplementation on the human immune system (Kelley et al. 2000, 2001a). In this study 17 women resided in a metabolic suite for 93 days (mean age 28, mean BMI 22.8). During the first 30 days the ladies were supplemented with 6g/d sunflower oil (placebo).
At day 30, 10 of the volunteers supplemented their diet with 3.9 g/d CLA (CLA isomers made up 65% weight total fatty acids, of which 22.6% trans-10, cis-12 CLA, 17.6% cis-9, trans-11 CLA, 59.8% other isomers). The remaining 7 women supplemented their diets with the sunflower oil (3.9 g/d). Although CLA concentration increased from 0.012 to 0.97% in PBMC lipids, it did not significantly alter the concentration of other fatty acids, ex vivo LPS-stimulated secretion of PGE$_2$, LTB$_4$, IL-1β, TNFα, PHA-induced IL-2, the % of T-cells producing IL-2, IFNγ or the % monocytes secreting TNFα (Kelley et al., 2000, 2001a). As part of the same study, CLA did not affect DTH responses to a number of recall antigens, antibody titers to influenza vaccine, numbers of circulating white blood cells, granulocytes, monocytes, lymphocytes, and PHA-stimulated lymphocyte proliferation (Kelley et al., 2000, 2001a). However, this study was of a very controlled nature with respect to food intake, exercise, had a very low sample size and did not allow for a washout period after the initial basal period of sunflower oil supplementation, prior to commencement of CLA intake. Additionally, the CLA used consisted of a heterogenous mix of four CLA isomers. The total CLA content was 65% weight total fatty acids present (24-17%, cis-11, trans-13, trans-10, cis-12, cis-9, trans-11 and trans-8, cis-10 in order of decreasing content) (Kelley et al., 2000, 2001a). Clearly, the content of the more rigorously investigated and biologically active isomers (cis-9, trans-11 and trans-10, cis-12) in this study was quite low; this may have influenced study outcome.

1.3.10 CLA and mechanisms of action.

A number of pathways have been proposed as to the mechanisms of action of CLA. Elongated and desaturated metabolites of CLA (e.g. conjugated 18:3, 20:3 and 20:4) have been identified in rat liver and mammary tissue (Ip et al., 1997, Banni et al., 2001) and in human serum (Belury, 2002). CLA is also readily oxidised to β-oxidation products (e.g. C16:1 and C16:2) (Ip et al., 1997). The biological activity of these metabolites is as yet unknown. However, they are known to accumulate in the phospholipids (Banni et al., 2001, Belury, 2002). It has been postulated that the formation of such metabolites might reduce/displace the accumulation of AA in the phospholipids, thereby reducing the availability of COX for AA and the production of downstream eicosanoid products (Liu & Belury 1998, Belury, 2002). The C20 metabolites of CLA may also function as substrates or antagonists of COX activity, thereby reducing the availability of enzyme for AA (Belury, 2002). Finally, CLA may decrease the production of arachidonate-derived eicosanoids by inhibiting COX-1 and / or COX-2 expression or activity (Belury, 2002).
However as yet, the true effects of CLA and its metabolites on eicosanoid pathways are unknown.

CLA is reported to function as an antioxidant (Ha et al., 1990, Ip et al., 1991, 1996, Nicolosi et al., 1997). However in two studies CLA did not function as an antioxidant (van den Berg et al., 1995, Banni et al., 1998) and indeed acted as a prooxidant (Chen et al., 1997, Cantwell et al., 1998). However, Leung & Liu (2000) reported in a gas chromatographic model of antioxidant activity that trans-10, cis-12 CLA acted as an antioxidant at all concentrations tested (2-200μM). Indeed it was more effective than cis-9, trans-11 CLA and α-tocopherol at lower concentrations (2-20μM). Cis-9, trans-11 CLA acted as an antioxidant at concentrations below 20μM, however at 200μM it acted as a strong pro-oxidant (Leung & Liu, 2000). The authors concluded that the discrepancies between previous studies may be due to the different isomeric mixes used in previous studies. Conversely, a CLA mixture (0-150μM for 24 h) increased glutathione peroxidase and phospholipid glutathione peroxidase mRNA and activity in HUVEC cells, thus indicating an increase in antioxidant activity (Farquharson et al., 1999).

CLA may operate by altering transcription factor activity. CLA is a known PPARα and PPARγ ligand (Houseknecht et al., 1998, Moya-Camarena et al. 1999ab, Yu et al., 2002). The majority of studies investigating the effect of CLA on PPAR activity have focused on PPARα and lipid metabolism, where CLA appears to share some of the activities of the PPARα ligand, WY14643 (Belury 2002). Recently, Yu and co-workers (2002) reported the constitutive expression of functional PPARγ (but not PPARα or β) in Raw264.7 macrophage cells. Using reporter assays and RT-PCR, the authors found CLA activated PPARγ, decreased IFNγ-induced COX-2, iNOS and TNFα mRNA expression and activity and reduced PGE2, NO, TNFα, IL-1β and IL-6 secretion. CLA also induced differentiation of the monocytic cell line HL60 (Yu et al., 2002). Transfecting dominant negative PPARγ into the RAW cells ablated the ability of various CLA isomers to regulate the iNOS reporter construct. The authors therefore concluded that the anti-inflammatory properties of CLA might be mediated (at least in part in RAW264.7 cells) via PPARγ.

Recently it was reported that CLA (0.18-1.78 x 10^-5 M) did not affect membrane PLC or PKC activity in the MCF-7 breast cancer cell line (Park et al. 2000). However, to date there is no information relating to the effect of CLA on the activity of other transcription factors (e.g NFκB, AP-1 etc).
1.4 Research objectives.

Previous authors have established that CLA possesses the ability to modulate immune function. However, the majority of studies have involved various animal and in vitro models and have included isomeric blends rather than the individual CLA isomers. Indeed only one study has investigated the influence of a CLA isomeric blend on human immune function. Furthermore there is a lack of information in relation to the molecular mechanisms of action of CLA; most studies have focused on the PPAR family of transcription factors.

The aims of this thesis are as follows;

1) To investigate the effects of dietary supplementation with two isomeric blends of CLA on various indices of human immune function.

2) To ascertain whether there is a difference in efficacy between the two CLA isomeric blends, or with LA (the control fatty acid in this study), on human immune function.

3) To determine the in vitro effects of the individual CLA isomers on cytokine production, lymphocyte proliferation and eicosanoid production in human PBMC's.

4) To investigate if changes in fatty acid-induced cytokine production were evident at the transcriptional (mRNA) level.

5) To compare the effects of the CLA isomers in vitro with two saturated fatty acids, one monounsaturated fatty acid and a polyunsaturated fatty acid.

6) To elucidate the molecular mechanisms of action of the fatty acids, in particular if fatty acids modulate the NF-κB family of transcriptional activator proteins.
Chapter Two.

Materials and Methods.
2.0 Collection of Blood Samples.

Blood samples were obtained by venupuncture, or, donated by the Irish Blood Transfusion and Services Board ('leukocyte-rich, buffy coat' preparations, Irish Blood Transfusion Services (IBTS), National Blood Centre, James’s Street, Dublin, Ireland). Venupuncture samples were collected in vacutainers containing heparin and lithium (Beckton Dickinson UK Ltd., Cowley, Oxford, UK). Serum was isolated from blood collected in silicone-coated additive-free vacutainers, while plasma was separated from blood collected in EDTA-containing vacutainers (Beckton Dickinson UK Ltd., Cowley, Oxford, UK). Where necessary, blood samples were placed on ice to prevent spontaneous TNFα release (Freeman *et al* 1990).

2.1 Isolation of peripheral blood mononuclear cells (PBMC).

Peripheral blood mononuclear cells (PBMC’s) were isolated as described by Boyum, (1968). For samples obtained by venupuncture, PBMC’s were carefully separated by diluting the heparinised blood in half with 25mM Hepes buffered Hanks Balanced Salt Solution cocktail (HBSS, Gibco, Grand Island, New York). The PBMC-rich preparations obtained from the IBTS were diluted 1:9 in Hepes buffered HBSS. Both mixtures were carefully layered onto ‘Lymphoprep’ ficoll-hypaque density gradient solution (density 1.077 g/L, ratio of diluted blood: lymphoprep 4:1, Nycomed Pharma, Oslo) and centrifuged at 1200 rpm for 30 min. The ‘buffy coat’ interface containing the PBMC’s was collected using a sterile pasteur pipette, diluted approx. 1:5 in the HBSS cocktail and centrifuged for a further 10 min at 1200 rpm. The supernatant was decanted, the cells gently washed in 10 ml fresh HBSS cocktail and centrifuged again at 1200 rpm for 5 min. Cells were re-suspended in Roswell Park Memorial Institute (RPMI) complete medium supplemented with 2 mmol L-glutamine/L, 100 mg streptomycin/ml and 100 µg penicillin/ml (Gibco, Grand Island, New York). After enumeration (Section 2.4), cells were seeded at the appropriate concentration for each experiment.

For all of the *in vitro* studies 10% heat inactivated Foetal Bovine Serum (FBS), was included in the RPMI-complete medium. FBS was heated at 54°C for 30 min to inactivate complement. The same batch of FBS was utilised in all of the *in vitro* cultures, thereby minimising variations in the serum of nutrients such as growth factors and lipids. Autologous serum (2.5% v/v) was used in the human intervention trial.
2.2 THP-1 cell line.

The human leukaemic cell line (THP-1) was obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury). Originally derived from a young boy with leukaemia, it is a monocytic cell line that retains its monocytic phenotype for fourteen months (Tsuchiya et al, 1980). THP-1’s were cultured in RPMI supplemented with 10% heat inactivated FBS, 2 mmol L-glutamine/L, 100 mg streptomycin/ml and 100 μg penicillin/ml, as for the PBMC’s. Cells were cultured in 75 cm² flasks at 37°C, 5% CO₂ and 95% air in a humidified incubator (Infrared CO₂ incubator, Forma Scientific, Ohio, USA). THP-1’s were passaged bi-weekly and passages below number 20 were used for all cell culture experiments. As THP-1’s are a suspension cell line, splitting of cells was achieved by simply dividing the cells 1:4 in fresh pre-warmed media. However, for all experiments the cells were gently centrifuged (1000 rpm for 10 min), the supernatant was decanted and the cells re-suspended in pre-warmed fresh media. Cells were counted (Section 2.4) and seeded at the appropriate concentration.

2.3 Freezing and reviving cells.

A cryopreservation solution was prepared containing 45% FBS, 50% RPMI and 5% Dimethyl sulfoxide (DMSO, Sigma –Aldrich chemical Co., Poole, Dorset, UK). THP-1’s were routinely harvested in the same manner as for subculture, the supernatant decanted and the cells resuspended in the cryopreservation media at approximately 2-4 x 10⁶ cells/ml. One ml of the cell suspension was immediately aliquoted into sterile cyrotubes (Nunc plasticware, Life Technologies, Roskilde, Denmark), gently placed on ice to chill, frozen at −20°C overnight, and, subsequently transferred to a −70°C freezer for long-term storage.

To revive the cells, cyrotubes were removed from the −70°C freezer and quickly thawed in the hand. Under sterile conditions, the cells were transferred to a 25cm² tissue flask containing pre-warmed media and allowed to culture at 37°C, 5% CO₂ and 95% air in a humidified incubator.

2.4 Cell enumeration and viability.

Cell viability and yield were assessed using 0.1% w/v ethidium bromide/acridine orange (EB/AO) (Lee et al 1975). Cells were counted on a Neubauer haemocytometer under ultraviolet (uv) light using a modification of the protocol of Hudson and Hay (1976). Under uv light, live viable cells fluoresce green whereas nonviable cells stain red/orange.
All enumerations were carried out in duplicate and cells were resuspended at the appropriate concentration.

2.5 Mitogen and antibody preparation.

The plant lectins, Concanavalin A (Con A) and Phytohemagglutinin-P (PHA) polyclonally stimulate human T lymphocyte proliferation and division. Bacterial endotoxin or lipopolysaccharide (LPS) stimulate macrophage proliferation. The mitogens Con A, PHA and LPS (Sigma- Aldrich, Poole, Dorset, UK) were made up in stock solutions of 10 mg/ml in RPMI cocktail and aliquots frozen at −20°C until required. For the intervention study, stocks were further diluted to yield working solutions of 200μg/ml and frozen at −20°C until needed. The murine monoclonal anti-human CD3 antibody OKT3 is an antibody for the CD3 molecule on the T-cell surface; it specifically activates T-cells. The murine monoclonal anti-mouse MHC antibody (anti-IE) was included in the experiments as an antibody (OKT3) control. Stock solutions of OKT3 (CRL 8001, ATCC, Rockville, MD, USA) and anti-IE (HB179, ATCC, Rockville, MD, USA) hybridoma supernatants were prepared at 200μg/ml and stored at −20°C. A large batch of all mitogens and antibodies was prepared prior to commencing each study; this stock of stimulants was used for all investigations within any one experiment.

2.6 Preparation of fatty acids.

At the start of in vitro experiments a stock of fatty acids was prepared in sterile DMSO. The concentration of DMSO in cultured cells was always <0.1%. The following acids were prepared at 100mM, aliquoted into small sterile vials and frozen at −20°C, protected from light:

- Cis-9, trans-11 conjugated linoleic acid (C18:2 cis-9, trans-11 CLA)
- Trans-10, cis-12 conjugated linoleic acid (C18:2 trans-10, cis-12 CLA)
- Linoleic acid (C18:2)
- Stearic acid (C18:0)
- Oleic acid (C18:1)
- Palmitic acid (C16:0)

Fresh fatty acids were prepared every 6 months. Both of the CLA isomers were supplied by Alexis chemicals (Cayman Chemicals, Ann Arbor, MI, US), while the remaining fatty acids were obtained from Sigma-Aldrich (Sigma-Aldrich, Poole, Dorset, UK).
2.7 *In vitro* Experimental Conditions.

For all of the *in vitro* experiments, except the lymphocyte proliferation assays, the fatty acids were added to the PBMC's and THP-1's at the time of plating and allowed to incubate for 48 h at 5% CO$_2$, 95% humidity and 37$^\circ$ C. After this 48 h culture period and where necessary, PHA (10µg/ml) was added to the PBMC's and LPS (1µg/ml) to the THP-1's, and culture continued for a varying periods of time depending on the assay of interest.

2.8 Lymphocyte Proliferation Assays.

Lymphocyte proliferation was measured by thymidine incorporation (Wood & Greally, 1976). Triplicate aliquots of cells (100 µl, 2 x 10$^6$ cells / ml) were seeded in 96 well round bottom plates for all PBMC proliferation assays. For the human supplementation trial, 5 µl autologous serum (2.5% v/v) was added to each well and PBMC's subsequently cultured in the presence and absence of the T- cell mitogens PHA and Con A (both at 10µg/ml), the antibody OKT3 (10µg/ml) and the OKT3 negative control, anti-IE (30µg/ml). Negative controls containing RPMI instead of mitogen were also included. Additional RPMI was added to each well to yield a total volume of 200 µl/ well and cell concentration of 1 x 10$^6$ cells / ml.

To investigate the effects of fatty acids on lymphocyte proliferation *in vitro*, fatty acids (100µM) were added directly to the plated PBMC's. 10% FBS was included in the culture media and PHA was the only mitogen used in these *in vitro* experiments. PHA (10µg/ml) was added at the same time as the fatty acids.

All plates were incubated at 37$^\circ$ C, 5% CO$_2$, 95% humidity for 72 h. Lymphocyte proliferation was assessed as the incorporation of $^3$[H] thymidine (0.3 µCi / well, specific action 6.7 Ci/mmol, New England Nuclear, Boston, MA) during the final 18 h of the culture period. Cells were either harvested onto glass fibre filters using a cell harvester (Inotech, Dottikon, Switzerland) and radiolabel uptake assessed using a liquid scintillation counting system (Wallac, Turku, Finland), or, frozen at –70$^\circ$ C until harvesting. Preliminary experiments indicated no effect of freeze-thaw on $^3$[H] thymidine activity and are presented in fig 2.1.

Lymphocyte proliferation results throughout this thesis are expressed as actual counts per minute (cpm) and/or as Simulation Index values where;

Simulation Index (SI) = $^3$[H] Incorporation in presence of mitogen

$^3$[H] Incorporation in the absence of mitogen.
**Fig 2.1.** The effect of a single freeze-thaw cycle on $^3$H thymidine activity (cpm), in human PBMC’s is presented below. Values represent the mean ± SD of three independent proliferation assays and indicate the averages between well.
2.9 Cytokine Assays (IL-2, TNFα and IL-4)

To ascertain ex vivo IL-2 and TNFα activity, 2 x 10^6 cells / ml in RPMI (500 μl) were cultured in 24 well flat bottomed plates, with 2.5 % v/v autologous serum (25 μl), and in the presence and absence of the mitogens PHA (10 μg/ml), LPS (10 μg/ml), OKT3 (10 μg/ml,) or anti-IE (30 μg/μl.). IL-4 production was investigated in PHA stimulated cells only. Additional RPMI was added to each well to yield a final volume of 1 ml and cell concentration of 1 x 10^6. At 24 h supernatants were collected and frozen at −70°C for analysis by enzyme-linked immunoassay (ELISA).

For in vitro assays, PBMC’s (5 x 10^6/5ml) and fatty acids were co-incubated for 48 h, PHA (10 μg/ml) added and culture continued for a further 24 h. Supernatants were aliquotted and frozen at −70°C for subsequent analysis of cytokine and eicosanoid (See Section 2.12) concentrations by ELISA.

For all experiments, IL-2, TNFα and IL-4 concentrations (pg/ml) were quantified using commercial ELISA kits (R & D Systems, Abingdon, Oxon, UK).

2.10 Principle of ELISA.

ELISA is based on a competitive binding technique. An anti-cytokine (TNFα, IL-2 or IL-4) antibody was coated onto polystyrene microtitre wells and allowed to adhere. High affinity microtitre plates (Costar EIA/RIA high binding plates) were utilised for the IL-4 assays. After an overnight incubation the wells were washed with phosphate buffered saline (PBS)-Tween-20 (1M PBS [8mM Na2HPO4,2H2O, 1.5mM KH2PO4, 137 mM NaCl, 2.7mM KCL], 0.05% Tween-20), and blocked for 1h in block buffer (1% BSA, 5% Sucrose in 1M PBS with 0.05% NaN3) to remove excess primary antibody and prevent non-specific binding. The plate was again washed in PBS-Tween 20 and cytokine standard, cell culture supernatant or spiked samples of known cytokine concentration, were added in duplicate and allow adhere. Control wells containing substrate and stop solution alone were included. Cytokine present bound to the antibody and unreacted components were removed by washing with PBS-Tween 20. Anti-cytokine monoclonal antibody was added which bound the captured cytokine. After a suitable incubation period and further washing with PBS-Tween, the enzyme alkaline phosphatase was added and formed a conjugate with the secondary anti-cytokine antibody. Unbound enzyme-conjugate was removed during a final washing step and tetramethylbenzidine (TMB) chromogen and hydrogen peroxide (1:1) solution was then added to the wells. The amount of coloured produce formed was in direct proportion to the amount of cytokine present in the sample.
Reaction was terminated by the addition of 2N sulphuric acid (stop solution) and absorbance measured using a multiwell scanning spectrophotometer at a wavelength in accordance with the manufacturers instructions. A standard curve was constructed for each assay by plotting mean absorbance values (optical density, OD) from the standard solutions versus known cytokine concentrations. Recovery was assessed using the spiked samples. Samples with a concentration greater than the standard curve were diluted and reassayed to obtain a precise concentration.

2.11 Soluble Cell Adhesion Molecule Expression.

5 ml of blood was drawn into additive-free vacutainers (Beckton Dickinson UK Ltd, Cowley, Oxford, UK). Serum was isolated and immediately frozen at –70 °C for subsequent analysis of sICAM concentrations by ELISA as above. (R & D Systems, Abingdon, Oxon, UK).

2.12 Eicosanoid Secretion.

For the clinical trial, blood was collected in EDTA-containing tubes, plasma separated and stored immediately at –70°C for subsequent analysis of PGE₂ and LTB₄ by ELISA (R & D Systems, Abingdon, Oxon, UK).

PGE₂ secretion in vitro was measured in the cell supernatants collected for ELISA, as outlined in Section 2.9.

2.13 Fatty Acid Analysis: Extraction of total lipids.

For fatty analysis of all samples (plasma and cell pellets), total lipids were isolated using the method of Folch et al, (1957).

For the CLA intervention trial, 400µl of plasma was transferred to 16 x 100 mm borosilicate glass test tubes containing 400µl of Tris-EDTA buffer and henceforth was processed in an identical manner to the PBMC cell pellets.

During in vitro experiments, PBMC’s (5 x 10⁶ cells / 5ml) were incubated with fatty acids (100µM) for 48h and stimulated with PHA (10 µg/ml) for a further 24 h. PBMC total lipids were isolated as follows. After the 72 h culture period, the mixed mononuclear cell population was pelleted and washed twice with HBSS. The cells were resuspended in 0.5 ml HBSS and this suspension pipetted into a 16 x 100 mm plain borosilicate glass test tube.
Both PBMC’s and plasma samples were subsequently processed in an identical manner. Lipid extraction was achieved by the addition of 2.5 ml chloroform : methanol (2:1) with 0.01% (w/v) butylated hydroxy toluene (BHT) to the cell suspension. Samples were vortexed for one minute and then centrifuged at 2500 rpm for 10 min to separate the aqueous and organic phases. The supernatants were removed using a glass pasteur pipette and transferred to a 12 x 75 ml borosilicate tube. Another lipid extraction was repeated by adding 2 ml chloroform with 0.01% (w/v) BHT to the PBMC sample, vortexing for approx. 30 sec and centrifuging the samples at 2500 rpm for 5 min. Supernatants were pooled with the first extract and the pooled organic phase was evaporated to dryness in a vortex evaporator (AGB Scientific Ltd., Dublin, Ireland). The dried samples were flushed with nitrogen (N\textsubscript{2}), sealed to prevent lipid oxidation and stored at −20° C.

Methyl esters were prepared by adding 0.5ml 0.01M NaOH in dry methanol according to the method of Jiang et al., (1999). Samples were vortexed for 30 seconds, flushed with N\textsubscript{2} and placed on a heating block at 60° C for 15 min. Boron Trifluoride Methanol (0.75 ml of 14% BF3, Sigma-Aldrich, Poole, Dorset, UK) was then added to the mixture, samples vortexed for 30 seconds and incubated at 60° C for 15 min. Lipids were extracted three times using 0.5 ml hexane, samples dried in a vortex evaporator and stored under N\textsubscript{2} at −20° C until analysis.

The fatty acid methyl ester composition of total plasma lipids were analysed for incorporation of CLA isomers using a Shimadzu GC-14A gas liquid chromatograph (Mason Technologies, Dublin), which was fitted with a Shimadzu C-16A integrator. A CP Sil 88 fused Silica Column (50 m x 0.22 m file thickness; Chrompack Ltd, Middleburg, The Netherlands) was installed. Nitrogen was used as the carrier gas. Oven temperature conditions for each run were an initial column temperature of 120° C, increasing to 180° C at a rate of 8° C per minute. Column temperature was held at 180° C for 40 mins, subsequently increased to 220° C at a rate of 4° C per min and held at 220° C for 15 mins. A fatty acid methyl ester standard spiked with known concentrations of the cis-9, trans-10 and trans -10, cis -12 isomers of CLA was used for peak identification. Fatty acids were identified with retention times compared to standards. Fatty acid compositions were calculated as a percentage of the total fatty acids.

2.14 MTT Assay.

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma-Aldrich, Poole, Dorset, UK) is a tetrazolium salt that is incorporated into the mitochondria of living cells. Solutions of MTT dissolved in phenol red-free cell culture medium are
yellowish. However in the presence of viable cells purple formazan crystals are formed, which are soluble in isopropanol. The amount of viable cells is directly proportional to the colour intensity formed and can be quantified spectrophotometrically (Mossman, 1983, Vistica et al, 1991).

5 x 10^5 PBMC’s were resuspended in 100μl phenol red-free culture medium, plated in a 96-well microtitre plate in the presence and absence of all fatty acids (50-200μM) and cultured for 48h at 5% CO₂, 95% humidity and 37°C. Control wells containing medium alone or blank (empty wells) were also included. 10μl of MTT was added to the wells and cells cultured for a further 4h. After this incubation period, the cultures were removed from the incubator and the resulting formazan crystals fully dissolved in 100 μl MTT solubilisation solution (Sigma-Aldrich, Poole, Dorset, UK) by repetitive pipetting and gentle rocking on a gyratory shaker. Absorbance was measured at 570 nm.

The optical density (OD) of the blank was subtracted from the average of the test results. Percentage cytotoxicity was calculated for all fatty acids at the different concentrations.

2.15 RNA extraction.

Total ribonucleic acid (RNA) was isolated from cultured cells using TRIZOL® Reagent (GIBCOBRL, Life Technologies™). TRIZOL® Reagent consists of a monophasic solution of phenol and guanidine isothiocyanate (GITC) and is an improvement to the single step method of Chomczynski and Sacchi (1987). TRIZOL® Reagent maintains the integrity of RNA while disrupting cells and dissolving cell components. Cells were pelleted in a 30 ml sterilin (Bibby Sterilin Ltd, UK), medium decanted and then cells lysed by repetitive pipetting with TRIZOL® Reagent (1- 1.5 ml TRIZOL® Reagent per 5-10 x 10⁶ cells). Samples were transferred to an eppendorff and incubated at room temperature for 15 min to allow complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added per initial 1ml reagent, tubes shaken vigorously by hand for 15 seconds and incubated at room temperature for 2-3 min. Samples were then centrifuged for 15 minutes at 12,000 rpm.

The upper aqueous phase containing the RNA was transferred to a fresh tube and RNA precipitated with 100% isopropanol (0.5 ml per 1ml Reagent used initially). Samples were incubated at room temperature for 10 minutes and then centrifuged at 12,000 rpm for 10 minutes. The supernatant was removed and the RNA containing pellet washed with 75% ethanol (1 ml per 1 ml initial TRIZOL Reagent). 75% ethanol was prepared in 0.1% diethyl pyrocarbonate (DEPC) treated water; DEPC-water is RNase free. Samples were
vortexed, centrifuged at 7,500 rpm for 5 minutes, supernatant removed and samples left to air-dry. Care was taken not to over-dry the RNA as this decreases the solubility of the RNA.

2.16 Quantitation of RNA.

The quantity and purity of the eluted RNA was determined spectrophotometrically (Eppendorff biophotometer, Germany) by measuring absorbance at 260nm and 280nm. Readings were performed using a 1:25 dilution in a quartz cuvette. RNase free water was used as the diluent control. An optical density (OD) of 1.0 represents an RNA yield of 40 μg/ml. RNA purity can be assessed from the OD’s at 230nm, 260nm and 280nm. An OD\textsubscript{260/280} of less than 1.6 implies partially dissolved RNA samples, 1.7-2.0 indicates good purity while an OD\textsubscript{260/280} of greater than 2 implies purity. An OD\textsubscript{260/230} greater than 2 indicates no residual guanidinium contamination.

2.17 RNA Agarose Gel Electrophoresis.

To check the integrity of total RNA, 1-5μg RNA was separated electroporetically using a formaldehyde denaturing agarose gel. The gel was prepared as followed: 1.2g agarose was dissolved in 87 ml DEPC-treated water in a microwave oven. 10 ml MOPS running buffer, pH 7.0 (0.2M MOPS (3-(N-morpholino) propanesulfonic acid), 10mM EDTA pH 8.0, 10mM sodium acetate pH 7.0) and 3 ml of 37% (w/v) formaldehyde were added in a fume hood to the agarose solution when it had cooled to approximately 55°C. The gel was allowed to set in a fume hood for 1 hr prior to electrophoresis.

Meanwhile, RNA samples to be analysed were prepared as follows: to a sterile microcentrifuge tube 1-5 μg total RNA in a volume of 2μl was added to 1μl 10X MOPS running buffer, 5 μl formamide, 2 μl formaldehyde (37% w/v), 1 μl ethidium bromide (400 μg/ml in DEPC treated water) and 1 μl RNA loading dye (50% glycerol, 1mM EDTA (pH 8.0), 0.1% bromophenol blue, 0.1% xylene cyanol FF). The samples were mixed well and heated to 65°C for 10mins. After this incubation, the samples were vortexed, centrifuged briefly and stored on ice. The gel wells were cleaned and the gel pre-run for 10 min at constant voltage (70V) to remove any residual formaldehyde prior to sample loading. The samples were loaded and the gel run at the constant voltage of 70V until the bromophenol blue tracing dye had run three-quarters of the length of the gel. The RNA was visualised using a UV trans-illuminator. Intact RNA is represented by two clear bands representing 18S and 28S RNA respectively.
2.18 Principle of Reverse Transcription of RNA for Taqman two step RT-PCR.

Taqman RT-PCR allows for the sensitive and quantitative measurement of mRNA. The initial step requires the conversion of RNA into single stranded cDNA using random hexamers from the TaqMan® Reverse Transcription Reagents. This cDNA is subsequently subjected to PCR amplification using primers designed to anneal to a particular region of the gene of interest. The PCR reaction exploits the 5' nuclease activity of AmpliTaq Gold® DNA Polymerase to cleave a TaqMan® probe (Holland et al, 1991, Gelfand et al, 1993). The Taqman® probe has a fluorescent reporter dye (usually FAM or VIC) attached to the 5'-end of the probe and a quencher dye (usually TAMRA) attached to the 3'-end of the probe. When the probe is intact, the proximity of the reporter and quencher dyes results in suppression of reporter fluorescence by Förster-type energy transfer ( Förster 1948, Lakowicz 1983). During the PCR reaction, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites. AmpliTaq Gold DNA Polymerase cleaves the probe if it is hybridised to the target, displacing the probe and separating the reporter and quencher dyes, thus increasing the fluorescence of the reporter dye. The polymerisation strand continues, however the 3'-end of the probe is phosphorylated to prevent extension of the probe during PCR. This process is maintained with every cycle and does not interfere with accumulation of PCR product. An increase in fluorescence signal emitted is detected only if the target sequence is complimentary to the probe and amplified during PCR, hence non-specific amplification is not detected. Accumulation of PCR product is detected directly by examining the increase in fluorescence emitted by the reporter dye. Figure 2.2 illustrates the polymerisation associated with the 5'-3' nuclease activity of AmpliTaq Gold to cleave a Taqman probe (Reference: TaqMan® Universal PCR Master Mix protocol, P/N 4303339, Applied Biosystems, Warrington, Cheshire, UK).

GAPDH was used as the housekeeping gene (endogenous control) for all experiments. GAPDH is used to normalize target gene levels and to account for differences in the amount of total RNA added to each reaction.

Accurate quantitation of mRNA expression is accomplished using a competitive strategy whereby a known amount of cDNA (standard) is added to the reaction plate and amplified for both housekeeping and target genes. This RT-PCR standard is generated from pooling RNA from the same set of samples and is thus reflective of the concentration of the samples under investigation. Analysis is based on the parameter threshold cycle time (Ct). Ct is the fractional cycle number at which the fluorescence passes a fixed threshold.
Fig 2.2. Stepwise representation of the structure-dependent polymerisation-associated 5'-3' nuclease activity of Amplitaq Gold DNA polymerase on a fluorogenic probe during one extension phase of PCR. (adapted from Applied Biosystems, TaqMan® universal Master Mix Protocol (1998).

1. Polymerisation.

Two fluorescent dyes, a reporter (R), and a quencher (Q), are attached to the 5’ and 3’ ends of a Taqman® probe.

2. Strand displacement.

When both dyes are attached to the probe, reporter dye emission is quenched.

3. Cleavage.

During each extension cycle, the TaqDNA polymerase cleaves the reporter dye from the probe.

4. Polymerisation completed.

Once separated from the quencher, the reporter dye emits its characteristic fluorescence.
level in the exponential phase of amplification (Higuchi et al, 1993). It is set separately for both the target and housekeeping genes. The exponential phase is reflective of real-time kinetics as none of the reaction components are limiting. Target and housekeeping genes were distinguished by the presence of the FAM or VIC fluorescent reporter dyes at the 5'-end of the probe in target genes (IL-2, IL-4 and TNFα) and housekeeping (GAPDH) genes respectively. A series of standard curves are calculated using a log scale of standard concentration against Ct, an equation of the line is generated and subsequent gene expression for both gene sets calculated from their respective curves. Samples are expressed normalised to GAPDH and relative to a control experiment. (Higuchi et al, 1993, Applied Biosystems 1998, User Bulletin No. 2. Relative Quantitation of gene expression. P/N 4303859.)

2.19 Preparation of RNA sample prior to reverse transcription.

The RNA samples were first treated with DNase I, amplification grade, (GibcoBRL, Life Technologies™) to eliminate any possible contamination of the RNA by genomic DNA.

4 μg of total RNA was incubated with 1 μl of 10X DNase I Reaction buffer (200mM Tris-HCL (pH 8.4), 20mM MgCl₂, 500mM KCl), and 1 μl of 10X DNase I, Amp Grade (1U/μl), in a final volume of up to 20 μl. This reaction was incubated at room temperature for 15 min. DNase I was then inactivated by the addition of one tenth the reaction volume of 25mM EDTA to the reaction mixture and heated to 65°C for 10 min.

2.20 Reverse Transcription: synthesis of first strand cDNA.

4 μg of DNase I treated RNA was reverse transcribed (RT) using the enzyme Moloney Murine Leukemia Virus Reverse Technologies (M-MLV RT, Promega Corporation, Madison, US). This enzyme is purified from an E. Coli strain expressing a recombinant clone (Roth et al, 1985). 1 μl of random primers (0.5 μg random primers/μl, Promega Corporation, Madison, US) were added to 4 μg of DNase I treated RNA and samples heated to 70°C for 5 min to melt secondary structure within the template. After this time samples were immediately placed on ice to prevent secondary structure reforming. Two other sample types were prepared: RNA for creating a standard curve and negative controls for the RT reaction (i.e. –RT’s), where water was added instead of enzyme. The following was then added to the DNase I treated RNA:

- 10 μl 5X M-MLV buffer (250mM Tris-HCL (pH 8.3 at 25°C), 375mM KCl, 15mM MgCl₂ and 50mM DTT, Promega Corporation),
2 μl 10X dNTPs (10mM each dATP, dCTP, dGTP, dTTP, Promega Corporation),
2 μl M-MLV RT enzyme (200 units enzyme/μl, 20mM Tris-HCL (pH 7.5) 200mM NaCl, 0.1mM EDTA, 1mM DTT, 0.01% Nonidet® P-40 and 50% glycerol, Promega Corporation) to the +RT samples and to the standard curve samples. 2 μl nuclease free water was added to the -RT samples.
DEPC treated water to bring a final volume of 50μl in both +RT and -RT samples.
Standard curve samples were brought up to a final volume of 40μl with DEPC treated water.
Samples were gently mixed and incubated for 60 min at 37°C followed by 70°C for 10 min to inactivate the enzyme.
As calculated from original RNA concentrations (4 μg/50μl for samples and 4μg/40μl for standards), sample concentrations were then 80 ng/μl for the +RT and -RT samples and 100ng/μl for the standard curve samples. +RT and -RT samples were diluted 1:10, aliquoted into RNase treated PCR tubes and frozen at −20°C for subsequent use. For the standard curve samples, the cDNA samples were diluted to form a standard curve of 50 ng/μl, 25 ng/μl, 12.5 ng/μl, 6.7 ng/μl and 3.3 ng/μl. For all subsequent RT-PCR reactions 2 μl of both the samples and the standard curve samples were added to the tube yielding final concentrations of 16 ng/μl +RT and −RT cDNA and 100, 50, 25, 12.5, 6.7 ng/μl cDNA for the standard curves reactions.

2.21 Taqman quantitative RT-PCR.
RT-PCR was preformed for the cytokines TNFα, IL2 and IL-4 using the Pre-Developed TaqMan® Assay Reagent kits for gene expression quantification (PDAR’s, Applied Biosystems, UK). Briefly these kits provide a pre-optimised primer/probe mix for detection and quantification of specific genetic sequences for the cytokine of interest. For all cytokines GAPDH was used as the housekeeping gene. Standards and −RT samples were analysed in triplicate for both target and housekeeping genes; samples were assayed in duplicate.
For all primer/probe sets (IL-2, IL-4, TNFα and GAPDH) a separate mastermix was prepared consisting of (for one 25 μl reaction):
12.5 μl 2X Taqman® Universal PCR Master mix (Applied Biosystems, Warrington, Cheshire, UK). This master mix contains the enzyme, AmpErase uracil-N-glycosylase, which prevents the reamplification of carryover-PCR products by removing any uracil incorporated into double-stranded DNA.
• 1.25 µl 20X Target Primers and probes (human IL-2, IL-4, TNFα and GAPDH PDAR target kits, Applied Biosystems, Warrington, Cheshire, UK).
• 9.25 µl DEPC-treated water.

This Primer-probe-mastermix was mixed well prior to addition of 23 µl per well to a MicroAmp® Optical 96-well reaction plate (Applied Biosystems, Warrington, Cheshire, UK). 2 µl of either DEPC-treated water (PCR negative control), -RT sample, standard or cDNA (+RT) (equivalent to 16 ng RNA/2µl), was added to the respective wells and the plate sealed with a MicroAmp® Optical caps (Applied Biosystems, Warrington, Cheshire, UK). After a brief gentle centrifugation step to ensure removal of any air bubbles present, the MicroAmp® plate was placed in an ABI prism 7700 sequence detection system (Applied Biosystems, Warrington, Cheshire, UK). PCR reaction thermal cycle conditions involved an initial step of 50°C for 2 mins, 95°C for 10 mins followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min. The 2 min 50°C step was necessary for optimal AmpErase uracil-N-glycolyase enzyme activity, while the 10 min 95°C step was required to activate AmpliTaq Gold DNA polymerase (Applied Biosystems, 2000). Analysis was conducted using the ABI prism 7700 sequence detection system (Applied Biosystems, Warrington, Cheshire, UK) after setting a threshold level in the exponential phase of amplification for both the target and housekeeping genes. A standard curve was constructed for each gene. Gene expression for unknown samples were calculated for both target genes and GAPDH using these standard curves. Samples are expressed normalised to GAPDH and relative to a control experiment.

2.22 Preparation of cytosolic fractions.

Cytosolic fractions were prepared as follows. PBMC's (8 x 10^6) were cultured for 48h in the presence and absence of cis-9, trans-11 CLA, trans-10, cis-12 CLA, LA, PA at 100 µM. PHA (10 µg/ml) was added and cells cultured for a further 3h. Two sets of controls containing (1) cells alone, and (2) cells and PHA were also included. An identical culture system was established for THP-1’s, however, the initial density of THP-1 monocytes was 2 x 10^6 / ml and cells were stimulated with LPS (1µg/ml) for 30 min and 1 hr. As this leukaemic cell multiplies by a factor of two approximately every 24 h, the final density of THP-1 monocytes at the end of the culture period averaged at 8 x 10^6.

At the end of the incubation periods, cells and media were harvested into a 30 ml sterilin and centrifuged at 2500 rpm for 5 min. Supernatant was then decanted. Tissue culture plates were washed with 5 ml ice-cold PBS to remove any residual cells and this cell-PBS suspension transferred to the sterilin. For the PBMC’s, the wells were gently
scraped with a blunt sterile pasteur pipette, during the washing step to ensure complete collection of cells. The pelleted cell-PBS mix was vortexed and centrifuged again at 2500 rpm for 5 min. PBS was decanted and the washing step repeated using 5 ml ice cold PBS. After another centrifugation step, the PBS was decanted, pelleted cells resuspended in 1 ml ice-cold PBS and transferred to an eppendorf. This PBS-cell suspension was centrifuged at 10,000 rpm for 10 min at 4°C, supernatant discarded and the pellet resuspended in 200 μl lysis buffer (1X PBS (8mM Na₂HPO₄, 2H₂O, 1.5mM KH₂PO₄, 137 mM NaCl, 2.7mM KCL), 1% Nonidet P-40, 1mM EDTA, 0.5mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/mL leupeptin hydrochloride). Samples were incubated on ice and vortexed every 5 min for approx. 60 min. After 1h, lysates were centrifuged for 10 min at 13,000 rpm at 4°C. The supernatant containing the cytosolic fraction was removed and stored at -20°C for future protein estimation.

2.23 Preparation of nuclear extracts.

Nuclear extracts were prepared from PBMC's and THP-1's as described by Osborn et al, (1989). Briefly, 8 x 10⁶ PBMC's (2 x 10⁶ THP-1's) were cultured in duplicate with and without fatty acids as described earlier. After 48 h incubation, PBMC's were stimulated with PHA (10 μg/mL), and cultured for a further 2h at 37°C, 95% humidity, 5% CO₂. THP-1's were activated with LPS (1μg/ml) for 30 – 60 min. At the same time, cells were cultured in the presence of fatty acid, but without PHA/LPS, to ascertain the effects of fatty acids on PBMC's and THP-1's in the resting state.

After this incubation period, 5 ml ice-cold PBS (1X) was added to each well to stop the reaction. All subsequent reactions and buffers were ice-cold. Buffers were prepared immediately prior to use. Cells were pelleted and resuspended in 1 ml hypotonic buffer (Buffer A, 10mM Hepes (pH 7.9), 1.5 mM MgCl₂, 10 mM KCL, 0.5mM phenylmethylsulfonyl fluoride (PMSF) and 0.5mM dithiothreitol (DTT)). After centrifugation in a minifuge for 10 min at 13,000 rpm and 4°C, the pellet was completely resuspended in 20 μl Buffer A containing 0.1% v/v Nonidet P-40. The cells were lysed in this buffer for 10 min on ice, followed by centrifugation (13,000 rpm, 10 min, 4°C). To prepare the nuclear extract, the pellet was re-suspended thoroughly in 15 μl high salt buffer (Buffer C, 20mM Hepes pH 7.9, 1.5mM MgCl₂, 420mM NaCl, 0.2mM EDTA, 25% glycerol, 0.5mM PMSF), placed on ice for at least 15 min (with repeated vortexing) and centrifuged (13,000 rpm, 10 min, 4°C). To the supernatant, 75 μl Buffer D (10mM Hepes pH 7.9, 50mM KCL, 0.2mM EDTA, 20% glycerol, 0.5mM DTT, 0.5mM PMSF) was
added. Protein concentrations were determined by Bradford assay (Section 2.24). Extracts retain their activity for up to 6 months when stored at $-70^\circ$C.

### 2.24 Determination of protein concentrations (Bradford Method).

The concentration of protein in cytosolic and nuclearextracts was determined by the method of Bradford (1976) using the Bio-Rad Protein Assay concentrate (Cat # 500-0006).

For cytoplasmic fractions, 799 µl of PBS and 1µl of protein sample (1:800 dilution) were mixed with 200 µl of dye concentrate in a sterile eppendorff and incubated for 5-30 min at room temperature. A standard curve was prepared as follows. A stock of 1 mg/ml BSA in PBS was prepared and serially diluted to yield standards at 50, 30, 25, 20, 15, 10, 5 and 0 µg/ml protein.

However, for protein estimation in nuclear extracts, samples were diluted 1:80 in Buffer D (10 µl sample, 790 µl Buffer D), and mixed with 200µl Bio-Rad Dye concentrate as before. A stock of 1 mg/mL BSA in buffer D was prepared and serially diluted in Buffer D to yield standards at 25, 20, 15, 10, 5 and 0 µg/mL protein.

The absorbance of the protein-solutions for both cytoplasmic and nuclear extracts were read at 595 nm in duplicate in an Eppendorff biophotomer. Readings were averaged for standards and samples, a standard curve constructed and the protein concentrations with reference to the standard curve. A spiked protein standard of known concentration was included in all assays to assess recovery (Sigma Protein Standard (1mg/ml), Sigma, Poole, UK).

### 2.25 Principle of Immunoblotting.

Immunoblotting combines the resolution of gel electrophoresis with the specificity of immunochemical detection. One-dimensional gel electrophoresis under denaturing conditions (i.e. in the presence of SDS, Laemmli, (1970)) separates proteins according to their molecular size as they move through a polyacrylamide gel matrix towards the anode. A discontinuous buffering system is utilised in these experiments. In addition, the proteins are denatured, by boiling, in the presence of β-mercaptoethanol to reduce the proteins to their subunits by reducing disulphide bonds. The electrophoresed proteins are then transferred to a sensitive membrane (e.g. polyvinylidene fluoride), blocked to prevent non-specific binding, and incubated with a primary antibody sensitive for the protein of interest. Following repeated washing, the membrane is then incubated with a suitable horseradish peroxidase conjugated secondary antibody. After an appropriate incubation period, the blot is washed and chemiluminescence reagent used to detect the expression of
the protein of interest. Molecular weight markers are included in all experiments so the molecular weight of the detected proteins and sensitivity of the antibody can be confirmed.

In this thesis, immunoblotting studies were performed on using the IκBα antibody (IκBα (C-21), sc-371, Santa Cruz Biotechnology, Inc) and NF-κB p65 antibody ((C20) sc-372, Santa Cruz Biotechnology, Inc) to determine the effects of fatty acids on IκBα and p65 expression in PBMC cytosolic extracts and in THP-1 cytosolic and nuclear fractions.

2.26 Preparation of samples for polyacrylamide gel electrophoresis (PAGE): acetone precipitation of protein.

The equivalent of 50-100μg of cytosolic protein, or 10μg of nuclear protein, was pipetted into labelled minifuge tubes, 5 volumes of ice-cold acetone added and the samples stored at −20°C for at least 60 min. After this incubation period, the tubes were centrifuged at 12,000 rpm for 5 min, the acetone aspirated and the pellet allowed air-dry to remove any residual acetone. The pellet was re-suspended in 10-15 μl of sample buffer (2% w/v SDS, 25 mM Tris (pH 6.8), 10% v/v glycerol, 0.3% v/v bromophenol blue (0.4%) in ethanol, 5% v/v β-mercaptoethanol, made up to 100ml with distilled water). Minifuge lids were pierced and samples boiled at 100°C for 5 min to denature the proteins to their subunits by reducing disulfide bonds. 5μl of the Sigmamarker protein marker (Sigmamarker wide molecular weight range, Cat#M4038, Sigma, St. Louis, US) was prepared in a similar manner. After the 5 min period, the tubes were removed, centrifuged briefly (30 sec) and placed on ice prior to immediate use for SDS-PAGE electrophoresis.

2.27 SDS polyacrylamide gel electrophoresis.

Proteins in the cytosolic and nuclear extracts were separated on reducing gels using a discontinuous buffer system (Semi-dry transfer buffer: 48mM Tris base, 39mM glycine, 0.037% w/v SDS dissolved in 400ml distilled water and then 100ml methanol) as described by Laemmli (1970) and adapted by Sambrook et al, (1989). 10% acrylamide gels were prepared (Resolving gel: 6.66g 30% acrylamide, bisacrylamide mix (BDH, Poole, UK), 5.0 ml 1.5M Tris (pH 8.8), 8.23 ml distilled water, 0.1ml 10% ammonium persulphate, 10μl TEMED, Stacking gel: 1.33 ml 30% acrylamide, bisacrylamide mix, 3.05 ml 1M Tris (pH 6.8), 5.55 ml distilled water, 50μl 10% ammonium persulphate and 10μl TEMED). Electrophoresis as carried out at 25mA per gel for 1-1.5h until the migration dye
front had reached approximately 5 cm from the gel base. The electrophoresed proteins were then ready for transfer.

2.28 Western blotting.

During the SDS-PAGE stage, Biotrace polyvinylidene fluoride (PVDF) transfer membrane (0.45µm, Pall Life Sciences) of dimensions 9 x 6.5 cm was immersed first in methanol for approx 1 min and then in transfer buffer on a rotating platform for at least 30 min. The semi-dry method for transfer of electrophoresed proteins to immobilizing membranes as described by Towbin (1979) was preformed using a Sammy semi-dry blot system (Schleicher & Schuell, Germany). Whatman 3mm CHR filter papers (Cat # 3030-861) cut to 9 x 6.5 cm were saturated in transfer buffer prior to transfer. The blot sandwich was prepared (per gel) as follows: cathode, 5 sheets of soaked Whatman filter papers, the PVDF membrane on top, followed by the gel, another 5 sheets of presoaked filter papers and finally the anode. Care was taken to ensure the absence of air bubbles and to note the orientation of the gel. Electrophoresis transfer was performed at 100 mA per gel for 60 min.

2.29 Detection of antigens.

After transfer, the blot sandwich was carefully opened. The lane containing the protein marker was carefully excised with a scalpel and placed in a light-protected sterilin containing Coomassie Brilliant Blue gel stain (0.025%w/v Coomassie Brilliant Blue R, 40% methanol, 7% acetic acid). After shaking for approx. 5 min at room temperature, the markers were subsequently destained in 50% methanol: water, until desired contrast was reached. The marker was removed and allow air dry.

The remaining PVDF membrane was washed in 1X PBS twice and incubated overnight at this stage if necessary in 1X PBS. Care was taken at all times not to touch any of the membrane with hands; forceps were used at all times. The membrane was incubated in approximately 20 ml blocking buffer (5% w/v Marvel non-fat dried milk in 1X PBS) for 1 h at room temperature with agitation. After repeated washing (2/3 times) with PBS, the nitrocellulose membrane was incubated with primary antibody (IκBa; 1:1000 dilution in blocking buffer, p65 1:5000 dilution for cytoplasmic extracts, 1:2000 for nuclear extracts) for 1.5 h at room temperature with agitation. After incubation with primary antibody, the blot was washed (x 3 for 5 min) with PBS-Tween-20 (0.05% v/v). Membrane was incubated with HRP-conjugated secondary antibody (polyclonal goat anti-rabbit IgG-HRP conjugated immunoglobulins, Dako, Cambridge, UK) diluted in blocking buffer for 1 h at
room temperature with agitation. All IkBα blots were incubated with secondary antibody at a 1:1000 dilution; p65 cytoplasmic and nuclear extracts were incubated in a 1: 20,000 and 1:10,000 dilution of antibody respectively. The membrane was again rinsed with PBS-Tween (x 3 for 5 min). Detection was preformed by incubating the membrane in a 10ml solution of 1:1 Luminol enhancer solution: stable peroxidase buffer (Supersignal® West Dura, Pierce) prepared fresh and then agitated gently for 10 min at room temperature. The membrane was covered in clingfilm, transferred to autoradiography cassette and exposed to Fuji RX film for 2 min – overnight depending on the exposure required. The films were developed using CURIX 60, AGFA, Type 9462/100/140 (Agfa-Gevaert AG Munich, Germany).

2.30 Reuse of Western Blots.

To probe for a different protein or if the initial Western Blot was unsatisfactory, the blot was stripped. After washing in PBS (4 x 5 min), the blot was incubated for 30 min at 50°C in stripping buffer (62.5mM Tris pH 6.8, 100mM β-Mercaptoethanol, 2% SDS). The blot was washed for a further 6 x 5 min in PBS. The blot was then ready to use again commencing with the blocking stage in western blot detection protocol.

2.31 Principle of Electrophoretic Mobility Shift Assay (EMSA).

The electrophoretic mobility shift assay (EMSA) detects the interaction of DNA binding proteins (e.g. NF-κB) with their cognate DNA recognition sequences (e.g. κB enhancer elements) in a qualitative and quantitative manner. Briefly, the purified nuclear proteins are incubated with a 32P-radiolabelled DNA probe (in this case specific for NF-κB) followed by separation of the complexes from the free probe through a non-denaturing PAGE. The DNA-protein bound complexes migrate at a slower rate than the free probe and can be easily detected. The gels are transferred to sturdy blotting paper by drying. Exposure of the dried gel to X-Ray film at −70°C, allows for detection of the DNA-protein complexes by autoradiography. Positive controls reactions containing the cell line HUT78 were included in all of the EMSA experiments. HUT78 constitutively expresses NF-κB.

2.32 EMSA for NF-κB.

PBMC and THP-1 nuclear extract were separated on 4% polyacrylamide non-reducing gels using the ATTO gel system (ATTO, Medical Supply Corporation, Japan). 4μg protein was used for all PBMC experiments and for resting THP-1’s. Due to the
abundance of NF-κB in LPS-activated THP-1's, 2μg protein was used in these EMSA's. Gel composition involved 3.125 mL Acrylamide mix (40%, Accugel 29:1 National Diagnostics, Atlanta, Georgia, US), 2.5 mL 10X Tris borate buffer (TBE buffer: 0.6M Tris Base, 0.87M Boric acid, 20mM EDTA), 0.05g Ammonium persulphate (nuclease free), 15μl TEMED, 0.5mM DTT and 19.4 ml distilled water. The gel was allowed polymerise for 30 min at room temperature prior to electrophoresis. Binding conditions were prepared in accordance with Sen and Baltimore, (1988). Nuclear extracts (4 or 2μg) were incubated with 10, 000 cpm of the 32P-labelled NFκB for 30 min at room temperature. The DNA-protein binding reaction therefore contained 4 (2) μg protein extract, 10, 000 cpm 32P-labelled oligonucleotide probe (Perkin-Elmer Life Sciences, Inc., Boston, MA), 2 μl Poly (dl-dC) (a non specific competitor which prevents non-specific binding of proteins to radiolabelled DNA, Amersham-Pharmacia, UK), 1μl binding reaction buffer (100mM Tris pH 7.5, 1M NaCl, 40% glycerol, 10mM EDTA, 50mM DTT, 1mg/mL nuclease free BSA (Sigma, Cat#B2518) and sterile water to make up a final volume of 20 μl. The binding reaction was arrested by the addition of one tenth of a volume of gel loading dye (0.25% Bromophenol blue, 30% glycerol in sterile water) prior to loading the samples onto the gel for electrophoresis.

The DNA-protein complexes were separated on 4% polyacrylamide gels that had been pre-run in 0.5X TBE for 30 min at 80 V. Samples were loaded and the gel run at 150 V for 1-1.5 h or until the bromophenol blue dye was three-quarters way down the gel. Gels were transferred with the appropriate precautions to a piece of firm blotting paper and wrapped in cling film, dried in an automatic drier for approx 30 min at 80°C and exposed to Fuji RX film in autoradiography cassettes (Kodak, UK) with intensifying screens for 24-48 h at ~70°C. Films were developed as for Western blots but with appropriate care.

2.33 Quantitation of autoradiographic bands.
Quantitation of both EMSA and western blotting autoradiographic bands was performed with the aid of the Kodak Image Station 440cf and analysed using the Kodak 1D Analysis Software. The position of the bands was confirmed by the inclusion of suitable molecular weight markers and/or positive controls during the experiments.
Chapter Three.

The effects of different blends of isomers of conjugated linoleic acid on immune function in healthy volunteers.
3.0 Introduction.

Over the past 15 years the influence of dietary fatty acids on immune function has been extensively investigated. The majority of studies have focused on the n-6 and n-3 polyunsaturated fatty acids (PUFA). For example, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the n-3 PUFA derived from fish oils, have been reported to modulate mitogen-stimulated lymphocyte proliferation (Endres et al, 1993), cytokine production (Endres et al, 1993, Caughey et al, 1996) and eicosanoid secretion (Kelley et al, 1999). Less is known about the immuno-modulatory benefits of the ‘novel’ n-6 PUFA, conjugated linoleic acid (CLA).

CLA is a collective term referring to a mixture of geometric and positional conjugated dieonic isomers of linoleic acid (C18:2, n-6). Dairy products and ruminant meats are the natural dietary sources of CLA. CLA is the product of endogenous synthesis by the action of the enzyme Δ9 desaturase on vaccenic acid in ruminants (Griinari et al, 1998, 2000). Smaller amounts are also formed during microbial biohydrogenation of linoleic and linolenic acids (Kepler et al, 1966). Most (~80%) of the CLA in meat and dairy products is present as the cis-9, trans-11 isomer (Chin et al, 1992, Kramer et al, 1997, Parodi, 1997), however lower levels of other isomers are also present in milk (Griinari et al, 1998). Estimated average daily intake of CLA in humans is reported to range from 95 to 200 mg/day (Chin et al, 1992, Ens et al, 2001, Ritzenthaler et al, 2001). CLA can also be produced by base-catalysed isomerisation of linoleic acid (Banni & Martin, 1998), resulting in a mixture in which the predominant isomer types include the cis-9, trans-11 isomer (~35%) and a trans-10, cis-12 isomer (~35%).

Scientific research has increasingly focused on the health benefits of CLA. In vitro and animal studies have demonstrated that CLA has anti-carcinogenic (Ip et al, 1991, Banni et al, 1999), hypolipidaemic (Nicolosi et al, 1997) and anti-atherogenic properties (Lee et al, 1994, Gavino et al, 2000, Stangl, 2000). Feeding diets enriched with CLA, improved insulin sensitivity and glucose tolerance in mice (Houseknecht et al, 1998). CLA is also reported to affect body composition; mice fed a CLA-enriched diet experienced rapid weight loss and had decreased fat stores (Park et al, 1999, Gavino et al, 2000). In contrast, it has been reported that CLA induced hyperlipidaemia and insulin resistance in mice (Tsuboyama-Kasaoka et al, 2000, Clement et al, 2002). These anti-adipogenic and pro-diabetic effects have been ascribed to the trans-10, cis-12 CLA isomer (Houseknecht et al, 1998, Clement et al, 2002, Roche et al, 2002).

In addition, CLA appears to possess intrinsic immuno-modulatory properties, particularly with respect to cell-mediated immune function. In vitro studies show that
CLA enhanced porcine blood lymphocyte proliferation in response to the T-cell mitogen phytohemagglutinin (PHA), inhibited concanavalin A (Con A)-induced Interleukin (IL)-2 production and suppressed the phagocytic activity of murine macrophages (Chew et al, 1997). In vivo studies in chickens, mice and rats also demonstrate a significant increase in mitogen-stimulated lymphocyte blastogenesis following CLA supplementation (Cook et al, 1993, Miller et al, 1994, Turek et al, 1998, Hayek et al, 1999). Indeed, dietary CLA exerts specific effects on the CD8+ T-cell subset in pigs. CLA ingestion expanded CD8+ number and function and enhanced the proliferation and cytotoxic potential of these T-cells (Bassaganya-Riera et al, 2000, 2002). CLA is also reported to depress macrophage phagocytosis (Cook et al, 1993, Miller et al, 1994) and to modulate eicosanoid production, particularly PGE_{2} (Li et al, 1998, Kavanaugh et al, 1999). In addition, CLA isomeric blends have been reported to enhance IL-2 production in mice (Wong et al, 1997, Hayek et al, 1999, Yang & Cook, 2003), but reduced IL-4, IL-6 and TNFα production in Balb/C mice and in Sprague-Dawley rats (Turek et al, 1998, Yang & Cook, 2003). Conversely Kelley et al, (2002) supplemented C57BL/6N mice with a diet enriched with purified cis-9, trans-11 CLA or trans-10, cis-12 CLA for eight weeks. At the end of the study, there was no difference between the study groups with respect to the number of immune cells in the circulation, spleen or bone marrow, *ex vivo* PGE_{2} secretion and the proliferation of Con A- and LPS- stimulated splenocytes (Kelley et al, 2002). However, both of the CLA isomers increased *ex vivo* LPS stimulated TNFα and IL-6 production and decreased Con A-induced IL-4 secretion. IL-2 production in response to Con A did not differ significantly between the groups, but tended to be lower in mice fed the cis-9, trans-11 rich diet. The authors concluded that the two CLA isomers had similar effects on the immune response variables tested (Kelley et al, 2002). However, it remains unclear whether the immuno-modulatory effects observed in the aforementioned studies can be ascribed to a particular CLA isomer. In addition, it is not known whether the CLA isomers will differentially affect human immune function.

There have been relatively few studies that have investigated the effect of CLA on humans. At the outset of this study, none were published. Kelley et al, (2000, 2001a) examined the effects on immune function of supplementing the human diet with a commercial CLA isomeric mix. They reported no effect of CLA on lymphocyte proliferation, serum antibody titers, delayed type hypersensitivity (DTH), *ex vivo* LPS-stimulated secretion of PGE_{2}, LTB_{4}, IL-1β, TNFα or PHA-induced IL-2 production. Likewise, the authors found no difference in the % of T-cells producing IL-2 and IFNγ, the % monocytes secreting TNFα or the numbers of circulating white blood cells.
granulocytes, monocytes, lymphocytes (Kelley et al, 2000, 2001a). Although the authors did report an increase in CLA concentration from 0.012 to 0.97% in peripheral blood mononuclear cell (PBMC) lipids, supplementation did not significantly alter the concentration of other fatty acids. (Kelley et al, 2001a). Whilst this study was conducted in a highly controlled metabolic suite, their findings were based on a very small sample size (n=10) and the CLA blend used consisted of a heterogenous mix of CLA isomers.

Relatively little is known in relation to the efficacy of the individual CLA isomers. Kelley et al, (2002) reported no difference between the isomers with respect to murine immune function. However, the trans-10, cis-12 isomer is credited with the aforementioned hypolipidaemic and body-composition effects (West et al. 1998, Park et al. 1999), while the cis-9, trans-11 isomer is frequently assumed to be the most biologically active form of CLA (Mac Donald, 2000).

The objective of the present study was to determine whether CLA modulates the immune response in humans. This double blind placebo controlled trial investigated the effects of dietary supplementation with two blends of CLA on lymphocyte function and cytokine production in healthy free-living volunteers. The CLA blends provided different levels of the cis-9, trans-11 and trans-10, cis-12 isomers so that the relative efficiency of the individual isomers on the immune response could be determined.
3.1 Subjects and methods.

3.1.1 Experimental Design.

The study was approved by the Ethics Committee of the Federated Dublin Voluntary Hospital. Written informed consent was obtained from all volunteers before commencing the trial. The study was conducted on an outpatient basis. Fifty-five healthy volunteers participated in this study; these subjects were recruited from the personnel of Trinity College Dublin and St. James’s Hospital, Dublin. A screening blood sample ensured that all subjects conformed with the following biochemical inclusion criteria:

- fasting plasma cholesterol < 6.5 mmol/l,
- plasma triglyceride < 2.0 mmol/l,
- glucose < 110 mg/dl,
- gammaglutamyltransferase < 60 IU/L,
- haemoglobin > 11 mg/dl
- BMI < 25 Kg/m²
- < 90 min strenuous exercise per week,
- Not habitual consumers of n-3 or n-6 PUFA or other dietary supplements
- No history of any inflammatory disorder
- Not regular smokers.
- Not on prescribed medication.

A basic lifestyle and dietary questionnaire was completed to screen for individuals with excessive cheese and meat consumption (CLA intake <300 mg/day as based on their dairy produce intake). This study was double-blinded and placebo controlled. Subjects were randomly assigned to one of three intervention groups who received 3g/d total fatty acids containing either an 80:20 cis-9, trans-11: trans-10, cis-12 CLA isomer blend (80:20), a 50:50 cis-9, trans-11: trans-10, cis-12 CLA isomer blend (50:50) or linoleic acid (control). The CLA isomer blends therefore provided different amounts of the cis-9, trans-11 and trans-10, cis-12 isomers. Each volunteer was allotted 6 x 0.5 g capsules per day. Compliance was verified by measurement of plasma total fatty acid composition by gas chromatography and by conducting a capsule count.
3.1.2 Study Subjects.

The study was carried out at the Nutrition Laboratory, Trinity Centre for Health Sciences, St. Jame’s Hospital, Dublin 8. Subjects arrived at the Laboratory between 07.30 and 08.00h at weeks 0, 4 and 8 for blood sampling after a 12h overnight fast. Subjects abstained from alcohol and refrained from strenuous exercise for 24 h before the investigation. Body weight and height were recorded on both occasions. All subjects received two 4-week allocations of capsules following blood sampling at weeks 0 and 4. All unused capsules were returned at week 4 and at the end of the study.

3.1.3 Laboratory methods.

At weeks 0 and 8, 50 ml blood was collected in 10 ml Lithium Heparin vacutainers (Beckton & Dickinson, Cowley, Oxford, UK) and PBMC’s isolated as described in Section 2.1. An additional 5 ml blood was collected in additive-free vacutainers (Beckton & Dickinson, Cowley, Oxford, UK). This sera was used both as a source of autologous serum in culture media, and aliquots stored immediately at −20°C until sICAM-1 analysis by ELISA (Section 2.11). Furthermore 10ml blood was collected in EDTA tubes, plasma separated and immediately frozen for analysis of circulating LTB4 and PGE2 concentrations and for gas chromatography analysis (Sections 2.12, 2.13). Cell culture experiments were set up for lymphocyte proliferation and cytokine production assays. The mitogens PHA and Con A and the antibodies OKT3 and anti-IE were included in lymphocyte proliferation assays. PHA, LPS, OKT3 and anti-IE were used in investigations of TNFα and IL-2 activity. PHA alone was included in experiments for IL-4 secretion. Lymphocyte proliferation and cytokine production were measured as outlined in Sections 2.8 and 2.9. The gas chromatography work was completed by my colleague Dr. Enda Noone.

3.1.4 Statistical Analysis.

Results are expressed as Means ± SEM for the number of observations indicated. All statistical analysis was performed with the Apple Macintosh compatible statistical package DataDesk 4.1 (Data Description Inc., N.Y., USA). The distribution of the data for each variable was assessed and some of the variables transformed to normalize the distribution of some of the data sets. Repeated measures ANOVA was used to investigate statistical changes in biochemical parameters as a result of dietary intervention in control and those receiving the isomeric blends of CLA.
3.2 Results.

3.2.1 Capsule composition.

The composition of the supplement capsules are presented in Table 3.1. The 80:20 and 50:50 blends consisted of 67.2% and 58.8% total CLA respectively. The cis-9, trans-11 and trans-10, cis-12 isomers were present in ratios of 80:20 and 50:50 respectively. The control fatty acid, linoleic acid, contained 66.9% pure linoleate with no detectable CLA. Total compliance as assessed by pill count of returned capsules at weeks 4 and 8 was 93%. There was no significant difference in compliance between the study groups (A: 93.7%, B: 93.3% and c: 92.18%).

3.2.2 Subjects.

All fifty-five volunteers who entered the study (20 male, 35 female) completed the trial. The total study group had a mean age 31.5 (SD 9.9) yrs, mean weight 69.25 (SD 12.1) kg and mean BMI 23.9 (SD 4.3) kg/m². The characteristics of each subject group are shown in Table 3.2. There was no significant difference in body weight and BMI between the treatment groups at baseline or after dietary supplementation. No untoward side-effects of ingesting the CLA or linoleate concentrate were reported.

3.2.3 Effect of CLA on lymphocyte proliferation.

Figs. 3.1 and 3.2 present the effects of the three treatments on PHA, Con A and OKT3-induced PBMC proliferation when expressed as actual counts per minute (cpm) and as a simulation index (SI, where SI = cpm of stimulated cells/cpm unstimulated (basal) cells). Repeated measures ANOVA demonstrated that supplementation with the 80:20 CLA isomer blend group significantly (P=0.03) increased PHA-induced proliferation when expressed as cpm and as SI. The 50:50 CLA isomer blend group significantly (P=0.03) decreased Con A-stimulated proliferation when lymphocyte proliferation was expressed as SI. The control fatty acid linoleic acid had no significant effect on PHA, Con A, OKT3 or anti-IE-induced proliferation.
Table 3.1. Capsule composition of the 50:50 and 80:20 cis-9, trans-11, trans-10, cis-12 isomer blends and of the control treatment, linoleic acid.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>50:50 CLA isomer blend</th>
<th>80:20 CLA isomer blend</th>
<th>Control (linoleic acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acid composition (%):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>ND¹</td>
<td>ND¹</td>
<td>3.7</td>
</tr>
<tr>
<td>C18:0</td>
<td>ND¹</td>
<td>ND¹</td>
<td>1.3</td>
</tr>
<tr>
<td>C18:1</td>
<td>22.7</td>
<td>28.8</td>
<td>24.2</td>
</tr>
<tr>
<td>C18:2</td>
<td>ND¹</td>
<td>ND¹</td>
<td>66.9</td>
</tr>
<tr>
<td>SFA²</td>
<td>7.0</td>
<td>5.9</td>
<td>ND¹</td>
</tr>
<tr>
<td>others</td>
<td>ND¹</td>
<td>ND¹</td>
<td>3.9</td>
</tr>
<tr>
<td>CLA isomers (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total CLA</td>
<td>67.2</td>
<td>58.8</td>
<td>ND¹</td>
</tr>
<tr>
<td>cis-9, trans-11</td>
<td>31.0</td>
<td>44.9</td>
<td>ND¹</td>
</tr>
<tr>
<td>trans-10, cis-12</td>
<td>31.5</td>
<td>11.0</td>
<td>ND¹</td>
</tr>
<tr>
<td>trans-10, trans-12</td>
<td>2.4</td>
<td>1.1</td>
<td>ND¹</td>
</tr>
<tr>
<td>Other isomers</td>
<td>2.3</td>
<td>1.8</td>
<td>ND¹</td>
</tr>
</tbody>
</table>

¹Not detectable (ND).
²Denotes saturated fatty acid.
³Materials and analysis details kindly provided by Loders Croklaan BV, The Netherlands.

Table 3.2. Subject characteristics pre and post supplementation with either a 50:50 or 80:20 cis-9, trans-11: trans-10, cis-12 CLA isomer blend or linoleic acid. Values represent mean ± SEM in parenthesis.

<table>
<thead>
<tr>
<th></th>
<th>50:50 CLA isomer blend</th>
<th>80:20 CLA isomer blend</th>
<th>Linoleic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=19)</td>
<td>(n=17)</td>
<td>(n=19)</td>
</tr>
<tr>
<td>wk 0</td>
<td>wk 8</td>
<td>wk 0</td>
<td>wk 8</td>
</tr>
<tr>
<td>wk 0</td>
<td>(n=19)</td>
<td>(n=17)</td>
<td>(n=19)</td>
</tr>
<tr>
<td>wk 8</td>
<td>(n=19)</td>
<td>(n=17)</td>
<td>(n=19)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>33.8</td>
<td>28.4</td>
<td>32.2</td>
</tr>
<tr>
<td>(2.8)</td>
<td>(1.4)</td>
<td>(2.4)</td>
<td></td>
</tr>
<tr>
<td>Height (m)</td>
<td>168.8</td>
<td>158.9</td>
<td>170.9</td>
</tr>
<tr>
<td>(1.4)</td>
<td>(1.9)</td>
<td>(2.3)</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>66.2</td>
<td>66.2</td>
<td>69.4</td>
</tr>
<tr>
<td>(1.9)</td>
<td>(1.7)</td>
<td>(2.9)</td>
<td>67.9</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.2</td>
<td>23.3</td>
<td>23.3</td>
</tr>
<tr>
<td>(0.7)</td>
<td>(0.6)</td>
<td>(0.6)</td>
<td>23.4</td>
</tr>
<tr>
<td>(0.6)</td>
<td>(0.8)</td>
<td>(0.7)</td>
<td></td>
</tr>
</tbody>
</table>
Fig 3.1. Effect of supplementation with either a 50:50 or 80:20 cis-9, trans-11: trans-10, cis-12 CLA blend or linoleic acid (3g/d for 8 weeks) on lymphocyte proliferation when expressed as counts per minute (cpm). Values represent mean ± SEM. * indicates a statistical difference between group mean values pre and post-supplementation, P=0.03, 2-way ANOVA.
**Fig 3.2.** Effect of supplementation with either a 50:50 or 80:20 cis-9, trans-11: trans-10, cis-12 CLA blend or linoleic acid (3g/d for 8 weeks) on lymphocyte proliferation when expressed as a simulation index (SI), where \( SI = \frac{cpm \text{ stimulated cells}}{cpm \text{ unstimulated/basal cells}} \). Values represent mean ± SEM. * indicates a statistical difference between group mean values pre and post-supplementation, \( P=0.03 \), 2-way ANOVA.
3.2.4 Cytokine production.

Table 3.3 shows the effects of supplementation with the 50:50 CLA isomer blend, the 80:20 CLA isomer blend and linoleic acid on IL-2 production with and without the mitogen PHA and anti-CD3 monoclonal antibody OKT3. The 50:50 CLA isomer mix significantly decreased IL-2 concentrations in unstimulated cells (P=0.0027), cells challenged with OKT3 (P=0.03) and following incubation with the antibody control anti-IE (P=0.001). IL-2 concentrations in PHA-stimulated cells were significantly increased in the 50:50 CLA group (P=0.0024). In the 80:20 CLA isomer blend group IL-2 production was significantly decreased in basal (unstimulated) cells (P=0.009) but significantly increased in PHA-challenged cells (P=0.003). The control treatment (linoleic acid) significantly decreased IL-2 concentrations in unstimulated cells (P=0.006) and in cells incubated with anti-IE (P=0.001), while PHA-induced IL-2 production was significantly increased (P=0.001).

Table 3.4 shows the effect of supplementation with a 50:50 CLA blend, an 80:20 CLA blend or linoleic acid on TNFα production with and without stimulation with PHA, LPS and OKT3. PHA-induced TNFα production was significantly increased in the 50:50 CLA isomer group (P=0.002), 80:20 CLA isomer group (P=0.001) and control group (linoleic acid) (P=0.001) after the eight week supplementation period. Supplementation with either the 50:50 CLA blend, the 80:20 CLA blend or with linoleic acid (control) had no significant effect on TNFα secretion in unstimulated, LPS or OKT3 challenged cells or in cells cultured with the antibody control, anti-IE.

There was no significant treatment effect with any of the three oils on PHA-induced IL-4 production (Table 3.5).

3.2.5 Soluble adhesion molecule expression and eicosanoid production.

The effect of supplementation on sICAM-1, LTB4 and PGE2 are presented in Tables 3.6 and 3.7. Although supplementation with all three fatty acids tended to decrease circulating sICAM-1 levels, this effect was not significant. Supplementation did not affect plasma PGE2 or LTB4 concentrations.
Table 3.3. Effects of supplementation with a 50:50 cis-9, trans-11: trans-10, cis-12 CLA isomer blend, an 80:20 cis-9, trans-11: trans-10, cis-12 CLA isomer blend or linoleic acid on IL-2 production (pg/ml). Values represent mean ± SEM. * indicates a statistical difference between wk 0 and wk 8 values, P=0.02, 2 way ANOVA.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>50:50 CLA isomer blend</th>
<th>80:20 CLA isomer blend</th>
<th>Linoleic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n 19)</td>
<td>(n 17)</td>
<td>(n 19)</td>
</tr>
<tr>
<td></td>
<td>wk 0       Mean  SEM   Mean  SEM   Mean  SEM   Mean  SEM</td>
<td>wk 0       Mean  SEM   Mean  SEM   Mean  SEM</td>
<td>wk 0       Mean  SEM   Mean  SEM   Mean  SEM</td>
</tr>
<tr>
<td>Control</td>
<td>11.2   1.5       9.2*  1.5       21.7  7.17       18.5*  6.8       10.3  1.9       8.4*  2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHA</td>
<td>90.9   14.2       189.9* 36.1     69.7  17.5       226.1* 36.4     101.2 24.3       246.2* 63.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OKT3</td>
<td>32.9   7.5        18.3*  3.1       54.1  14.8       31.3  25.6       32.8 10.6       21.3  4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-IE</td>
<td>8.6    0.8        5.0*  0.5       7.7   2.8        6.6   7.6        8.1   0.7        4.7*  0.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.4. Effects of supplementation with a 50:50 cis-9, trans-11: trans-10, cis-12 CLA isomer blend, an 80:20 cis-9, trans-11, trans-10, cis-12 CLA isomer blend or linoleic acid on TNFα production (pg/ml). Values represent mean ± SEM. * indicates a statistical difference between wk 0 and wk 8 values, P=0.02, 2 way ANOVA.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>50:50 CLA isomer blend (n 19)</th>
<th>80:20 CLA isomer blend (n 17)</th>
<th>Linoleic acid (n 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wk 0 Mean SEM</td>
<td>wk 8 Mean SEM</td>
<td>wk 0 Mean SEM</td>
</tr>
<tr>
<td>Control</td>
<td>216.2 52.4</td>
<td>191.2 46.1</td>
<td>169.1 36.4</td>
</tr>
<tr>
<td>PHA</td>
<td>1293.7 128.4</td>
<td>2216.2* 245.3</td>
<td>1316.7 163.1</td>
</tr>
<tr>
<td>LPS</td>
<td>1224.8 172.0</td>
<td>1556.9 166.6</td>
<td>1743.5 322.8</td>
</tr>
<tr>
<td>OKT3</td>
<td>674.3 67.27</td>
<td>647.6 80.9</td>
<td>636.9 96.14</td>
</tr>
<tr>
<td>Anti-IE</td>
<td>254.3 69.1</td>
<td>185.5 39.8</td>
<td>138.1 45.95</td>
</tr>
</tbody>
</table>
Table 3.5. Effects of supplementation with either a 50:50 or 80:20 cis-9, trans-11: trans-10, cis-12 CLA blend, or linoleic acid (3g/d for 8 weeks) on PHA-stimulated IL-4 production (pg/ml). Values represent mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>50:50 CLA isomer blend</th>
<th>80:20 CLA isomer blend</th>
<th>Linoleic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n 19)</td>
<td>(n 17)</td>
<td>(n 19)</td>
</tr>
<tr>
<td>wk 0</td>
<td>wk 8</td>
<td>wk 0</td>
<td>wk 8</td>
</tr>
<tr>
<td>Mean</td>
<td>30.6</td>
<td>27.8</td>
<td>29.0</td>
</tr>
<tr>
<td>SEM</td>
<td>4.5</td>
<td>5.5</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Table 3.6. Effects of supplementation with either a 50:50 or 80:20 cis-9, trans-11: trans-10, cis-12 CLA blend, or linoleic acid (3g/d for 8 weeks) on sICAM-1 concentrations (pg/ml).

<table>
<thead>
<tr>
<th></th>
<th>50:50 CLA isomer blend</th>
<th>80:20 CLA isomer blend</th>
<th>Linoleic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n 19)</td>
<td>(n 17)</td>
<td>(n 19)</td>
</tr>
<tr>
<td>wk 0</td>
<td>wk 8</td>
<td>wk 0</td>
<td>wk 8</td>
</tr>
<tr>
<td>Mean</td>
<td>325.6</td>
<td>313.3</td>
<td>302.4</td>
</tr>
<tr>
<td>SEM</td>
<td>14.1</td>
<td>13.5</td>
<td>9.5</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM.
Table 3.7. Effects of supplementation with a 50:50 cis-9, trans-11: trans-10, cis-12 CLA isomer blend, an 80:20 cis-9, trans-11, trans-10, cis-12 CLA isomer blend or linoleic acid (3g/d for 8 weeks) on plasma PGE$_2$ and LTB$_4$ concentrations (pg/ml). Values represent mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>50:50 CLA isomer blend ($n=19$)</th>
<th>80:20 CLA isomer blend ($n=17$)</th>
<th>Linoleic acid ($n=19$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wk 0</td>
<td>wk 8</td>
<td>wk 0</td>
</tr>
<tr>
<td><strong>PGE$_2$</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>11.7</td>
<td>12.1</td>
<td>12.1</td>
</tr>
<tr>
<td>SEM</td>
<td>0.5</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td><strong>LTB$_4$</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>60.2</td>
<td>60.4</td>
<td>57.6</td>
</tr>
<tr>
<td>SEM</td>
<td>4.2</td>
<td>1.7</td>
<td>5.2</td>
</tr>
</tbody>
</table>
3.2.6 Fatty acid composition of total plasma lipids.

Total plasma fatty acid composition was determined a measure of compliance, hence is reflective of total plasma lipids rather than PBMC fatty acid content. The fatty acid composition of total plasma lipids are presented in Table 3.8. There was a significant increase in the level of C18:2 cis-9, trans-11 isomer in the groups who received the 50:50 CLA (P<0.001) and the 80:20 CLA isomers (P<0.0001) respectively. The relative increase in cis-9, trans-11 CLA in the CLA supplement groups was associated with a significant decrease in C18: 3, n-3 in the 50:50 CLA group (P=0.01), and C20:5, n-3 in the 80:20 CLA treatment group (P=0.02). No other significant changes in fatty acid composition in either of the CLA groups were observed. Supplementation with linoleic acid did not significantly affect the fatty acid composition of total plasma lipids. The trans-10, cis-12 CLA isomer was not detected in the week 0 total plasma lipids in this study and was only detected in some of the week 8 samples.
Table 3.8. Fatty acid composition (w/w %) of total plasma lipids as a result of dietary supplementation using isomeric blends of CLA and linoleic acid at 3g/d for 8 weeks. Values represent mean ± SEM in parenthesis. Significance difference between week 0 and week 8 *P=0.01; **P=0.02, †P=0.001, ‡P=0.0001, 2 way ANOVA.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>50:50 CLA isomer blend (n 19)</th>
<th>80:20 CLA isomer blend (n 17)</th>
<th>Control (Linoleic acid) (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wk 0</td>
<td>wk 8</td>
<td>wk 0</td>
</tr>
<tr>
<td>C16:0</td>
<td>25.4</td>
<td>24.8</td>
<td>25.1</td>
</tr>
<tr>
<td></td>
<td>(0.5)</td>
<td>(0.5)</td>
<td>(1.9)</td>
</tr>
<tr>
<td>C16:1</td>
<td>2.3</td>
<td>2.1</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>(0.2)</td>
<td>(0.2)</td>
<td>(0.9)</td>
</tr>
<tr>
<td>C18:0</td>
<td>9.2</td>
<td>8.6</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>(0.5)</td>
<td>(0.3)</td>
<td>(1.2)</td>
</tr>
<tr>
<td>C18:1</td>
<td>21.4</td>
<td>22.8</td>
<td>21.1</td>
</tr>
<tr>
<td></td>
<td>(0.9)</td>
<td>(0.6)</td>
<td>(2.3)</td>
</tr>
<tr>
<td>C18:2 n-6</td>
<td>28.5</td>
<td>27.3</td>
<td>28.8</td>
</tr>
<tr>
<td></td>
<td>(0.97)</td>
<td>(0.8)</td>
<td>(3.4)</td>
</tr>
<tr>
<td>C18:3 n-6</td>
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<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>(&lt;0.1)</td>
<td>(&lt;0.1)</td>
<td>(&lt;0.1)</td>
</tr>
<tr>
<td>C18:3 n-3</td>
<td>0.7</td>
<td>0.5*</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>(&lt;0.1)</td>
<td>(0.1)</td>
<td>(0.3)</td>
</tr>
<tr>
<td>C18:2</td>
<td>0.3</td>
<td>0.6†</td>
<td>0.4</td>
</tr>
<tr>
<td>c9,t11 CLA</td>
<td>(&lt;0.1)</td>
<td>(&lt;0.1)</td>
<td>(0.2)</td>
</tr>
<tr>
<td>C20:2</td>
<td>0.3</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>(&lt;0.1)</td>
<td>(&lt;0.1)</td>
<td>(&lt;0.1)</td>
</tr>
<tr>
<td>C20:3 n-6</td>
<td>1.5</td>
<td>1.2</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>(0.2)</td>
<td>(0.1)</td>
<td>(0.4)</td>
</tr>
<tr>
<td>C20:4 n-6</td>
<td>7.3</td>
<td>6.5</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>(0.3)</td>
<td>(0.4)</td>
<td>(1.6)</td>
</tr>
<tr>
<td>C20:5 n-3</td>
<td>1.0</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>(0.1)</td>
<td>(0.1)</td>
<td>(0.5)</td>
</tr>
<tr>
<td>C22:4 n-6</td>
<td>0.3</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>(&lt;0.1)</td>
<td>(&lt;0.1)</td>
<td>(0.3)</td>
</tr>
<tr>
<td>C22:6 n-3</td>
<td>2.3</td>
<td>2.3</td>
<td>2.2</td>
</tr>
<tr>
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<td>(0.2)</td>
<td>(0.2)</td>
<td>(0.7)</td>
</tr>
</tbody>
</table>
3.3 Discussion.

The results of the present study show that supplementation with two isomeric blends of CLA and linoleic acid modulated the human immune response. However the only significant difference between the CLA isomers and linoleic acid was in relation to lymphocyte proliferation. Our study showed that PHA-induced lymphocyte proliferation was significantly enhanced by the 80:20 CLA isomer, while the 50:50 CLA isomer blend significantly decreased Con A-induced blastogenesis, but only when expressed as a simulation index. Linoleic acid did not affect lymphocyte proliferation. Cytokine production was also affected by CLA supplementation. Nevertheless the control treatment (linoleic acid) was also associated with changes in the cytokine response. Careful consideration was given to the choice of control oil used in the present study. Whilst every fatty acid can modulate the immune system, linoleic acid was chosen since it represented the non-conjugated control for CLA. All treatments enhanced PHA-stimulated IL-2 and TNFα production but attenuated basal IL-2 production. OKT3, a T-cell stimulator significantly reduced IL-2 production in the 50:50 CLA group with no significant change in the 80:20 CLA or control (linoleic acid) groups. There was no effect of CLA or linoleic acid supplementation on PHA-induced IL-4 secretion, plasma PGE2 and LTB4 or serum sICAM-1 concentrations.

To date, the majority of studies investigating the effect of CLA on the immune system have been *in vitro* or animal studies. One animal study reported no effect of dietary CLA supplementation on lymphocyte proliferation (Kelley *et al.*, 2002). However, the remaining studies have shown that CLA enhances lymphocyte proliferation (Cook *et al.*, 1993, Miller *et al.*, 1994, Chew *et al.*, 1997, Turek *et al.*, 1998, Hayek *et al.*, 1999), which agrees with the effect of the 80:20 CLA blend on PHA-induced lymphocyte proliferation. In contrast, the 50:50 CLA isomer blend attenuated Con A induced lymphocyte proliferation, however this was only evident when results were expressed as a SI. Linoleic acid had no affect on lymphocyte proliferation. There are considerable methodological considerations with respect to the lymphocyte proliferation assay, especially when studying the effects of CLA. The aforementioned studies conducted by other groups used included foetal or bovine calf sera in their culture systems. Not only are these sera known to mask lymphocyte proliferation (Yaqoob *et al.*, 1994), they are also endogenous sources of CLA (Park & Pariza, 1998) and this may have concealed the true effect of exogenously added CLA. Our study, which utilised autologous serum rather than foetal calf serum, showed that the 80:20 CLA isomer blend rich in cis-9, trans-11 CLA, enhanced PHA-
induced blastogenesis when results were expressed as cpm and as a simulation index. The 50:50 CLA isomer blend attenuated Con A induced lymphocyte proliferation when results were expressed as a SI. Hence the apparent differences between isomeric blends may perhaps be ascribed to an isomer specific immuno-modulatory effect. This is in contrast to the study of Kelley et al. (2002) who reported no difference between the CLA isomers with respect to murine mitogen-stimulated splenocyte proliferation. In this murine study, both isomers tended to decrease LPS-stimulated proliferation. However, it is noteworthy that the purified \textit{trans}-10, \textit{cis}-12 CLA tended to reduce proliferation to a greater degree than the \textit{cis}-9, \textit{trans}-11 CLA at lower doses of mitogen (Kelley et al. 2002). In addition 5\% FBS was included in the culture medium and this may have influenced the results obtained. However, there is a paucity of information in relation to the effects of the individual CLA isomers on lymphocyte proliferation, particularly in humans and further investigations are required.

The only published CLA human intervention trial to date (Kelley et al. 2000, 2001a), reported no effect of supplementation (3.9 g/day of a CLA mix for 62d) on PHA induced lymphocyte proliferation. The CLA capsules used contained approximately 2.5g/d pure CLA, however it was a heterogenous mix of CLA isomers (22.6\% \textit{trans}-10, \textit{cis}-12, 17.6\% \textit{cis}-9, \textit{trans}-11, 23.6\% \textit{cis}-11, \textit{trans}-13, 16.6\% \textit{trans}-8, \textit{cis}-10 and 19.6\% other isomers). Linoleic acid (approx 2.8 g/d pure linoleate) was used as the control fatty acid. Nevertheless, the study consisted of a very small sample size of just 10 young healthy volunteers in the CLA group and 7 in the linoleic acid group. Volunteers resided in a metabolic suite throughout the study where they consumed a calorie controlled diet and had controlled exercise patterns. In addition all subjects consumed a basal diet supplemented with linoleic acid for 30 d prior to supplementation with CLA or linoleic acid. No wash-out period was allowed, despite the fact that the effects of fatty acids, in particular fish oils, are reported to exert immuno-modulatory effects for 10 weeks after termination of supplementation (Endres et al, 1989). Finally all subjects consumed 100 mg $\alpha$-tocopherol every five days. Although maintenance of vitamin E status is required to prevent oxidative stress, recent studies suggest co-supplementation with vitamin E may mask the immuno-modulatory effects of fatty acids (oleic acid, fish oils, evening primrose oil, coconut and safflower oils) (Yaqoob et al, 2000). Indeed vitamin E is reported to directly affect immune function (Van Tits et al, 2000).

Otherwise, the difference in lymphocyte proliferation between CLA treatment groups in our study could be a due to the different stimulators of proliferation. PHA and Con A are two polyclonal T-cell mitogens, which ultimately result in a rapid increase in
intracellular free calcium concentrations. Protein Kinase C (PKC) activation and subsequent T-cell proliferation (Altman et al., 1990, Goldsmith & Greene, 1996). Activation of the CD3-TCR complex is an inherent component of T-cell activation upon antigen recognition. (Clevers et al., 1988, Altman et al., 1990). Con A has been reported to bind to both the $\alpha$ and $\beta$ chains of the TCR and to the CD3 receptor complex in a manner analogous to the monoclonal antibody OKT3 (Van Wauwe et al., 1980, Landegren et al., 1984, LiCastro et al., 1993). PHA, also binds directly to the $\alpha$ and $\beta$ chains of the TCR (Kanellopoulos et al., 1985, Chilson et al., 1989). It has been demonstrated that PHA-P, the purified form of the lectin used in our study, induces calcium mobilisation and T-cell proliferation following activation of the CD2 pathway (O’Flynn et al., 1985, 1986), an alternative pathway of T-cell activation which can occur in the absence of the CD3-TCR complex (Leca et al., 1986, Ohno et al., 1991). Simultaneous activation of CD2 and CD3-TCR may regulate (Holter et al., 1988) or have a synergistic effect on T-cell activation (Webb et al., 1990). Although we did not investigate the fatty acid composition of PBMC lipids in our study, it is possible that the incorporation of CLA into the phospholipid bilayer of the plasma membrane may have altered the expression, number, density or orientation of the TCR/CD3 complex and CD2. Increased surface expression of the CD2 molecule would enhance CD2-mediated lymphocyte proliferation. In such an instance, PHA-P induced proliferation would be enhanced and expected to exceed that of Con A and OKT3. Wong et al. (1997) also reported a significant increase in PHA-induced proliferation whilst Con A induced proliferation was reduced in Balb/C mice following three weeks supplementation with CLA at 0.3 and 0.9% CLA. Interestingly, in the study of Kelley et al. (2001), the CLA content of PBMC’s increased from 0.012 to 0.97% post-supplementation. However, further research is necessary to investigate whether cell surface molecule expression is affected by CLA incorporation.

IL-2 promotes T-cell proliferation and differentiation (Goldsmith & Greene, 1996), B-cell differentiation (Waldman et al., 1984), monocytic and natural killer cell (NK) activity and cytokine expression (Hillman & Haas, 1995). TNF$\alpha$ is a Th1 type derived cytokine and a potent mediator of inflammation and cellular immune responses. TNF$\alpha$ influences a plethora of responses; from induction of other pro-inflammatory cytokines (e.g. IL-1 and IL-6), activation and differentiation of monocytes and macrophages, to co-ordination of normal tissue remodelling and an increased host defence (Tracey & Cerami, 1994, Hillman & Haas, 1995). Three animal feeding trials showed that CLA significantly increased Con A and PHA induced IL-2 production in mice (Wong et al. 1997, Hayek et al. 1999, Yang & Cook, 2003), while one study reported no effect of the
CLA isomers on Con A-stimulated IL-2 in C57BL/6N mice (Kelley et al. 2002). Our study would agree with the potential immuno-stimulatory effects of CLA, however similar increases were observed with linoleic acid. The effects of CLA on TNFα production were investigated in three animal studies. Dietary CLA supplementation decreased basal TNFα production but had no effect on LPS-induced TNFα secretion in rat peritoneal macrophages (Turek et al., 1998). Similarly, Yang & Cook, (2003) reported that dietary CLA decreased plasma TNFα, but did not affect LPS-induced macrophage TNFα production in Balb/C mice. Conversely, supplementation with diets rich in cis-9, trans-11 CLA or trans-10, cis-12 CLA significantly enhanced LPS-stimulated TNFα secretion in C57BL/6N mice (Kelley et al., 2002). In the aforementioned human study of Kelley et al. (2000, 2001), the authors reported no significant effect of CLA supplementation on ex vivo LPS-stimulated secretion of IL-1β, TNFα, PHA-induced IL-2, the % of T-cells producing IL-2 and IFNγ or the % monocytes secreting TNFα. Our results show that both CLA and linoleic acid enhanced the capacity of PHA-stimulated PBMC’s to secrete IL-2 and TNFα, which is suggestive of enhanced cell-mediated immunity. This increase in TNFα production concurs with the murine study of Kelley et al. (2002). In contrast, the 50:50 CLA isomer blend significantly reduced OKT3 stimulated IL-2 expression; OKT3 treatment also moderately decreased lymphocyte proliferation and TNFα secretion in this group only. OKT3 is a monoclonal antibody, which binds specifically to the CD3/TCR complex hence it may be more representative of the inflammatory T-cell response in vivo (Van Wauwe et al., 1980, Meuer et al., 1984). This may suggest that the 50:50 CLA blend attenuates the immune response, however further human trials using purer forms of the CLA isomers are required. Both isomeric blends significantly depressed IL-2 secretion in basal/ unstimulated and anti-IE treated cells. Anti-IE is an irrelevant isotypic monoclonal antibody, which prevents proliferation and was used in this cell culture system as a negative control for the OKT3 antibody. We are unsure why the concentrations of IL-2 in anti-IE treated cells decreased in both the 50:50 and linoleic acid groups, however it would appear to mirror the reduction in IL-2 observed in both these study groups in basal/unstimulated cells. This reduction may reflect the down-regulation of the inflammatory response, which has been associated with diets rich in n-6 PUFA (Calder, 1998).

IL-4 production is indicative of Th2-like phenotype or humoral immune response; it also antagonises the Th1 T-cell response. IL-4 is principally produced by activated CD4+ helper T-cells, mast-cells (Milanese et al., 1982) and by PHA-stimulated T-cells
(Gearing, 1996). It promotes the growth and differentiation of B- and T-cells, antibody production and mediates the regulatory effects on macrophages (Puri & Siegel, 1993). Constitutive concentrations are much lower than either IL-2 or TNFα. Yang & Cook, (2003) showed that a CLA-enriched diet decreased Con A-induced splenocyte IL-4 secretion in Balb/C mice. Similarly, dietary supplementation with purified cis-9, trans-11 CLA or trans-10, cis-12 CLA decreased Con A-stimulated IL-4 production in C57BL/6N mice (Kelley et al. 2002). However, neither linoleic acid nor CLA supplementation had a significant effect on PHA-induced IL-4 production in this human study.

Intercellular adhesion molecule-1 (ICAM-1, CD54), a transmembrane adhesion molecule expressed on endothelial cells, polymorphonuclear cells and fibroblasts (Kevil & Bullard, 1999). ICAM-1 expression enhances leukocyte adhesion and transendothelial migration (Meager, 1999). Over-expression plays a role in the inflammatory component of atherosclerosis. sICAM-1 is the product of proteolytic cleavage of the extra-cellular region of leukocyte ICAM-1 (Ghaisas et al. 1997). It has been demonstrated that sICAM-1 is elevated in patients with coronary vascular disease (CVD) (Ridker et al. 1998). sICAM-1 is postulated as a useful monitor of disease activity (Gearing & Newman, 1993) and an easily available molecular marker for early atherosclerosis (Ghaisas et al. 1997). We investigated the effect of CLA on sICAM-1 expression due to its speculative properties as potential indicator of inflammation and future atherosclerotic risk (Gearing & Newman, 1993, Ridker et al. 1998). There was no effect of CLA or linoleic acid supplementation on circulating sICAM-1 concentrations in this study group who were young, normolipidaemic non-smokers and had no history of inflammatory conditions. The effect of CLA and sICAM-1 expression should be re-assessed in high CVD risk groups with elevated basal levels of sICAM-1.

PGE_2 and LTB_4 are arachidonic acid (C20:4 n-6; AA) derived metabolites that are members of the eicosanoid family of local acting hormones and lipid mediators of inflammation (Goodwin & Cueppens, 1983). PGE_2 inhibits lymphocyte proliferation and IL-2 secretion, whereas LTB_4 stimulates lymphocytes at low concentrations but is inhibitory at higher concentrations (Kinsella et al. 1990). Dietary strategies, involving PUFA, can alter immune cell membrane phospholipid composition and affect PGE_2 and LTB_4 production (Sperling et al. 1998). Although CLA lowered serum PGE_2 and ex vivo splenic LTB_4 expression in rats (Sugano et al. 1998), CLA supplementation did not affect ex vivo LPS-stimulated secretion of PGE_2, LTB_4 in the human trial of Kelley et al (2001a). Similarly, no difference in PGE_2 secretion was observed in mice fed diets rich in the purified cis-9, trans-11 or trans-10, cis-12 CLA isomers (Kelley et al. 2002). We found no
significant effect of supplementation with either CLA blend or linoleic acid on plasma PGE$_2$ and LTB$_4$ concentrations. Whilst PBMC phospholipid fatty acid composition would be the most suitable tool to investigate the effect of CLA supplementation on the fatty acid precursors of eicosanoid metabolism, it is also interesting to note that plasma AA levels (w/w %) were not significantly altered by CLA supplementation in our study. Hence it is probable that PBMC AA levels were not affected and this may account for the lack of observed effect with respect to PGE$_2$ and LTB$_4$.

Plasma fatty acid composition was used in this study to check compliance to the CLA supplement. There was a significant increase of 90% (P< 0.001) and 87% (P<0.001) in the cis-9, trans-11 isomer was observed in total plasma lipids in both the 80:20 and 50:50 groups, which would confirm compliance. However, the cis-9, trans-11 isomer composed <1% of total fatty acids in total plasma lipid fatty acid composition. The trans-10, cis-12 isomer was not detected in the week 0 total plasma lipids in this study and was only detected in some of the week 8 samples. It would appear that the trans-10, cis-12 isomer is not incorporated efficiently into total plasma lipids. It has been reported that the trans-10, cis-12 isomer is catabolised into C20:4 5,8,12,14 and C20:3 8,12,14 via desaturation and elongation pathways, metabolites which cannot be detected by gas chromatography (Sebedio et al. 1997). Martin et al. (2000) hypothesised that the trans-10, cis-12 isomer is more easily oxidised due to its structure, thereby allowing it to bypass a number of rate limiting steps in the peroxisomal β-oxidation pathway and making it more difficult to detect this isomer in plasma.

In conclusion, this study highlighted CLA as a potential modulator of the immune response. However, it did not differ dramatically in its effects from linoleic acid. Clearly several areas need clarification and further research using purer forms of the CLA isomers is needed before the full effects and therapeutic potential of CLA as an immuno-modulatory substance can be evaluated.
Chapter Four.

The effects of fatty acids, including CLA, on cytokine gene expression and protein concentration in human peripheral blood mononuclear cells.
4.0 Introduction.

There is an abundance of experimental evidence indicating that the concentration and type of dietary fat can alter immune response. However, the majority of in vitro studies have used animal models rather than human peripheral blood mononuclear cells (PBMC’s). The advent of sensitive new techniques (e.g. Taqman RT-PCR), allows us to examine the mechanisms of action of fatty acids. In particular, we can investigate whether fatty acids can affect the transcriptional and translational regulation of proteins involved in immune cell responses (e.g. cytokines).

T-cell activation is characterised by a series of highly organised interactions involving antigens, co-stimulatory molecules and intra-cellular signalling events. Briefly, antigen presented in the context of an antigen presenting cell (APC), binds to the T-cell receptor (TCR): CD3 complex and initiates a series of intracellular signalling events involving the protein kinases (e.g. Lck, Fyn, ZAP-70, MAPK), membrane proteins (e.g. LAT), adapter molecules (e.g. Grb2) and enzymes (e.g. PLCγ1, PI-3K and PKC). Ultimately, this signalling cascade results in the activation of transcription factors including NF-AT, AP-1 and NF-κB and hence regulates the transcription of a particular set of genes (see chapter 1 for review). Interactions between costimulatory molecules (e.g. CD4/CD8, CD28, ICOS, CD40 etc.) on both the APC and T-cell surface are essential for T-cell activation and cytokine secretion (e.g. IL-2). Indeed expression of the IL-2 receptor α (IL-2Ra, CD25) and the secretion of IL-2 are critical in generating intracellular signals to drive the T-lymphocyte through the cell cycle (Mills et al, 1993).

Cytokines and eicosanoids represent critical signalling networks and feedback loops between cells of this immune system. Investigations of the effects of fatty acids on cytokine production generally focus on cytokines secreted by CD4αβ+ T-lymphocytes, pro-inflammatory cytokines (e.g. TNFa, IL-1β and IL-6), or on the eicosanoids PGE2 or LTB4. Antigen-naive T-cells expressing the CD4αβ receptors are termed precursor of T-helper cells (Thp, Seder & Paul, 1994) and upon exposure to antigen in the context of an APC can undergo differentiation to an uncommitted cell termed Th0 (Bucy et al, 1995, Zhai et al, 1999). The presence of particular cytokines dictates how this Th0 T-cell responds and is presented in Fig 4.1. Th0 T-cells presented with antigen in the presence of IL-4 results in the formation of a primed Th2 T-lymphocyte necessary for immune cell responses such as B-cell activation, antibody production and allergic reactions (Nelms et al, 1999). On the contrary exposure to antigen in the presence of IL-12 leads to the formation of Th-1 cells, which are involved in inflammation, graft rejection and
Fig 4.1. Brief schematic illustrating the differentiation pathway of CD4αβ+ T-helper lymphocytes.
delayed-type hypersensitivity (Hsieh et al., 1993). Th-2 cells secrete IL-4, IL-5 and IL-13, whereas Th-1 cells secrete IFN-γ, IL-2 and TGFβ. The nature of the T-helper cell response can be classified as Th1 or Th2 according to the cytokine profile they produce following antigenic stimulation (Seder & Paul, 1994). Negative feedback exists between the two T-helper subsets. However, Thp/Th0 cells are capable of producing IL-2 (Dutton et al., 1998), while both Th-1 and Th-2 human T-cell subsets secrete IL-10 (Zhai et al., 1999).

TNFα is the first cytokine to be released in response to bacterial endotoxin. It is produced by macrophages and monocytes and to a lesser extent, lymphocytes (Rock & Lowry, 1991). TNFα activates neutrophils, T- and B- cells, induces other pro-inflammatory cytokines (e.g. IL-1β and IL-6) and mediates the systemic effects of inflammation including fever and synthesis of acute phase proteins (Tracey & Cerami, 1994). The eicosanoid PGE₂ is derived from the fatty acid arachidonic acid, and is conferred with anti- and pro-inflammatory effects. It is credited with inducing fever, pain, oedema and vascular dilation but has also been shown to inhibit lymphocyte proliferation and cytokine production (including IL-1, TNFα) (Tilley et al., 2001).

Ex vivo studies show that high-fat diets or diets rich in n-3 PUFA can inhibit mitogen induced lymphocyte proliferation (Virella et al., 1991, Brouard & Pascaud, 1993, Khalfoun et al., 1996a, Purasiri et al., 1997). Indeed 100μM fatty acids added to human peripheral blood mononuclear cells (PBMC’s) in vitro was found to significantly reduce Con A-induced lymphocyte proliferation in the order of eicosapentaenoic acid (EPA) > arachidonic acid (AA) > docosahexaenoic acid (DHA) > α-linolenic acid (ALA) > stearic acid > linoleic acid (Calder & Newsholme, 1992a). Studies in rat splenic and cervical lymph node lymphocytes revealed similar patterns of inhibition (Calder et al., 1991, Calder & Newsholme, 1992b). Palmitic acid inhibited proliferation in rat lymph node lymphocytes (Calder & Newsholme, 1992a) but not in human PBMC’s or in rat splenocytes (Calder et al., 1991, Calder & Newsholme, 1992b). In addition, oleic acid did not inhibit proliferation of human PBMC’s (Calder et al., 1991, Calder & Newsholme, 1992). An in vitro study with a conjugated linoleic acid (CLA) isomeric blend (50: 50 cis-9, trans-11 CLA, trans-11, cis-12 CLA) reported a dose-dependent increase in porcine PHA-stimulated lymphocyte proliferation (Chew et al., 1997).

Many in vitro studies looking the effects of fatty acids on immune function have included an analysis of cytokine production. Once again, most of these investigations have examined the effect of the n-3 fatty acids (ALA, EPA and DHA) on cytokine production and have focused on the lymphokine IL-2. In vitro cultures rich in these fatty acids

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decreased mitogen-stimulated IL-2 secretion in human blood (Das, 1994, Devi & Das, 1994, Purasiri et al, 1997, Santoli & Zurier, 1989, Calder & Newsholme, 1992a). Calder & Newsholme (1992ab) found that PBMC’s and rat lymph node lymphocytes incubated for 24 or 48 h with DHA, EPA, ALA, arachidonic acid, linoleic acid and oleic acid in vitro, and subsequently stimulated with Con A, secreted significantly less IL-2 than control cells. Palmitic acid and stearic acid were without effect in these bioassays (Calder & Newsholme, 1992). The decrease in IL-2 in the rat lymphocytes was accompanied by a reduction in the expression of the transferrin receptor (important in IL-2 signalling), but not of the IL-2 R (Calder & Newsholme, 1992b). CLA decreased Con A-induced IL-2 production in porcine lymphocytes in vitro (Chew et al, 1997).

However, there is a paucity of information with respect to the effect of fatty acids on cytokines other than IL-2 (both at mRNA and protein level) in vitro. Toborek et al (2002) reported olive oil to decrease TNFα mRNA expression in a HUVEC cell line, while murine RAW 264.7 macrophages cultured in an EPA-rich medium expressed significantly less TNFα mRNA and produced less TNFα (Lo et al, 1999a, Novak et al, 2003). Yang & Cook, (2003) incubated RAW 264.7 macrophages with purified cis-9, trans-11 CLA, trans-10, cis-12 CLA or an isomeric blend for 24 h. The cis-9, trans-11 CLA isomer caused a dose-dependent decrease in LPS-stimulated TNFα production. The CLA blend also decreased TNFα secretion, while the trans-10, cis-12 CLA isomer was least effective (Yang & Cook, 2003). Unfortunately, the authors did not investigate mRNA expression in this research paper. The remainder of studies have involved animal feeding trials whereby n-3 PUFA have been found to decrease IL-2Rα mRNA in C57Bl/6 mice (Jolly et al, 1998), IL-1βmRNA in Balb/C mice (Robinson et al, 1996) and IL-12p40 mRNA and IFN-γ mRNA in CBH/Hen mice infected with Listeria monocytogenes (Fritsche et al, 1999). Wallace et al, (2001) fed C57Bl/6 mice a high-fat diet or a low fat diet rich in coconut oil, safflower oil or fish oil for six weeks. A non-significant decrease in Con A –induced splenic lymphocyte IL-2, IL-4 and IFN-γ mRNA was observed following the fish oil-rich diet. With respect to cytokine secretion, the safflower oil and fish oil diets decreased Con A-induced IFN-γ whereas the coconut oil-rich diet enhanced IL-2 production. IL-4 was not affected (Wallace et al, 2001). As an adjunct to this study, murine splenic lymphocytes were incubated with exogenous fatty acids in vitro. Stearic, oleic and linoleic acids were all found to significantly decrease Con A-induced IL-2 secretion after a 48 h incubation period relative to control cells (no fatty acid added). Palmitic acid was without effect (Wallace et al, 2001).
Finally, *in vitro* studies with EPA and DHA reported a decrease in lipopolysaccharide (LPS) or calcium ionophore stimulated PGE$_2$ in RAW 264.7 macrophages and human endothelial cells (Lo *et al.*, 1999b, Urquhart *et al.*, 2001), but not in human T- cells (Santoli *et al.*, 1990). In the study of Calder *et al.*, (1991), PGE$_2$ levels were measured in the culture media of rat splenic lymphocytes cultured with various fatty acids. PGE$_2$ concentrations were similar between the various fatty acids treatments, however a significant increase in PGE$_2$ secretion was noted in cells cultured with arachidonic acid. (Calder *et al.*, 1991). CLA isomeric blends are reported to decrease PGE$_2$ secretion in cultured keratinocytes (Liu & Belury, 1997, 1998), but not in MCF-7 breast cancer cells (Park *et al.*, 2000b).

The aim of this study was to investigate the effects of various saturated fatty acids (SFA) and n-6 PUFA, including the *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA isomers, on mitogen-stimulated lymphocyte proliferation, cytokine secretion and PGE$_2$ production. In particular we hoped to ascertain whether the immuno-modulatory effects of fatty acids were evident at the level of mRNA and protein.
4.1 Materials and methods.

4.1.1 Cell culture and experimental design.

Human peripheral blood mononuclear cells (PBMC’s) were isolated from whole blood donated either by healthy human volunteers or from ‘leukocyte-rich, buffy coat’ preparations donated by the Irish Blood Transfusion Services Board as outlined in Sections 2.0 and 2.1. PBMC’s were cultured in RPMI supplemented with 10% FCS, glutamine and penicillin-streptomycin. The density of PBMC’s plated varied with each particular experiment. (Section 2.7). In general, the fatty acids (100µM) were added to culture systems at the time of plating and culture continued for a further 48 h (or 72 h depending on assay) to allow cellular incorporation of the fatty acids, particularly into the cell membrane. At the end of this period, PHA (10µg/ml) was added to the experiments and culture continued for the appropriate amount of time depending on the assay of interest. Fatty acids (100µM) investigated in all experiments included:

- Cis-9, trans-11 CLA.
- Trans-10, cis-12 CLA.
- Linoleic acid.
- Palmitic acid.
- Stearic acid.
- Oleic acid.

Controls were established containing cells alone and DMSO in a concentration equivalent to that present in all other treatments (<0.1%).

4.1.2 In vitro lymphocyte proliferation assays.

Lymphocyte proliferation assays were performed as outlined in Section 2.8. Fatty acids (100µM) and PHA (10 µg/ml) were added to the PBMC’s (2 x 10^5 cells/well) at the time of plating and cultured for 72 h at 37°C, 5% CO_2, 95% humidity. Lymphocyte proliferation was assessed by \(^{3}\text{H}\) thymidine uptake during the last 18 h of the culture period and radiolabel uptake assessed by a liquid scintillation counting system (Section 2.8). Six independent experiments were conducted.

4.1.3 Cytokine protein assays (ELISA: IL-4, IL-2 and TNFα).

5 x 10^6 PBMC’s were plated with fatty acids for 48 h, PHA (10µg/ml) was added and culture continued for a further 24 h. After this time the supernatants were collected by centrifugation, samples aliquoted and stored at -70°C until analysis. IL-2, IL-4 and TNFα
concentrations, (pg/ml), were quantified by commercial ELISA as outlined in sections 2.9. Six independent experiments were conducted. DMSO did not significantly affect cytokine production hence is omitted from the analysis.

4.1.4 Cytokine mRNA assays (RT-PCR: IL-4, IL-2 and TNFα).

A second culture system was established with the same cells as for the protein experiments. This second set of cells was used for determination of cytokine mRNA expression. Briefly, 5 x 10^6 PBMC’s were plated with fatty acids for 48 h, PHA (10μg/ml) was added and culture continued for a further 4 h (Barth et al, 2000, Stordeur et al, 2002). RNA was extracted as per section 2.15 and quantitated spectrophotometrically (Section 2.16). The integrity of the RNA was assessed by RNA agarose gel electrophoresis (Section 2.17). RNA was DNase treated and subsequently reverse transcribed to synthesise cDNA (Sections 2.19 and 2.20). Taqman® RT-PCR was preformed for IL-2, IL-4 and TNFα using pre-developed Taqman® Assay Reagent Kits for gene expression quantification. GAPDH was used as the housekeeping gene. Results are expressed normalised to GAPDH and relative to control experiment (i.e. cells and PHA; no fatty acids), (See Section 2.21) Six independent experiments were carried out. DMSO did not significantly affect cytokine mRNA expression, hence is omitted from the analysis.

4.1.5 Eicosanoid (PGE$_2$) assays.

Aliquots of the supernatants from section 4.1.5 were stored at −20°C for analysis of PGE$_2$ concentration by commercial ELISA (Section 2.12). Six independent experiments were preformed.

4.1.6 MTT assay.

5 x 10^5 PBMC’s were cultured in the presence and absence of all fatty acids at 50, 100, 150 and 200μM for 48 h prior to conducting the MTT assay. The MTT assay was performed as per section 2.14 and was used to assess cytotoxicity and cellular viability (detailed in section 2.14). Results are expressed as a % viability of control cells. Six independent experiments were performed.

4.1.7 Gas chromatography.

5 x 10^5 PBMC’s were incubated with fatty acids for 48 h prior to addition of PHA (10μg/ml). Culture was continued for a further 24 h. Lipid extraction was performed immediately as outlined in Section 2.13. Methyl esters were prepared and gas-liquid
chromatography was performed. Spiked samples were included to identify all the fatty acids and fatty acid compositions were calculated as a percentage of total fatty acids (Section 2.13). Six independent experiments were conducted.

4.1.8 Statistical Analysis.

Results are expressed as Mean ± SEM for the number of observations indicated. Statistical analysis was performed with the statistical package Data Desk 6.0 (Data Description Inc., N.Y., USA). The distribution of the data for each variable was assessed. If required variables were transformed to normalise the distribution of the data. Multiple comparisons were preformed by one-way-ANOVA. If ANOVA highlighted significant intergroup differences, individual differences were subsequently examined by Fishers least significant difference test.
4.2 Results.

4.2.1 Lymphocyte Proliferation.

Fig 4.2 illustrates the effects of fatty acids on lymphocyte proliferation. The saturated fatty acid stearic acid significantly decreased (P<0.001) lymphocyte proliferation relative to control cells (PHA alone) and all other fatty acid treatments. However there was no difference in proliferation between any of the other fatty acids.

Fig 4.2. The effect of fatty acids on lymphocyte proliferation in vitro. Values represent Mean ± SEM for 6 independent observations. Statistically significant inhibition between stearic acids and other treatments: *P>0.001, one way ANOVA with LSD. PHA: phytohaemagglutinin, DMSO: dimethyl sulfoxide, c9t11 CLA: cis-9, trans-11 CLA, t10c12 CLA: trans-10, cis-12 CLA, LA: linoleic acid, PA: palmitic acid, SA: stearic acid, OA: oleic acid.
4.2.2 Cytokine ELISA data (IL-4, IL-2 and TNFα).

The influence of fatty acids on IL-4, IL-2 and TNFα protein expression is presented in Table 4.1. For IL-4, palmitic acid alone caused a significant (P<0.05) increase in IL-4 protein concentrations in cell supernatant. Linoleic, stearic and oleic acids tended to decrease IL-4 secretion compared to cells treated with PHA alone, however these reductions were not significant (-10%, -19% and -21% respectively). On the other hand, cis-9, trans-11 CLA, trans-10, cis-12 CLA, linoleic acid and oleic acid all significantly (P<0.05) decreased IL-2 secretion compared to control cells. Oleic acid was most effective at decreasing IL-2 secretion (-48%). Whilst palmitic acid significantly increased TNFα secretion compared to oleic acid (P<0.05), none of the other fatty acids significantly affected the production of this cytokine.

4.2.3 Cytokine mRNA expression (IL-4, IL-2 and TNFα).

Table 4.2 presents the effects culturing human PBMC’s with a range of fatty acids on IL-4, IL-2 and TNFα mRNA expression. The cis-9, trans-10 CLA isomer, oleic acid and stearic acid all significantly (P<0.05) decreased IL-4 mRNA expression in PBMC’s compared to the control cells and the cells treated with the other fatty acids. However, there was no difference between cis-9, trans-11 CLA, stearic and oleic acids. Trans-10, cis-12 CLA tended to decrease IL-4 mRNA expression (-37%) relative to control cells (i.e. cells treated with PHA alone). Nevertheless this change did not reach significance (P=0.053). Cis-9, trans-11 CLA isomer and stearic acid significantly (P<0.05) decreased IL-2 mRNA expression relative to all other treatments. Again, the trans-10, cis-12 CLA tended to decrease IL-2 mRNA expression (-36%), as did linoleic and oleic acids (by -42 and -48% of control cells respectively), but these changes did not reach significance. Conversely, palmitic acid significantly (P<0.05) increased IL-2 mRNA expression relative to all other treatments. Palmitic acid alone affected TNFα mRNA expression, significantly (P<0.05) increasing TNFα mRNA compared to all other treatments.
Table 4.1. The effect of fatty acids on IL-4, IL-2 and TNFα protein expression in human PBMC's. Values represent Mean ± SEM for 6 independent observations. abc Mean values not sharing a common superscript letter were significantly different: P<0.05. PHA: phytohaemagglutinin, c9t11 CLA: cis-9, trans-11 CLA, t10c12 CLA: trans-10, cis-12 CLA, LA: linoleic acid, PA: palmitic acid, SA: stearic acid, OA: oleic acid.

<table>
<thead>
<tr>
<th></th>
<th>IL-4 (pg/ml)</th>
<th>IL-2 (pg/ml)</th>
<th>TNFα (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells &amp; PHA</td>
<td>109 (^a)</td>
<td>2417 (^a)</td>
<td>2519 (^{ab})</td>
</tr>
<tr>
<td>c9t11 CLA</td>
<td>108 (^a)</td>
<td>1619 (^b)</td>
<td>2354 (^{ab})</td>
</tr>
<tr>
<td>t10c12 CLA</td>
<td>105 (^a)</td>
<td>1607 (^b)</td>
<td>2443 (^{ab})</td>
</tr>
<tr>
<td>LA</td>
<td>99 (^a)</td>
<td>1662 (^b)</td>
<td>2623 (^{ab})</td>
</tr>
<tr>
<td>PA</td>
<td>150 (^b)</td>
<td>2307 (^a)</td>
<td>3166 (^{b})</td>
</tr>
<tr>
<td>SA</td>
<td>90 (^a)</td>
<td>1699 (^a)</td>
<td>2371 (^{ab})</td>
</tr>
<tr>
<td>OA</td>
<td>88 (^a)</td>
<td>1247 (^b)</td>
<td>1969 (^{ac})</td>
</tr>
</tbody>
</table>

Table 4.2. The effect of fatty acids on cytokine (IL-4, IL-2 and TNFα) mRNA expression in human PBMC's. Values represent Mean ± SEM for 6 independent observations. abc Mean values not sharing a common superscript letter were significantly different: P<0.05. PHA: phytohaemagglutinin, c9t11 CLA: cis-9, trans-11 CLA, t10c12 CLA: trans-10, cis-12 CLA, LA: linoleic acid, PA: palmitic acid, SA: stearic acid, OA: oleic acid.

<table>
<thead>
<tr>
<th></th>
<th>IL-4 : GAPDH</th>
<th>IL-2 : GAPDH</th>
<th>TNFα : GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells &amp; PHA</td>
<td>1.00 (^a)</td>
<td>1.00 (^a)</td>
<td>1.00 (^a)</td>
</tr>
<tr>
<td>c9t11 CLA</td>
<td>0.41 (^b)</td>
<td>0.50 (^b)</td>
<td>1.43 (^a)</td>
</tr>
<tr>
<td>t10c12 CLA</td>
<td>0.63 (^a)</td>
<td>0.64 (^a)</td>
<td>1.10 (^a)</td>
</tr>
<tr>
<td>LA</td>
<td>1.04 (^a)</td>
<td>0.58 (^a)</td>
<td>1.01 (^a)</td>
</tr>
<tr>
<td>PA</td>
<td>1.06 (^a)</td>
<td>1.29 (^c)</td>
<td>2.13 (^b)</td>
</tr>
<tr>
<td>SA</td>
<td>0.38 (^b)</td>
<td>0.51 (^b)</td>
<td>0.88 (^a)</td>
</tr>
<tr>
<td>OA</td>
<td>0.43 (^b)</td>
<td>0.52 (^a)</td>
<td>0.94 (^a)</td>
</tr>
</tbody>
</table>
4.2.4 PGE$_2$ secretion.

None of the fatty acids tested significantly affected PGE$_2$ concentrations in cell supernatants. Stearic acid was omitted due to space constraints on the ELISA plate.

**Fig 4.3.** Fatty acids do not influence PGE$_2$ secretion by PBMC's *in vitro*. Values represent Mean ± SEM for 6 independent observations. PHA: phytohemagglutinin, c9t11 CLA: *cis*-9, *trans*-11 CLA, t10c12 CLA: *trans*-10, *cis*-12 CLA, LA: linoleic acid, PA: palmitic acid, OA: oleic acid.
4.2.5 MTT assays.

At 100µM none of the fatty acids tested significantly affected cell viability relative to control cells. (fig 4.4). The dose response effect of fatty acids on cell viability is presented in fig 4.5. It showed that fatty acid concentrations up to 200µM did not adversely affect % cell viability. Nevertheless, high concentrations of vehicle (DMSO) >150µM did significantly (P<0.05) reduce % cell viability.

Fig 4.4. The effect of fatty acids (100µM) on cell viability (% control cells). Values represent Mean ± SEM for 6 independent observations. DMSO: dimethyl sulfoxide, c9t11 CLA: cis-9, trans-11 CLA, t10c12 CLA: trans-10, cis-12 CLA, LA: linoleic acid, PA: palmitic acid, SA: stearic acid, OA: oleic acid.
Fig 4.5. Dose response study investigating the effects of fatty acids (and vehicle control, DMSO) on % cell viability. Values represent Mean ± SEM for 6 independent observations. Statistically significant inhibition between palmitic and stearic acids and other treatments; *P>0.001, one way ANOVA with LSD. DMSO: dimethyl sulfoxide, c9t11 CLA: cis-9, trans-11 CLA, t10c12 CLA: trans-10, cis-12 CLA, LA: linoleic acid, PA: palmitic acid, SA: stearic acid, OA: oleic acid.
4.2.6 Gas chromatography

PBMC fatty acid composition was determined by gas-liquid chromatography to ensure cellular incorporation of the fatty acids. Table 4.3 details the total lipid fatty acid composition (% w/w) of PBMC's incubated with cis-9, trans-11 CLA, trans-10, cis-12 CLA, linoleic acid, palmitic acid, stearic acid or oleic acid for 48 h and subsequently stimulated with PHA for 24 h.

Cis-9, trans-11 CLA supplementation was associated with a significant (P<0.001) increase in this fatty acid compared to control (cells treated with PHA alone) and the other fatty acid treatments. This treatment was also associated with a significant reduction in the levels of C18:0 (P<0.05), C18:1 (P<0.01), C18:2 n-6 (P<0.01), C18:3 n-3 (P<0.05), C20:3 n-6 (P<0.01), C20:5 n-3 (P<0.001) and C22:6 n-3 (P<0.01) compared to controls.

PBMC's incubated with the trans-10, cis-12 CLA isomer contained significantly (P<0.001) more of this fatty acid relative to control cells and the other treatments. Incubation with this CLA isomer significantly decreased the amounts of C18:0 (P<0.05), C18:1 (P<0.01), C18:2 n-6 (P<0.05), C20:3 n-6 (P<0.001), C20:5 n-3 (P<0.001) and C22:6 n-3 (P<0.001) in the total lipid fatty acid composition compared to controls.

Linoleic acid treatment was associated with a significant (P<0.001) enrichment of this fatty acid compared to control PBMC's and those treated with the other fatty acids. Linoleic acid supplementation significantly reduced the amounts of C18:0 (P<0.05), C18:1 (P<0.01), C18:3 n-3 (P<0.05), C20:3 n-6 (P<0.01), C20:5 n-3 (P<0.001) and C22:6 n-3 (P<0.01) relative to controls.

In a similar fashion palmitic acid supplementation significantly (P<0.001) increased the proportion of this fatty acid in the PBMC total lipid fraction compared to control and the other fatty acid treatments. This increase was accompanied by a significant decrease in C18:0 (P<0.001), C18:1 (P<0.001), C18:2 n-6 (P<0.001), C18:3 n-3 (P<0.01), C20:3 n-6 (P<0.001), C20:5 n-3 (P<0.001) and C22:6 n-3 (P<0.001) relative to control.

Treatment with stearic acid significantly enhanced the concentration of this fatty acid compared to the other fatty acid treatments (P<0.001), but not to control cells. Nevertheless, stearic acid supplementation significantly decreased the amount of C18:2 n-6 (P<0.001), C18:2 110e12 CLA (P<0.001), C20:3 n-6 (P<0.001), C20:5 n-3 (P<0.001) and C22:6 n-3 (P<0.001) compared to control cells.

Finally oleic acid supplementation was associated with a significant (P<0.001) enrichment of this fatty acid compared to controls and the other fatty acid treatments. Supplementation with this fatty acid was accompanied by a significant decrease in C18:0 (P<0.001), C18:2 n-6 (P<0.05), C20:3 n-6 (P<0.01), C20:5 n-3 and C22:6 n-3 (P<0.001).
compared to controls. C18:2 c9t11 CLA and C18:2 t10c12 CLA were not detected in these oleic acid treated cells.

Table 4.4 outlines a summary of the fatty acid composition of the total lipid fraction of PBMC's cultured in the presence of fatty acids. As would be expected, the ratio of saturated fatty acids (SFA): unsaturated fatty acids (USFA) was significantly increased by palmitic acid (P<0.001) and stearic acid (P<0.05) relative to control cells and the other fatty acid treatments. In addition, the proportion of n-6/n-3 fatty acids were significantly (P<0.001) increased following culture with cis-9, trans-11 CLA, trans-11, cis-12 CLA and linoleic acid compared with cells incubated with PHA alone or with palmitic and stearic acid.
Table 4.3. Fatty acid composition (w/w %) of the total lipid fraction of PBMC's following incubation with fatty acids (100μM for 48 h), prior to stimulation with PHA, (10 μg/ml for 24 h). Values represent Mean ± SEM in parenthesis of six separate experiments. Statistically significant difference in fatty acid composition found between fatty acid treatments and control (PHA) values * P<0.05, **P<0.01, †P<0.001. nd: not detectable.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>PHA (control)</th>
<th>PHA c9t11</th>
<th>PHA t10c12</th>
<th>PHA Linoleic</th>
<th>PHA Palmitic</th>
<th>PHA Stearic</th>
<th>PHA Oleic</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0</td>
<td>23.4 (0.8)</td>
<td>16.7 (7.9)</td>
<td>18.9 (10.5)</td>
<td>13.6 (7.4)</td>
<td>78.8† (7.9)</td>
<td>22.5 (17.9)</td>
<td>12.1* (5.6)</td>
</tr>
<tr>
<td>C16:1</td>
<td>1.0 (0.2)</td>
<td>1.3 (0.6)</td>
<td>1.6 (0.8)</td>
<td>2.3 (1.7)</td>
<td>0.4 (0.2)</td>
<td>3.4 (2.7)</td>
<td>3.6 (2.6)</td>
</tr>
<tr>
<td>C18:0</td>
<td>30.2 (1.1)</td>
<td>15.4* (3.3)</td>
<td>17.9* (3.8)</td>
<td>18.5* (4.4)</td>
<td>8.2† (4.9)</td>
<td>47.9† (16.6)</td>
<td>11.5† (3.8)</td>
</tr>
<tr>
<td>C18:1</td>
<td>24.1 (0.8)</td>
<td>12.1** (2.1)</td>
<td>13.2** (2.2)</td>
<td>9.8** (4.4)</td>
<td>3.7† (2.7)</td>
<td>19.5 (14.5)</td>
<td>58.7† (15.0)</td>
</tr>
<tr>
<td>C18:2 n-6</td>
<td>9.3 (0.5)</td>
<td>3.9** (0.2)</td>
<td>4.9* (1.4)</td>
<td>60.2† (6.7)</td>
<td>2.6† (0.3)</td>
<td>2.3† (0.9)</td>
<td>5.0* (1.7)</td>
</tr>
<tr>
<td>C18:3 n-6</td>
<td>0.7 (0.3)</td>
<td>0.4 (0.1)</td>
<td>0.4 (0.2)</td>
<td>0.6 (0.3)</td>
<td>0.3 (0.1)</td>
<td>0.6 (0.5)</td>
<td>2.5 (1.5)</td>
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<tr>
<td>C18:3 n-3</td>
<td>1.8 (0.2)</td>
<td>0.7* (0.2)</td>
<td>1.0 (0.5)</td>
<td>0.7* (0.3)</td>
<td>0.5** (0.2)</td>
<td>1.0 (0.9)</td>
<td>1.1 (0.5)</td>
</tr>
<tr>
<td>C18:2 c9</td>
<td>0.4 (&lt;0.1)</td>
<td>47.3† (5.5)</td>
<td>0.9 (0.3)</td>
<td>0.6 (0.4)</td>
<td>0.2 (&lt;0.1)</td>
<td>0.9 (1.5)</td>
<td>nd</td>
</tr>
<tr>
<td>C18:2 t10</td>
<td>0.9 (&lt;0.1)</td>
<td>0.6 (0.2)</td>
<td>36.4† (14.1)</td>
<td>0.4 (0.2)</td>
<td>0.5 (0.3)</td>
<td>0.1† (0.3)</td>
<td>nd</td>
</tr>
<tr>
<td>C20:3 n-6</td>
<td>4.3 (0.2)</td>
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<td>2.2† (0.8)</td>
<td>2.5** (0.6)</td>
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<tr>
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<td>0.1 (0.1)</td>
<td>1.5 (0.9)</td>
<td>0.3 (0.1)</td>
<td>1.1 (0.1)</td>
<td>0.2 (0.3)</td>
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<tr>
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<td>1.1 (0.2)</td>
<td>0.5† (&lt;0.1)</td>
<td>0.6† (0.2)</td>
<td>0.6† (0.2)</td>
<td>0.6† (0.3)</td>
<td>0.2† (&lt;0.1)</td>
<td>0.6† (&lt;0.1)</td>
</tr>
<tr>
<td>C22:5 n-3</td>
<td>3.4 (0.3)</td>
<td>2.3** (0.3)</td>
<td>1.3† (0.5)</td>
<td>2.2** (0.5)</td>
<td>1.3† (0.3)</td>
<td>1.4† (0.3)</td>
<td>1.5† (0.3)</td>
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Table 4.4. Summary of the fatty acid composition of total lipid fraction of human PBMC’s cultured in the presence of fatty acids. Values represent Mean ± SEM in parenthesis of four separate experiments. Statistically significant difference in fatty acid composition found between fatty acid treatments and control (PHA) values \* P<0.05, †P<0.001.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>SFA/USFA</th>
<th>n-6/n-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA alone</td>
<td>1.1</td>
<td>2.4</td>
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4.3 Discussion.

In this study we investigated the effects a range of fatty acids on PHA-induced lymphocyte proliferation in human PBMC's *in vitro*. Stearic acid alone was found to inhibit lymphocyte proliferation at the concentration tested (100μM). We studied the influence of fatty acids on the secretion of the lymphokines IL-4 and IL-2 and the pro-inflammatory cytokine TNFα. *Cis*-9, *trans*-11 CLA, stearic acid and oleic acid all significantly reduced IL-4 mRNA expression relative to controls. However these decreases were absent at the protein level. In contrast the saturated fatty acid palmitic acid significantly increased IL-4 protein levels. IL-2 mRNA expression was significantly reduced by the *cis*-9, *trans*-11 CLA isomer and stearic acid, but increased by palmitic acid treatment. At the protein level, *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, linoleic and oleic acids all significantly decreased IL-2 secretion. The SFA, palmitic acid, was associated with a significant increase in TNFα mRNA and increased TNFα protein levels relative to oleic acid. None of the fatty acids tested affected PGE₂ secretion. Cellular viability was not compromised by the concentrations of fatty acids used (100μM). Indeed a dose response study highlighted that cellular viability was only affected by the vehicle control (DMSO) at concentrations above that used in this study (150 and 200μM). Finally gas-liquid chromatography experiments confirmed the cellular incorporation into total cell lipids of the fatty acids in our cell culture system.

The decrease in PHA-induced lymphocyte proliferation following *in vitro* supplementation with stearic acid is consistent with that observed in other *in vitro* studies. In the studies of Calder and colleagues (Calder *et al.*, 1991, Calder & Newsholme, 1992a b), *in vitro* incubation of human PBMC's and rat lymphocytes with stearic acid (100μM) significantly decreased Con A-induced proliferation. In our culture system, treatment with palmitic acid and oleic acid did not affect lymphocyte proliferation. This concurs with the *in vitro* study of Calder & Newsholme (1992b) where palmitic acid and oleic acid (100μM) were added directly to human PBMC's in culture. In contrast, rat lymphocytes incubated with these fatty acids *in vitro* exhibited a significant reduction in Con A-induced proliferation (Calder *et al.*, 1991, Calder & Newsholme, 1992a). Finally, neither of the CLA isomers nor linoleic acid affected lymphocyte proliferation in our study. It has been reported elsewhere that linoleic acid supplementation depresses Con A-induced lymphocyte proliferation *in vitro* in human PBMC's (Mertin *et al.*, 1974, Weyman *et al.*, 1975, Calder & Newsholme, 1992b) and rat splenic lymphocytes (Calder *et al.*, 1991, Calder & Newsholme, 1992a). In contrast, it has been shown that a CLA isomeric blend (50:50 *cis*-9, *trans*-11 CLA: *trans*-10, *cis*-12 CLA) significantly enhanced porcine PHA-
stimulated lymphocyte proliferation in vitro (Chew et al., 1997). We are unsure why these fatty acids failed to affect lymphocyte proliferation as unsaturated fatty acids are known to inhibit lymphocyte proliferation to a greater degree than saturated fatty acids (Calder et al., 1991, Calder & Newsholme, 1992ab, Calder, 1997). In our culture system, we added the mitogens and fatty acids to the PBMC's at the same time. It has been observed that the immuno-modulatory effects of fatty acids (including lymphocyte proliferation) are more pronounced if fatty acids are added 20-24h prior to lymphocyte stimulation (Calder et al., 1991, Khalfoun et al., 1996a). Perhaps more significant effects on lymphocyte proliferation would have been observed had our culture system included a 'pre-incubation' period with cells and fatty acids alone prior to addition of mitogen. In addition, we included 10% foetal calf serum in our culture media. This was included as a source of nutrients and as a vehicle for presenting the exogenous fatty acids to the cells. FCS contains proteins capable of binding and transporting fatty acids to the cell (Tronstad et al., 2001). However, FCS is also known to contain exogenous lipids (Kaldjan et al., 1992), which could have masked the true effect of the lipids on lymphocyte proliferation. Indeed it is reported that fatty acids caused a more profound inhibition of lymphocyte proliferation in vitro in rat splenic lymphocytes cultured in serum-free media (Calder et al., 1991). In all our experiments we used the same batch of foetal calf serum to minimise inter-batch variation in lipids, growth hormones etc., however it is possible that inclusion of FCS may have masked or reduced the effects of fatty acids on lymphocyte proliferation.

There is very little information with respect to the effects of fatty acids on IL-4 mRNA expression and secretion in human PBMC's in vitro. To date, studies investigating the influence of fatty acids on IL-4 have focused on animal feeding trials. IL-4 mRNA expression was not affected in C57Bl/6 mice fed coconut oil, safflower oil or fish oil rich diets (Wallace et al., 2001). Likewise, IL-4 production was not affected in C57Bl/6 mice fed coconut oil, safflower oil or fish oil rich diets or in MF1 mice fed diets rich in coconut oil, safflower oil, fish oil or olive oil (Yaqoob et al., 1995, Wallace et al., 2001). Recently, decreases in Con A-stimulated IL-4 production were reported following supplementation of C57Bl/6N mice with a CLA isomeric mix (Yang & Cook, 2003), purified cis-9, trans-11 CLA or trans-10, cis-12 CLA (Kelley et al., 2002). We demonstrated that cis-9, trans-11 CLA, stearic and oleic acids significantly decreased IL-4 mRNA expression in vitro, however this effect was absent when examined at the protein level. In response to palmitic acid, IL-4 production was significantly increased. IL-4 mRNA expression was likewise enhanced, though not significantly.
In our study IL-2 mRNA expression was significantly reduced by the *cis*-9, *trans*-11 CLA isomer and stearic acid, whereas palmitic acid increased IL-2 mRNA levels. IL-2 mRNA expression was not affected in the aforementioned study of Wallace *et al.* (2001) or in a feeding trial whereby C57Bl/6 mice were fed diets rich in EPA or DHA (Jolly *et al.*, 1998). However, Jolly *et al.* (1998) reported a significant decrease in IL-2Rα mRNA expression in the mice fed the EPA- and DHA-rich diets. Nevertheless, the pattern of the effects of fatty acids on IL-2 protein secretion in this study were similar to that reported previously. Both of the CLA isomers, linoleic acid and oleic acid significantly decreased IL-2 secretion, whereas palmitic acid and stearic acid did not affect IL-2 production. It has been frequently reported that USFA, (particularly linoleic and oleic acid) decrease the production of IL-2 in animal and human models *in vitro* (Calder *et al.*, 1991, Calder & Newsholme, 1992ab, Wallace *et al.*, 2001) and *ex vivo* (Yaqoob *et al.*, 1995, Jolly *et al.*, 1998, Wallace *et al.*, 2001). CLA isomeric blends decreased Con A-stimulated IL-2 production in porcine lymphocytes *in vitro* (Chew *et al.*, 1997), but increased its production in an animal feeding study (Yang & Cook, 2003). These reductions in IL-2 are consistent with the results obtained with the two individual CLA isomers in our study. In agreement with the study of Calder & Newsholme (1992b), IL-2 production was not affected by palmitic and stearic acids.

Combining the IL-4 and IL-2 data, it would appear that the SFA palmitic acid induced a rapid induction of gene transcription. This was translated into greater amounts of protein for IL-4 but not IL-2. Conversely, although the *cis*-9, *trans*-11 CLA isomer, stearic and oleic acid decreased IL-4 mRNA expression, they did not affect the translation of the protein. The *cis*-9, *trans*-11 CLA isomer alone decreased IL-2 at both the level of mRNA expression and protein. Therefore it would appear, at least in our culture system, that perhaps fatty acids interfere with cytokine production both at a transcriptional and post-translational level, and possibly in a cytokine specific manner. However, further experiments are necessary to examine this possibility. We investigated cytokine mRNA and protein expression at one time-point only (4 h and 24 h post-PHA stimulation for mRNA and protein respectively). Preliminary experiments in our lab indicated that these were the times of maximal cytokine mRNA expression and protein secretion. However, perhaps a time-course experiment investigating the rate of induction and degradation of both mRNA and protein would have been more useful. Indeed Bird *et al.*, (1998) reported secretion of IL-4 to require at least four cell divisions, whereas IL-2 is secreted earlier by activated naïve T-cells (Dutton *et al.*, 1994, Sad & Mossman, 1994, Seder & Paul, 1994). IL-2 is also reported to have a biphasic pattern of secretion (Morvan *et al.*, 1995).
Inclusion of Actinomycin D (an antibiotic that binds to DNA and inhibits RNA chain elongation), in future experiments would clarify whether changes in mRNA expression were due to a decrease in gene transcription rather than accelerated transcript degradation. With respect to the changes in IL-2 protein secretion, it is possible that processing via a post-translational mechanism could alter the rates of proteolytic cleavage (Jiang et al. 1997), or indeed a decrease in IL-2Rα or transferrin receptor expression would result in a decreased IL-2 secretion (Calder & Newsholme, 1992a, Jolly et al. 1998). The transcription factors NF-κB, NF-AT, AP-1 and Octamer proteins are involved in the activation of IL-2 and IL-2Rα, whereas STAT6 and NFAT and AP-1 are necessary for IL-4 gene induction (Burlinson et al. 1997, Murphy et al. 2000, for review see chapter one). Further experiments are needed to clarify if any of the fatty acid-induced changes in cytokine mRNA expression are related to alterations in transcription factor activity.

With respect to TNFα, palmitic acid alone increased the expression of this pro-inflammatory cytokine at the level of mRNA expression and protein secretion. Purasiri and colleagues (1997) showed that EPA, DHA and GLA significantly decreased TNFα production in human PBMC’s in vitro, while an EPA-rich medium was found to reduce TNFα mRNA production in LPS-stimulated RAW 264.7 macrophages (Lo et al. 1999a, Novak et al. 2003). However, the majority of studies investigating the effect of fatty acids on TNFα secretion have focused on high-fat diets or diets rich in n-3 PUFA. High-fat diets have been found to increase TNFα secretion in murine splenocytes (Hardardottir & Kinsella, 1991, Fong Lin et al. 1996) whereas fish oil decreased TNFα secretion in rat splenocytes (Tappia & Grimble, 1994) and human PBMC’s (Endres et al. 1989, 1993, Meydani et al. 1991, 1993, Kelley et al. 1999, Grimble et al. 2002). Diets rich in the cis-9, trans-11 CLA or trans-10, cis-12 CLA isomers increased LPS-stimulated TNFα production in C57Bl/6N mice, whereas the cis-9, trans-11 CLA and trans-10, cis-12 CLA decreased LPS-induced TNFα production in RAW macrophages in vitro (Yang & Cook, 2003). Neither of the CLA isomers affected PHA-induced TNFα production in our culture system. The increases in TNFα observed in this study suggest that the SFA palmitic acid induces a pro-inflammatory environment in our culture system.

None of the fatty acids investigated in this study affected PGE₂ secretion. Calder et al. (1991) measured the concentration of Con A-induced PGE₂ in the culture medium of rat splenocytes following incubation with palmitic, stearic, oleic and linoleic acid (100μM) in vitro. The level of PGE₂ production was similar between the fatty acid treatments (Calder et al. 1991). PGE₂ is reported to inhibit lymphocyte proliferation (Goodwin et al. 1974) and cytokine production (e.g. IL-2, TNFα, IFN-γ) (Gordon et al. 1976, Knudsen et al. 153
However, the immuno-modulatory effects of fatty acids are reported to be PGE\(_2\) independent (Santoli et al. 1990, Calder et al. 1992, Soyland et al. 1993, Rotondo et al. 1994). Due to space constraints on the ELISA plate, we did not analyse the effect of stearic acid on PGE\(_2\) secretion. It would have been interesting to see if the decrease in lymphocyte proliferation observed following incubation with stearic acid was coupled with an increase in PGE\(_2\) production. However, it would appear that the effects of fatty acids on lymphocyte proliferation, cytokine mRNA expression and cytokine production are PGE\(_2\) independent.

The MTT assay confirmed that the effects of fatty acids on cytokine production were not associated with a significant decrease in cell viability. The lowest cellular viability was reported with stearic acid (93%). However this was not statistically significant from control cells. The two CLA isomers, linoleic, palmitic and oleic acids returned cellular viabilities of >97% relative to control cells (i.e. cells without fatty acid). Therefore we can conclude that the effects mediated by fatty acids observed in this study were not associated with a decrease in cellular viability. A dose response study was conducted to ascertain the effects of fatty acids (0-200\(\mu\)M) on cellular viability. DMSO (vehicle control) alone significantly reduced cellular viability at 150 and 200 \(\mu\)M, however this was above the levels used on our study. None of the fatty acids were toxic to the cells at any of the concentrations tested. Consequently, in our culture system, fatty acids up to 200\(\mu\)M are not toxic to human PBMC’s and do not interfere with cellular viability.

Gas-liquid chromatography was conducted on total cell lipids to confirm the uptake of exogenous fatty acids by the PBMC’s. The total fatty acid composition of PBMC’s stimulated with PHA is comparable to that reported previously for total fatty acid composition of PHA-activated human T-lymphocytes (Anel et al. 1990ab). The principal fatty acids included palmitic acid, stearic acid, oleic acid and linoleic acid as reported elsewhere (Anel et al. 1990ab). The fatty acid composition of the PBMC’s in our study contained more stearic acid and less arachidonic acid than reported in the study of Anel et al. (1990ab). Incubation with all of the fatty acids resulted in a significant increase in the amount of that fatty acid in the total lipid fraction of the PBMC’s. This confirms uptake of these exogenous fatty acids by the PBMC’s in our culture system. Although incubation with stearic acid did increase the content of this fatty acid in the total cell lipids, the gas chromatography results would suggest that it was incorporated to a lesser extent than the other lipids. However, the increase in stearic acid was accompanied by an increase in oleic acid that was much greater than treatment with the CLA isomers, linoleic or palmitic acids. This would suggest the elongation and desaturation of the stearic acid in our culture system.
to oleic acid by the action of Δ-9 desaturase as reported elsewhere (Anel et al., 1990b). As expected the ratio of saturated fatty acids: unsaturated fatty acids was much greater in the palmitic and stearic acid treated cells. This concurs with the pattern of incorporation noted in rat splenic lymphocytes phospholipid fraction (Calder et al., 1994). Finally, the fatty acid treatments used in this study were accompanied by a significant reduction in the n-3 PUFA content of the cell. In conclusion, the gas chromatography data confirm the uptake of the fatty acids by the PBMC’s in our culture system.

The implications of altering PBMC membrane fatty acid composition are far greater than was possible to address within this study. They suggest that these fatty acids could have altered the membrane fluidity and permeability of these cells. Indeed, palmitic and stearic acids are reported to decrease fluidity, while unsaturated fatty acids increase the fluidity of rat splenic lymphocytes (Calder et al., 1994). Recently, there have been fundamental advances in the role of plasma membrane lipid-rich ‘rafts’ in cell signalling and membrane functioning (Brown & London, 1998, Langlet et al., 2000). These lipid rafts play a critical role in protein mobility and interactions. They contain T-cell receptors (e.g. TCR), T-cell associated signalling proteins (e.g. lck, fyn, LAT, PI-3K), IL-2 receptor subunits, and are known to ‘clump’ or ‘patch’ upon immune-cell activation (Xavier et al., 1998, Goebel et al., 2002, Matko et al., 2002, Mitchell et al., 2002, Simons & Ehehalt, 2002). Although the IL-2, IL-4 and TNFα receptors are embedded in the plasma membrane (Janeway & Travers, 1997), it is not known if they are incorporated in or interact with these lipid rafts. However, it is possible that the incorporation of the fatty acids into the PBMC total lipid fraction may have affected the composition of these lipid rafts and possibly altered the degree of protein mobility, interactions and downstream signalling events. Indeed Stulnig et al. (1999) reported biosynthetic incorporation of PUFA in T-cells displaced the Src family kinases from the T-cell lipid rafts and was accompanied by a decrease in T-cell signalling. However, further experiments are required to look at the effects of physiological incorporation of fatty acids into lipid rafts, protein interactions and downstream signalling events.

In conclusion, this study shows that fatty acids affect the production of the cytokines IL-4, IL-2 and TNFα, both at the level of mRNA and protein. The cis-9, trans-11 CLA isomer was most potent with respect to lowering IL-4 mRNA and IL-2 mRNA and protein levels. Palmitic acid alone affected TNFα production. We did not observe any influence on lymphocyte proliferation or PGE₂ secretion. MTT assay confirmed that the effects on cytokine production were not associated with a decrease in cell viability, while gas chromatography confirmed the incorporation of the fatty acids into the total cell lipids.
Further experiments are needed to ascertain whether these fatty acids are acting at the level of the transcription factor (e.g. NF-κB, AP-1, NF-AT) or at a post-translational level.
Chapter Five.

The effects of dietary fatty acids on the NF-κB signalling pathway.
5.0 Introduction.

The effects of various fatty acids on immune function and the inflammatory response have been extensively researched and are reviewed in chapter one. Fatty acids are known to modulate such markers of immune functions as lymphocyte proliferation, cytokine and eicosanoid production and adhesion molecule expression (Calder et al, 1998, 2000, Kelley et al, 1998). However, less is known about the molecular mechanisms underlying these immuno-modulatory effects. Therefore this chapter investigates how fatty acids modulate the Nuclear Factor kappa B (NF-κB) family of transcriptional activator proteins.

Nuclear Factor kappa B (NF-κB) is a family of transcription factors that are central to inflammation, tumorogenesis and cellular processes such as proliferation and apoptosis (Karin & Ben-Neriah, 2000, Ghosh & Karin, 2002). NF-κB is a heterodimer of proteins of the Rel family including c-rel, RelB, p65 (Rel-A), p50 (and it’s precursor p105), p52 (and it’s precursor p100) (Ghosh et al, 1998, Karin & Ben-Neriah, 2000). The most common form is a p65/p50 heterodimer and it is retained in an inactive form due to its association with inhibitors of kappa B (IκB) (Karin & Ben-Neriah, 2000). Several IκB family members have been identified, however the predominant and most extensively form is IκBα. In resting cells, IκBα is attached to the p65 subunit of the NF-κB heterodimer, (Huxford et al, 1998, Jacobs & Harrison, 1998). NF-κB activation is controlled by IκBα. Upon signal-induced activation, IκBα is phosphorylated, ubiquinated and ultimately degraded. After IκBα degradation, the NF-κB dimers are free to translocate to the nucleus, bind to κB-responsive elements in target genes and activate transcription. Fig 5.1 depicts a schematic of NF-κB activation. Cellular stress (induced by cytokines, growth factors, oxidative stress, TCR ligation etc.) results in the phosphorylation of this IκBα: NF-κB complex by a family of IκB kinases (IKK). At least three members of this IKK family have been identified to date, namely IKK-α, -β and -γ (recently reviewed by Karin & Ben-Neriah, 2000, Ghosh & Karin, 2002). These kinases are activated by a host of upstream activators (including PKC, NIK etc) and result in the phosphorylation of IκBα at serines 32 and 36 (Brown et al, 1995, DiDonato et al, 1996). This phosphorylation marks the IκBα for polyubiquination by a specific ubiquitin ligase (β-Trcp) belonging to the SCF (Skp-1/Cul/F box) family (Ben-Neriah, 2002) and subsequent degradation by the proteasome (Ghosh & May, 1998, Ghosh & Karin, 2002). The NF-κB heterodimer is then free to bind to DNA and activate transcription.

Traditionally, it was thought that the NF-κB: IκBα complex was ubiquinated and degraded in the cytoplasm, however recent research proposes the NF-κB: IκBα complex to
**Fig 5.1.** A schematic model of NF-κB activation.

- **Cytokine receptors e.g.** TNFα, IL-1, LPS, dsRNA, Ionizing Radiation, TPA, ROS, Okadac acid etc

- **IKKα, IKKβ**

- **NF-κB responsive genes e.g.** IL-1, TNFα, IL-2, chemokines, immunoreceptors, Adhesion molecules, Acute phase proteins, COX

- **UV Others?**

- **NF-κB responsive gene**

- **Proteasome degradation**
shuttle continuously in and out of the nucleus, even in resting cells (Huang et al. 2000, Johnson et al. 1999, Rodriguez et al. 1999, Ghosh & Karin, 2002). This mechanism is reliant upon the presence of nuclear localisation signals (NLS) and nuclear export signals (NES) on the IkBα and the p65/p50 structures. Nuclear import is mediated by NLS and crystallographic studies reveal IkBα to completely mask the NLS on p65, but not the p50 subunit (Huxford et al. 1998, Jacobs & Harrison, 1998). This ‘unmasked’ NLS would therefore allow the NF-κB: IkBα complex to enter the nucleus (Ghosh & Karin, 2002). Upon nuclear entry and in resting cells, NES work in conjunction with a nuclear export protein called chromosome maintenance region 1 (CRM-1) resulting in removal of the IkBα: NF-κB complex from the nucleus (Ossareh-Nazari et al. 1997, Ghosh & Karin, 2002). The combined presence of a NES on IkBα and of an additional NES on the p65 subunit (Harhaj and Sun, 1999) ensure the immediate nuclear expulsion of the NF-κB: IkBα complex and prevent NF-κB activation in resting cells (Ghosh & Karin, 2002). However in activated cells, the IkBα already phosphorylated at serines 32 and 36 by the cytoplasmic IKK’s, is primed for further polyubiquination (Brown et al. 1995, DiDonato et al. 1996). It has also been suggested that IkBα ubiquination occurs in the nucleus upon recognition by the nuclear receptor β-Trcp, which then targets the phosphorylated IkBα for ubiquination, subsequent proteasome-mediated degradation and ultimately NF-κB activation (Davis et al. 2002).

Given the proven immuno-modulatory effects of fatty acids on cytokines etc., it is possible that these effects are mediated via the NF-κB signalling pathway. Although these investigations are still in an early stage, it has been shown that fatty acids, principally linoleic, stearic, linolenic, arachidonic, oleic and eicosapentaenoic acids may mediate transcription factor activity particularly in endothelial cells and macrophages. It has been demonstrated that linoleic acid increases NF-κB transcription in porcine endothelial cells (Hennig et al. 1996, 2000). Indeed a hierarchy of activation was reported in these endothelial cells of the order linoleic acid > stearic acid > linolenic acid > oleic acid (Hennig et al. 2000). Another study showed that linoleic and linolenic acids increase the transcriptional activation of NF-κB reporter genes (Toborek et al. 2002). Human endothelial cells pre-incubated with oleic acid and subsequently challenged with LPS demonstrated decreased NF-κB activity (Carluccio et al. 1999). Fatty acids have also been shown to modulate macrophage NF-κB. In U937 monocytes and J774 macrophages arachidonic acid and eicosapentaenoic acid (EPA) had differential effects on NF-κB activity: arachidonic acid enhanced NF-κB activity whereas EPA was without effect (Camandola et al. 1996). However, RAW 264.7 macrophages incubated with EPA for 48
h and subsequently stimulated with LPS had decreased NF-κB activation (Lo et al., 1999a). More recently LPS-activated RAW 264.7 macrophages incubated with an omega-3 fatty acid emulsion exhibited decreased NF-κB activity, which was secondary to an inhibition of IκBα phosphorylation. (Novak et al., 2003).

Hence it appears that fatty acids may mediate their effects on immune function through the transcription factor NF-κB. However, the majority of investigations to date are in endothelial cells and macrophages. There is no information regarding the effects of fatty acids on transcription factor activity in human peripheral blood cells or on the monocytic cell-line THP-1’s. Similarly there is an absence of information with respect to the effect of conjugated linoleic acid (CLA) on NF-κB transcriptional activity. Therefore the focus of this study was to provide mechanistic clues regarding the immuno-modulatory functions of the fatty acids cis-9, trans-11 CLA, trans-10, cis-12 CLA, linoleic acid (LA) and palmitic acids (PA).
5.1 Materials and methods.

5.1.1 Cell culture and experimental design.

THP-1 monocytes were obtained from EACC and cultured in pre-warmed RPMI media supplemented with FCS and penicillin/streptomycin as outlined in Section 2.2. PBMC’s were isolated from 80 ml of fresh whole blood donated by healthy volunteers in Trinity College Dublin as outlined in sections 2.0 and 2.1. For experiments involving THP-1 monocytes, cells were seeded at a density of $2 \times 10^6$. As this leukaemic cell multiplies by a factor of two approximately every 24 h, the final density of THP-1 monocytes at the end of the culture period averaged at $8 \times 10^6$. PBMC’s were cultured at a density of $8 \times 10^6$ cells. Fatty acids were directly added to both cell systems and culture continued for 48h. The following fatty acids (100μM) were included in all experiments: Cis-9, trans-11 CLA (c9, t11 CLA), Trans-10, cis-12 CLA (t10, c12 CLA), Linoleic acid (L.A), Palmitic acid (PA).

Control wells were established including cells alone and cells and DMSO in a concentration equivalent to that present in all other treatments (0.1%).

5.1.2 NF-κB : DNA binding experiments (Electrophoretic mobility shift assays).

After the initial 48 h incubation period, THP-1’s were activated for 1 h with LPS (1μg/ml) while PBMC’s were stimulated with PHA (10μg/ml) for 2 h. Similar experiments were conducted to investigate the effects of fatty acids on NF-κB: DNA binding in resting cells, i.e cells incubated with fatty acids but without LPS/PHA. Nuclear extracts were prepared as described in Section 2.23 and protein concentration quantitated by the Bradford method (Section 2.24). The binding of NF-κB to κB enhancer elements on DNA was measured by EMSA as outlined in Section 2.32.

5.1.3 IκBα and p65 (Rel A) Immunoblotting.

Experiments were performed to examine the effects of fatty acids on nuclear and cytoplasmic IκBα and p65 expression in THP-1 monocytes and cytoplasmic IκBα and p65 expression in PBMC’s. After the initial 48 h incubation period THP-1’s were stimulated with LPS (1μg/ml) for 30 and 60 min and PBMC’s with PHA (10μg/ml) for 2hr. As for the EMSA experiments, the effects of fatty acids on IκBα and p65 expression were investigated in resting cells (i.e. in the absence of LPS/PHA) (Section 2.7.). Cytoplasmic
and nuclear extractions were performed (Section 2.22, 2.23), protein concentration measured by the Bradford method (Section 2.24) and samples acetone-precipitated for separation of proteins by SDS-PAGE and western blotting (Sections 2.26, 2.27, 2.28). 10µg of protein was loaded for nuclear extracts and 50µg protein for cytoplasmic fractions. The antibodies IκBα (C-21), sc-371, (Santa Cruz Biotechnology, Inc, USA) and p65, (C-20), sc-372, (Santa Cruz Biotechnology, Inc, USA) were used to detect IκBα and p65 expression in both fractions (Section 2.29).

5.1.4 Statistical Analysis.

Quantitation of both EMSA and western blotting autoradiographic bands was performed with the aid of the Kodak Image Station 440cf and analysed using the Kodak 1D Analysis Software. The position of the bands was confirmed by the inclusion of suitable molecular weight markers and/or positive controls during the experiment (Section 2.33). Results are expressed as Means ± SEM for the number of observations indicated. All experimental procedures were repeated at least 3 to 6 times. Statistical analysis was performed with the statistical package DataDesk 6.0 (Data Description Inc., N.Y., USA). The distribution of the data for each variable was assessed and variables transformed if necessary to normalise the distribution of the data. Multiple comparisons were performed by one-way ANOVA. Individual differences were subsequently tested by Fishers least significant difference (LSD) test after demonstration of significant intergroup differences by ANOVA. In some instances independent t-tests were conducted. A statistical probability of P< 0.05 was considered statistically significant.
5.2 Results.

5.2.1 NF-κB: DNA binding.

Figures 5.2a and b, show that there was a dramatic decrease in NF-κB: DNA binding following incubation of resting THP-1 monocytes with the cis-9, trans-11 CLA isomer, trans-10, cis-12 CLA isomer and linoleic acid. Quantitative analysis of the individual blots revealed that NF-κB: DNA binding was significantly (P<0.001) reduced by 0.6 fold following pre-incubation with cis-9, trans-11 CLA relative to control (cells alone). Treatment with trans-10, cis-12 CLA also caused a significant (P<0.05) reduction (0.4 fold) in NF-κB: DNA binding. Linoleic acid was associated with a significant (P<0.01) decrease (0.5 fold) in NF-κB activity after the 48 h pre-incubation period. In contrast, palmitic acid had no significant effect on NF-κB: DNA binding compared to control cells. The cis-9, trans-11 CLA isomer was the most potent fatty acid at reducing NF-κB: DNA binding; it significantly (P<0.05) decreased binding relative to palmitic acid. No differences were observed between control cells (i.e. cells alone) and cells co-cultured with DMSO.

The effect of pre-incubating THP-1 monocytes with fatty acids prior to stimulation with LPS are presented in Figures 5.3a and 5.3b. Cis-9, trans-11 CLA was most effective at decreasing (P<0.01, independent t-test, 0.5 fold) LPS-induced NF-κB: DNA binding relative to control cells (i.e. cells treated with LPS alone). It also decreased NF-κB binding relative to palmitic acid (P<0.05, independent t-test, 0.8 fold). Trans-10, cis-12 CLA and linoleic acid marginally decreased mean LPS-activated NF-κB activity (by 0.2 and 0.1 fold respectively). Conversely pre-incubation with palmitic acid caused a 0.38 fold increase in NF-κB: DNA binding relative to control cells, but this failed to reach statistical significance.

To confirm the aforementioned effects observed in THP-1 monocytes, the effects of fatty acids on NF-κB: DNA binding was investigated in human PBMC’s. In agreement with the effects observed in the THP-1 monocytic cell line, human PBMC’s pre-incubated with fatty acids for 48 h demonstrated decreased NF-κB: DNA binding. (figs 5.4a, 5.4b). Once more, the cis-9, trans-11 CLA isomer caused a significant (P<0.001) reduction (0.5 fold) in NF-κB: DNA binding compared to control cells. NF-κB: DNA binding was also significantly reduced by trans-10, cis-12 CLA (P<0.05, 0.3 fold), linoleic acid (P <0.001, 0.5 fold) and palmitic acid (P<0.01, 0.4 fold) relative to the control cells. There was no significant difference between control and DMSO treated cells.

PBMC’s incubated with fatty acids and activated with PHA revealed a similar picture to that observed in the LPS-stimulated THP-1’s. (See fig 5.5a, 5.5b). The fatty acids...
acid treatments decreased PHA-induced NF-κB : DNA binding, nevertheless there was
greater inter-experimental variation that observed with the THP-1 studies. Cis-9, trans-11
CLA decreased PHA-induced NF-κB activity by 0.2 fold, trans-10, cis-12 CLA by 0.3 fold
and linoleic acid by 0.2 fold. However, none of these decreases were statistically
significant. NF-κB : DNA was also reduced (0.3 fold) in PBMC’s treated with palmitic
acid prior to PHA activation relative to control PBMC’s stimulated with PHA alone.
**Fig 5.2a.** A representative gel illustrating the effect of incubating resting THP-1 monocytes with fatty acids (48 h) on NF-κB activation, as assessed by EMSA. Ctrl: control, dmso: DMSO, c9, t11 CLA: cis-9, trans-11 CLA, t10, c12 CLA: trans-10, cis-12 CLA, LA: linoleic acid, PA: palmitic acid, +ve control: positive control (nuclear extracts of the NF-κB constitutively expressing cell-line, HUT-78).

![NF-κB gel](image)

**Fig 5.2b.** Quantitative analysis of the blots was performed by scanning densitometry. Results represent Mean ± SEM for 5 independent experiments. Statistically significant inhibition of NF-κB : DNA binding is observed between ctrl, dmso and treatments: *P<0.05; **P<0.01, ***P<0.001. Significant difference between cis-9, trans-11 CLA and palmitic acid: †p<0.05

![Fold induction relative to control](image)
**Fig 5.3a.** A representative gel showing the effect of incubating THP-1 monocytes with fatty acids (48 h), followed by LPS-activation (1 h), on NF-κB activity as assessed by EMSA. Ctrl: control, c9, t11 CLA: cis-9, trans-11 CLA, t10, c12 CLA: trans-10, cis-12 CLA, LA: linoleic acid, PA: palmitic acid, +ve control: positive control (nuclear extracts of the NF-κB constitutively expressing cell-line, HUT-78).

**Fig 5.3b.** Quantitative analysis of the blots was performed by scanning densitometry. Results represent Mean ± SEM for 5 independent experiments. Cis-9, trans-11 CLA statistically inhibited NF-κB: DNA binding relative to control *P<0.01 and palmitic acid †p<0.05 (Independent t-test).
**Fig 5.4a.** A representative gel illustrating the effect of incubating resting human PBMC’s with fatty acids (48 h) on NF-κB activation, as assessed by EMSA. Ctrl: control, dmso: DMSO, c9, t11 CLA: cis-9, trans-11 CLA, t10, c12 CLA: trans-10, cis-12 CLA, LA: linoleic acid, PA: palmitic acid, +ve control: positive control (nuclear extracts of the NF-κB constitutively expressing cell-line, HUT-78).

**Fig 5.4b.** Quantitative analysis of the blots was performed by scanning densitometry. Results represent Mean ± SEM for 5 independent experiments. Statistically significant inhibition of NF-κB : DNA binding is observed between ctrl and treatments: *P<0.05; **P<0.01, †p<0.001
**Fig 5.5a.** A representative gel showing the effect of incubating human PBMC’s with fatty acids (48 h), followed by PHA-activation (2 h), on NF-κB activity as assessed by EMSA. Ctrl: control, c9, t11 CLA: cis-9, trans-11 CLA, t10, c12 CLA: trans-10, cis-12 CLA, LA: linoleic acid, PA: palmitic acid, PHA: phytohemaglutinin, +ve control: postive control (nuclear extracts of the NF-κB constitutively expressing cell-line, HUT-78).

**Fig 5.5b.** Quantitative analysis of the blots was performed by scanning densitometry. Values represent Mean ± SEM for 6 independent experiments. A non-statistically significant trend towards decreased NF-κB activity was observed with PHA & cis-9, trans-11 CLA, PHA & trans-10, cis-12 CLA, PHA & linoleic acid and PHA & palmitic acid.
5.2.2 \( \text{IkB}_\alpha \) expression.

The effect of fatty acids on cytoplasmic and nuclear \( \text{IkB}_\alpha \) expression was studied in both resting and LPS stimulated THP-1 monocytes. Fatty acids did not significantly affect cytoplasmic \( \text{IkB}_\alpha \) expression in resting THP-1 monocytes (Figure 5.6a). Increased expression of cytoplasmic \( \text{IkB}_\alpha \) is indicative of decreased NF-\( \kappa \)B activity. To investigate whether the decrease in NF-\( \kappa \)B activity observed in LPS- treated monocytes was secondary to an increase in cytoplasmic \( \text{IkB}_\alpha \) expression, cytoplasmic \( \text{IkB}_\alpha \) expression was measured in monocytes at 30 and 60 min post LPS stimulation. At 30 min post LPS stimulation, palmitic acid caused a significant (\( P<0.05 \)), increase (0.7 fold of control) in cytoplasmic \( \text{IkB}_\alpha \) expression, however this was not evident at 60 min post LPS stimulation. None of the other fatty acid treatments affected \( \text{IkB}_\alpha \) expression. (See fig 5.6a). Fig 5.6b depict quantitative denistometric data for each of the aforementioned westerns.

More profound changes were observed with respect to the effects of fatty acids on nuclear \( \text{IkB}_\alpha \) expression in both resting and LPS-activated THP-1 monocytes (figs 5.7a, 5.7b). Nuclear \( \text{IkB}_\alpha \) expression was significantly (\( P<0.03 \), 0.8 fold) increased in resting THP-1 monocytes cultured with cis-9, trans-11 CLA relative to control cells. Nuclear \( \text{IkB}_\alpha \) expression following cis-9, trans-11 CLA treatment was also significantly greater than that after treatment with trans-10, cis-12 CLA (\( P<0.01 \), 1.1 fold) and palmitic acid (\( P<0.001 \), 1.3 fold). Linoleic acid likewise increased nuclear \( \text{IkB}_\alpha \) expression relative to control cells (\( P<0.01 \), 0.4 fold), cells incubated with trans-10, cis-12 CLA (\( P<0.02 \), 0.8 fold) and cells incubated with palmitic acid (\( P<0.01 \), 0.9 fold). To confirm the effects of fatty acids on nuclear \( \text{IkB}_\alpha \) expression in LPS-activated cells, experiments were conducted whereby nuclear \( \text{IkB}_\alpha \) expression was measured 60 min post LPS stimulation. THP-1 monocytes stimulated with LPS for 60 min revealed identical nuclear \( \text{IkB}_\alpha \) expression patterns. cis-9, trans-11 CLA increased LPS- induced \( \text{IkB}_\alpha \) expression relative to control cells (8 fold, \( P<0.001 \)), trans-10, cis-12 CLA (5.3 fold, \( P<0.005 \)) and palmitic acid (6.5 fold, \( P<0.002 \)). Linoleic acid enhanced LPS-induced \( \text{IkB}_\alpha \) expression relative to control cells (11.6 fold, \( P<0.001 \)), trans-10, cis-12 CLA (6.5 fold, \( P<0.001 \)) and palmitic acid (10.5 fold, \( P<0.001 \)). Nuclear \( \text{IkB}_\alpha \) expression was not detectable in THP-1 monocytes at 30mins.

Cytoplasmic \( \text{IkB}_\alpha \) expression was likewise investigated in resting and PHA activated PBMC extracts. As would be expected, a greater degree of variability was observed between experiments than with the THP-1 monocytes. In resting cells, 48 h incubation with cis-9, trans-11 CLA. trans-10, cis-12 CLA and palmitic acid caused a significant decrease (by 0.2, 0.2 and 0.1 fold respectively, \( P<0.05 \)) in cytoplasmic \( \text{IkB}_\alpha \)
relative to cell alone (control). (See fig 5.8a, 5.8b) In human PBMC's pre-incubated with fatty acids and activated with PHA for 2hr, there was no significant difference in cytoplasmic \( \text{IkB} \alpha \) expression between fatty acids and cells treated with PHA alone (fig 5.8a, 5.8b). Experiments were conducted to examine the effect of fatty acids on nuclear \( \text{IkB} \alpha \) expression in human PBMC's. Significant inter-individual variation and inconsistent \( \text{IkB} \alpha \) expression was observed therefore yielding inconclusive results.
**Fig 5.6a.** Representative western blots showing the relative IkBa expression in i) resting, ii) 30 min LPS-activated and iii) 60 min LPS-stimulated THP-1 cytoplasmic extracts. ctrl: control, c9t11 CLA: cis-9, trans-11 CLA, t10, e12 CLA: trans-10, cis-12 CLA, LA: linoleic acid, LPS: Lipopolysaccharide, PA: palmitic acid.

i) Resting cells.

ctrl  dmso  c9 t11  t10 e12  LA  PA
     CLA   CLA

37 kDa

ii) LPS stimulated cells (30 min)

LPS c9 t11  t10 e12  LA  PA
    CLA   CLA

37 kDa

iii) LPS stimulated cells (60 min)

LPS c9 t11  t10 e12  LA  PA
    CLA   CLA

37 kDa
Fig 5.6b. Quantitative analysis of THP-1 cytoplasmic IκBα expression western blots was performed for each timepoint. (i; resting cells, ii; 30 min and iii; 60 min). Values represent Mean ± SEM for 3 independent experiments. Statistically significant inhibition is observed between ctrl and treatments: *P<0.05.

i) Resting cells.

![Graph showing fold induction relative to control for resting cells and various treatments.]

ii) 30 min post LPS-stimulation

![Graph showing fold induction relative to LPS (control) for LPS and various treatments.]

iii) 60 min post LPS-stimulation

![Graph showing fold induction relative to LPS (control) for LPS and various treatments.]

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**Fig 5.7a.** Representative western blots showing the relative THP-1 nuclear IκBα expression in i) resting and ii) 60 min LPS-activated THP-1 monocytes. ctrl: control, c9, t11 CLA: cis-9, trans-11 CLA, t10 c12 CLA: trans-10, cis-12 CLA, LA: linoleic acid, LPS: Lipopolysaccharide, PA: palmitic acid.

i) Resting cells.

![Western blot of resting cells](image)

<table>
<thead>
<tr>
<th>ctrl</th>
<th>dmso</th>
<th>c9 t11 CLA</th>
<th>t10 c12 CLA</th>
<th>LA</th>
<th>PA</th>
</tr>
</thead>
</table>

37 kDa

ii) 60 min post LPS stimulation

![Western blot of LPS-activated cells](image)

<table>
<thead>
<tr>
<th>LPS</th>
<th>c9 t11 CLA</th>
<th>t10 c12 CLA</th>
<th>LA</th>
<th>PA</th>
</tr>
</thead>
</table>

37 kDa
**Fig 5.7b.** Quantitative analysis of nuclear IκBα expression western blots was performed for each timepoint: i; resting cells, ii; 60 min post LPS stimulation. Values represent Mean ± SEM for 4 independent experiments. Statistically significant inhibition of nuclear IκBα expression is observed between *cis*-9, *trans*-11 CLA and treatments: *P<0.03; **P<0.01, ***P<0.001, ****P<0.005. Significant inhibition between linoleic acid and treatments †P<0.02, ††P<0.01 † † † †P<0.001

i) Resting cells.

![Graph showing fold induction relative to control for various treatments including ctrl, dms, c9t11 CLA, t10 c12 CLA, LA, and PA.]

ii) 60 min post LPS stimulation.

![Graph showing fold induction relative to LPS for treatments including LPS, LPS c9t11 CLA, LPS t10c12 CLA, LPS LA, and LPS PA.]

**Fig 5.8a.** Representative western blots showing the relative IκBα expression in i) resting and ii) 2h PHA-activated human PBMC cytoplasmic extracts. ctrl: control, c9, t11 CLA: cis-9, trans-11 CLA, t10, c12 CLA: trans-10, cis-12 CLA, LA: linoleic acid, PA: palmitic acid, PHA: phytohemagglutinin.

i) Resting cells

ii) 2h post PHA stimulation.

**Fig 5.8b.** Quantitative analysis of human PBMC cytoplasmic IκBα expression western blots was performed for each timepoint. (i; resting cells and ii; 2 h). Values represent Mean ± SEM for 6 independent experiments. Statistically significant inhibition is observed between ctrl and treatments: *P<0.05.

i) Resting cells.

ii) 2 h post PHA stimulation.
5.2.3 p65 expression.

Fatty acids had no significant effect on cytoplasmic or nuclear p65 expression in resting or LPS-stimulated (30 and 60 min) THP-1 monocytes. (See fig 5.9a, 5.9b, 5.10a, 5.10b). In human PBMC’s, although greater variation was observed, fatty acids had no significant effect on p65 expression in either resting or PHA-stimulated PBMC’s. (Fig 5.11a, 5.11b).

Fig 5.9a. Representative western blots showing the relative THP-1 cytoplasmic p65 expression in i) resting, ii) 30 min and iii) 60 min LPS-activated THP-1 monocytes. ctrl: control, c9, t11 CLA: cis-9, trans-11 CLA, t10, c12 CLA: trans-10, cis-12 CLA, LA: linoleic acid, LPS: Lipopolysaccharide, PA: palmitic acid.

i) Resting cells

```
ctrl  dmso  c9  t11  t10  c12  LA  PA  
CLA  CLA
```

65 kDa

ii) 30 min post LPS stimulation

```
LPS

LA  PA
CLA  CLA
```

65 kDa

iii) 60 min post LPS stimulation

```
LPS

c9  t11  t10  c12  LA  PA  
CLA  CLA
```

65 kDa
Fig 5.9b. Quantitative analysis of cytoplasmic THP-1 p65 expression western blots was performed for each timepoint: i; resting cells, ii; 30 min, iii; 30 min post LPS stimulation. Values represent Mean ± SEM for 4 independent experiments.

i) Resting cells

![Graph showing fold induction relative to control for resting cells.]

ii) 30 min post LPS stimulation.

![Graph showing fold induction relative to control (LPS) for 30 min post LPS stimulation.]

iii) 60 min post LPS stimulation.

![Graph showing fold induction relative to control (LPS) for 60 min post LPS stimulation.]

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**Fig 5.10a.** Representative western blots showing the relative nuclear p65 expression in i) resting THP-1's, ii) 30 min and iii) 60 min post LPS stimulation. ctrl: control, c9, t11 CLA: cis-9, trans-11 CLA, t10, c12 CLA: trans-10, cis-12 CLA, LA: linoleic acid, LPS: Lipopolysaccharide, PA: palmitic acid.

i) Resting cells.

![Western blot of resting cells](image)

ii) 30 min post LPS stimulation.

![Western blot of 30 min post LPS stimulation](image)

iii) 60 min post LPS stimulation.

![Western blot of 60 min post LPS stimulation](image)
**Fig 5.10b.** Quantitative analysis of THP-1 nuclear p65 expression western blots was performed for each timepoint: i) resting cells, ii) 30 min and iii) 60 min post LPS stimulation. Values represent Mean ± SEM for 3 independent experiments.

i) Resting cells.

![Graph showing fold induction relative to control for resting cells](image)

ii) 30 min post LPS stimulation.

![Graph showing fold induction relative to control for 30 min post LPS stimulation](image)

iii) 60 min post LPS stimulation.

![Graph showing fold induction relative to control for 60 min post LPS stimulation](image)
**Fig 5.11a.** Representative western blots showing the relative PBMC cytoplasmic p65 expression in i) resting and ii) 2 h PHA stimulated human PBMC’s. ctrl: control, c9, t11 CLA: cis-9, trans-11 CLA, t10, c12 CLA: trans-10, cis-12 CLA, LA: linoleic acid, PA: palmitic acid, PHA: phytohemagglutinin.

i) Resting cells

![Image of western blots]

<table>
<thead>
<tr>
<th>Ctrl</th>
<th>dmsO</th>
<th>c9t11</th>
<th>t10c12</th>
<th>LA</th>
<th>PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLA</td>
<td>CLA</td>
<td>CLA</td>
<td>CLA</td>
<td>LA</td>
<td>PA</td>
</tr>
</tbody>
</table>

ii) 2h post PHA stimulation.

![Image of western blots]

<table>
<thead>
<tr>
<th>Ctrl</th>
<th>dmsO</th>
<th>c9t11</th>
<th>t10c12</th>
<th>LA</th>
<th>PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLA</td>
<td>CLA</td>
<td>CLA</td>
<td>CLA</td>
<td>LA</td>
<td>PA</td>
</tr>
</tbody>
</table>

**Fig 5.11b.** Quantitative analysis of PBMC cytoplasmic p65 expression western blots was performed for each timepoint: i; resting cells and ii; 2 h post PHA stimulation. Values represent Mean ± SEM for 6 independent experiments.

i) Resting cells.

![Graph of fold induction relative to control for resting cells]

**Treatment**

- ctrl
- dmsO
- c9t11 CLA
- t10c12 CLA
- LA
- PA

ii) 2h post PHA stimulation.

![Graph of fold induction relative to control (PHA)]

**Treatment**

- PHA
- PHA c9t11 CLA
- PHA t10c12 CLA
- PHA t10c12 CLA
- PHA PA
5.3 Discussion.

In the present study we report that fatty acids down-regulate NF-κB: DNA binding in THP-1 monocytes and human PBMC’s. These effects were more apparent in resting cells. In addition there was a difference between fatty acids. An order of efficacy (cis-9, trans-11 CLA > linoleic acid > trans-10, cis-12 CLA > palmitic acid) was shown in THP-1’s. In PBMC’s that order was slightly different (cis-9, trans-11 CLA > linoleic acid > palmitic acid > trans-10, cis-12 CLA). In LPS- and PHA- activated THP-1’s and PBMC’s, the degree of down-regulation was less pronounced. Cis-9, trans-11 CLA alone significantly decreased LPS-induced NF-κB activity in THP-1 monocytes. Although, this decrease was also observed in PBMC’s, it failed to reach significance. However, the suppressive effect of cis-9, trans-11 CLA on NF-κB was remarkably consistent in these two cell types under resting and activated conditions. Treatment with cis-9, trans-11 CLA resulted in an average fold reduction of 0.55 in THP-1’s and 0.35 in PBMC for both basal and stimulated NF-κB activity. In the case of IkBα expression, our study shows fatty acids failed to affect cytoplasmic IkBα expression in THP-1 monocytes. In contrast cis-9, trans-11 CLA and linoleic acid dramatically increased nuclear IkBα expression in resting and LPS activated (1h) monocytes. Cytoplasmic IkBα expression tended to decrease in resting PBMC’s co-cultured with fatty acids; nevertheless this effect was lost upon PHA activation. Finally, fatty acids did not affect p65 cytoplasmic or nuclear expression in THP-1 experiments, or cytoplasmic p65 expression in human PBMC’s.

The majority of studies investigating the effects of fatty acids on NF-κB activity have studied endothelial cells and macrophage cell lines, and have focused on linoleic, linolenic, arachidonic or oleic acids. It has been demonstrated that linoleic acid (90μM) increased NF-κB: DNA binding and the transcriptional activity of NFκB-responsive genes (Hennig et al, 1996, 2000, Toborek et al, 1996, 2002). In another study arachidonic acid, but not EPA (45μM for 1 h), enhanced NF-κB expression in U937 monocytic and J774 macrophage cells (Camandola et al, 1996). Supershift analysis revealed that this increased expression was due to enhanced p50 and p65 activity (Camandola et al, 1996). In addition, a ω-3 fatty acid emulsion decreased LPS-induced NF-κB: DNA binding and transcriptional activity in RAW 264.7 macrophages; NF-κB activity in cells treated with a ω-6 emulsion did not differ from cells alone (control cell) (Novak et al, 2003). More recently 72 h pre-treatment with sodium oleate (50-100μM) was found to decrease LPS-induced NF-κB: DNA binding in HUVEC’s (Carluccio et al, 1999). Apart from the oleic acid study, the majority of these investigations were conducted in cells co-cultured with fatty acids for relatively short periods of time (mainly 1-6h). In the studies of Toborek,
Hennig and colleagues, transcription was investigated mainly at 6h. This time point provided maximal rates of transcription in their culture systems. However, one of their earlier investigations reveals insignificant levels of NF-κB activation following incubation with linoleic acid for 2 h, 4 h or 24 h (Hennig et al, 1996). In our study we incubated both THP-1 monocytes and PBMC’s with fatty acids for 48 h prior to stimulation with LPS/PHA if required. We intended to encourage fatty acid uptake by the cells before probing for transcription factor activity. This difference in experimental design may explain the disparity of results with respect to linoleic acid between our study and other published data. In agreement with this discrepancy, studies with oxidised-LDL reveal short-term cultures to increase NF-κB transcription, whereas longer-term incubations actually decrease NF-κB activation in both THP-1 monocytes and primary human macrophages (Ohlsson et al, 1996, Mitkita et al, 2001, Brand et al, 1997).

There is no published information to date with respect to the effect of CLA on NF-κB activity. However, CLA has been reported to directly regulate the expression of NF-κB-target genes such as p53 and Bcl-XS (Pahl, 1999, Majumder et al, 2002). We report that cis-9, trans-11 CLA consistently decreased NF-κB activity in resting and LPS-activated THP-1 cells. Confirmation of this suppressive effect of the cis-9, trans-11 CLA isomer, on NF-κB transcriptional activity, was provided by the replication of results with the EMSA’s in resting PBMC’s.

This is a lack of information regarding the effects of fatty acids on IkBα and p65 expression. Recently Novak et al. (2003), reported a decrease in the expression of the phosphorylated form of IkBα following incubation of LPS-stimulated RAW 264.7 macrophages with a ω-3 fatty acid emulsion. A ω-6 fatty acid emulsion did not alter phosphorylated IkBα expression and neither fatty acid emulsion type affected total cell IkBα expression. In agreement with this study, fatty acids did not increase cytoplasmic IkBα expression in our culture system. However, cis-9, trans-11 CLA and linoleic acid significantly increased nuclear IkBα expression in resting and LPS-activated THP-1 monocytes. Newly synthesised IkBα is known to enter and accumulate in the nucleus, whereby it terminates NF-κB-dependent transcription (Arenzana-Seisdedos et al, 1995). This is due to the higher affinity of the NF-κB complex for IkBα than for DNA, resulting in the retrograde transport of the IkBα: DNA complex out of the nucleus by a mechanism involving nuclear export sequences (NES) on IkBα and p65, and a nuclear export protein called CRM1. (Arenzana-Seisdedos et al, 1995, Ossarch-Nazari et al, 1997, Rodriguez et al, 1999, Renard et al, 2000). The observed increase in nuclear IkBα expression in this study is consistent with our findings of decreased NF-κB binding. However, IkBα is an
NF-κB responsive gene and increases in IkBα mRNA are dependent on the binding of NF-κB to the κB sequences present in the promoter of the IkBα gene (de Martin et al., 1993, Sun et al., 1993). As we witnessed decreases in NF-κB activity with the cis-9, trans-11 CLA and linoleic acid, this would suggest these fatty acids do not activate IkBα transcription in a NF-κB-dependent manner. Similarly, we did not observe an increase in cytoplasmic IkBα expression. Hence it would appear that fatty acids do not affect the entry of IkBα into the nucleus.

The NF-κB:IkBα complex is thought to shuttle continuously in and out of the nucleus by a mechanism involving nuclear import and export sequences in both resting and activated cells (Huang et al., 2000, Johnson et al., 1999, Rodriguez et al., 1999, Ghosh & Karin, 2002). The fact that we observed increased nuclear expression of IkBα in THP-1 monocytes would indicate that perhaps fatty acids may have interfered with this nuclear import-export flux, possibly by affecting the phosphorylation status of IkBα. As mentioned previously, Novak et al., (2003), reported a decrease in the expression of the phosphorylated form of IkBα following incubation of RAW 264.7 macrophages with a ω-3 fatty acid emulsion. Down-regulation of IkBα phosphorylation would allow the IkBα: NF-κB complex to enter the nucleus, but it may have been shielded from subsequent ubiquination and degradation. If phosphorylation was affected, this may have been at the level of IKK activity or have involved phosphorylation of IkBα residues other than serines 32 and 36 (Schubert et al., 2002). In addition, any influence on the phosphorylation status of IkBα may have been effected at an earlier stage in cell signalling, possibly involving kinases e.g. PKC and MAPK, toll receptors or even PPAR’s. Fatty acids, in particular PUFA, are reported to inhibit the TCR-triggered MAP kinases ERK1 and ERK2 (Denys et al., 2001) along with various PKC’s, including PKCθ, which is expressed in lipid rafts and co-localises with IKKβ (Khoshan et al., 2000). PUFA were found to depress NF-κB activation mediated by toll-like receptor 4 (Lee et al., 2001) and are direct ligands of the PPAR transcription family (Forman et al., 1997, Kliewer et al., 1997). PPAR ligands are reported to negatively interfere with the NF-κB signalling pathway (Delerive et al., 1999, 2000, Yang et al., 2000). Alternatively, the fatty acids may have interfered with the ubiquination or degradation process of IkBα. Clearly, there are a variety of ways by which fatty acids could affect NF-κB/IkBα signalling. Our results imply the cis-9, trans-11 CLA and linoleic acid to increase nuclear IkBα expression; further research is required to elucidate their exact mechanism of action on IkBα.

We did not observe any effect of fatty acids on p65 expression either in cytoplasmic or nuclear THP-1 extracts suggesting that fatty acids do not mediate NF-κB
activity by affecting p65 protein levels. However, it is possible that the fatty acids may have affected p65 phosphorylation and/or acetylation status and hence NF-κB transcriptional activity. p65 contains a 'transactivation domain' (TAD), which allows for post-translational regulation of this NF-κB subunit (Lienhard Schmitz et al., 1995). The TAD allows for inducible phosphorylation of p65 by kinases including PKA (principally the catalytic subunit, PKAc), casein kinase II, PI-3K and Atk (Ghosh & Karin, 2002, Vermeulen et al., 2002). PI-3K is of particular interest with respect to fatty acids as it is intrinsically linked to phospholipids and phosphoinositides (Hwang & Rhee, 1999). These p65 TAD phosphorylation sites are only available upon IkBα degradation, but allow for enhanced DNA binding and provide an additional interaction site for NF-κB coactivators including CBP/p300 (Zhong et al., 1998, 2002). Additionally, acetylation of p65 is known to affect the efficiency of interaction with IkBα. Acetylation of p65 (probably mediated by CBP/p300 and histone acetyl transferases) controls the duration of NF-κB transcription; deacetylation of p65 (mediated by histone deacetylases) results in enhanced p65 binding to newly synthesised IkBα and subsequent nuclear export (Vermeulen et al., 2002). Although, we did not see a difference in p65 protein levels, it is possible that fatty acids may have influenced either the phosphorylation or acetylation of this rel-subunit and affected subsequent DNA-binding or interaction with IkBα.

In our study we looked at NF-κB activity, IkBα and p65 expression in THP-1 monocytes and human peripheral blood mononuclear cells. Whilst the results obtained in PBMC's tended to reflect those procured in THP-1 monocytes, there was a greater degree of inter-individual variation in all experiments. It is possible that some of this variation may be attributable to polymorphisms in the NF-κB and IkBα promoter regions (Ota et al., 1999, Curran et al., 2002, Parker et al., 2002); recently it has been shown that the ability of fish oil to decrease TNFα production ex vivo is influenced by polymorphisms in the TNFα gene (Grimble et al., 2002). In addition the fact that some of our sample population were smokers (n=2) and the heterogeneity of the cell type involved may have contributed to the variation observed. Perhaps if we had grown semi-transformed T-cell blasts (Cantrell & Smith, 1984) or purified a particular mononuclear cell type (e.g. monocytes), results would have been less variable. Resting PBMC's expressed lower cytoplasmic IkBα when treated with fatty acids, which would differ from the lack of effect observed in THP-1's. However, these decreases were lost when cells were activated with PHA. It is also possible that slightly different results may have been obtained due to the difference in stimulus used; i.e. LPS v PHA. PHA-P is a polyclonal mitogen, thought to act in particular through the CD2 surface molecule (O’Flynn et al., 1985, 1986). LPS is known to signal
through a CD14 and toll receptor pathway (Medzhitov & Janeway, 2000). Further experiments are necessary to elucidate if such a difference exists, however it would appear that the effects on NF-κB are stimulus-independent. Nevertheless, it is reassuring that the overall results obtained were comparable between a tumour cell line and PBMC’s from healthy donors.

In short, we found fatty acids, particularly cis-9, trans-11 CLA and linoleic acid to decrease NF-κB transcriptional activity in resting and activated THP-1 monocytes and human PBMC’s. These fatty acids also enhanced nuclear IκBα expression in resting and LPS-activated THP-1 monocytes. None of the fatty acids tested affected nuclear or cytoplasmic p65 expression. The cis-9, trans-11 CLA isomer was the most potent of all the fatty acids tested, whereas its ‘sister’ isomer trans-10, cis-12 CLA and the saturated fatty acid, palmitic acid were least effective. Further research is required to elucidate how exactly these fatty acids are mediating their effects on NF-κB signalling (e.g. affecting phosphorylation/kinase activity).
Chapter Six.

General Discussion.
This thesis was initiated with the aim of exploring the influence of the CLA isomers (cis-9, trans-11 CLA and trans-10, cis-12 CLA) on human immune function. Immunological and inflammatory processes including lymphocyte proliferation and cytokine production are sensitive to, and their functions subsequently affected by, alterations in their exogenous lipid supply. The immuno-modulatory benefits of the ‘novel’ fatty acid, CLA, are poorly characterised in humans. A human intervention trial and in vitro studies were carried out to fully examine the immuno-modulatory benefits of the CLA isomers, with some interesting results.

In chapter three, the intervention trial examined the influence of two isomeric blends of CLA on human immune function. Linoleic acid was included as a control fatty acid. Ex vivo measures indicated that the isomeric blend rich in the cis-9, trans-11 CLA isomer enhanced PHA-induced lymphocyte proliferation, whereas the trans-10, cis-12 CLA-rich blend depressed Con A-stimulated lymphocyte proliferation. However, as PHA and Con A are both T-cell mitogens, the differences observed in the lymphocyte proliferation results may have been influenced by the mitogen used. The concentrations of mitogens used in this study were chosen due to their maximal effects on lymphocyte proliferation during preliminary in-house experiments. However, it is possible that the concentrations used were too strong, and masked any CLA-induced effect. Additional studies, possibly involving a mitogen dose-response curve, are required to investigate this apparent disparity. All of the fatty acid treatments increased PHA-stimulated IL-2 and TNFα and decreased basal IL-2 secretion. However, there was no difference in efficacy between the CLA isomers or linoleic acid. Similarly, no difference existed between the three treatments with respect to mitogen stimulated IL-4 production, circulating PGE$_2$, LTB$_4$ and sICAM-1. Unfortunately, we did not measure the concentrations of these cytokines in the intra-cellular milieu. It would have been useful to examine the % of cells secreting each of the aforementioned cytokines and to see if the intra-cellular environment reflected the levels of cytokines detected in the supernatant.

The human intervention study suggested that the CLA isomeric mixes can influence human immune function, however they were similar in efficacy to the n-6 PUFA linoleic acid. At the time of commencing this study, purer forms of the CLA isomers were unavailable. Experiments using the individual isomers are necessary to investigate further if the observed differences in lymphocyte proliferation were due to an isomer-specific effect. We focused on two CLA isomers, the cis-9, trans-11 CLA and trans-10, cis-12 CLA. Chemical CLA synthesis leads to a predominance of these two CLA isomers. The cis-9, trans-11 CLA is also the most abundant naturally occurring isoform, comprising
approximately 75-80% total CLA in milk fat. However, another CLA isomer, trans-7, cis-9 CLA, is the second most prevalent CLA isomer in milk fat. The immuno-modulatory influence of this naturally occurring CLA isomer is as yet unknown and should not be ignored. It is also possible that we did not provide volunteers with an adequate dose of CLA or for a long enough period of time to observe an immunological effect. To clarify the effects of CLA on human immune function ex vivo, further supplementation trials are required, involving higher doses of CLA, the individual CLA isomers, and perhaps in a different population group (e.g. older individuals, males only etc.) for a longer period of time.

The in vitro studies in chapter four revealed that all of the fatty acids tested were incorporated into the total cell lipids and were capable of modulating cytokine transcription and translation. However, the precise mechanisms involved remain to be defined. We observed more pronounced effects on the lymphokine IL-2. Cis-9, trans-11 CLA consistently decreased both IL-2 mRNA expression and production, whereas trans-10, cis-12 CLA, linoleic acid and oleic acid all reduced IL-2 secretion. This may suggest that fatty acids are more influential at reducing naïve T-cell (Th0/Thp cells) and/or Th1 type T-cell activity. Experiments examining the effects of fatty acids on IFN-γ, (the proto-typical Th1 type cytokine), are required to examine this hypothesis. Our results also suggest that fatty acids are less influential on the Th2 type cytokine IL-4. Only three of the fatty acid treatments (cis-9, trans-11 CLA, stearic acid and oleic acid) decreased IL-4 mRNA expression. However, IL-4 production is reported as being cell-cycle dependent; hence a more pertinent analysis would involve studying IL-4 production over successive cell divisions. Of greater concern, the saturated fatty acid palmitic acid commonly found in cocoa butter, animal fats, milk, eggs and meat, consistently increased TNFα production. Appropriate amounts of IFNγ are required for pathogen clearance, however inappropriate overproduction is associated with a pathological response in vivo. Our results indicate an enhanced pro-inflammatory response following palmitic acid treatment in our culture system.

When investigating the influence of any effector on cytokine production, it is important to remember that cytokines function as part of an interacting network rather than in isolation. Our study focused on the lymphokines IL-2 and IL-4; perhaps it would have been of value to investigate the production of other cytokines e.g. IL-10. The cytokine IL-10 is produced exclusively by Th1 cells in mice and by both Th1 and Th2 cells in humans. IL-10 is characterised by its suppressive effects on the immune response. Indeed it is reported to increase IkBα expression and to decrease NF-κB activity. Hence, the influence
of fatty acids, including the CLA isomers on other cytokines, including IL-10, warrants further study in the aforementioned cell culture systems.

Chapter five explored the hypothesis that the effects of fatty acids on cytokine production were related to a decrease in the activity of the transcription factor NF-κB. The cis-9, trans-11 CLA isomer, and linoleic acid, decreased NF-κB activity in resting human PBMC’s, and in THP-1’s. In addition, the cis-9, trans-11 CLA isomer reduced LPS stimulated NF-κB activity in THP-1’s. These observed decreases in NF-κB activity, following treatment with cis-9, trans-11 CLA and linoleic acid, were coupled with an increase in nuclear IκBα expression in THP-1’s. However cytoplasmic IκBα, nuclear p65 and cytoplasmic p65 expression were not affected.

With respect to the effects of palmitic acid on TNFα, NF-κB activity and IκBα expression, the mechanisms of action are less clear. Palmitic acid resulted in significantly enhanced TNFα mRNA expression relative to all of the other fatty acid treatments, and increased TNFα production compared to oleic acid, in PHA-stimulated PBMC’s. In addition, palmitic acid significantly increased NF-κB: DNA binding and decreased nuclear IκBα expression in resting and LPS-stimulated THP-1 monocytes. However, NF-κB activity and IκBα expression were not influenced by palmitic acid in the human PBMC experiments. Hence it would appear that the effects on TNFα mRNA expression and production in human PBMC’s are independent of NF-κB. One possible mechanism whereby palmitic acid could influence TNFα production may lie at the level of the cellular membrane. It is possible that incorporation of this saturated fat into the cell membrane would alter the fluidity of the membrane (and the composition of the cellular lipid raft) and therefore the activity/orientation/density/number of the cell membrane-associated receptors (including the TNFα p55 and p75 receptors). Such an action may alter the subsequent receptor-mediated signalling pathways (e.g. PKC, TRAF etc.) and indeed the eventual transcription and translation of the TNFα protein. However, more research is needed to elucidate how exactly palmitic acid affects TNFα production in human PBMC’s.

These results suggest that the cis-9, trans-11 CLA isomer and linoleic acid may have affected the phosphorylation of IκBα and/or p65 or the remaining NF-κB subunits. If phosphorylation was affected this could have occurred at an earlier stage in cell signalling and may have influenced IκB kinases, MAP kinases or enzymes such as PKC. Further experiments are required to elucidate where exactly fatty acids are exerting their influence on this signalling pathway. To confirm that the decrease in NF-κB: DNA binding is also present at the level of transcription, future experiments using reporter assays specific for NF-κB dependent genes are necessary. We focused on one transcription factor, NF-κB,
which is ubiquitously expressed and critical for immune functions including lymphocyte proliferation, eicosanoid secretion and cytokine production. However, NF-κB works in conjunction with other transcription factors including AP-1, NF-AT, PPARs, STATs, and octamer proteins to regulate immune cell activity. We observed pronounced effects on IL-2 production. The IL-2 promoter contains binding sites for AP-1, NF-AT, STAT5b and Octamer proteins; hence the effect of fatty acids on these transcription factors is also worthy of attention. Fatty acids, including CLA, are reported to be direct ligands for the transcription factors PPARα and PPARγ. In particular PPARγ is reported to induce a decrease in the expression of pro-inflammatory cytokines by antagonising the activity of NF-κB, STAT1 and the AP-1 activating kinases JNK and p38. It is possible that the effects we observed on cytokine gene expression, production and NF-κB activity were caused by an increase in PPARγ activity. The influence of fatty acids on all of these transcription factors, including PPARγ, must be investigated so a more comprehensive picture of the effects of fatty acids on the transcriptional machinery can be drawn.

In conclusion, this thesis suggests a disparity in the immuno-modulatory effects of CLA ex vivo and in vitro. A predominance of the cis-9, trans-11 CLA isomer was associated with an enhanced T-cell proliferative response ex vivo. However in vitro culture with the purified form of this isomer was associated with a significant decrease in IL-4, IL-2, NF-κB activation, and an increase in nuclear IκBα expression. It is interesting to note that linoleic acid also reduced IL-2 production, NF-κB activity and increased nuclear IκBα expression. Although linoleic acid is the non-conjugated form of CLA, our results suggest its unsuitability as a placebo or control in future studies of CLA. This may also explain why we did not observe any real difference in effects between the CLA blends and linoleic acid in the human trial. The pro-inflammatory nature of the saturated fatty acid palmitic acid, was confirmed in this thesis. Whilst the trans-10, cis-12 CLA, stearic and oleic acid influenced immune function, their effects were secondary to those of the cis-9, trans-11 CLA isomer. From the in vitro studies, we can conclude that the purified cis-9, trans-11 CLA isomer is a more potent modulator of immune function than its sister isomer, trans-10, cis-12 CLA. Therefore, this thesis suggests that the cis-9, trans-11 CLA isomer has the ability to interact with the nuclear regulatory proteins controlling cytokine gene transcription, and to down-regulate the human immune response in vitro.

Following on from the studies presented in this thesis, a variety of other factors may have influenced the results obtained and warrant further investigation. Little knowledge exists regarding the influence of fatty acids on the co-stimulatory molecules and lipid raft composition. Activation of co-stimulatory molecules, particularly CD28, is a

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pre-requisite for cytokine production. CD28 is an NF-κB responsive gene and is associated in lipid rafts. CD28 is critical for increased IL-2 mRNA expression and stability, IL-4 receptor sensitivity and IL-2 production. The influence of fatty acids on this molecule deserves further attention. Equally, the area of lipid rafts and lipid raft composition is a new and exciting development. Along with CD28, the TCR and the IL-2 receptor subunits, a range of TCR associated molecules are embedded in these rafts or become associated upon T-cell activation. As the fatty acids used in this study modulated cellular total lipid composition, it would be interesting to explore if the lipid raft composition was affected also.

The \textit{in vitro} cell culture models used were designed to explore the effects of fatty acids in a mononuclear cell suspension (containing T- and B-lymphocytes, monocytes, macrophages, etc.) and in a transformed monocytic cell line. It would be worthwhile to investigate if the fatty acids are exerting a universal effect on all cell types or are targeting one particular cell type, e.g. the antigen-presenting monocyte, more so than another. Similarly, in these culture systems we included SFA, MUFA and n-6 PUFA. The influence of n-3 PUFA on the immune function is regarded as superior to that of the n-6 PUFA. It would be useful to include both EPA and DHA in future experiments so a hierarchy of efficacy could be established.

Human pathologies including Inflammatory Bowel Disease (IBD) and rheumatoid arthritis are characterised by an exacerbated Th1 type response. The term IBD comprises the diseases ulcerative colitis and Crohn's disease and its severity is affected by the amount and composition of fat in the diet. Clinically, IBD is associated with enhanced mucosal inflammation, caused in part by an increase in Th1 type cytokines (e.g. IFN-γ) and CD4+ T-cells. Rheumatoid arthritis is likewise associated with a skew towards a Th1 type cytokine profile. In our culture systems, \textit{in vitro} treatment with \textit{cis}-9, \textit{trans}-11 CLA was associated with a down regulation of NF-κB activity and decreases in IL-2 production. Although beyond the scope of this thesis, it has been reported elsewhere that differences exist in TCR mediated-NF-κB activation between Th1 and Th2 clones, whereby NF-κB is more potently activated in Th1 clones, resulting in IL-2 production and secretion. The down regulation of NF-κB activity, coupled with the decrease in IL-2 production observed following treatment with the \textit{cis}-9, \textit{trans}-11 CLA isomer, suggests a possible therapeutic role for this isomer with respect to these diseases. There is a need for further investigations looking at the effect of the \textit{cis}-9, \textit{trans}-11 CLA, individually and as an isomeric blend, in \textit{in vitro} and animal models of IBD and rheumatoid arthritis. An appropriate \textit{in vitro} model of IBD is the Caco-2 cell line. The human colon carcinoma cell line, Caco-2, is regarded as
the most relevant *in vitro* model for the study of differentiation and regulation of intestinal functions. This *in vitro* model could be easily adapted to investigate the influence of fatty acids, e.g. CLA, on intestine integrity and function. Appropriate animal models of experimental IBD include IL-10 knockout mice, and dextran sulfate sodium or bacterial-induced colitis in rats and pigs. CLA feeding studies and subsequent analysis of immunological, histological and molecular markers, would ascertain whether the CLA isomers, particularly *cis*-9, *trans*-11 CLA, improve IBD onset and inflammation. They would also provide a valuable insight into a potential therapeutic role for CLA in the treatment of human IBD, prior to commencing any type of clinical intervention. Similarly, *in vitro* and animal experimental models of rheumatoid arthritis exist (e.g. collagen induced arthritis in rats), and provide useful tools for examining the influence of CLA on this chronic inflammatory disease. Finally, if a positive effect is observed with CLA in the aforementioned studies, the challenge remains to identify whether this fatty acid is active in preventing disease initiation and/or progression, can ameliorate concomitant and undesirable side-effects (e.g. pain/swelling), and to identify its mechanisms of action.
Chapter Seven.

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