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Effects of Polyunsaturated Fatty Acids on Mediators of Inflammation associated with Atherosclerosis

A thesis submitted to the University of Dublin, Trinity College, for the degree of Doctor of Philosophy (Ph.D)

by

Anne Mullen

July 2005

Department of Clinical Medicine,
University of Dublin,
Trinity College
Dedicated to my parents, Margaret and Tim
DECLARATION

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

Anne Mullen

5 January 2006
Date
SUMMARY

The inflammatory pathogenesis of atherosclerosis has been defined, largely by the "response to injury" hypothesis. The morphological development of atherosclerosis has also been defined. Monocytes and lymphocytes accumulate in infancy at lesion-prone sites and are believed to initiate the atherosclerotic process in response to endothelial injury. They transmigrate across dysregulated endothelium from the circulation to the subendothelial space. Monocytes differentiate to macrophages, and the foam cell derivative is a major contributor to the necrotic core or "gruel" of the lesion. The secretory products of resident inflammatory cells mediate lesion progression. Lymphocytes, monocytes, macrophages and dendritic cells potentiate the dysregulation of endothelial cells and induce the proliferation and transmigration of the normally quiescent smooth muscle cell. The immune cells are vital by their protective capacity and their accumulation at the arterial wall is not clinically significant. Their reaction to endothelial injury, however, changes the dynamic in their function from physiological to pathological. Modulation of the inflammatory phenotype of cells that initiate and promote atherosclerosis could, potentially, mute the aggressive and dysregulated processes of lesion development. Fatty acids are known to modulate immune responses. This thesis investigates the effects of a number of polyunsaturated fatty acids (PUFA) on inflammatory mediators of lymphocytes, monocytes and macrophages associated with atherosclerosis, in vivo, ex vivo and in vitro.

The effects of conjugated linoleic acid (CLA) on systemic and peripheral blood mononuclear cell inflammatory mediators were investigated in Chapter 3. A human intervention study showed that supplementation with an isomeric blend of CLA modulates elements of the activated Th1 lymphocyte response ex vivo. The predominant isomers, cis 9, trans 11-CLA and trans 10, cis 12-CLA suppressed the production of pro-inflammatory cytokines in activated monocytes in vitro. These isomers have elsewhere been shown to reduce the nuclear transactivational binding of the pro-inflammatory transcription factor NF-κB. CLA acid has also been demonstrated to ligate to the anti-inflammatory transcription factor PPAR.

Elevated non-esterified fatty acids (NEFA), originating from triglyceride-rich lipoproteins and adipose tissue, are atherogenic. The fatty acid composition of circulating
NEFA may modulate this atherogenicity. A blend of fatty acids was designed in Chapter 4 to compositionally represent NEFA typically found in the serum of those at risk of developing cardiovascular disease. The saturated fatty acid (SFA) component was partially replaced by mono-unsaturated fatty acids (MUFA) and the n-3 long chain (LC) PUFA eicosapentanoic (EPA) and docosahexanoic (DHA) acids. The effects of altering the composition of NEFA on inflammatory mediator production in activated macrophages were investigated in vitro. The addition of the n-3 LC-PUFA appeared to confer some anti-inflammatory potential relative to the other treatments and control although generalised fatty acid effects common to all NEFA blends were also observed.

The n-3 LC-PUFA have been widely reported to exert anti-inflammatory effects in vitro, although the use of “fish oil” emulsions has obscured understanding of the specific effects of EPA and DHA. EPA and DHA were shown to potently suppress the production of pro-inflammatory mediators by activated macrophages in Chapter 5. EPA and DHA decreased cytoplasmic liberation, increased cytoplasmic retention and decreased nuclear translocation of NF-κB. However, it is likely that EPA and DHA also affect other transcription factors and related kinases to suppress pro-inflammatory cytokine production since, in Chapter 5, neither fatty acid appeared to affect the nuclear transactivational binding of the active NF-κB p65 subunit. Further in vitro work is required to elucidate other mechanisms that may complement the suppressive effects of EPA and DHA on the NF-κB signalling pathway.
ACKNOWLEDGEMENTS

I would like to thank Dr. Helen Roche for supervising my Ph.D. I am grateful for her direction, example and support, and for the opportunities I was given to travel with my work.

I appreciate the advice and support of Prof. Michael Gibney, Dr. Aideen McKevitt, Jo Gibney, Mary Moloney and Dr. Nick Kennedy in the Dept. of Clinical Medicine.

Thanks to everyone I’ve worked with in the Nutrition Lab and in the Nutrigenomics Research Group – Dr. John Browne, Eve Draper, Dr. Niamh Durcan, Dr. Christine Loscher, Jolene MacMonagle, Fiona Moloney, Dr. Enda Noone, Dr. Anne Nugent, Dr. Catherine Phillips, Audrey Tierney, Marianne Walshe and Dr. Sinéad Weldon. I’d like to thank Anne and Sinéad for introducing me to tissue culture, ELISA and RT-PCR and Christine for Western Immunoblotting. A special thanks to Fiona and Sinéad for their friendship and support.

I am grateful to Dr. Lucy Norris, Dr. Sharon O'Toole and Janette Brosnan, Dept. of Obstetrics & Gynaecology, for assistance with the fibrinogen clotting assay, for use of the microplate reader and for emergency freezer space.

I’d like to thank Dr. Paul Spiers, Dept. of Pharmacology, for instruction on gelatin zymography and for allowing me use of equipment and lab-space. Thanks also to Ken Scott and Dr. Pierce Kavanagh in the Dept. of Pharmacology for advice and assistance.

I would like to thank Dr. Yvonne Williams, Dr. Mohamed Abdel-Laff, Dr. Anthony Davies and Dr. Yuri Volkov for assistance with the Cellomics KineticScan Reader. I appreciate being given the opportunity to use this technology and to work with this group in the Institute for Molecular Medicine.

Thanks to the staff of the IMM and the Trinity Centre for Health Sciences. A special thanks to everyone in the Phase III labs – I’ve really enjoyed working with you. I’m grateful to the Cancer Research, Thoracic Oncology and Thrombosis and Haemostasis groups for shared equipment and facilities, and to the Dept. of Surgery for use of tissue culture facilities in my first year.

I am indebted to the volunteers of the CLA study, for generosity with time (and blood!), for dedication to the study and for perennial good humour.

Thank you to Mr. Charles Reade and the bell-ringers of St. Patrick’s Cathedral and St. Audeon’s, Dublin. I can’t thank you enough for the joy you’ve given and shared with me.

Thanks to my friends – you’ve been amazing.

To my parents, Margaret and Tim, for everything. Thanks Sara, Darby, Desrae, Tim and Mary.
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<th>Description</th>
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<tr>
<td>ABCA1</td>
<td>ATP binding cassette A1</td>
</tr>
<tr>
<td>ACAT</td>
<td>Acyl Co A transferase</td>
</tr>
<tr>
<td>ADHD</td>
<td>Attention-deficit hyperactivity disorder</td>
</tr>
<tr>
<td>AHA</td>
<td>American Heart Association</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presentation cells</td>
</tr>
<tr>
<td>ATL</td>
<td>Adult T cell leukaemia</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CE</td>
<td>Cholesteryl ester</td>
</tr>
<tr>
<td>CEPT</td>
<td>Cholesteryl ester transfer protein</td>
</tr>
<tr>
<td>cIAP</td>
<td>inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>CLA</td>
<td>Conjugated linoleic acid</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
</tr>
<tr>
<td>CM</td>
<td>Chylomicron</td>
</tr>
<tr>
<td>COMA</td>
<td>Committee of Medical Aspects of Food Policy</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CRP</td>
<td>C reactive protein</td>
</tr>
<tr>
<td>CSF</td>
<td>Cytokine synthesis inhibitory factor</td>
</tr>
<tr>
<td>CT</td>
<td>Cariotrophin</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
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<td>DD</td>
<td>Death domain</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexanoic acid</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-dimethylbenz(a)anthracene</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DR</td>
<td>Death receptor</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed type hypersensitivity</td>
</tr>
<tr>
<td>EB/AO</td>
<td>Ethidium bromide/acridine oragne</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electromobility shift assay</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentanoic acid</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
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<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty acid methyl ester</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GCSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone deacetyltransferase</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks balanced salt solution</td>
</tr>
<tr>
<td>HCS</td>
<td>High content screening</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HDC</td>
<td>Histidine decarboxylase</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone sensitive lipase</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>HSVEC</td>
<td>Human saphenous vein endothelial cell</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>ICE</td>
<td>IL-1β-converting enzyme</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of κB</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor κB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1RacP</td>
<td>IL-1 receptor accessory protein</td>
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<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<tr>
<td>IRAK</td>
<td>IL-1R associated kinase</td>
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<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>LAK</td>
<td>Lymphocyte-activated killer</td>
</tr>
<tr>
<td>LBP</td>
<td>LPS-binding protein</td>
</tr>
<tr>
<td>LC</td>
<td>Long chain</td>
</tr>
<tr>
<td>LCAT</td>
<td>Lecithin:cholesterol acyltransferase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>Symbol</td>
<td>Term</td>
</tr>
<tr>
<td>--------</td>
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</tr>
<tr>
<td>LIF</td>
<td>Leukaemic inhibitory factor</td>
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<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X receptor</td>
</tr>
<tr>
<td>m</td>
<td>Membrane-bound</td>
</tr>
<tr>
<td>MAG</td>
<td>Monoacylglycerol</td>
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<tr>
<td>MALT</td>
<td>Mucosal-associated lymphoid tissue</td>
</tr>
<tr>
<td>MARCO</td>
<td>Macrophage receptor with collagenous structure</td>
</tr>
<tr>
<td>MbCD</td>
<td>Methyl-b-cyclodextrin</td>
</tr>
<tr>
<td>MC</td>
<td>Medium chain</td>
</tr>
<tr>
<td>MDGF</td>
<td>Monocyte-derived growth factor</td>
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<td>MCP</td>
<td>Monocyte chemotactrant protein</td>
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<td>MCSF</td>
<td>Macrophage colony-stimulating factor</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage inhibitory factor</td>
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<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MT</td>
<td>Membrane type</td>
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<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acid</td>
</tr>
<tr>
<td>MyD88</td>
<td>Molecule myeloid differentiation factor 88</td>
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<tr>
<td>NAK</td>
<td>NF-kB-activating kinase</td>
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<td>NEFA</td>
<td>Non-esterified fatty acid</td>
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<td>NEMO</td>
<td>NF-kB essential modulator</td>
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<td>NF-kB</td>
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<td>NF-kB-inducing kinase</td>
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<td>Natural killer</td>
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<td>Oncostatin M</td>
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<td>PAF</td>
<td>Platelet activating factor</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAI</td>
<td>Plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemaglutinin</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLAD</td>
<td>Pre-ligand assembly domain</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome-proliferator</td>
</tr>
<tr>
<td>PPP</td>
<td>Platelet-poor plasma</td>
</tr>
<tr>
<td>PPRE</td>
<td>Peroxisome-proliferator</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet-rich plasma</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>r</td>
<td>Receptor</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel homology domain</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>s</td>
<td>Soluble</td>
</tr>
<tr>
<td>SAA</td>
<td>Serum amyloid A</td>
</tr>
<tr>
<td>SC</td>
<td>Short-chain</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated fatty acid</td>
</tr>
<tr>
<td>SFP</td>
<td>Super-family protein</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signalling</td>
</tr>
<tr>
<td>SRA</td>
<td>Scavenger receptor A</td>
</tr>
<tr>
<td>SRB</td>
<td>Scavenger receptor B</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element</td>
</tr>
<tr>
<td>TACE</td>
<td>TNFα-converting enzyme</td>
</tr>
<tr>
<td>TAD</td>
<td>Transactivational domain</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TAK</td>
<td>TGFB activating kinase</td>
</tr>
<tr>
<td>TBP</td>
<td>TNF-binding protein</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper lymphocyte</td>
</tr>
<tr>
<td>TIF</td>
<td>TNFα inhibitory factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1R</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethyl benzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Tr</td>
<td>T regulatory lymphocyte</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR-associated death domain</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF-associated factor</td>
</tr>
<tr>
<td>TRL</td>
<td>Triglyceride-rich lipoprotein</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling</td>
</tr>
<tr>
<td>TVA</td>
<td>Trans vaccenic acid</td>
</tr>
<tr>
<td>UFA</td>
<td>Unsaturated fatty acid</td>
</tr>
<tr>
<td>URA</td>
<td>Unexplained recurrent abortion</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VALT</td>
<td>Vessel-associated lymphoid tissue</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VLA</td>
<td>Very late antigen</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
1.1 CARDIOVASCULAR DISEASE
Cardiovascular disease (CVD), encompassing ischaemic heart disease, coronary artery disease and peripheral vascular disease, is the most prevalent cause of death in the Western world (Collins, 1996). 39% of deaths in Irish people in 2003 related to CVD (Central Statistics Office, Ireland). Atherosclerosis, the principal cause of CVD, is responsible for 50% of all mortality in the USA, Europe and Japan (Ross, 1993). Clinical presentations of the disorder include myocardial infarction (MI), stroke, transient ischaemic attacks, claudication, angina and gangrene.

CVD has traditionally been addressed as a disease of lipid deposition. Cholesterol testing is commonly used to identify those at high risk. However, only 50% of subjects in the Framingham Heart Study cohort who sustained an MI had increased total cholesterol (de Ferranti & Rifai, 2002; Mullenix et al. 2005). Less than 50% of coronary artery disease can be ascribed to traditional risk factors, including family history, hypercholesterolaemia, smoking, diabetes, obesity and hypertension (Verma et al. 2002). Plasma concentrations of the acute phase inflammatory reactant C reactive protein (CRP) correlated significantly with heart disease risk by the Framingham Calculated Risk Score and correlated only minimally with traditional components of coronary risk (Albert et al. 2003). The risk of coronary disease is about 90% greater in those with raised circulating concentrations of CRP and about 40% greater in those with raised total blood leukocyte counts (Malik et al. 2001). Systemic acute inflammatory reactants appear to independently determine prognosis of acute ischaemic syndromes (Liuzzo, 2001). There is substantial evidence implicating an inflammatory process in the pathogenesis of acute coronary syndromes (Fan & Watanabe, 2003; Langheinrich & Bohle, 2005; Mullenix et al. 2005; Mulvihill & Foley, 2005).

1.2 ATHEROSCLEROSIS: AN INFLAMMATORY DISEASE
The presence of inflammation in atherosclerosis was recognised in the 19th century by European pathologists. The autopsies (nearly 100,000) of Von Rokitansky (1804-1878) revealed inflammation within the atherosclerotic vessel wall. Aschoff (1866-1942) described inflammatory nodules and phagocytic activity within heart muscle and Virchow (1821-1902) argued a primary role for inflammation in the disease (Encyclopaedia Britannica, 1982). However, a role of inflammation in the pathogenesis of atherosclerosis has only recently been established, defined largely by the “response to injury” theory propounded by Ross (1993).
In this seminal paper Ross proposed that the lesions of atherosclerosis represent a series of highly specific cellular and molecular responses that, in aggregate, characterise atherosclerosis as an inflammatory disease. Inflammatory cells, which accumulate from infancy at the arterial wall, are activated (the “response”) by endothelial dysfunction (the “injury”) arising from insults related to CVD risk factors including elevated and modified LDL, free radicals caused by cigarette smoking, hypertension, diabetes mellitus, elevated plasma homocysteine and infectious microorganisms. The immune response that ensues is mediated chiefly by monocyte-derived macrophages and T lymphocytes and, in dysregulation, advances the injury in a series of defined pathologies.

1.3 DEVELOPMENT OF THE ATHEROSCLEROTIC LESION

The normal arterial wall is lined with an endothelial cell monolayer upon a basement membrane that provides a sensory interface between the bloodstream and the vessel wall (Houston et al. 2001). The underlying intima of is composed of connective tissue and some smooth muscle cells (SMCs). The media is muscular, of variable thickness and consists of SMCs. The adventitia is a supporting connective tissue layer which embeds the vessel in its surrounds (Collins, 1996; Wick et al. 2004). The predominant sites of atherosclerosis in order of frequency are the abdominal aorta and iliac arteries, the proximal coronary arteries, the thoracic aorta, the femoral and popliteal arteries, the internal carotid arteries and the vertebral, basilar and middle cerebral arteries (Collins, 1996).

The American Heart Association (AHA) has classified the development of the atherosclerosis (lesion Types I to VI) (Stary et al. 1994). A second classification system (Phases 1 to 5) has also been described (Fuster, 1994). Discrete lesional features define disease progression.

1.3.1 Early lesions

The early, non-clinical, lesions of atherosclerosis are classified by the AHA as Types I and II (Stary et al. 1994) or elsewhere, in aggregate, as Phase 1 (Fuster, 1994). Type I lesions are present in the coronary arteries of 45% of infants in the first eight months of life. They are composed of small isolated groups of lipid-laden macrophages, known as foam cells. The successive Type II lesions are visible as raised, yellow-coloured streaks, patches or spots on the arterial surface, known as “fatty streaks”. Fatty streaks are
usually found in the aorta in the first decade of life, the coronary arteries in the second and the cerebral arteries in the third or fourth decades (Lusis, 2000). They are composed primarily of stratified macrophage foam cells, some T lymphocytes and have a subendothelial accumulation of foam cells, lipid-laden SMCs and small amounts of extracellular lipid (Ross, 1993; Stary et al. 1994; Collins, 1996). Early lesional lipids are primarily (77%) cholesterol esters of oleic and linoleic acids, cholesterol and phospholipids. The small group of Type II lesions that progress to Type III co-localise with specific adaptive intimal thickenings in predictable locations and are progression-prone or Type IIa lesions. A larger subgroup of Type II lesions, found in intima that is thin and contains few SMCs, are progression-resistant or Type IIb lesions (Stary et al. 1994). Disruption to the matrix, intimal architecture, adventitia and adjacent media is absent or minimal in Types I and II, or Phase 1, early lesions (Ross, 1993). It is suggested that fatty streaks may be reversible (Libby et al. 1996).

1.3.2 Intermediate lesions
Type III lesions, alternatively termed intermediate, transitional or pre-atheromatous, form the morphological bridge between Type IIa lesions and atheroma. They are composed of layers of macrophages and SMCs (Ross, 1993) under which lie extracellular lipid pools that replace intercellular matrix proteoglycans and fibres, create "gruel", drive SMCs apart and disrupt their structural coherence (Stary et al. 1994). They belong to the Phase 1 classification (Fuster, 1994).

1.3.3 Advanced lesions
Lesion Types IV and Va, also characterised as Phase 2, are contained beneath a thin plaque. Type IV lesions have a predominance of diffuse extracellular lipid while in Type Va lesions the high lipid content is localised (Fuster, 1994). These lesions are firm, grey in colour and elevated. They are composed of proliferating SMCs, macrophages, lymphocytes, foam cells and extracellular matrix (Collins, 1996). The plaques become increasingly complex with calcification, ulceration, haemorrhage, fissure and thrombosis (Ross, 1993; Lusis, 2000). Neovascularisation occurs at the lesion cap and shoulders. The expression of leukocyte adhesion molecules has been shown to be more prevalent on intimal neovasculature than on arterial luminal enodothelium (O’Brien et al. 1996). Nodular deposits of calcification lend a pipe-like rigidity to the vessel wall. The plaque is associated with loss of elastic tissue and
increased vessel wall fragility (Collins, 1996). Types IV and Va lesions may evolve over time to the stenotic and fibrotic Types Vb and Vc. Plaque rupture and the subsequent thrombus formation characterises Phases 3 (mural thrombus) and 4 (occlusive thrombus), or genral Type VI lesion. The Phase 3 increase in stenosis may cause angina; the Phase 4 occlusive thrombus may result in unstable angina, MI or ischaemic sudden death. The Phase 5 lesion is severely stenotic and occlusive (Fuster, 1994). The advanced lesion, characterised by a lipid-rich core, stenotic, thrombotic and ischaemic complications distinguishes the disease seen in industrialised populations from that found in the developing world, where clinical events, per se, do not arise (Hegyi et al. 1996).

1.3.4 Rupture-prone lesions

The risk of rupture and thrombogenicity depends on the composition of the plaque rather than the percent stenosis of the vessel (van der Wal et al. 1994; Corti et al. 2004). Angiograms before and after administration of thrombolytic therapy reveal that the vast majority of MIs do not occur in the most stenotic lesions, but rather in those with stenoses of ≤70% (Plutzky 2001). Plaque morphology of necropsy specimens shows that in 50% to 75% of cases the culprit lesions have coronary thrombus, whereas the remainder show stable coronary plaques with >75% cross-sectional area luminal narrowing (Kolodgie et al. 2004). Such culprit lesions tend to be relatively soft, have larger lipid and necrotic cores, have thinner fibrous caps, are extremely numerous and more vulnerable to passive disruption because of their high lipid content (Fuster, 1994; Plutzky 2001; Corti et al. 2004). The plaque cap lacks underlying collagen support and is heavily infiltrated by macrophages, foam cells and T lymphocytes, although SMC content within the cap is sparse (Fuster, 1994; van der Wal et al. 1994; Kolodgie et al. 2004). In addition, macrophages and foam cells secrete matrix degrading metalloproteinase enzymes that can render a lesion susceptible to destabilisation and rupture (van der Wal et al. 1994; Corti et al. 2004). An in vitro study using a thermogenetic catheter has demonstrated an increased thermogenetic heterogenousity in rupture-prone compared to stable plaques (Lind, 2003), indicating that vulnerable plaques have a greater metabolically active cell population than stable plaques, which appear to be more inert. Vulnerable lesions tend to be thin-capped fibroatheromas with a heavy inflammatory infiltrate and the least luminal narrowing. Highly stenosed lesions have thick, fibrous caps, contain fewer macrophages and appear, ultimately, to be less
prone to rupture (Kolodgie et al. 2004). Several additional pathophysiological mechanisms may play a significant role in the process of plaque ulceration and rupture, including rheological factors, circumferential wall stress, circadian variation and vasoconstriction (Moreno et al. 1994).

1.4 CELLS OF THE ATHEROSCLEROTIC LESION
Structural and inflammatory functions of the major cells of the atherosclerotic microenvironment marry the “response to injury” hypothesis with the morphologically defined progression of the disease (Section 1.3).

1.4.1 Endothelial cells
The endothelium lining the entire vascular system is a monolayer of about $1 \times 10^{13}$ cells, weighing approximately 1 to 1.5kg and covering $700m^2$ (Sumpio et al. 2002; Laroia et al. 2003). Endothelial cells are the gatekeepers of vascular function and under basal conditions maintain a non-thromogenic blood-tissue barrier. The endothelium regulates thrombosis and thrombolysis, platelet adherence, vascular tone, inflammatory responses and controls leukocyte and platelet interactions with the cell wall (Sumpio et al. 2002). Endothelial injury results in a pro-atherogenic and pro-thrombotic phenotype characterised by increased leukocyte adhesion, promotion of SMC migration and proliferation, modulation of extracellular matrix composition, modulation of vascular tone and susceptibility to thrombus formation (Vogel, 1999). Of particular interest is the pro-adhesive quality of dysfunctional endothelium, particularly with regard to the recruitment of leukocytes and platelets from the circulation.

Circulating leukocytes roll along and are transiently tethered to dysregulated endothelial cells, initially mediated by selectins (Krieglstein & Granger, 2001; Malik et al. 2001). E-selectin is expressed exclusively on activated endothelium, induced by inflammatory cytokines and transcriptionally activated by nuclear factor-κB (NF-κB). P-selectin is expressed mainly on platelets, stored in α-granules, but also on activated endothelial cells, stored in Weibel-Palade bodies (Parker et al. 2001; Blankenberg et al. 2003). L-selectin is expressed on leukocytes. By interacting with their ligands selectins create weak bonds between activated endothelial cells and leukocytes.

A stronger attachment of leukocytes to endothelial cells is mediated by ICAM-1 and VCAM-1 (Malik et al. 2001). Sites of atheroma formation display augmented expression of these cell adhesion molecules (Libby et al. 1996). ICAM-1, the most
widely expressed cell adhesion molecule, is found on leukocytes, fibroblasts, epithelial and endothelial cells. Its expression is upregulated in response to lipopolysaccharide (LPS), phorbol esters, pro-inflammatory cytokines and LDL exposure (Yuan et al. 2001). Ligands for ICAM-1 include the leukocyte specific β2-integrins (Blankenberg et al. 2003). VCAM-1 is transcriptionally induced on endothelial cells but can also be expressed by other cell types like macrophages, myoblasts and dendritic cells (DCs). It interacts with α4β1-integrin, known also as very late antigen-4 (VLA-4). Engagement of VCAM-1 and its ligand induces conformational change in the endothelial barrier and allows leukocyte transmigration (Matheny et al. 2000). PECAM-1 is densely found at the junctions between endothelial cells where it is involved in homophilic binding between adjacent cells (Newton et al. 1997). It is involved in endothelial integrity and extravasation of cells from the blood (Blankenberg et al. 2003). The processes mediated by adhesion molecules that culminate in leukocyte transmigration are presented in Figure 1.1.

---

Figure 1.1 Cell adhesion molecule mediation of leukocyte rolling, tethering, arrest and subsequent transmigration of endothelial cells. Adapted from Blankenberg et al. 2003
In humans, focal expression of adhesion molecules has been consistently observed in atherosclerotic plaques (Malik et al. 2001; Blankenberg et al. 2003). Soluble forms of cell adhesion molecules are shed from activated endothelial. Clinical studies show that elevated serum ICAM-1, VCAM-1, E and P selectin may be independent risk factors for CVD. Circulating concentrations of soluble cell adhesion molecules, particularly ICAM-1 and E-selectin reflect the level of established CVD risk factors such as smoking, waist-hip ratio, blood pressure, total and LDL cholesterol in healthy men and women (Demerath et al. 2001). P-selectin knock-out (−/−) and ICAM-1 −/− mice develop fewer arterial lesion compared to controls in the apoE −/− mouse model (Collins et al. 2000). The British Regional Heart Study demonstrated that although concentrations of soluble adhesion molecules were significantly associated with some classical coronary risk factors only elevated ICAM-1 in the upper tertiles offered predictive value for relative risk of coronary heart disease (Malik et al. 2001). Elsewhere, soluble P-selectin, but not E-selectin, ICAM-1 or VCAM-1, was found to be an independent predictor of unstable coronary syndrome in patients with CVD, whereas E-selectin was associated only with extent of atherosclerosis (Parker et al. 2001).

1.4.2 Smooth Muscle Cells

SMCs of the vascular media do not proliferate or migrate in the quiescent state. This contractile phenotype, maintained in part by endothelial-derived nitric oxide (NO), is characterised by the presence of α-actin and the inability to undergo cytokinesis (Moiseeva 2001; Reusch et al. 2003; Lavezzi et al. 2005). Platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), activators of vascular SMC proliferation, are upregulated after endothelial injury (Law et al. 1996) and SMC proliferation is believed to be one of the fundamental mechanisms in the pathophysiology of the atherosclerotic process. Activated vascular SMCs proliferate and express interleukin (IL)-1 and IL-6, growth factor endothelin-1 and the receptor for PDGF (Reusch et al. 2003). There is a loss of contractile function, a switch in actin expression from α to β, an increase in rough endoplasmic reticulum and an increase in synthetic function. Proliferative SMCs produce collagen, elastin and extracellular matrix within the atherosclerotic lesion (Ross, 1999). Lavezzi et al. (2005) have demonstrated the activation of the c-fos gene in foetal SMCs. Activation of c-fos causes a loss of SMC differentiation and increased migration towards the intima, indicating a pre-natal beginning of human atherosclerosis. In addition, proliferating cell nuclear
antigen (PCNA), a marker of proliferative disease, was detected in SMCs from the vasculature of infants and young people, whereas SMC apoptosis characterised advanced lesions of adulthood (Lavezzi et al. 2005). Vascular calcification has been recently associated with the SMC transitional phenotype (Steitz et al. 2001; Tyson et al. 2003). Bobryshev (2005) has reported that SMCs in the necrotic core of human atherosclerosis transdifferentiate into chondrocyte-like cells whose membrane-bound vesicles appear to undergo calcification. In the atherosclerotic-like lesions of the apo E-/- mouse transdifferentiation of SMCs into chondrocytes was implicated in atherosclerotic calcification (Bobryshev, 2005).

Both innate and adaptive immune responses play a role in the development of atherosclerosis. Innate responses are not very specific and involve receptors that recognise evolutionary conserved patterns on antigens, such as scavenger receptors and Toll-like receptors (TLRs). Cells of the innate immune system involved in atherosclerosis include DCs, monocytes, macrophages and macrophage foam cells. The highly specific adaptive immune response involves clonal selection and expansion of T and/or B lymphocyte subsets following antigen recognition. T helper lymphocytes contribute to an adaptive immune response in the development of atherosclerosis.

1.4.3 Dendritic cells
Sentinel DCs reside in vessel-associated lymphoid tissue (VALT) distributed throughout the subendothelial layer of the arterial intima (Cao et al. 2003). Immature DCs sample their antigenic microenvironment by macropinocytosis and receptor-mediated endocytosis, undergo maturation and mobilisation, and migrate to zones of T lymphocyte residence to function as antigen-presentation cells (APCs). DCs have been identified within human and murine atherosclerotic lesions (VanderLann & Reardon, 2005). They express leukocyte adhesion molecules ICAM-1 and VCAM-1 and high levels of both class I and class II major histocompatibility complex (MHC) which enable them to proximate naïve T cells. They co-localise with T lymphocytes in the shoulder regions of rupture-prone plaques and in regions of neovascularisation (Lord & Bobryshev, 2001; Cao et al. 2003; Yilmaz et al. 2004). It is suggested that plasmacytoid DCs induce Th1 differentiation while myeloid DCs promote Th2 differentiation. It is also suggested that the activation status, rather than lineage, of the DC determines the T lymphocyte subtype it primes (Mills & McGuirk, 2004). IL-12 secreted by DCs is a
potent and obligatory inducer of Th1 lymphocyte differentiation (Moser & Murphy, 2000). DCs are more potent activators of T lymphocytes than macrophages and lesional monocytes may differentiate to DCs rather than macrophages depending on microenvironmental stimuli, such as the presence of oxidised LDL. DCs may express immunoglobulin and complement receptors, such as FcγR and C1q (although monocyte-derived DCs are C1q negative), which mediate the uptake of antigen-immune complexes (Lord & Bobryshev, 2001; Cao et al. 2003). Nicotine has been shown to dose-dependently activate DCs, leading to increased Th1 cytokine secretion (Aicher et al. 2003). It has been suggested that vascular DCs are principally responsible for T cell activation in atherosclerosis (Cao et al. 2003).

1.4.4 Monocytes, macrophages and foam cells
Expression of cell adhesion molecules by endothelial cells (P-selectin, E-selectin, ICAM-1 and VCAM-1) draws circulating monocytes to the arterial site of injury. In addition, a gradient of monocyte chemoattractants such as monocyte chemoattractant protein (MCP)-1, tumour necrosis factor (TNF) α, IL-1 and macrophage colony-stimulating factor (MCSF) are expressed by endothelial cells, SMCs and already resident monocytes/macrophages (Raines et al. 1996; Heygi et al. 2001). Lysophosphatidylcholine, a component of oxidised LDL, is also a potent monocyte chemoattractant. Following adherence to the endothelium, monocytes spread and crawl between endothelial cells and may localise in the intima (Raines et al. 1996). Monocytes may differentiate to C1q negative DCs (Schmitz et al. 1997; Cao et al. 2003) and some may egress back into the circulation (Libby et al. 1996). Classically, however, the intimal monocyte accumulates lipid and differentiates into the macrophage foam cell in response to factors such as MCSF and granulocyte CSF (GCSF) (Raines et al. 1996). In early lesion development, it is most likely that the macrophage response is protective (Ross, 1999). With sustained inflammation and continued monocyte influx the dynamic between protective and pathological changes and the aggressive inflammatory phenotype displayed by the classically activated macrophage is operative in lesion progression.

Macrophages are the predominant inflammatory cell of atherosclerosis, in number and pleiotrophy, with initiating, exacerbating and constitutive roles in lesion development. Mice defective in the GCSF receptor (ie: low macrophage number), MCP-1, CCR2 (the MCP-1 receptor) and class A or class B scavenger receptor (lipid
receptors) are all defective in atherogenesis (Song et al. 2001). Macrophages have been identified in atheromatous gruel, sclerotic tissue and thrombus of the atherosclerotic process (Moreno et al. 1994) and particularly at the periphery or "shoulder" and lipid-rich core of plaques that are prone to rupture (Heygi et al. 2001; Okazaki et al. 2002). The main roles of the macrophage in this context are as a scavenger cell, an antigen presentation cell and as a secretory cell. Macrophage apoptosis within the lesional micro-environment is an emerging concept in the aetiology of the atherosclerosis.

1.4.4a Macrophage scavenger receptors

The glycoprotein scavenger receptors of macrophages have been referred to as "molecular fly-paper" and enable resolution of most inflammatory situations by scavenging debris from interstitial tissue (Raines et al. 1996). In addition, this removal of antigen is normally enhanced by the migration of macrophages from the site of injury. Scavenger receptors have a host of antigenic ligands, including native and modified lipoproteins. The principal scavenger receptors of the macrophage and their lipoprotein ligands are presented in the Table 1.1.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>SRA-I &amp; II</th>
<th>MARCO</th>
<th>SRB-I</th>
<th>CD36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidised LDL</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acetylated LDL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Native LDL</td>
<td>-</td>
<td>ND</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>HDL</td>
<td>-</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VLDL</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
</tbody>
</table>

The class A scavenger receptor was initially identified to have two isoforms, SRA-I and SRA-II. A third splice variant, SRA-III, and another member, macrophage receptor with collagenous structure (MARCO), have been described. The principal class B scavenger receptors are SRB-I and CD36 (Peiser & Gordon, 2001). Foam cells are generated by the uptake of modified lipoproteins, particularly oxidised LDL, through SRA and CD36.
In vitro exposure of a murine macrophage cell-line and ex vivo peritoneal macrophages to native, acetylated and oxidised LDL induced persistent CD36 expression with the greatest induction by oxidised LDL. Incubation with HDL reduced CD36 expression (Han et al. 1997). Expression of CD36 was increased in vitro on monocytes by MCP-1 (Tabata et al. 2003). Macrophages expressing SRA-I and SRA-II have been identified in early and mildly advanced plaques, where CD36+ cells were rare. In advanced atherosclerotic lesions CD36+ cells were detected in the core regions, with SRA isoforms distributed more in the peripheral regions and closer to the luminal surface of the plaque, indicating that CD36 and SRA might contribute differently to plaque development in atherosclerosis (Nakagawa-Toyama et al. 2001). However, atheroma-associated foam cells do not exhibit suppression of scavenger receptors, allowing an exaggerated accumulation of lipids (Libby et al. 1996). In a protective capacity lipid-laden macrophages may transfer cholesterol to lymphocytes, evidenced by a 111% increase in lymphocyte total cholesterol content following co-culture, which may suppress lymphocyte proliferation activity (de Bittencourt Jnr. & Curi, 1997).

1.4.4b Macrophage antigen presentation

Macrophages within the subendothelium and advanced plaques express major histocompatibility complex (MHC) class II antigen and are found in close apposition with T lymphocytes. Macrophages serve as APCs to adaptive immune elements, although they are not as potent in this regard as DCs.

1.4.4c Macrophage secretory products

The role of the macrophage most pertinent to this thesis is that of a secretory cell. CD14+ macrophages are a major constituent of the lesional macrophage infiltrate (Raines et al. 1996). CD14+ is a co-receptor for NF-κB signalling which induces transcription of pro-inflammatory mediators. In a protective capacity these mediators have a direct anti-pathogen activity or contribute to the activation of the effector cells. The macrophage-derived cytokine milieu can influence the proliferation and differentiation of T helper lymphocytes (Trinchieri, 1997). Lesional macrophages produce pro-inflammatory cytokines such as IL-1β, TNFα and IFNγ. They secrete monocyte-derived growth factor (MDGF) and PDGF, which induce proliferation of SMCs from the contractile to the synthetic phenotype. Macrophages secrete vascular
endothelial growth factor (VEGF) which induces neovascularisation at the base of advanced plaques (Heygi et al. 2001). Macrophages and foam cells secrete matrix metalloproteinases (MMPs) which degrade extracellular matrix and may compromise plaque stability, predisposing to rupture (Libby et al. 1996; Lind, 2003). Migration inhibitory factor (MIF) is secreted constitutively and ubiquitously. It is abundantly expressed by various cells of the human atherosclerotic early and advanced lesion including macrophages, lymphocytes, vascular endothelial cells and SMCs (Burger-Kentishcher et al. 2002; Lue et al. 2002) and was upregulated in a macrophage model by oxidised LDL (Atsumi et al. 2000). MIF is a “cytozyme”, a cytokine (one of the first discovered, in 1966) with enzymatic tautomerase and oxidoreductase properties. It inhibits the random migration of macrophages (Lue et al. 2002). Mentioned earlier, the efficient removal of antigenic debris by scavenging macrophages depends upon cell migration away from the area of damage. This may potentially be inhibited by MIF. Furthermore, MIF increased oxidised LDL uptake and adhesion molecule expression on a HUVEC (human umbilical vein endothelium cell) model (Burger-Kentishcher et al. 2002), indicating that it may potentiate recruitment of monocytes to the lesion. MIF-stimulated macrophages secrete bioactive TNFα (Calandra et al. 1994). Histamine, a vasoconstrictor, is chronically produced in atherosclerotic lesions and elevated levels are associated with acute coronary events. Macrophages in human atherosclerotic lesions express histidine decarboxylase (HDC), a histamine-producing enzyme (Sasaguri & Tanimoto, 2004). The major secretory products of macrophages and foam cells relating to atherosclerosis development are listed in Table 1.2.

Table 1.2 Major secretory products of macrophages. Data from Raines et al. 1996

<table>
<thead>
<tr>
<th>IL-1</th>
<th>Connexin</th>
<th>Thrombospondin</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>Osteopontin</td>
<td>Serum amyloid A</td>
</tr>
<tr>
<td>TNFα</td>
<td>Osteonectin</td>
<td>Matrix Gla protein (MGP)</td>
</tr>
<tr>
<td>MMPs</td>
<td>Factor XIIIa</td>
<td>Plasminogen activator inhibitor (PAI)</td>
</tr>
<tr>
<td>TIMP-1 &amp; 2</td>
<td>15-LOX</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>MCP-1</td>
<td>Complement C3b</td>
</tr>
<tr>
<td>VCAM-2</td>
<td>MCSF</td>
<td>Leukaemic inhibitory factor (LIF)</td>
</tr>
<tr>
<td>Endothelin-1</td>
<td>PDGF</td>
<td>Lipoprotein lipase (LPL)</td>
</tr>
</tbody>
</table>
1.4.4d Macrophage apoptotic cell death

At initial activation macrophages are resistant to apoptotic cell death (Boscá et al. 2005). Secretory products of macrophages including TNFα, IL-1β and IFNγ, when synthesised at high concentrations, are promoters of apoptosis. After cumulative damage, or when synthesis of inflammatory mediators decrease, terminally differentiated macrophages display down-regulation of c-fos, the receptor for the apoptosis-suppressant MCSF, and undergo the characteristic mitochondrial cell death program (Raines et al. 1996; Boscá et al. 2005). Apoptosis has been described in both macrophages and SMCs at all stages of the atherosclerotic lesion by TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling) staining (Heygi et al. 1996; Heygi et al. 2001). Apoptosis of macrophage foam cells in atherosclerosis appears to be pathological, contributing to the accumulation of gruel, plaque instability and release of cytotoxic products (Heygi et al. 1996; Inagaki et al. 2002; Lusis, 2002). Oxidised LDL is reported to be cytotoxic to macrophages, initiating and propagating cell injury and apoptosis (Heygi et al. 2001). In addition, IFNγ has been demonstrated to induce apoptotic cell death of macrophages. IFNγ up-regulated TNFR1 and caspase-8, but did not affect bax and bcl-2, indicating that macrophage apoptosis is induced in this instance through a cell death receptor rather than a mitochondrial pathway (Inagaki et al. 2002). Apoptosed macrophages and foam cells are not cleared from the atherosclerotic lesion. The overlying fibrous cap and increased expression of MIF limits the migratory potential of the macrophages. Heat-shock protein (HSP)-70, an indicator of cellular metabolic stress, is elevated in atherosclerotic lesions, particularly at sites of necrosis and lipid accumulation. Trapped macrophages and foam cells may die by apoptosis, hypoxia or cytotoxicity (Raines et al. 1996) and potentiate inflammation by releasing toxic oxidised products of LDL and lysosomal proteases.

1.4.5 T lymphocytes

T lymphocytes are found in human atherosclerotic lesions (Mazzone et al. 1999; Song et al. 2001; de Boer et al. 2003) and appear to be of multiple origin. Lymphocyte recruitment to specialised microenvironments, such as the atherosclerotic plaque, is believed to be trafficked or “homed” by adaptive decision processes which allow an integrated and controlled system of lymphocyte function, life span and population dynamics (Picker, 1994; Butcher & Picker, 1996). Lymphocytes are homed to specific endothelial sites by expression of specialised adhesion molecules (von Andrian
Mackay, 2000). Circulating lymphocytes expressing β-integrins and VLA-4 interact with vascular endothelial cell adhesion molecules (Blankenberg et al. 2003) and transmigrate to tissue. In advanced plaques, T lymphocytes cluster in proximity of neovascularisation at the base of the lipid core and shoulders of plaques (O'Brien et al. 1996), indicating T lymphocyte infiltration from both the periphery and core of the lesion. An unexpectedly high proportion of lesional T lymphocytes (10 to 15%) were shown to be T cell receptor (TCR) γδ+, compared with 1 to 2% of peripheral blood lymphocytes, and a large proportion of lesional lymphocytes expressed the TCR Vδ1-chain typical of human mucosal-associated lymphoid tissue (MALT) (Wick et al. 2004). Constituting up to 20% of the total cell population within the plaque, 70% of lesional T lymphocytes are CD4+ cells (Lord & Bobryshev, 2001; Wick et al. 2004). CD4+ cells recognise antigen phagocytosed, processed and presented by MHC class II molecules of APCs. The remainder of lesional T lymphocytes are CD8+ cytotoxic cells, which recognise antigen in terms of class I MHC and will not be addressed in this thesis. The immunological memory of the CD4+ T helper (Th) lymphocyte enables specific antigenic recognition, activating clonal expansion and an appropriate cytokine secretory response that promotes effector cells (Mazzone et al. 1999; Song et al. 2001). Th lymphocytes represent an adaptive immune response. They communicate antigenic information to effector cells and initiate an immune response adaptive to specific antigenic stimuli, as interpreted by the Th memory.

T lymphocytes are active in the development of atherosclerosis. When CD40, a potent T lymphocyte activator, is blocked, there is approximately 60% reduction in atherosclerosis. The atherosclerotic-prone LDL receptor -/- mouse crossed with the lymphocyte-deficient RAG1 mouse displays a decreased VLDL cholesterol content and reduced atherosclerotic lesions on a Western-type diet compared to LDLR -/- controls (Song et al. 2001).

1.4.5a T helper lymphocytes (Th1/Th2)

In the classical model of T helper cell differentiation naïve Th0 cells respond to antigen presentation, by cells such as mature DCs and macrophages expressing MHC class II or the cytokine milieu at the site of immune response, by polarising to a Th1 or Th2 pathway of adaptive immunity (Del Prete, 1998; Lord & Bobryshev, 2001). The polarised Th1 lymphocyte secretes pro-inflammatory cytokines such as IL-2, IFNγ, TNFα and TNFβ (Trinchieri et al. 1996; Lord & Bobryshev, 2001). The polarised Th2
lymphocyte secretes cytokines such as IL-3, IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Trinchieri et al. 1996; Pohl-Koppe et al. 1998). Polarisation may be influenced in subjects with a particular genetic background or under the influence of strong and/or chronic micro-environmental signals. Modes of immunization (parenteral or inhalation/ingestion), antigen forms (soluble or corpusculate) and doses influence Th polarisation (Romagnani, 1996). The presence of IL-12, produced by DCs and macrophages, or IL-4, released by human bone marrow non-T non-B cells, at the time of antigen presentation is critical in determining polarisation to Th1 or Th2, respectively (Trinchieri et al. 1996; Mazzone et al. 1999). However, Th cells expressing Th1 and Th2 phenotypes, secreting both IFNγ and IL-4, (effectively matured Th0 cells) have been identified in terminal differentiation (Kamogawa et al. 1993; Seder & Paul, 1994; Kelso 1995). Th cells secreting both IFNγ and IL-10 have been reported in patients with chronic Borrelia burgdorferi infection (Pohl-Koppe et al. 1998). A model of Th differentiation is presented in Figure 1.2.

![Figure 1.2 Th0 differentiation model. Naive Th0 cells classically differentiate towards either a Th1 or Th2 response, although a mature Th0 phenotype has been identified](image-url)
Strict Th1/Th2 polarisation is a useful, but simplified, model of definition. Furthermore, this model is based on murine immune responses and is less applicable to the human CD4+ lymphocyte, where expression of some cytokines is less restricted by polarisation. The potential of human Th1 and Th2 to secrete classically polarised cytokines is presented in Table 1.3.

### Table 1.3 Secretion of classically polarised cytokines from human Th1 and Th2 lymphocytes

The Th1/Th2 polarisation model is useful in definition, but cytokines are produced less exclusively *in vivo*. Adapted from Romagnani, 1996.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Th1</th>
<th>Th2</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>IL-3</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>IL-4</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>IL-5</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>IL-10</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>IL-13</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>IFNγ</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>TNFα</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>TNFβ</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Th1 cells are more suitable for protection against intracellular parasites because of their production of cytokines that promote opsonising and complement-fixing antibodies, antibody-dependent cell cytotoxicity, macrophage and cytolytic T cell activity, and delayed type hypersensitivity (DTH) (Romagnani, 1996). Th1 cytokines, particularly IL-2 and IFNγ, are the most prevalent in human atherosclerotic plaques (Frostegård *et al.* 1999) and may promote atherosclerosis (Song *et al.* 2001; Mallat *et al.* 2003; Mallat & Tedgui, 2004). Lesion development in atherosclerosis-prone mice can be inhibited by pharmacological blocking of the Th1 response (Laurat *et al.* 2001). Secretion of IL-2, IFN and TNF results in growth inhibition and expression of adhesion molecules by a variety of cells (Seder & Paul, 1994; Frostegård *et al.* 1999). IFNγ induces the expression of MHC class II and activation of macrohages, antagonises the production of stabilising collagen and when blocked an approximately 60% reduction in
Atherosclerosis has been observed (Song et al. 2001). Activation of macrophages by Th1 cytokines potentiates an aggressive inflammatory phenotype.

Th1 and Th2 responses offer a dynamic in the progression of atherosclerosis. A Th1-driven response has been shown to promote atherosclerosis whereas immune modulation through the Th2 pathway is believed to have the potential to impede the disease process (Laurat et al. 2001; Lord & Bobryshev, 2001; Song et al. 2001; Mallat & Tedgui, 2004).

Th2 cells promote humoral immune responses such as B lymphocyte differentiation and antibody production, IgE and IgG1 isotype switching, mucosal immunity, mast cell and eosinophil growth and differentiation, and facilitation of IgA synthesis (Romagnani, 1996; Lord & Bobryshev, 2001). Th2 cytokines such as IL-4, IL-10 and IL-13 antagonise the Th1 response and several macrophage functions. IL-10 is considered potently antiatherogenic. However, the Th1 inducer IL-12 dominates over IL-10, and transcripts of IL-4, IL-5 and IL-10 are not generally found in the atherosclerotic plaque (Frostegård et al. 1999).

As detailed earlier, the polarisation of Th responses is a simplified, but useful, model. Human Th1 and Th2 lymphocytes are not mutually exclusive in the cytokines they secrete (Romagnani, 1996) and the mutual antagonism of these responses is not complete. A balanced, rather than biased, immune response is necessary for host protection and the resolution of infection. A number of factors are involved in the regulation of T helper responses.

The memory function of T lymphocytes allows reduction in their sentinel numbers. Encounter with antigen induces proliferation of clones, yielding approximately 1,000 fold more descendants of identical antigenic specificity (von Andrian & Mackay, 2000). When the antigen is cleared, the T lymphocyte cohort is condensed once more by death of the majority and survival of a few antigen-experienced memory cells.

Timing may be a factor in determining cytokine polarisation. Kinetic studies in Mycobacterium bovis and Onchocerca ochengi infected cattle have demonstrated reciprocal IFNγ and IL-4 responses, a fluctuation of cytokine responses over time, periods of polarisation and periods of non-polarisation before a definite Th1 or Th2 response is established (Rhodes & Graham, 2002).

Th1/Th2 response switching occurs to limit or prevent a typical, but inappropriate, Th response in some instances. A Th1 response observed in moderately
hypercholesterolaemic apoE -/- mice was switched to a less inflammatory Th2 response in severely hypercholesterolaemic mice (Zhou et al. 1998) indicating damage limitation. Th1 effector mechanisms play a role in acute allograft rejection. Allograft tolerance is characterised in vivo by decreased IL-2 and IFNγ, and increased IL-4 and IL-10, expression (Steele & Strom, 1994). The embryo expresses paternal MHC antigens and should thus induce a Th1 response, similar to an allograft. Significant increases in Th1-like clones have been reported in the deciduas of women suffering unexplained recurrent abortion (URA). It has been proposed that a Th1/Th2 switch occurs at the materno-foetal interface to sustain pregnancy. Progesterone favours the development of Th2-type cells and inhibits production of IFNγ and TNFα by peripheral blood mononuclear cells (PBMC) of women with URA (Romagnani, 1996).

Anergy is a state in which cells persist in a hyporesponsive state. T lymphocyte function, in anergy, is downregulated despite antigen encounter. Clonal anergy, or arrested development, arises from incomplete T cell activation. Adaptive tolerance, representing a more generalised inhibition of proliferation and effector functions, arises from stimulation in an environment deficient in co-stimulation or high in co-inhibition (Schwartz, 2003).

1.4.5b T regulatory cells
T regulatory (Tr) cells have important immunosuppressive properties, particularly of Th1 immune responses. A number of T regulatory (Tr) cells have been described. Tr1 cells secrete both IL-10 and TGFβ, Th3 cells primarily secrete TGFβ, CD8+Tr cells secrete either IL-10 or TGFβ and CD4+CD25+ cells inhibit immune responses through cell-cell contact (McGuirk & Mills, 2002; Mills & McGuirk, 2004). The secretion of IL-10, as mentioned earlier, may suppress the aggressive inflammatory phenotype typical in the atherosclerotic lesion. CD4+CD25+ lymphocytes have recently been confirmed as Tr cells in humans, comprising 6% of the circulating T lymphocyte population (Dieckmann et al. 2001). They are the best characterised of the Tr cells to date. CD25 is a receptor for IL-2. CD4+CD25+ lymphocytes inhibit pro-inflammatory IL-2 production by Th1 lymphocytes. The source of Tr cells remains undetermined. It is most likely that CD4+ regulatory T cells originate in the thymus in a lineage separate from effector T cells. Cells are found in the newborn thymus with markers and functional properties of Tr cells before there are many T cells of any kind detected in the spleen (Itoh et al. 1999). Immature CD4-CD8- thymocytes injected into the
thymuses of irradiated mice develop into CD4+CD25+ T cells (Schwartz, 2005). It is also speculated that Tr cells develop in the periphery and circulate back to the thymic medulla after antigenic stimulation. Cytokines appear to influence development of Tr cells. Tr cells express Foxp3, and CD25-CD4+ T cells can be converted to CD25+Foxp3 cells in the presence of cytokines during antigenic stimulation (Chen et al. 2003). CD4+ T cells primed with IL-10 and IFNγ differentiate into Tr1 cells (Levings et al. 2001). Culture of murine T cell precursors with TGFβ promotes induction of Th3 cells (Weiner, 2001). The Th1-suppressive effects of Tr lymphocytes and their potential differentiation by the cytokine milieu of a microenvironment are of interest in atherosclerosis, although Tr lymphocytes may suppress both Th1 and Th2 responses (VanderLaan & Reardon, 2005). Tr1 cells expanded \textit{in vitro} and administered, with their cognate antigen, to apoE -/- mice induced a lower accumulation of macrophages and T cells in the atherosclerotic plaque and a significant reduction in lesion size (Mallat et al. 2003).

McGuirk & Mills (2002) ask if the balance between Th1 and Th2, effector and regulator cells, should be intentionally upset, as it may lead to inflammatory pathology or increased susceptibility to other disease. Certainly, the regulatory machinery of the Th1/Th2 paradigm is extensive (effector cell clearance, anergy, Th1/Th2 switching and Tr lymphocytes). However, a dysregulated Th1 inflammatory response pervades in human atherosclerosis. A therapy that suppresses Th1, promotes Th2 or restores balance between Th1/Th2 pathways may have potential in the treatment of atherosclerosis.

1.5 INFLAMMATORY MEDIATORS OF ATHEROSCLEROSIS

The mediators of atherosclerosis-associated inflammation investigated in the experimental chapters of this thesis are addressed individually in this section. These include monocyte, macrophage and T helper lymphocyte cytokines, matrix-degrading enzymes and systemic acute phase reactants.

1.5.1 Interleukin 1

IL-1 has been described, along with TNF, as a prototypic inflammatory cytokine. It represents, however, a family of pro-inflammatory and antagonistic ligands of which IL-1α and IL-1β are the predominant forms. In addition to IL-18 and IL-1 receptor agonist (IL-1ra), six other members have recently been identified. IL-1 genes are mapped to the human chromosome 2, between the IL-1α and IL-1β 2q loci (2q13-q21)
with the exception of IL-18, which is mapped to chromosome 11. The phenotype induced by a cytokine such as IL-1 depends on ligation with a specific receptor. The IL-1 receptor (IL-1R) has at least 9 members, including the signal-transducing IL-1R accessory protein (IL-1RacP). The human IL-1Rs 1 to 7 genes are located on chromosome 3, SIGIRR on chromosome 11 and IL-1Rs 8 and 9 on the X chromosome (Sims et al. 2002). The specificity of IL-1 ligand and receptor binding is represented in Figure 1.3.

![Diagram showing preferential binding of IL-1 ligands and receptors.](image)

**Figure 1.3 Preferential binding of IL-1 ligands and receptors.** Data from Sims et al. 2001; Sims et al. 2002

IL-1α and IL-1β share 25% amino acid sequence homology across their precursor structure, 22% amino acid sequence homology over their mature segments and exercise the same biological effects (March et al. 1985). The IL-1α pro-cytokine is a 33kDa, 271-amino acid peptide with bioactivity. This precursor is enzymatically cleaved by calpain into the mature 17kDa, 159-amino acid IL-1α and a 112-amino acid pro-sequence (Kobayashi et al. 1990; Watanabe & Kobayashi, 1994). The human and mouse pro-cytokine have 54% amino acid sequence homology and mature IL-1α shows 58% identity (March et al. 1985). IL-1α is expressed by a multitude of cells, including
fibroblasts, hepatocytes, keratinocytes, brown fat adipocytes, monocytes and macrophages (Buryšek et al. 1993; Dinarello, 1994; Tsukui et al. 1994; Ghanekar et al. 1996; Raines et al. 1996). During the differentiation of monocytes to macrophages, a transition from IL-1β to IL-1α production is displayed (Beuscher et al. 1992). The IL-1β precursor is a 48.5kDa, 269-amino acid peptide. It is cleaved by a cell-surface 45kDa cysteine-protease called IL-1β-converting enzyme (ICE) into the mature 17kDa, 153-amino acid IL-1β and a 116-amino acid pro-segment (Thornberry et al. 1992; Wewers et al. 1997). The IL-1β pro-form may also be cleaved by the main proteases released at sites of inflammation, such as elastases, cathepsin G and collagenases. The human and mouse mature IL-1β have a 78% amino acid homology (Gray et al. 1986). Monocytes are the predominant source of IL-1β but other cells known to express the ligand include adrenal cortical cells, macrophages, DCs, endothelial cells, keratinocytes, osteoblasts, trophoblasts and T lymphocytes (Gonzalez-Hernandez et al. 1995; Ghanekar et al. 1996; Jokhi et al. 1997; Wewers et al. 1997).

Human IL-1R1 and IL-1R2 are 80kDa, 552-amino acid and 60-68kDa, 385-amino acid transmembrane glycoproteins, respectively. Both show affinity for IL-1α and IL-1β. They show 28% amino acid sequence homology in their extracellular regions (about 320 amino acids) but differ markedly in their cytoplasmic regions. IL-1R1 has a 213-amino acid residue cytoplasmic region which was until recently considered as a domain of signal transduction. IL-1R2 has a 29-amino acid cytoplasmic tail and is believed to act as a neutralising decoy to the cellular effects of IL-1 ligation. Signal is generated when IL-1R1 interacts with IL-1RacP, the 66kDa, 550-amino acid transmembrane glycoprotein and member of the IL-1R family. The 200kDa complex of IL-1/IL-1R1/IL-1RacP complex is internalised initiates downstream NF-κB activation (Greenfeder et al. 1995a; Greenfeder et al. 1995b; Bowie & O’Neill, 2000). IL-1R1 is expressed mainly, but not exclusively, on fibroblasts and T lymphocytes. IL-1R2 is found predominantly on B cells and neutrophils (Greenfeder et al. 1995a; Greenfeder et al. 1995b).

IL-1 is capable of inhibiting or promoting its own synthesis, depending on conditions and cell types. IL-1α and IL-1β block the binding of each other to IL-1R. IL-1ra also is competitive in this regard but its ligation to IL-1R does not elicit an inflammatory response. Its classical antagonism of other IL-1 ligands is considered anti-inflammatory. Soluble (s)IL-1R1 preferentially binds IL-1ra whereas sIL-1R2 shows 2000-fold less affinity for IL-1ra. The ligation of IL-1 with its soluble receptor
has a neutralising effect. Therefore, IL-1α and IL-1β are more available for cellular ligation when sIL-1R1 is antagonised by IL-1ra and less available for cellular ligation when they are antagonised by sIL-1R2 (Greenfeder et al. 1995a; Hallegua & Weisman, 2002). The interaction between IL-1, IL-1ra, sILR, membrane-bound (m)IL-1R2, mIL-1R1 and IL-1RacP are summarised in Figure 1.4.

Figure 1.4 Classical interactions between IL-1 ligand, soluble and membrane-bound receptors and accessory protein. Binding of IL-1 to a soluble receptor is antagonistic (1). Binding of IL-1ra to the soluble receptor increases the likelihood of IL-1 binding to membrane-bound receptors (2). Binding of IL-1ra to the membrane-bound receptor decreases potential for IL-1 ligation (3). IL-1R2 is considered as a decoy receptor and IL-1 ligation is redundant (4). IL-1 ligation to membrane-bound IL-1R1 recruits IL-1RacP (5) and the complex is internalised (6).
IL-1 is unusual in that the intracellular precursors do not contain a recognizable hydrophobic secretory signal sequence that would allow extracellular release of the protein by classical secretory pathways involving the endoplasmic reticulum and golgi system. The mechanism of its release is unresolved. It is postulated that a small fraction of intracellular IL-1 colocalises with inactive procaspase-1 in specialised secretory lysosomes. Upon cellular activation, procaspase-1 is converted to active caspase-1 by a complex of proteins termed the IL-1 inflammasome. K+ effluxes out of the cell, Ca2+ influxes and phospholipases are thus activated. Phosphatidylcholine-specific phospholipase C facilitates lysosomal exocytosis and the subsequent release of IL-1 at the cell membrane (Dinarello, 2005). IL-1 synthesis is induced by TNFa, IFNα, IFNβ, IFNγ, bacterial endotoxins, viruses, mitogens and antigens. The synthesis of IL-1 is suppressed by IL-1ra, lipoproteins, lipids, α2-macroglobulins, PGE_2 and glucocorticoids. Activators of PPARγ modulate IL-1 synthesis in phorbol myristate acetate (PMA)-activated monocytes. PGJ_2 inhibited IL-1 secretion and increased IL-1ra production. TZDs barely inhibited IL-1 secretion and strongly enhanced IL-1ra production (Meier et al. 2002). Acute phase reactants such as CRP and α1-anti-trypsin have been shown to augment the synthesis of IL-1ra from mononuclear cells (Fiotti et al. 1999). MMP-1, 2, 3 and 9 were demonstrated to degrade IL-1β but not IL-1α in activated connective tissue cells (Ito et al. 1996).

IL-1 stimulates adjacent immune and lesional cells in a microenvironment. It stimulates the induction of Th1 lymphocytes to secrete IL-2, and in conjuction with macrophage-derived IL-12 stimulates NK cells to secrete IFNγ. It induces endothelial cells to secrete MCP-1 and upregulates E-selectin, ICAM-1 and VCAM-1, enhancing leukocyte adhesion. Resident fibroblasts are induced to express MMPs by IL-1 and it promotes the proliferation of B cells and their synthesis of immunoglobulins. IL-1 stimulates the proliferation and activation of NK cells, fibroblasts, thymocytes and glioblastoma cells and enhances the metabolism of arachidonic acid (C20:4n-6), especially of prostocyclin and PGE_2, in inflammatory cells (Hallegua & Weisman, 2002). Mice with -/- or disrupted IL-1R1 are resistant to high doses of LPS, develop less severe pancreatitis, exhibit an impaired acute phase response and are resistant to fever development when challenged with turpentine (Fantuzzi et al. 1996; Norman et al. 1996; Zheng et al. 1995). This suggests that the response to challenge in these instances, such as IL-1 activation and inflammation, is the mediator of pathogenesis. Administration of IL-1ra has been shown to exert anti-inflammatory effects in vivo and
in experimental models of disease, including septic shock, arthritis and colitis (Meier et al. 2002). However, IL-1β-/- mice display an impaired defence response to infection with influenza (Kozak et al. 1995). The inflammatory phenotype induced by IL-1 is a vital component of immune defence in this instance. IL-1 alters endothelial function in vivo and in vitro. By promoting thrombotic processes and attenuating anticoagulatory mechanisms it plays a pathological role in venous thrombosis, arteriosclerosis and vasculitis (Brody et al. 1992). Although IL-1 supports monocyte-mediated tumour cytotoxicity and has been reported to induce tumour regression it appears to be an autocrine growth modulator for human gastric and thyroid carcinoma. IL-1α has been shown to accelerate the recovery of platelets after carboplatin therapy and may be clinically useful in preventing or treating thrombocytopenia induced by chemotherapy. IL-1 may be used as a stimulator of haematopoiesis and may have a protective effect on chemotherapy-induced myelosuppression (Dullens et al. 1991; Johnson, 1992). The use of IL-1 receptor antagonists appears to be more promising than the use of IL-1 due to the very high toxicity of this compound in diseases such as RA and OA and septic shock in animals, and only recombinant IL-1ra, anakinra, is approved from use in humans (Hallegua & Weisman, 2002; Dinarello, 2005). IL-1 activates osteocasts and appears to be involved in the generation of lytic bone lesions. IL-1 stimulates the hypothalamic-pituitary-adrenal axis, leading to production of ACTH, growth hormone, vasopressing and somatostatin. IL-1β-immunoreactive hypothalamic nerve fibres innervate endocrine and autonomous nuclei controlling central aspects of the acute phase reaction (Hallegua & Weisman, 2002). IL-1 acts on hepatocytes to enhance the synthesis of acute phase proteins (Tsukui et al, 1994). In the central nervous system (CNS), IL-1 is involved in the induction of the so-called slow-wave sleep. IL-1β can prevent the depression of antibody responses observed during sleep deprivation. It has been shown that IL-1 promotes wound healing through effects on angiogenesis, promotion of fibroblast proliferation and chemotactic activity on neutrophils (Hallegua & Weisman, 2002). The production of MMPs is enhanced by IL-1β that is secreted from activated macrophages and other cell types (Ito et al. 1996). IL-1 is also associated with bone formation and remodelling, insulin secretion, appetite regulation, fever induction and neuronal phenotype. It is also known as lymphocyte activating factor, endogenous pyrogen, catabolin, haemopoietin-1, melanoma growth inhibition factor and osteoclast activating factor. Some of the biological activities of IL-1 are mediated indirectly by the
induction of the synthesis of other mediators including ACTH, PGE₂, platelet factor 4, CSF, IL-6 and IL-8.

1.5.2 Interleukin 2

IL-2 is inextricably associated with the T helper lymphocyte. It is a major secretory product of the activated Th1 cells and is required for T cell activation and proliferation, clonal expansion and differentiation (Zhang & Nabel, 1994). The human IL-2 gene is mapped to chromosome 4q band 26-28 (Siegel et al. 1984). It is a 15.4kDa, 133-amino acid peptide bearing 65% amino acid sequence homology to murine IL-2 (Kashima et al. 1985). The human IL-2 receptor is composed of three transmembrane glycoproteins. The α subunit is a 55kDa, 251-amino acid chain containing a short 13-amino acid cytoplasmic domain. It is alternatively known as IL-2R, IL-2Ra, CD25, p55 and Tac antigen. The β subunit is a 70kDa, 525-amino acid chain containing an extensive 286-amino acid cytoplasmic domain. The γ subunit is a 64kDa, 247-amino acid chain containing an 86-amino acid cytoplasmic domain. The affinity of the α subunit for ligand appears to be low and it has no signal transducing capability. The β subunit initiates signal transduction through Jak1, Syk and p56lck, a member of the src family. Jak3 is activated by the γ subunits, which is common to the IL-4, IL-7, IL-9 and IL-15 receptors. The αβγ receptor is of high affinity and is expressed by activated T cells and monocytes. Ligation to the βγ receptor initiates signal transduction in NK cells, neutrophils, monocytes and CD8+ cells. The αβ receptor is not generally believed to transduce signal (Goldsmith & Green, 1996; Lin & Leonard, 1997), although γ subunit -/- embryonic fibroblasts are reported to show responsiveness to IL-2. A soluble 42kDa fragment of the α subunit is continuously secreted by activated T cells, concentrations varying markedly in infectious and immune pathologies, and is an indicator of T cell activation (Honda et al. 1990).

Expression of IL-2 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 (Shaw et al. 1988; Hoyos et al. 1989; Serfling et al. 1989). IKKγ has recently been demonstrated to play an essential role in the expression of IL-2. Jurkat T cells lacking the enzyme failed to induce IL-2 due to a selective loss in IKK activity, IκBα degradation and NF-κB nuclear binding, although AP-1 and NFAT binding activities in the IL-2 promoter were unaffected (He & Ting, 2003). Protein kinase C (PKC) may also be involved in the

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transcription of IL-2. T lymphocytes express the PKC isoforms α, β, δ, ε, ζ, η and θ (Long et al. 2001). PKCe, and PKCa to a lesser degree, regulate AP-1 and NFAT (Genot et al. 1995). PKCθ is a regulator of IL-2 promoter activation in Jurkat T lymphocytes (Ghaffari-Tabrizi et al. 1999) and synergises with p85 in the activation of NF-κB (Dienz et al. 2000). PKCβ and PKCδ are reported to participate in the regulation of IL-2 synthesis. Neutralisation of PKCa and PKCβ inhibits IL-2R expression. Long et al. 2001, demonstrated that when PKCβ was introduced into PKCβ-deficient K4 cells, the ability of the cells to secrete their translated IL-2 was restored (Long et al. 2001), indicating that PKCβ is essential for release of the cytokine. Kawakami & Parker (1992) reported that PKC was important in Th1 rather than Th2 T-lymphocyte signalling. However, Noble 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.

The effects of secreted IL-2 are regulated, as with other cytokines, by the availability of environmental receptors. Mentioned above, the γ subunit is a common component of cytokine receptors. IL-4 has been shown to selectively suppress IL-2-induced monocyte tumoricidal activity and pro-inflammatory cytokine secretion (Bosco et al. 1995). In addition, IL-4 inhibited IL-2-binding to monocyte cell surface despite an increase in the expression of the γ subunit receptor. The availability of the γ subunit appears to be a limiting factor in the response of monocytes to IL-2, IL-4 or IL-7 (Espinoza-Delgado et al. 1995).

IL-2 is a growth and activating factor for many cells. It stimulates the proliferation and differentiation of all T lymphocyte subpopulations. IL-2 can induce the expression of the Th2 regulatory cytokines IL-4 and IL-10, and at high concentrations it enhances IL-6 and TNF production and histamine release from basophils. In vivo, trials of IL-2-immunotherapy of malignancies demonstrate an association of the cytokine with elevated serum concentrations of IL-6 and CRP although in vitro IL-2-activated PBMC showed reduced potential to stimulate acute phase reactant release from hepatocytes (Wigmore et al. 2002). IL-2 activates monocytes/macrophages (Espinoza-Delgado et al. 1995), stimulates proliferation and differentiation of B cells and augments the cytolytic activity of natural killer (NK) and lymphocyte-activated killer (LAK) cells. IL-2-deficient mice show delayed but biologically functional T helper cell responses, reduced but inducible NK cell activity and high levels of class-switched IgG1 and IgE isotypes, indicating compensation for and non-essentiality of IL-2 in these instances. However, IL-2-deficient mice develop
an unlimited autoimmune inflammatory bowel disease with striking clinical and biological similarity to human ulcerative colitis (Lin & Leonard, 1997).

As a growth and proliferatory factor, IL-2 appears pro-inflammatory when expressed in the microenvironment of the atherosclerotic lesion. T lymphocytes are found in large numbers in the human plaque and elevated IL-2 levels have been observed in patients with unstable angina (Mazzone et al. 1999). Pre-incubation of vascular SMCs with IL-2 increased intracellular Ca2+ and stimulated glycosaminoglycan and prostacyclin synthesis in response to angiotensin II (Nabata et al. 1997), indicating that IL-2 may play a role in the pathogenesis of atherosclerosis by sensitising vascular SMCs to provocative agents. Furthermore, aortic sections from apo E -/- mice injected intraperitoneally with IL-2 showed a profound atherogenic burden relative to placebo-injected controls, whereas and anti-atherogenic effect was demonstrated with injection of anti-IL-2 (Upadhya et al. 2004).

IL-2 is a growth factor for T lymphocytes and is capable of worsening adult T cell leukaemia (ATL) (Fujimura et al. 2004). It can damage the integrity of CNS endothelium and the blood brain barrier. Neuropsychiatric side effects of IL-2 therapy include fatigue, disorientation and depression (Anisman et al. 2005). Transgenic mice harbouring the IL-2 or IL-2R gene show growth retardation and a selective loss of Purkinje cells in the cerebellum (Ishida et al. 1989). Transgenic expression of IL-2 in murine pancreatic β cells induces pancreatic distuction and death from an infiltrating and predominating macrophage inflammatory response (Allison et al. 1994).

1.5.3 Interleukin 6
IL-6 has alternatively been named IFNβ2, IL-1-inducible protein, hepatocyte-stimulating factor, cytotoxic T cell differentiation factor, B cell differentiation factor and/or B cell stimulatory factor 2. IL-6 is a member of a cytokine family that also includes leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), Oncostatin M (OSM), IL-11 and cariotrophin 1 (CT-1), all of which induce hepatic expression of acute phase proteins (Hirano et al. 1997).

Human pro-IL-6 is a 22-27kDa, 212-amino acid molecule and maps to the human chromosome 7 bands p21-p14 (Kishimoto, 1992; Hirano et al. 1994; Hibi et al. 1996). Mature IL-6 is a 185-amino acid peptide. Human and murine IL-6 share 62% amino acid sequence homology. Cells known to express IL-6 include CD8+ T cells, fibroblasts, synoviocytes, adipocytes, osteoblasts, megakaryocytes, endothelial cells,
sympathetic neurons, cerebral cortex neurons, chromaffin cells of the adrenal medulla, mast cells, keratinocytes, Langerhans cells, astrocytes, neutrophils, monocytes, eosinophils, colonic epithelial cells and, most likely, pancreatic islet B cells (Cichy et al. 1996; Fried et al. 1998; Miyazawa et al. 1998). It is estimated that one third of circulating IL-6 comes from adipose tissue. It is suggested that higher levels of IL-6 mRNA and protein are expressed in the fibroblast and preadipocyte than in the mature, basal state adipocyte. Catecholamine-induced release of IL-6 from adipose tissue may account for the elevation by stress of IL-6 concentration (Yudkin et al. 2000).

The human IL-6 receptor is composed of two transmembrane glycoproteins, the gp30 and IL-6R (gp80) subunits. The signal transducing subunit, gp130, is a 130-145kDa, 896-amino acid peptide with a 597-amino acid extracellular, a 22-amino acid transmembrane and a 277-amino acid cytoplasmic region. The IL-6-binding subunit, IL-6R, is an 80kDa, 468-amino acid peptide with a 358-amino acid extracellular, a 28-amino acid transmembrane and an 82-amino acid cytoplasmic region. The human IL-6R gene is located on chromosome 1 band q21 and shares 53% identity with the rat hepatic IL-6R amino acid sequence (Keller et al. 1996). The ligation of IL-6 to IL-6R is of low-affinity prior to the recruitment of gp130. Cells known to express IL-6R include CD4+ and CD8+ T cells, hepatocytes, CD34+ stem cells, neurons, monocytes and osteoblasts. gp130 is also expressed in cells that do not express IL-6 receptors. It has been found to be a component of other receptors including those for IL-11, LIF, OSM, CNTF and CT1. A soluble form of the IL-6R has been described that also interacts with gp130. The cytoplasmic region of IL-6R appears redundant, since mutant IL-6R lacking the intracytoplasmic domain is functional. Furthermore, a soluble (s)IL-6R lacking the transmembrane and intracytoplasmic domains associates with gp130 in the presence of IL-6 and mediates its function (Taga et al. 1989). A hexamer composed of two IL-6, two IL-6R and two gp130 associates upon ligation. IL-6 activates the transcription factors STAT1, STAT3 and STAT5, and multiple signalling molecules. JAK1, JAK2 and Tyk2 associate constitutively with gp130 and are tyrosine-phosphorylated in response to IL-6, CNTF, LIF, OSM or IL-11 ligation. JAK is activated through two conserved motifs, “box1” and “box2”, of gp130. STAT5 is activated by IL-6 at the same membrane proximal region (Hirano et al. 1997). CNTF, LIF, OSM and IL-6 induce tyrosine phosphorylation of SHP2 (a phosphotyrosine phosphatase), which is a mammalian homologue of Drosophila corkscrew, pp120, Shc, Grb2, Raf1, ERK-1 and ERK-2. SHP2 is inducibly associated with gp130 and JAK2. The Ras-MAPK pathway
is activated by the IL-6 cytokine family and is possibly mediated by SHP2 and/or Shc. STAT3 activation and the tyrosine phosphorylation of SHP2 depend on the tyrosine residues of the gp130 cytoplasmic domain (Hirano et al. 1997). SHP2 is activated by the phosphorylation of Tyrosine 759 of the gp130 cytoplasmic domain. STAT3 is activated by the phosphorylation of any one of the four tyrosines (Y767, Y814, Y905 or Y915) in the carboxy terminus of gp130 (YXXQ). Src-family tyrosine kinases such as Btk, Tec, Fes and Hck are activated by the IL-6 cytokine family. Tec may be one of the adapter molecules linking the cytokine receptor to PI-3 kinase (Hirano et al. 1997).

Figure 1.5 Signal transduction pathways regulating cell growth, differentiation and death effected by IL-6 ligation. Adapted from Hirano et al. 1997

Human IL-6 synthesis is stimulated by LPS, PDGF, OS, IL-1, TNFα and hypoxia, and is inhibited by glucocorticoids (Keller et al. 1996). Unlike most soluble receptors, sIL-6R does not have an antagonistic role in IL-6 metabolism and appears to enhance the response to IL-6 (Romano et al. 1997; Sakamoto et al. 2003). Catecholamines stimulate IL-6 release from adipose tissue. Psychological stress can elevate circulating IL-6
concentrations (Yudkin et al. 2000). IL-6 and sIL-6R levels are significantly elevated in surgical patients in positive association with the degree of surgical stress and complications (Sakamoto et al. 2003). The circadian variation of IL-6 in serum and urine is well recognised. Sothern et al. (1995) demonstrated that serum IL-6 concentrations were higher during the night with a peak at 1:00hrs and lowest during the day with a nadir at 10:00hrs. IL-6 concentrations in urine were highest during the day with major and minor peaks at 17:30hrs and 8:30hrs, respectively, and a nadir at 23:30hrs in healthy subjects (Sothern et al. 1995). IL-6 may be a mediator of several infectious and autoimmune diseases including HIV, rheumatoid arthritis, Castleman’s disease and the paraneoplastic symptoms of cardiac myxoma. Elevated concentrations of IL-6 in serum and cerebrospinal fluid are found in sepsis (Keller et al. 1996).

The primary role of systemic IL-6 is the activation of hepatic acute phase proteins including CRP, serum amyloid A (SAA), fibrinogen, complement and a-l-antitrypsin (Keller et al. 1996; Harris et al. 1999; Yudkin 2000). IL-6 differentiates B cells and is an activation factor for T lymphocytes. In the presence of IL-2, IL-6 induces the differentiation of immature T cells into cytotoxic T cells. IL-6 regulates the growth and development of trophoblasts of embryonic stem cells. A combination of sIL-6R and IL-6 activates the induction of chemokines and ICAM-1 expression on endothelial cells. IL-6-/- mice show impaired leukocyte accumulation in subcutaneous air pouches (Romano et al. 1997). IL-6 may induce the proliferation of malignant cells. Melanoma, renal cell carcinoma, prostate carcinoma, ovarian carcinoma, lymphoma, leukaemia, multiple myeloma and Kaposi’s sarcoma are stimulated by IL-6 (Keller et al. 1996; Romano et al. 1997). An increase in serum IL-6 indicates inferior survival periods in adult T cell leukaemia/lymphoma (Yamamura et al. 1998). IL-6 may promote osteoporosis. IL-6 stimulates osteoclast proliferation. IL-6 deficient mice are protected from oestrogen depletion-induced bone loss (Elhage et al. 2001). Conditioned media from marrow cultures of patients with Paget’s disease stimulated osteoclast-like cell formation in normal human marrow cultures and was reversed by the addition of IL-6 antibody (Keller et al. 1996). IL-6 is produced by a variety of cells involved in the progression of atherosclerosis, including endothelial cells, monocytes, macrophages and T lymphocytes (Yudkin et al. 2000). IL-6 mRNA and protein are expressed in human and murine atherosclerotic plaques and increased concentrations have been implicated in angiogenesis and arterial remodelling (Elhage et al. 2001; Romano et al. 1997). Serum IL-6 concentrations are positively associated with aortic diameter in patients
with aortic aneurysm (Rohde et al. 1999). Elevated IL-6 has been associated with hypercholesterolaemia (Bennet et al. 2003). However, IL-6 -/- mice develop a metabolic syndrome characterised by maturity-onset obesity, abnormal blood lipid and carbohydrate metabolism and increased plasma leptin concentrations, reversed by intracerebroventricular IL-6 replacement (Wallenius et al. 2002). The −573C/G C allele and the −174G/C C allele of the IL-6 promoter are associated with increased transcription of IL-6 in an inflammatory situation but not necessarily serum concentrations, which vary greatly (Bennet et al. 2003). The −174G/C C allele is associated with elevated plasma CRP concentrations, which are highly heritable and unchanging (Vickers et al. 2002). The −174G/C polymorphism is associated with systemic inflammation but not with risk of MI (Bennet et al. 2003). A microsatellite CA-repeat polymorphism in the IL-6R locus, and the frequency of the common 149bp allele, are both associated with obesity (Escobar-Morreale et al. 2003). The principal inducers of systemic IL-6 (red) and the cellular, systemic and metabolic effects of elevated IL-6 (blue) are presented in Figure 1.6.

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![Diagram of Inducers of Systemic IL-6 Production](image)

**Figure 1.6 Inducers of systemic IL-6 production.** Cellular, systemic and metabolic effects of elevated circulating IL-6. Data from Yudkin et al. 2000; Simpson et al. 1997
1.5.4 Interleukin 10

IL-10, or cytokine synthesis inhibitory factor (CSIF), is a homodimer with subunits of 160 amino acid residues in length and a monomeric molecular weight of 18.5kDa (Lalani et al. 1997). The human IL-10 gene maps to chromosome 1 and the protein bears 73% amino acid sequence homology to murine IL-10. IL-10 bears only 20 to 26% amino acid sequence homology, but unmistakable structural homology, to the other members of its cytokine family including IL-19, IL-20, IL-22, IL-24 and IL-26 (Dumoutier & Renauld, 2002; Fickenscher et al. 2002). The V-shaped IL-10 homodimer is similar in structure to IFNγ (Lalani et al. 1997), AK155 (a herpesvirus saimiri-induced gene) and murine mob-5 (homology of human mda-7, which inhibits growth of certain tumour cell lines) (Moore et al. 2001). The human IL-10 receptor gene maps to chromosome 11 and consists of two subunits. IL-10R1 (alternatively IL-10Ra) is a 90-100kDa transmembrane glycoprotein, of limited cell-type expression with a STAT3 docking site and JAK1 association region within the cytoplasmic domain (Liu et al. 1994; Kotenko & Pestka, 2000) IL-10R1 binds IL-10 with high affinity. The accessory subunit, IL-10R2 (alternatively IL-10Rb) contributes little to IL-10 binding affinity but appears crucial in signal transduction though the cell membrane by recruiting two protein kinases of the JAK family (Mocellin et al. 2004). The IL-10/IL-10R interaction engages the JAK family tyrosine kinases Jak1 and Tyk2. IL-10 induces tyrosine phosphorylation and activation of STAT1, STAT3 and STAT5 (Moore et al. 2001). The suppressor of cytokine signalling (SOCS)-1 and SOCS-3 may be two of the genes targeted by IL-10-activated STAT proteins (Mocellin et al. 2004). IL-10 is secreted by monocytes, macrophages, B lymphocytes, CD8+ T lymphocytes, CD4+ T lymphocytes, Tr cells, DCs, NK cells mast cells and keratinocytes (Zdanov et al. 1996; Pinderski-Oslund et al. 1999; Mocellin et al. 2001). It was initially classified as a Th2 cytokine as it is frequently associated with IgE-mediated hypersensitivity (Guerkov et al. 2003). Tr cells are a major producer of IL-10. IL-10 is perhaps the most important physiological suppressor of inflammation.

IL-10 inhibits Th1 proliferation, chemotaxis and expression of IL-2 and IFNγ (Schottelius et al. 1999; Mocellin et al. 2004), although this was observed only when macrophages were used as accessory cells to resting or concanavalin A (Con A) activated murine T lymphocytes (Ding et al. 1993). IL-10 did not inhibit the antigen presentation function of B cells and does not impair the presentation of antigens to stimulate Th2 clones (Fiorentino et al. 1991). The inhibition of Th1 cytokine production
by IL-10 is believed to be indirect, via inhibition of accessory cells (Moore et al. 2001). IL-10 has been reported to inhibit NF-κB activation in CD4+ T cells, indirectly via accessory cells, and to activate AP-1 and NF-κB in CD8+ T cells (Moore et al. 2001; Mocellin et al. 2004). T lymphocyte anergy can be induced in CD4+ and CD8+ lymphocytes in the presence of IL-10-treated DCs (Mocellin et al. 2004). IL-10 secreted by Tr cells inhibits antigen presentation cells. IL-10 inhibits the production of IL-12 by DCs and limits their function as antigen presentation cells to Th1 lymphocytes. IL-10 inhibits the production of IL-1, IL-6, IL-8, IL-12, TNF, GMCSF and MHC class II in monocytes (Schottelius et al. 1999; Guerkov et al. 2003) and induces release of the IL-1ra (Terkeltaub 1999). IL-10 pretreatment of monocytes suppressed TNF-induced IkBα and IL-8, which are positively upregulated by NF-κB, blocks IKK and inhibits NF-κB binding to DNA (Schottelius et al. 1999). In primary human macrophages, however, IL-10 has demonstrated no effect on IkBα degradation or NF-κB nuclear binding, despite inhibiting TNFα (Denys et al. 2002). It is speculated that IL-10 may interact with the AU-rich ARE regions of the TNFα gene 3’ untranslated region (UTR), which is associated with posttranscriptional control. The AU-rich ARE regions are implicated in regulation of mRNA stability and turnover and IL-10 may inhibit TNFα by posttranscriptional destabilisation (Terkeltaub 1999). IL-10 −/− mice develop a chronic Crohn’s-like enterocolitis, arthritis and overexpression of TNFα in response to LPS activation (Schottelius et al. 1999; Denys et al. 2002). IL-10 −/− mice show increased inflammatory response to skin irritants and produced increased levels of NO, IL-12, IL-1β, TNFα and IFNγ, but not IL-18, upon infection with Toxoplasma gondii resulting in shock, tissue damage and death (Zediak & Hunter, 2003).

The immunosuppressive properties of IL-10 are not universal. As indicated above, IL-10 can activate AP-1 and NF-κB in CD8+ T cells. IL-10 can induce leukocyte chemotaxis and induction of endothelial cell adhesion molecules. In healthy volunteers the IV administration of human recombinant IL-10 given after the induction of endotoxaemia produces pro-inflammatory effects by enhancing release of IFNγ and its inducible-monokines. IL-10 treatment of patients affected with acute myelogenous leukaemia increased plasma levels of TNFα and IL-1. IL-10 may play a role in early DC maturation by inhibiting their migration, increasing TLR expression and promoting antigen uptake. IL-10 promotes survival of normal B cells, which correlates with an increased expression the anti-apoptotic protein bcl2. IL-10 is also a potent cofactor for B cell proliferation (Mocellin et al. 2004).
IL-10 has been referred to as “an immunological scalpel for atherosclerosis” (Terkeltaub 1999). It is considered anti-inflammatory and protective (DePalma et al. 2003) although it is not universally expressed in atherosclerotic lesions (Uyemura et al. 1996). IL-10 induces the production of the tissue inhibitor of metalloproteinases (TIMP) 1 and inhibits MMP-9 activity, promoting plaque stability (Waehre et al. 2002). It inhibits monocyte and macrophage production of tissue factor, MCP-1, pro-inflammatory cytokines and COX-2 (Tzakias et al. 2003). IL-10 downregulates IL-12 and suppresses the Th1 immune response typical in aggressive lesion development (Terkeltaub 1999). Overexpression of IL-10 is characterised by smaller, more stable atherosclerotic plaques in animal studies. IL-10 -/- animals display increased neutrophil recruitment, elevated plasma TNFα concentration and high expression of ICAM-1. In vivo, IL-10 inhibited the mitogenic effects of oxidised lipids on human aortic endothelial cells, as assessed by monocyte binding (Tzakias et al. 2003). Angina patients, particularly those with unstable angina, have markedly increased serum TNFα concentrations, without a corresponding rise in IL-10, suggesting a net inflammatory phase in these subjects (Waehre et al. 2002). Following acute MI, subjects exhibit higher circulating IL-10 than controls or those with unstable angina, indicating that IL-10 is expressed in the circulation after acute coronary events. A strong positive correlation between IL-10 and HDL was found in subjects with unstable angina (Tzakias et al. 2003). Elsewhere, highly oxidised LDL has increased IL-10 production in human monocytes (Uyemura et al. 1996; Pinderski-Oslund et al. 1999).

Several polymorphisms of the human IL-10 gene 5'-flanking sequence have been identified, including point mutations at -1082G/A, 819C/T and -592C/A (Moore et al. 2001). The -1082G/A G allele is associated with higher Con A-induced IL-10 production, exacerbation of systemic lupus erythematosus (SLE), a higher rate of mortality in meningococcal disease and a lower efficacy of IFNα therapy in patients with chronic hepatitis C infection (Moore et al. 2001; Mocellin et al. 2004).

1.5.5 Tumour Necrosis Factor

Tumour necrosis factor was recognised in 1975 as a bacterially induced circulating factor with strong tumour regression capacity (Carswell et al. 1975). Expressed sequence tags (ESTs) indicated that TNF and the TNF receptor constitute gene superfamilies (Locksley et al. 2001). The moniker TNF can be misleading; tumouricidal
activity of the cytokine is generally by apoptosis rather than necrosis, and the cytokine acts in promoting cell survival as well as in a cytocidal capacity.

There are at least 17 members of the TNF ligand superfamily, of which TNFα prevails. Pro-TNFα is a 26kDa, 233-amino acid transmembrane peptide with a 176-amino acid extracellular, a 28-amino acid transmembrane and a 29-amino acid cytoplasmic domain. The mature, soluble 17kDa, 157-amino acid TNFα is created by proteolytic cleavage of the pro-cytokine by the 85kDa sheddase TNFα-converting enzyme (TACE) also known as ADAM-17 (Gruss & Dower, 1995; Black et al. 2003). The soluble TNFα circulates as a homo-trimer (Armitage, 1994). Human and murine pro-TNFα shows 79% amino acid sequence homology (Pennica, 1985). Mature TNF ligands show 25 to 30% amino acid sequence identity, limited to internal aromatic residues rather than the receptor specific external surfaces (Locksley et al. 2001). TNFα is expressed by macrophages, CD4+ and 8+ lymphocytes, adipocytes, keratinocytes, mammary and colonic epithelium, osteoblasts, mast cells, DCs, pancreatic β-cells, astrocytes, neurons, monocytes and steroid-producing cells of the adrenal zona reticularis.

There are 500 to 10,000 high affinity receptors for TNFα expressed in all somatic cell types except erythrocytes. The hallmark of the TNFR superfamily, of which there are at least 25 members, is the scaffold of disulphide bonds, which form cysteine-rich domains (Locksley et al. 2001). TNFR1 and TNFR2 are the predominant forms. TNFR1 is expressed particularly on cells susceptible to the cytotoxic action of TNF and possesses a death domain that interacts with adaptor molecules such as TRADD and RIP. TNFR2 is also present on many cell types, especially those of myeloid origin, including stimulated T and B lymphocytes. As with other cytokines, the activity and function of the TNF ligand depends heavily on its ligation with a specific receptor. Specific affinities between ligands and receptors are represented in Figure 1.7 below. Several soluble proteins that bind TNF have been described, including TNF-binding protein (TBP)-1 and 2, which inhibit binding of TNF to its receptor (Adolf & Fruhbeis, 1992).
The synthesis of TNFα is induced by IFN, IL-2, GMCSF, bradykinin, inhibitors of COX and platelet activating factor (PAF). TNFα production is inhibited by IL-6, TGFβ, vitamin D3, PGE2, dexamethasone, cyclosporin A and antagonists of PAF. In addition, TNF and TNFR activity are restrained by feedback inhibition, regulated expression of receptors, soluble processing of membrane-tethered ligands and receptors into soluble forms, the induction of nonsignalling decoy receptors, TACE and/or related protease cleavage of TNFRs, generating soluble receptors capable of inhibiting TNF (Locksley et al. 2001). Two cellular responses to TNF have been well documented; the induction

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Figure 1.7 Preferential binding of TNF ligands and receptors. Adapted from Locksley et al. 2001
of cell death and the activation of gene transcription for cell survival (Leong & Karson, 2000).

The former function of TNFα is generally mediated by apoptosis. Apoptosis, or "programmed" cell death, occurs during embryonic development, tissue remodelling and immune regulation and features a cardinal "set-list" of membrane blebbing, cellular shrinkage and condensation of chromatin. Binding of homo-trimeric TNF to the extracellular domain of a receptor harbouring either a cytoplasmic TNF-associated factor (TRAF) or death domain (DD)-binding motif leads to recruitment of a signalling adapter. Ligation to TNFR1 induces oligomerisation of the TNFR1 receptor DD and recruitment of the signalling adapter TNFR-associated death domain (TRADD). Ligation of Fas to its receptor recruits the signalling adapter FADD. The caspase cascade is induced, leading to the signalling of cellular toxicity (Schotte et al. 2001). TNFR that lack DDs can potently modulate the response to death receptors (DR). TNFR2 markedly enhances TNFR1-induced T cell death and CD40 can augment Fas-induced B cell death. Recent evidence has shown that TNFR chains preassemble into complexes on the cell surface prior to ligand binding. This region, termed PLAD (pre-ligand assembly domain) is necessary and sufficient for the self-assembly. The PLAD is distinct from the ligand-binding domain. PLAD interactions are highly specific and usually only receptor homotrimers are formed. Receptor preassembly is essential for ligand binding and signal transmission. Apoptotic pathways are involved in the killing mechanism of cytotoxic cells and in spatial homeostasis, but do not normally induce inflammation (Locksley et al. 2001) as cellular apoptotic fragments are removed by phagocytosis (Schotte et al. 2001). The mobility of phagocytes within the atherosclerotic lesion is limited by entrapment beneath the plaque and the expression of adhesion molecules and MIF. Apoptotic cell death has been reported within the atherosclerotic lesion. TNFα-mediated apoptosis is involved in the protective immune response against Mycobacterium tuberculosis. It induces alveolar macrophage apoptosis, limiting the growth of this intracellular pathogen. Alveolar macrophage infection with either the virulent H37Rv or attenuated H37Ra strains induce comparable levels of TNFα production but TNFα bioactivity is reduced in supernatants of virulent-infected macrophages due to an increased release of soluble TNFR2 with formation of inactive TNFα-TNFR2 complexes (Balcewicz-Sablinska et al. 1998). Pulmonary macrophages from BALB/c mice were found to undergo apoptosis after infection with
M. tuberculosis in a TNF-dependent manner, and this was inhibited by anti-TNF antibodies (Keane et al. 2002).

Necrosis, or “accidental/traumatic” cell death, is induced when the plasma membrane is damaged, the cell swells and bursts and cellular contents are released. TNF-mediated necrosis is less prevalent than TNF-mediated apoptosis. Cells infected by vaccinia virus, for example, are killed by TNF-mediated necrosis.

TNFa activates a number of genes, mediated by the induction of NF-κB and AP-1 (Leong & Karsan, 2000). Engagement of the TNFR1 leads to induction of ICAM-1, E-selectin, VCAM-1 and α2-integrin. TNFa stimulates the biosynthesis of collagenases in endothelial and synovial cells. TNFa induces macrophage synthesis of IL-1, IFNγ and PGE2. It enhances the proliferation of T lymphocytes in the absence of IL-2, and in the presence of IL-2 it promotes the proliferation and differentiation of B cells. TNF mediates part of the cell-mediated immune response against obligate and facultative bacteria and parasites and confers protection against Listeria monocytogenes. In transgenic animals overexpression of human TNFa precipitates chronic inflammatory polyarthritis. Transgenic mice expressing human TNFa in their T lymphocyte compartment have marked histologic and cellular changes locally in their lymphoid organ and a lethal wasting syndrome associated with widespread vascular thrombosis and tissue necrosis. Murine transgenic expression in pancreatic β-cells results in severe and permanent insulitis without evolution towards diabetes. TNFR1 -/- mice, which still express TNFR2, are resistant to lethal doses of bacterial LPS or enterotoxins, but readily succumb to infections with L. monocytogenes (Locksley et al. 2001). Mice deficient in LTb or its receptor LTbR do not develop secondary lymphoid organs such as lymph nodes or Peyer’s patches and have defective spleen structure and humoral immunity. Deletions of RANKL or RANK result in the absence of all peripheral and mesenteric lymph nodes, but Peyer’s patches remain intact and splenic architecture is unaffected. TNF/TNF superfamily proteins (SFPs) are involved in lymphoid organ definition. In germinal centres, B cells are stimulated and somatically hypermutate their antigen receptor genes; those with better antigen avidity are selected and can undergo heavy chain class switching to produce different antibody subclasses. These processes depend on antigen stimulation followed by engagement of CD40 on the B cells by CD40L on T lymphocytes. CD40 or CD40L deficiency impairs CD4+ T cell priming, follicular dendritic cell differentiation, germinal centre formation and class switching. IgM-expressing cells cannot undergo isotype conversion to IgG expression, leading to hyper
IgM syndrome. Mice deficient for LTα, LTβ, TNF and TNFR1 have severe defects in follicle and germinal centre formation. BlyS from activated DCs has been found to interact with the TAC1 and BCMA receptors on B cells and promote their survival. Transgenic mice overexpressing BlyS develop increased B cell numbers and autoimmunity. T lymphocyte activation is also regulated by TNF/TNFR SFPs (Locksley et al. 2001).

TNF specifically attacks malignant cells. It may safely be administered to patients without serious toxicity, shock or cachexia within a dose range that has failed to demonstrate significant improvement in cancer treatment. Adjuvants such as lithium chloride can increase the direct cytotoxicity of TNF on tumour cells *in vitro* and in animal studies without inducing significant side effects (Schotte et al. 2001). Treatment of human myeloid cell lines, including THP-1 and U937, with DMSO may potentiate the antiproliferative and cytotoxic effects of TNFα (Depraetere et al. 1995).

Adipose tissue is a significant source of TNFα and its endogenous elevation in models of rodent obesity mediates peripheral insulin resistance. TNFα induces insulin resistance, in part, through inhibition of intracellular signalling from the insulin receptor. Elevation of TNFα in adipose tissue in human obesity correlates strongly with hyperinsulinemia, a marker of insulin resistance (Hotamisligil et al. 1995). Exposure of cultured adipocytes inhibits insulin-stimulated glucose uptake. There is a concurrent decrease in insulin-stimulated autophosphorylation of the insulin receptor and a dramatic decrease in the phosphorylation of the insulin receptor substrate (IRS)-1. TNFα directly interferes with signalling of insulin through its receptor, blocking its biological actions (Hotamisligil et al. 1994). Sterol regulatory element binding protein (SREBP)-1 gene expression is decreased in the subcutaneous adipose tissue and skeletal muscle of subjects with type 2 diabetes and in the adipose tissue of obese normoglycaemic subjects. Exposure of isolated human adipocytes to TNFα produces a decrease in SREBP-1c mRNA and completely blocks the insulin-induced cleavage of SREBP-1 protein to the active form (Sewter et al. 2002). Plasma sTNFR2 concentrations were proportionate to BMI and leptin concentrations in a cohort of type 2 diabetic and control subjects (Fernandez-Real et al. 2000). In addition, carriers of the TNFR2 A2 allele had significantly greater BMI, fat mass, waist-to-hip ratio, total and VLDL triglyceride (TAG) concentration, leptin concentration and had a lower insulin resistance that non-carriers of the A2 variant (ie: carriers of A1, A3 and A4 alleles). The TNFα –863A allele is associated with a lower expression and secretion of the TNFα
gene and protein. In a group of first-degree relatives of type 2 diabetics, although plasma concentrations of sTNFR2 were higher and insulin sensitivity lower than in controls, neither the TNFα-863A allele nor sTNFR2 independently determined insulin sensitivity (Costa et al. 2003).

High expression of TNFα mRNA has been found in vascular cells, with most intense positivity at sites of endothelial disruption and thrombosis (Keso et al. 2001). In vivo, TNFα promotes lesion development and thrombosis by inhibiting anticoagulatory mechanisms of the vascular endothelium, by inducing chemotaxis in SMCs and lymphocytes and by stimulating macrophages to secrete the matrix degrading MMPs (Keso et al. 2001). TNF decreases lipoprotein lipase activity in cultured adipocytes and increases de novo hepatic fatty acid synthesis, potentiating hypertriglyceridemia (Keso et al. 2001). A direct regulation of TNF transcription rate by exposure of macrophages to LDL particles has been reported, supported by in vivo studies showing increased TNFα secretion in hypercholesterolaemic rabbits, in LDL-receptor -/- mice and in human subjects with CHD (Elneihoum et al. 1996; Vendrell et al. 2003; Zhao et al. 2003). However, it has also been reported that lipid accumulation from exposure of macrophages to acetylated LDL suppresses mRNA expression of TNFα and activity of the AP-1. Oxidised LDL stimulated AP-1 and PPARγ but inhibited NF-κB from activated macrophages (Ares et al. 2002). Gemfibrozil, a fibrate PPARα activator and lipid-lowering drug, significantly inhibits TNFα secretion from PBMC of subjects with CVD (Zhao et al. 2003).

The variation of TNFα expression between individuals may be problematic in clinical trials. Variable expression of TNFα in humans is a consequence of variability of the TNFα gene. The TNFα –308G/A A allele has been associated with increased transcription of TNFα in lymphocytes, a parental history of MI and an increased frequency of obesity (Herrmann et al. 1998). In a Mediterranean population, the frequency of the TNFα –308G/A A allele was higher in subjects with CHD than controls (Vendrell et al. 2003). However, the expression of this allele did not affect coronary stenosis or the frequency of coronary events in a study of 700 male autopsied specimens (Keso et al. 2001).

1.5.6 Matrix Metalloproteinase
The matrix metalloproteinases (MMPs) or matrixins, are key zinc-endopeptidases of extracellular matrix homeostasis. The timely breakdown of extracellular matrix is
essential for embryonic development, morphogenesis, reproduction, tissue resorption and vascular remodelling (Nagase & Woessner, 1999). Dysregulation of their activity advances the pathology of diseases such as atherosclerosis, arthritis, glomerulonephritis, periodontitis, cancer and multiple sclerosis (Galis et al. 1994; Matsumoto et al. 1998).

MMPs are synthesised as prepro-enzymes and secreted as pro-enzymes in most cases. The pro-peptide domain possesses a unique sequence characterised by the “cysteine switch” where a cysteine residue binds the catalytic zinc ion and maintains latency of the pro-enzymes (Nagase & Woessner, 1999; Rauch et al. 2002). The catalytic domain of the MMPs forms a “Met-turn” structure similar to that of other “metzincins” including astacins, repolysins (ADAMs) and serralysins (Nagase & Woessner, 1999). The dissociation of the pro-enzyme from the catalytic domain can occur by autocatalysis or catalyses by furin, plasmin or other MMPs. MMP-2 is activated through an alternative mechanism. Its prototypical form, pro-MMP-2, is activated by membrane bound MT1-MMP (MMP-14), thrombin or integrin α,β3 (Rajavashisth et al. 1999; Rauch et al. 2002). Most MMPs are secreted proteins but the recently described MT-MMPs are membrane-bound with a transmembrane and cytoplasmic domain. The main MMPs and their alternative names, indicating function, are presented in Table 1.4.

Table 1.4 The MMPs and their alternative names/functions

<table>
<thead>
<tr>
<th>MMP &amp; Alternative name/function</th>
<th>MMP &amp; Alternative name/function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MMP-1</strong></td>
<td>Interstitial collagenase</td>
</tr>
<tr>
<td><strong>MMP-2</strong></td>
<td>Gelatinase A</td>
</tr>
<tr>
<td><strong>MMP-3</strong></td>
<td>Stromelysin 1</td>
</tr>
<tr>
<td><strong>MMP-7</strong></td>
<td>Matrilysin</td>
</tr>
<tr>
<td><strong>MMP-8</strong></td>
<td>Collagenase 1</td>
</tr>
<tr>
<td><strong>MMP-9</strong></td>
<td>Gelatinase B</td>
</tr>
<tr>
<td><strong>MMP-10</strong></td>
<td>Stromelysin 2</td>
</tr>
<tr>
<td><strong>MMP-11</strong></td>
<td>Stromelysin 3</td>
</tr>
<tr>
<td><strong>MMP-12</strong></td>
<td>Metalloelastase</td>
</tr>
<tr>
<td><strong>MMP-13</strong></td>
<td>Collagenase 3</td>
</tr>
<tr>
<td><strong>MMP-14</strong></td>
<td>MT1-MMP</td>
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<td><strong>MMP-15</strong></td>
<td>MT2-MMP</td>
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<tr>
<td><strong>MMP-16</strong></td>
<td>MT3-MMP</td>
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<tr>
<td><strong>MMP-17</strong></td>
<td>MT4-MMP</td>
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<tr>
<td><strong>MMP-19</strong></td>
<td>Gelatinase</td>
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<tr>
<td><strong>MMP-20</strong></td>
<td>Enamelysin</td>
</tr>
</tbody>
</table>

The pro-peptide MMPs and endogenous inhibitors, such as α-macroglobulins and TIMP, tightly regulate activity of the enzyme. The activity of MMPs is downregulated by TGFβ, glucocorticoids and heparin (Bellosta et al. 1998; Nagase & Woessner, 1999).
PPARγ agonists downregulate MMP-9 in human monocytes \textit{in vitro}, potentially through transrepression of AP-1 or NF-κB sites in the MMP-9 promoter. No PPRE has been located in the human MMP-9 promoter, although two possible elements have been identified in the rat (Worley \textit{et al.} 2003).

MMP genes are inducible by growth factors such as PDGF, cytokines such as CD40, TNFa, IL-1 and M-CSF, chemical agents such as TPA and LPS, physical stress and haemodynamic forces, oncogenic cellular transformation, infection and increased expression of tenascin C (Galis \textit{et al.} 1995; Bellosta \textit{et al.} 1998; Shah & Galis 2001). MT-MMPs can cause MMP activation, leading to the hypothesis of a pericellular MMP-activation cascade (Rauch \textit{et al.} 2002). MMP-2 can be activated by MT1-MMP or thrombin. Cleavage of pro-MMP-2 has been induced by the presence of coagulation factors II, Va, VIIa and Xa in HUVECs and by PDGF in rodent SMCs (Rauch \textit{et al.} 2002).

There is a preponderance of evidence linking MMP activation to atherosclerotic tissue remodelling and plaque rupture. In normal human arterial specimens MMP-9 and TIMP stain uniformly, whereas in atherosclerosis the plaque shoulders and regions of foam cell accumulation display increased expression and activation of MMP-1, 2, 3 and 9 (Galis \textit{et al.} 1995). MMP-1, 3, 7 and 12 have been reported to be increased in thin, rupture-prone plaque of patients undergoing endarterectomy, with MMP-12 significantly increased in ruptured plaques and MMP-9 transcript level unchanged between AHA-defined lesion types (Morgan \textit{et al.} 2004). Elsewhere, MMP-9 has been reported to be more common in atherectomy samples from subjects with unstable angina (Bellosta \textit{et al.} 1998), a presentation of advanced atherosclerosis. Lipid-laden macrophages, but not alveolar macrophages, released MMP-1 and 3 spontaneously in culture (Galis \textit{et al.} 1995). MT1-MMP expression co-localises with SMCs and macrophages within the atheroma and its mRNA and protein expression increased 2 to 4 fold in SMCs exposed to IL-1α, TNFα and oxidised LDL (Rajavashisth \textit{et al.} 1999). MMP-12, an elastase that targets the basement membrane, was increased in response to pro-atherogenic feeding in rabbits and in response to acetylated LDL exposure of human macrophages \textit{in vitro} (Matsumoto \textit{et al.} 1998). Reactive oxygen species (ROS) are known to react with thiol groups such as those that maintain MMP latency. ROS generated from \textit{ex vivo} macrophage foam cells of hypercholesterolaemic rabbits have increased MMP activity of vascular cells under high oxidative stress (Rajagopalan \textit{et al.} 1996). The excessive degradation of extracellular matrix scaffold by MMPs is a major
molecular mechanism in the atherosclerotic process, but the identity of key responsible MMPs remains elusive. Fibrillar collagen, a major component that confers tensile strength to the atherosclerotic plaque, is degraded by traditional collagenases and also by the gelatinases MMP-2 and 9. The entrapment of macrophages within the lesion, and their inability to migrate from the site of injury, is central to the dysregulation of the immune response in atherosclerosis. MMPs, paradoxically, create space within the lesion by degradation of extracellular matrix. This does not promote macrophage migration but, rather, liberates SMCs and enables their proliferation and migration to the intima.

1.5.7 Acute phase proteins
An acute phase reactant is a protein whose plasma concentration increases (positive acute phase proteins) or decreases (negative acute phase proteins) by at least 25% during inflammatory disorders (Gabay & Kushner 1999). Among cytokines, IL-6 is the chief stimulator for the hepatic production of most acute-phase proteins. Among acute phase proteins, the role of CRP in atherosclerosis is best defined.

1.5.7a CRP
CRP was discovered in 1930 and so-named because it reacted with the pneumococcal C-polysaccharide in the plasma of patients during the acute phase of pneumococcal pneumonia (Gabay & Kushner 1999). It is a pentameric protein of five 23kDa identical units and the human gene maps to chromosome 1 (Alonso-Martinez et al. 2002; Labarrere & Zaloga, 2004).

CRP has a half-life of about 19 hours and a serum concentration in healthy populations of <2mg/L (de Ferranti & Rifai, 2002). Under physiological conditions CRP is synthesised at low rates and retained in the endoplasmic reticulum by resident carboxylesterases (Volanakis 2001). With illnesses such as rheumatoid arthritis or sepsis, concentrations can increase to 300mg/L. Generally, circulating CRP concentrations display stability, lack of diurnal variation and lack of influence of gender or age (Calabró et al. 2003). Ockene et al. (2001) found that 63% of CRP values in healthy individuals remained constants over three months. The main stimuli for the hepatic release of CRP are IL-6 and IL-1. Circulating levels are positively associated with obesity, smoking, blood pressure, heart rate, serum fibrinogen, TAG, apolipoprotein B and fasting glucose concentrations and negatively associated with
serum HDL (Rohde et al. 1999). Serum CRP concentration is a strong predictor of atherosclerotic lesion development, future cardiovascular events (Haverkate et al. 1997; Martin-Paredero et al. 1998; Madsen et al. 2001, Verma et al. 2002) and mortality in the elderly (Harris et al. 1999; Evrin et al. 2005). Individuals in the highest quartile of CRP concentration were predicted to have a 2.8 fold increase in risk of sudden cardiac death compared to those in the lowest quartile (Kuller et al. 1996). CRP levels correlate with calculated level of coronary heart disease risk by the Framingham calculated risk score (Albert et al. 2003). Albert et al. (2003) demonstrated that CRP levels correlated minimally with most traditional risk factors of CVD. CRP concentration has been shown to have a stronger relationship with stroke and MI than total cholesterol, LDL, apolipoprotein A1, Lp(a), homocysteine and sICAM-1 (Rerkasem et al. 2002). CRP represents a novel clinical marker of atherosclerosis. Experimental evidence indicates that CRP is more than a marker of disease, and has an active and independent role in progression of atherosclerosis.

CRP is thought to stimulate tissue factor production, to opsonise LDL and VLDL at their Ca2+-dependent phosphocholine binding sites, to activate the classical complement pathway via C1q recognition of ligand-complexed CRP and to mediate the uptake of native LDL into macrophages via the low-affinity CD32 receptor (Volanakis 2001; Zwaka et al. 2001). CRP recognises phosphocholine of bacterial and fungal polysaccharide and the unusual phospholipid constituents of damaged cells (where membrane phospholipid “flip-flop” occurs) and acts as an opsonin to promote their clearance (Gabay & Kushner 1999; Volanakis 2001). CRP induces ICAM-1 and VCAM-1 on human saphenous vein endothelial cells (HSVECs) in vitro and increases MCP-1 production (Verma et al. 2002). CRP is reported to induce E-selectin on endothelial cells, inhibit endothelial NO synthase (eNOS), activate NF-κB in endothelial and foamy cells and stimulate the secretion of macrophage foam cell pro-inflammatory cytokines (de Ferranti & Rifai, 2002; Labarrer & Zaloga 2004). However, in transgenic mice producing large amounts of CRP, the net effect of the acute phase reactant is anti-inflammatory, with suppressed expression of L-selectin, inhibition of superoxide production by neutrophils and the synthesis of IL-1Ra by mononuclear cells (Gabay & Kushner 1999). CRP co-localises with the terminal complement complex and foamy cells upon deposition in the arterial wall (Torzewski et al. 1998; Zwaka et al. 2001; Alonso-Martinez et al. 2002; Wieland et al. 2002). It is present in early human sclerotic lesions, in foamy cells and in the fibromuscular layer of
the intima populated predominantly by SMCs (Torzewski et al. 1998). CRP is also produced within the atherosclerotic lesion, although this has not been fully characterised. Human coronary artery SMCs, but not HUVECs, have been shown in vitro to produce CRP in response to inflammatory cytokines, particularly TNFα and a combination of IL-6 and IL-1β, and also by LPS (Calabrò et al. 2003).

CRP levels, in the absence of pathology, exhibit high heritability. CRP could potentially be used as an intermediate phenotype for genetic analysis that might be more tractable than the complex phenotype of clinical CVD, which would be expected to result from a wider variety of genetic and environmental factors (Vickers et al. 2002). A G/C polymorphism at nucleotide 1059 of the CRP gene has recently been identified (Cao & Hegele, 2000). Heterozygous (GC) carriers of the CRP 1059 polymorphism displayed significantly lower CRP levels than wild-type (GG) controls although the polymorphism is believed to be silent and does not predict clinical events relating to atherosclerosis (Zee & Ridker, 2002). Carriers of two GT(16) alleles, two GT(21) alleles or GT(16/21) heterozygotes of the intron of the CRP gene displayed two-fold lower CRP levels than those with other genotypes (Szalai et al. 2002). After strenuous exercise and post-CABG, CRP levels are higher in homozygotes of the +1444T allele than in carriers of the +1444C allele of the CRP gene (Brull et al. 2003). The presence of the −176G/C polymorphism of the IL-6 promoter is associated with higher baseline CRP levels (Vickers et al. 2002). Carriers of the +3954 allele 2 polymorphism of the IL-1β gene have twice the median CRP levels of carriers of the +3954 allele 1 genotype (Eklund et al. 2003).

1.5.7b Fibrinogen
The acute phase reactant, fibrinogen, is produced by hepatocytes in response to IL-6 (Martin-Paredero et al. 1998). Elevated fibrinogen has been established as an independent risk factor for coronary, cerebral and peripheral vascular disease (Kannel et al. 1987; Yarnell et al. 1991; Lowe et al. 1993). Fibrinogen levels are associated with risk factors of CVD including plasma cholesterol concentrations, smoking, hypertension, obesity, stress and diabetes (Martin-Paredero et al. 1998). The extent of atherosclerosis has been associated with age, TAG and fibrinogen concentrations (Levenson et al. 1995). Although fibrinogen and CRP levels are also associated (Mendall et al. 1996), fibrinogen is an independent risk factor in the development of atherosclerosis (Levenson et al. 1995; Rohde et al. 1999).
Fibrinogen is the main plasma coagulation protein and a cofactor for platelet aggregation (Rohde et al. 1999) and is considered pro-atherogenic in its thrombotic capacity. Dysregulation of coagulation and fibrinolysis contribute to the development and progression of atherosclerosis. Elevated levels of fibrinogen, factors VII and VIII, von Willebrand factor, thrombomodulin, tissue plasminogen activator and plasminogen activator inhibitors are associated with increased CVD-related morbidity (Krysiak et al. 2003).

Fibrinogen appears to play a role in the inflammatory progression of the atherosclerotic plaque. Binni et al. (1989) report that D-dimer deposition of fibrinogen in the arterial wall is associated with increasing severity of atherosclerosis. SMC proliferation and migration are stimulated by fibrinogen and its degradation products (Levenson et al. 1995). Fibrinogen induces endothelial cell proliferation and expression of adhesion molecules (Gabay & Kushner 1999, Tsakadze et al. 2002).

### 1.6 CELLULAR SIGNALLING: THE NF-κB PATHWAY

An efficient immune response is characterised by rapid, transient and multifunctional signalling events, condensed signalling machinery with expansive potential for de novo protein synthesis and in-built regulatory machinery sensitive to efficacy and potential for autotoxicity. By these standards, the system is energetically costly. The NF-κB pathway of signal transduction is such system. NF-κB was first described in 1986, by Sen & Baltimore, as a DNA-binding protein bound to a decameric consensus motif in the immunoglobulin κ light-chain gene enhancer (Roshak et al. 2002). It is evolutionarily conserved (Karin & Delhase, 2000), ubiquitously expressed and the related proteins Dorsal, Dif and Relish have been identified in *Drosophila* (Baldwin, 2001).

#### 1.6.1 NF-κB subunits

The five known members of the mammalian NF-κB family are Rel A (p65), c-Rel, RelB, p50 and p52. They are characterised by an N-terminal conserved 300 amino acid Rel homology domain (RHD) which mediates dimerisation, nuclear translocation, DNA binding and interaction with NF-κB inhibitors (Baldwin, 2001; Collins & Cybulsky, 2001). p65, c-Rel and RelB possess C-terminal transcriptional activation domains (TADs) and are capable of directly inducing transcription of target genes (Roshak et al. 2002; Vermeulen et al. 2002). p50 and p52 are synthesised as p105 and p100 precursors.
that contain the C-terminal ankyrin repeat motif of NF-κB inhibitors. Mature p50 and p52 lack a TAD, and as homodimers they have no intrinsic ability to activate transcription (Saccani et al. 2003).

**Figure 1.8 NF-κB subunits p65, c-Rel, RelB, p50 and p52.** The pro-forms of p50 and p52, p105 and p100 respectively, possess the ankyrin repeat domain of IkB. Mature p50 and p52 lack a transactivation domain. The cleaved ankyrin repeat domain is phosphorylated and polyubiquitinated, as for IkB. Adapted from Perkins, 1997

### 1.6.2 NF-κB dimers

Active NF-κB is found dimerised. Most members (except RelB) can homo- or heterodimerise (Tak & Firestein, 2001). The multiplicity of NF-κB subunits and their capacity for dimerisation reflects the pleiotropic nature of this signalling pathway. However, dimer effects are tightly regulated. Each dimer has a preferred DNA-binding site, thus specifying and limiting target gene transcription. Dimers associate selectively with each other. RelB dimerises only with p50 or p52 (Baldwin, 1996). p50 and p52 homodimers are inactive but they form active heterodimers with p65, cRel and RelB (Saccani et al. 2003). The expression of dimers is also time-responsive, with p65 being
found in "early" active NF-κB complexes and c-Rel, whose expression is stimulated by NF-κB itself, associated with "late" complexes (Perkins, 1997). The most intensively studied dimer is p50/p65. p65 deficient mice lose the ability to induce NF-κB regulated genes such as TNF, IκBa and GMSF, and -/- mice exhibit embryonic lethality due to widespread hepatic apoptosis (Baldwin, 1996). Both p50 and p65 play a role in constitutive IL-6 production in rheumatoid arthritis synovial fibroblasts while p65 activation by thrombin regulates ICAM-1 expression in endothelial cells. RelB plays a role in the development and differentiation of DCs and a mutation disrupting RelB impairs antigen presentation. Mice lacking p50 or RelB are immunodeficient but otherwise develop normally to adulthood. p50/52 double -/- mice exhibit impaired development of osteoclasts and B cells (Tak & Firestein, 2001). The p50/p50 and p52/p52 homodimers repress the transcription of their target genes (Zhang & Ghosh, 2001). Overexpression of p50 blocks transcription of TNFα in LPS-stimulated macrophages (Baer et al. 1998). p50 accumulates with delayed kinetics in macrophages following LPS stimulation and preferentially bind to three κB elements in the murine TNFα promoter, which appears to attenuate TNFα gene transcription. In fact, a genetic polymorphism that disrupts binding of p50 dimers to the κB1 site in the human TNFα gene causes increased transcription from this promoter (Wessell et al. 2004). However p52 can activate transcription when complexed to bcl3, an IκB-like molecule. The p52/RelB dimer is insensitive to IκBa and over time replaces p50/p65 binding to DNA, and may therefore sustain an inflammatory response (Saccani et al. 2003).

### 1.6.3 Regulation by Inhibitor of κB (IκB)

A key point in the activation and regulation of the NF-κB signalling pathway involves the natural inhibitor of the subunits, IκB. These inhibitors are characterised by an ankyrin repeat domain. Multiple mammalian forms exist, including IκBa, IκBβ, IκBδ (p100), IκBε, IκBγ (p105), p102, bcl3 and the recently described IκBζ. (Bowie & O’Neill, 2000; Roshak et al. 2002). The *Drosophila* homologue, Cactus, is under the control of Toll, a homologue of the IL-1 receptor (Baldwin, 2001). IκB binds to NF-κB dimers, masks their nuclear localisation sequences and retains the entire complex in the cytoplasm (Collins & Cybulsky, 2001). NF-κB dimers have inhibitor specificity. Dimers containing p65 or cRel associate with the IκBa (Baldwin, 1996). RelB/p52 does not associate with the IκBs and is retained in the cytoplasm by p100 (Saccani et al. 2003).
IkBα is the best characterised IkB member (Roshak et al. 2002). It can be divided into three structural domains; the 70-amino acid N-terminal region, a 205-amino acid internal region that is composed of ankyrin repeats that allow interaction with the RHD (Zhang & Ghosh, 2001), and a C-terminal 42-amino acid region that contains a proline (P)-, glutamic acid (E)-, serine (S)- and threonine (T)- rich (PEST) region (Ernst et al. 1995; Baldwin, 1996). Deletion of the N-terminal region does not inhibit the ability of IkBα to interact with NF-κB but deletion of the C-terminal region blocks its ability to inhibit DNA binding of NF-κB (Ernst et al. 1995; Jaffray et al. 1995). IkBα associates with the p50/p65 heterodimer and the p50 homodimer, but does not inhibit p50 homodimer DNA-binding (Tak & Firestein, 2001). 3D structures of IkBα in complex with the RHD of p50 and p65 show the core of the IkB molecule is positioned next to the nuclear localisation signal and strictly hinders it binding to karyopherin carrier proteins, which transport NF-κB to the nucleus (Karin & Delhase, 2000).

IkBα−/− mice enter a wasting phase and die about 7 days after birth as NF-κB is constitutively activated and its target genes constitutively expressed (Beg et al. 1995). IkBα is itself a target gene of NF-κB. Within an hour of activation IkBα levels recover within the cytoplasm and its sequestrant capacities are restored. In addition, IkBα shuttles between the cytoplasm and nucleus where, in the unstimulated state, it can inhibit and remove NF-κB by a potential nuclear export sequence (Baldwin, 1996). Thus, IkBα is associated with transient NF-κB activation (Tak & Firestein, 2001). IkBα is targeted by signalling pathways induced by TNF, IL-1, LPS and PMA.

IkBβ is targeted by signalling pathways initiated by IL-1 and LPS. It inhibits p50/p65 more strongly than it does p50/RelB and p50/c-Rel. Its resynthesis after degradation is relatively slow following NF-κB induction and thus allows a persistent activation of NF-κB to follow (Thompson et al. 1995).

p100 and p105 are often described as members of the NF-κB subunit family due to their RHD. However, strictly, the RHD belongs to p50 and p52 components. The ankyrin repeat motif, a character of the IkB family, defines p100 and p105 as intramolecular IkBs. The ankyrin repeat domains of full length p100 and p105 form stable dimers with other Rel proteins and cause their cytoplasmic retention (Karin & Delhase, 2000). p105 and p100 undergo limited and slow processing by, as with other IkBs, ubiquitinisation and degradation (Baldwin, 1996).
1.6.4 Regulation by Inhibitor κB kinase (IKK)

A further point of regulatory machinery is involved in the disassociation of the NF-κB dimer and the IκB inhibitor complex IKK. IKK was identified in 1997. The 900kDa complex has three main subunits. IKKα and IKKβ are 86kDa and 87kDa peptides respectively, have 52% homology and contain an N-terminal kinase domain, a leucine zipper and a helix-loop-helix motif. IKKα and IKKβ homo- and heterodimerise and are the catalytic components of the complex. IKKβ activation, rather than IKKα, appears to participate primarily in the NF-κB signalling pathway (Delhase & Karin, 1999; Karin & Delhase, 2000). IKKα -/- mice do not have affected NF-κB activation. IKKβ -/- fibroblasts fail to activate NF-κB and -/- mice display embryonic lethality (Roshak et al. 2002). IKKγ (NEMO; NF-κB essential modulator) is a 50-52kDa doublet containing several N-terminal helical regions, a leucine zipper and a Zn-finger at its docking-site C-terminus. It lacks kinase activity and is a regulatory protein necessary for, without enhancing, IKK activation. It preferentially binds IKKβ (Saccani et al. 2003). IKK activation depends upon the phosphorylation of the catalytic subunit by kinases such as NF-κB-inducing kinase (NIK) and the IKK-related NF-κB-activating kinase (NAK). The complex phosphorylates IκBα on serines 32 and 36 (Karin & Delhase, 2000; Baldwin, 2001; Tak & Firestein, 2001).

1.6.5 Activators of NF-κB

NF-κB is typically activated by signals related to pathogens or stress. Common stimuli include LPS, TNF, IL-1, T lymphocyte activation, UV radiation, growth factors and viral infection. NF-κB may also be induced by the redox state of the cells (Collins, 1993; Baldwin, 1996; Tak & Firestein, 2001). Agents that activate NF-κB have been shown to elevate levels of ROS. Antioxidants can inhibit NF-κB activation (Li & Karin, 1999). Elevated levels of native and oxidised LDL have been suggested to act as pro-oxidant signals in activating NF-κB target gene expression and this effect has been ascribed to lipid peroxidation products (Maziere et al. 1996). Angiotensin II, a potent vasoconstrictor elevated in hypertension and related to atherosclerosis, activates NF-κB in endothelial cells (Pueyo et al. 2000) and heart tissue (Theuer et al. 2002). Hyperglycaemia and elevated homocysteine are also reported to activate NF-κB (Collins & Cybulsky, 2001). LPS-activated NF-κB was significantly greater in macrophages from old mice compared to those from young mice due to a greater IκB degradation in the cytoplasm and p65 nuclear translocation, indicating that advanced
age may also be a factor in NF-κB activation. Addition of ceramide to macrophages from young mice increased NF-κB activation (Wessell et al. 2004). Mycoplasmal lipopeptides activate NF-κB. Opioid peptides, endomorphin-1 and 2 inhibited IL-10, IL-12 and H$_2$O$_2$ production, limited chemotactic and phagocytic function and potentiated NF-κB binding to DNA in a macrophage-differentiated human cell line (Azuma & Ohura, 2002). LPS is considered to be the most potent activator of the macrophage secretory response. When monocytes are differentiated to macrophages they appear to exhibit an enhanced sensitivity to LPS. In parallel with multiple changes in cell morphology and function during the differentiation process, there is an accumulation of functional proteins from the NF-κB family in the cytoplasm of the cell. Stimulation with LPS induces the translocation of the NF-κB proteins from the cytoplasm into the nucleus where it can bind DNA and activate target genes (Takashiba et al. 1999).

1.6.6 Exogenous inhibitors of NF-κB
The inhibition of the NF-κB signalling pathway by fatty acids is discussed in Chapter 5. Aspirin, or salicylate, is a potent inhibitor of the NF-κB signalling pathway (Kim et al. 2001; Shoelson et al. 2003) and acts by binding IKKβ (Liu et al. 2001). Vitamin C blocked IL-1 and TNF-mediated degradation and phosphorylation of IκBa by inhibiting IKK activation mediated by p38 MAPK at normal physiological concentrations (Bowie & O’Neill, 2000). β-adrenergic agonists have bronchodilatory and cardiotonic effects and have been shown to inhibit activation and nuclear translocation of NF-κB in monocytes activated by LPS, potentially via elevation of cAMP (Farmer & Pugin, 2000). IL-10 was shown to inhibit the induction of NF-κB by LPS in human PBMC (Wang et al. 1995). Glucocorticoids are reported to inhibit NF-κB by inhibiting activated subunits or by upregulating IκBa. Cyclosporin A and rapamycin are also reported to inhibit the activation of NF-κB in T lymphocytes. NO has been reported to inhibit the activation of NF-κB in endothelial cells (Baldwin, 1996). A20 is an NF-κB inhibitory protein with two κB elements in its promoter. Its transcription terminates activation of NF-κB (Heyninck & Beyaert, 2005). This negative-feedback inhibition establishes a potential anti-apoptotic role of NF-κB (Perkins, 1997).

1.6.7 NF-κB activation mechanism
The NF-κB signalling pathway is addressed in three stages: IκBa phosphorylation and degradation, NF-κB translocation and nuclear regulation, and signalling upstream of
IKK. Also discussed in this section are LPS activation of NF-κB through TLRs, fatty acids and TLR-signalling, and lipid rafts.

Figure 1.9 NF-κB signalling pathway and upstream induction. From website of BIOMOL, Hamburg (www.biomol.de)

1.6.7a IkBa phosphorylation and degradation
A key point in the NF-κB signal transduction pathway is inducible IkB degradation. In most IkB homologues, the ankyrin repeat core is preceded by an N-terminal regulatory domain with two essentially invariant serines. These are denoted S32 and S36 in IkBa.
These serines undergo rapid phosphorylation by IKK. Phosphorylated IκBα undergoes polyubiquitinisation at one or two lysines, also within the N-terminal regulatory domain, by a specialised ubiquitin ligase complex, E3, which recognises a docking-site created by the serine-phosphorylation of IκBα. E3 ubiquitinating ligase belongs to the Skp-1/Cul/Fbox (SCF) family. Polyubiquitinated IκBα is rapidly degraded by the 26S proteasome. The phosphorylation of IκBα is the only regulated step in this process; polyubiquitination and degradation are constitutive (Baldwin, 1996; Karin & Delhase, 2000; Roshak et al. 2002; Saccani et al. 2003).

1.6.7b NF-κB translocation and nuclear regulation

Liberated NF-κB dimers are free to translocate to the nucleus following dissociation from cytoplasmic IκB, transported by karopherin proteins. NF-κB appears rapidly in the nucleus following its dissociation and target gene transcription may begin within minutes following activation (Baldwin, 1996). NF-κB subunits generally interact with κB elements in the promoter regions of inflammatory response target genes and activate their transcription (Liu et al. 2001), although a κB element is not always required. The RHD of NF-κB associates with a large number of b-Zip containing transcription factors. p65 stimulates AP-1 DNA binding and activation through AP-1 sites in the absence of a κB element. In addition, nuclear binding of NF-κB does not indicate transactivation. NF-κB is capable of binding to DNA without being transcriptionally active. The p38 MAPK inhibitor SC203580, bcl2 and bcl-xL have been shown to inhibit transactivation without affecting the nuclear localisation of NF-κB (Perkins 1997).

In the nucleus, NF-κB must gain access to promoters and enhancers of the genes it regulates. These interactions involve the modification of the transcription factor and the surrounding chromatin architecture, which represents a repressive structure into which eukaryotic DNA is packaged (Perkins, 1997; Ma et al. 2004). Modifications, including acetylation, ubiquitination, methylation and SUMOylation, induce conformational changes, intramolecular allostERIC switches or attraction of additional adaptor proteins to the transcription factor-promoter complex (Vermeulen et al. 2002). Acetylation of lysine residues within amino-terminal tails of nucleosomal histones is linked to chromatin disruption and transcriptional activation of genes. NF-κB-dependent transcription requires multiple co-activators possessing histone deacetyltransferase (HAT) activity, such as CREB-binding protein (CBP). Histone deacetylase (HDAC) inhibitors enhance NF-κB-dependent gene expression in the presence of TNFα.
Interestingly, along with hydroxamic acid, benzamide and cyclic peptides the fatty acid derivative sodium butyrate is a prominent HDAC inhibitor (Rahman et al. 2003). The apoptotic and anti-proliferative properties of these compounds show potential in the treatment of solid and haematological malignancies. Unidentified acetylation events lead to prolonged IKK activity, prolonging the presence of NF-κB in the nucleus and enhancing NF-κB-dependent transactivation. Many co-activators of the NF-κB:κB promoter function, such as CREB-binding protein (p300), SRC-1 and P/CAF, possess a HAT domain and co-repressors possess HDAC activity, including HDAC1, HDAC2, HDAC3 and HDAC6 (Quivy & Van Lint, 2004). NF-κB and its related molecules, such as IKK, associate with these co-activators and co-repressors to modulate the transcriptional activity of the NF-κB:κB promoter complex.

In addition, in vitro studies demonstrate that dimer binding to κB sites may be preferential. However, the same κB site can be bound by different dimers with high affinity, causing potential competition and increasing flexibility and specificity in promoter responses. During DC maturation, rapidly activated dimers (eg: p50/p65) bound to target promoters are gradually replaced over time by slowly activated dimers such as p52/RelB, which is insensitive to IκBa. This allows fine-tuning of the response over time and can sustain inflammation by removing the potential of IκBa modulation (Saccani et al. 2003).

IκBa is a target gene of NF-κB transactivation and this represents another point of control in the system. IκBa transcription is activated by NF-κB binding. The newly synthesised IκBa enters the nucleus and removes NF-κB from its DNA-binding sites, transporting it back to the cytoplasm and thereby terminating NF-KB-dependent transcription.

1.6.7c Signalling from membrane to IKK

Binding of TNF and an aggregation of three membrane TNFR1s induces an association of the cytosolic region of the receptor with TRADD. TRADD recruits TRAF2, which interacts with NIK to activate IKK. Binding of IL-1 to IL-1R recruits the IL-1RacP, inducing and associating with IL-1R associated kinase (IRAK) via the adaptor molecule myeloid differentiation factor 88 (MyD88). IRAK activates TRAF6, which associates with the MAPK-kinase-kinase TGFβ activating kinase (TAK)-1 leading to activation of NIK and, subsequently, IKK (Bowie & O’Neill, 2000).
Binding of pathogen-associated molecular patterns (PAMPs), such as LPS, bacterial lipoprotein, lipoteichoic acid and lipoarabinomannan of mycobacteria to pattern recognition receptors (PRRs), such as CD13, β2-integrins and macrophage scavenger and complement receptors, represents a relatively invariant innate immune interaction. TLRs are such a group of evolutionarily conserved PRRs and the major transducer of their signal is the NF-κB pathway (Zhang & Ghosh, 2001). TLRs are a family of transmembrane receptors characterised by multiple copies of leucine-rich repeats in the extracellular domain and a cytoplasmic Toll/IL-1R (TIR) motif, bearing homology to the intracellular signalling domain of IL-1R1.

1.6.7d LPS activation of NF-κB through TLR4

In the late nineties, TLR4 was identified as the LPS receptor. It also acts as a receptor for PAMPs including lipoteichoic acid, fibronectin, fusion protein of respiratory syncytial virus and the plant diterpene taxol (Pålsson-McDermott & O’Neill, 2004). LPS-binding protein (LBP) is an acute phase protein that forms a high-affinity complex with the lipid A moiety of LPS. The LBP-LPS complex associates with soluble or membrane-bound CD14, which was originally identified as the LPS-receptor. CD14 presents LPS to TLR4 and MD2, a necessary extracellular adaptor protein. MD2 −/− mice are unresponsive to LPS (Nagai et al. 2002). TLR4-mediated response to LPS can be divided into an early MyD88-dependent response and a delayed MyD88-independent response (Shinohara et al. 2005). LPS binds to TLR4/MD2, recruiting the adaptor molecules MyD88 and Mal to the complex. As in IL-1-induction of the NF-κB signalling pathway, MyD99 recruits IRAK. Hyperphosphorylated IRAK dissociates from the complex and interacts with TRAF6. TRAM structurally resembles Mal and, in MyD88-independent signalling, acts as a bridge between TLR4 and TRIF. In the delayed activation of NF-κB via TLR4, TRIF binds to TRAF6 with subsequent associations of the higher NF-κB signalosome, as previously described. Tollip may also be involved as a negative adapter molecule in TLR signalling (Pålsson-McDermott & O’Neill, 2004; Takeda, 2005).

1.6.7e Fatty acids and TLR signalling

The lipopeptide composition of PAMPs appears to influence TLR binding and, hence, their immunoadjuvanticity. The activity of PAMP lipopeptides is strongly influenced by the number and character of fatty acids. Removal of the acylated saturated fatty acids
from lipid A of LPS results in a complete loss of endotoxic activity (Lee et al. 2003). Single-chain lipopeptides did not elicit a cellular response and activity was reduced for lipopeptides carrying short-chain fatty acids. Ester bound oleic acid and/or linoleic acid presence resulted in greatest lipopeptide activity as measured by IL-8 release (Spohn et al. 2004). Lipopeptides containing C10, C14 and C16 saturated fatty acid moieties effectively induce ERK-1 and 2 activation in bone marrow-derived macrophages (Müller et al. 2004). Saturated fatty acids (SFA) induce and unsaturated fatty acids (UFA) inhibit TLR4 activation as determined by NF-κB activation (Lee et al. 2001; Lee et al. 2003). SFA and LPS have been reported to induce NF-κB signalling through TLR4 and a common MyD88-dependent pathway. SFA have been reported to activate TLR4 and a MyD88-independent signalling pathway, whereas the long chain polyunsaturated fatty acid (LC-PUFA) DHA inhibited the activation of all TLRs tested (Lee et al. 2003).

1.6.7f Lipid rafts and signalling

Lipid rafts are detergent insoluble/resistant membrane domains rich in cholesterol and sphingolipids. These lipid-ordered domains exhibit less fluidity than the surrounding plasma membrane (Cuschieri, 2004) and they are postulated to be involved in the recruitment and concentration of molecules involved in signalling (Olsson & Sundler, 2005). Assembly of the LPS-binding complex appears to occur on lipid raft microdomains (Cuschieri, 2004). Cholesterol-depletion using methyl-b-cyclodextrin (MbCD) has proved useful in examining the components of lipid rafts. After LPS stimulation of murine macrophages, CD14 and the MAPK family members ERK-2 and p38, but not JNK, translocated to lipid rafts. MyD88 was present only in detergent soluble fractions. Cholesterol-depletion of lipid rafts did not inhibit TNFα production (Olsson & Sundler, 2005). Cholesterol-depletion of human macrophages had no effect on cell surface expression of TLR4 or TLR4 mobilisation to lipid rafts. Treatment with MbCD induced a sustained LPS-mediated activation of the MAPK family members p38 and JNK/SAPK and attenuated activation of ERK-1 and 2. This was also associated with a down-regulation of TNFα and enhancement of IL-10 production. The lipid raft appears to influence cellular signalling molecules, particular the MAPK family, and cytokine production (Cuschieri, 2004).
1.6.8 NF-κB and mediators of inflammation

Commonly cited target genes with κB promoter elements are listed in Table 1.5 (Baldwin, 1996; Perkins, 1997; Collins & Cybulsky, 2001; Tak & Firestein, 2001; Zhang & Ghosh, 2001).

Table 1.5 Target genes of NF κB.

<table>
<thead>
<tr>
<th>Target Genes</th>
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<tbody>
<tr>
<td>IL-1β</td>
</tr>
<tr>
<td>GM-CSF</td>
</tr>
<tr>
<td>IL-2</td>
</tr>
<tr>
<td>COX-2</td>
</tr>
<tr>
<td>IL-2R2</td>
</tr>
<tr>
<td>NOS</td>
</tr>
<tr>
<td>IL-6</td>
</tr>
<tr>
<td>MMP-13</td>
</tr>
<tr>
<td>IL-8</td>
</tr>
<tr>
<td>MCP-1</td>
</tr>
<tr>
<td>IL-12p40</td>
</tr>
<tr>
<td>Tissue factor</td>
</tr>
<tr>
<td>IFNβ</td>
</tr>
<tr>
<td>VCAM-1</td>
</tr>
<tr>
<td>IFNγ</td>
</tr>
<tr>
<td>ICAM-1</td>
</tr>
<tr>
<td>TNFα</td>
</tr>
<tr>
<td>E-selectin</td>
</tr>
<tr>
<td>TNFβ</td>
</tr>
<tr>
<td>MadCAM-1</td>
</tr>
<tr>
<td>Fas ligand</td>
</tr>
<tr>
<td>SAA</td>
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</tbody>
</table>

NF-κB regulates a number of transcription signalling molecules, some from its own pathway, and apoptotic genes including c-Myc, p53, p100/52, p105/50, cRel, IkBα (Perkins, 1997), inhibitor of apoptosis protein (cIAP)-1 and 2, and cyclin D1. Evolutionary conserved antimicrobial peptides such as β-defensins are regulated by NF-κB (Zhang & Ghosh, 2001). NF-κB inhibition in fibroblasts and vascular SMCs prevented upregulation of MMP-1, MMP-3 and MMP-9 (Bond et al. 1999; Bond et al. 2001). MMP-1 and MMP-3 expression were induced by NF-κB activation in macrophage-derived foam cells but constitutive MMP-9 was not (Chase et al. 2002). TNFα inhibitory factor (TIF), which is up-regulated by LPS activation of macrophages, has been shown to cause the selective nuclear accumulation of the inhibitory NF-κB p50 homodimers (Wessell et al. 2004), showing a self down-regulating mechanism within this inflammatory cascade.
1.6.9 NF-κB and atherosclerosis

NF-κB activity is poorly detected in SMCs of blood vessels devoid of atherosclerosis (Collins & Cybulsky, 2001). Brand et al. (1996) detected activated NF-κB in the intima and media of atheromatous areas of atherosclerotic lesions, expressed in SMCs, macrophages and endothelial cells. Hajra et al. (2000) found that the expression of key NF-κB/IκB components is markedly increased in a region of the ascending aorta and aortic arch highly predisposed to atherosclerotic lesion formation in mice. Atherosclerosis-prone mice exhibit activation of NF-κB when fed an atherogenic diet (Liao et al. 1994). Additionally, the atherosclerotic lesion develops in response to diverse stimuli that have in common the ability to create oxidative stress and activate the NF-κB system. Conversely, agents that inhibit lesion formation often act as antioxidants and stabilise the system (Collins & Cybulsky, 2001). Berliner et al. (1995) propose that oxidised lipids trapped in the subendothelial space may activate NF-κB and induce transcription of pro-inflammatory or apoptotic genes. NF-κB activates a variety of genes relevant to the pathophysiology of the vessel wall including cytokines, chemokines, leukocyte adhesion molecules and genes that regulate cell proliferation. Collectively the coordinated induction of NF-κB-dependent genes may exert a substantial atherogenic effect on the vessel wall (Collins & Cybulsky, 2001). An NF-κB decoy has reduced expression of ICAM-1, IL-2, TNF and inducible NO synthase (iNOS) in heart tissue and competed with endogenous κB sites for NF-κB binding, mimicking a sequesterisation similar to IκB (Yokoseki et al. 2001). Activation of NF-κB is associated with inhibited cell death through the expression of genes that promote resistance to apoptosis, such as c-IAP1 and 2 and XIAP as well as TRAF1 and 2. NF-κB suppresses mitochondrial cytochrome c release through the induction of a bcl-2 family member. Inhibition of NF-κB causes a reduction in cyclin D1-associated kinase activity and retarded G1/S transition, thus inhibiting cell-cycle progression. Endothelial cell proliferation in the arterial system is generally low. Foci of proliferation are, however, associated with areas of disturbed flow, laminar shear stress and NF-κB activation (Collins & Cybulsky, 2001). TLR4 expression has been described in endothelial cells and macrophages of atherosclerotic lesions (Xu et al. 2001; Edfeldt et al. 2002) and adventitial fibroblasts (Vink et al. 2002). Two polymorphic variants of the TLR4 gene, Asp299Gly and Thr399Ile, were associated with reduced extent and progression of carotid atherosclerosis (Kiechl et al. 2002). In another study TLR4 genotype was not associated with progression in established atherosclerosis, but
Asp299Gly-carriers treated with pravastatin demonstrated a lower risk of cardiovascular events than non-carriers (Boekholdt et al. 2003).

1.7 FATTY ACIDS

Fats in the Irish diet constitute 36.9% of total energy intake (North/South Ireland Food Consumption Survey, 1997 to 1999). Intake of triacylglycerol (TAG) in the Western diet may amount to 100g or more per day, digested and absorbed with more than 95% efficiency (Mu & Hoy, 2004). The TAG molecule consists of a glycerol backbone to which three fatty acids are acetylated, at positions sn1, sn2 and sn3. Fatty acids are hydrocarbon chains with a carboxyl group at one end and a methyl group at the other. They are of 2 to 24 carbon atoms in length with saturated or up to 6 unsaturated bonds. Generally, the fatty acids in the sn1 and sn3 position are different.

1.7.1 Classification and structure of fatty acids

Fatty acids may be classified according to the length of their hydrocarbon chain, the stereo-orientation of their bonds and the number of double-bonds they possess.

Short-chain fatty acids (SCFA) have less than 8 carbon atoms and are generally water soluble (Arab, 2003). Colonic bacteria generate SCFA such as acetate (2 carbon chain), propionate (3 carbon chain) and butyrate (4 carbon chain) from dietary carbohydrate, which pass by diffusion into enterocytes and may be used as an energy source. Medium-chain (MC-) fatty acids have 8 to 15 carbons (e.g. caprylic and capric acids with 8 and 10 carbon chains, respectively) and are intermediates in the biosynthesis of long-chain (LC-) fatty acids, the principle fatty acids of human metabolism and those addressed in this thesis.

Double-bonds are either cis or trans in stereo-orientation, representing a minor classification for fatty acids. The orientation of the double bond influences structure, melting point, membrane incorporation and the receptor-ligation potential of fatty acids. Cis bonds involve the adjacent positioning of hydrogens either side of the bond. Trans
bonds involve the opposite positioning of hydrogens either side of the bond (Figure 1.10).

![Figure 1.10 Stereo-orientation of cis and trans double bonds within hydrocarbon chain](image)

Figure 1.10 Stereo-orientation of cis and trans double bonds within hydrocarbon chain

*Trans* fatty acids are produced naturally by rumination and bacterial processing but are present in food largely by the industrial hydrogenation of polyunsaturated fatty acids (PUFA). *Cis* (c) and *trans* (t) double bonds are characteristic of the conjugated isomers of linoleic acid c9, t11-CLA and t10, c12-CLA.

The main classification of fatty acids is by the number of double-bonds they possess. SFA acids have no double bonds, e.g. palmitic acid (C16:0) and stearic acid (C18:0). Monounsaturated fatty acids (MUFA) contain one double bond, e.g. oleic acid (C18:1) has a double bond located at carbon 9 and, thus, is further classified as an n-9 fatty acid. PUFA contain more than one double bond. They are in liquid (oil) form at room temperature. Examples include linoleic acid (C18:2 n-6), eicosapentanoic acid (EPA; C20:5 n-3) and docosahexanoic acid (DHA; C22:6 n-3). The structures of these fatty acids and the CLA isomers, which feature in this thesis, are presented in Figure 1.11 overleaf.
Figure 1.11 Fatty acids used in the experimental chapters of this thesis
1.7.2 Dietary and biosynthetic sources of principal fatty acids

The dietary sources of principal fatty acids are presented in the Table 1.6.

Table 1.6 Principal dietary fatty acids and their sources. Adapted from Calder, 1998

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lauric acid (12:0)</td>
<td>coconut oil</td>
</tr>
<tr>
<td>Myristic acid (14:0)</td>
<td>Milk</td>
</tr>
<tr>
<td>Palmitic acid (16:0)</td>
<td>milk, eggs, animal fats, meat, cocoa butter, palm &amp; fish oils</td>
</tr>
<tr>
<td>Stearic acid (18:0)</td>
<td>milk, eggs, animal fats, meat &amp; cocoa butter</td>
</tr>
<tr>
<td>Oleic acid (18:1n-9)</td>
<td>olive oil, milk, eggs, animal fats, meat &amp; cocoa butter</td>
</tr>
<tr>
<td>Linoleic acid (18:2n-6)</td>
<td>some milk, eggs, animal fats, meat, green leaves, maize, sunflower, safflower &amp; soybean oils</td>
</tr>
<tr>
<td>α-Linoleic acid (18:3n-3)</td>
<td>green leaves, rapeseed, soyabean &amp; linseed oils</td>
</tr>
<tr>
<td>γ-Linolenic acid (18:3n-6)</td>
<td>borage &amp; evening primrose oils</td>
</tr>
<tr>
<td>Eicosapentaenoic acid (20:5n-3)</td>
<td>fish oils</td>
</tr>
<tr>
<td>Docosahexaenoic acid (22:6n-3)</td>
<td>fish oils</td>
</tr>
</tbody>
</table>

There are two main classes of PUFA, n-3 and n-6, with α-linolenic and linoleic acids as the respective family precursors. Unlike plants, mammals do not possess Δ12 and Δ15 desaturase enzymes that convert oleic acid to linoleic acid, and linolenic acid to α-linolenic, respectively. In 1929, Burr and Burr described a deficiency of these fatty acids from dietary fat exclusion; thus, α-linolenic and linoleic are termed essential fatty acids. Linoleic acid is converted to arachidonic acid, from which eicosanoids are principally derived in animal tissue. α-linolenic is converted to the LC-PUFA EPA and DHA using the same enzymes as n-6 fatty acid metabolic pathway (Figure 1.12, page 64). The n-3 and n-6 pathways are, in theory, competitive (Yaqoob, 2003). However, the efficiency of the n-3 pathway is questionable and little α-linolenic is supposed to proceed along the entire pathway to DHA (Burdge et al. 2003; Yaqoob 2003). In addition, linoleic acid is more prevalent in the Western diet than α-linolenic (although both are consumed in excess to requirements) and the n-6 pathway is thus quantitatively more important (Calder, 1998). The minimum human requirements for linoleic and α-linolenic acids are estimated to be 1 and 0.2% of total energy, respectively. On average,
in the UK diet, linoleic and α-linolenic acids contribute 5 and 0.6% of total energy, respectively (Calder, 2004). The relative inefficiency of the n-3 pathway in mammals and the shunting of enzymes towards the predominant n-6 pathway indicate that dietary sources of the LC-PUFA EPA and DHA are more biologically relevant than endogenous sources. EPA and DHA can be sourced from marine plants, especially the unicellular algae in phytoplankton, via elongation and desaturation of α-linolenic acid. The formation of these n-3 LC-PUFA by marine algae and their transfer through the food chain to fish account for the n-3 LC-PUFA in oily fish and fish iver oils (Calder, 1997; Calder 1998).
Figure 1.12 The n-3 and n-6 PUFA biosynthetic pathway. EPA, DHA and arachidonic acid may be biosynthesised from the essential α-linolenic and linoleic acids. Adapted from Calder 1998 & Yaqoob 2003
1.7.3 Fat digestion, fatty acid absorption and lipoprotein metabolism

Digestion of dietary lipids begins in humans in the stomach, although in rodents lingual lipases secreted from Ebner’s salivary glands initiates digestion of fat. Gastric lipases partially hydrolyse 10 to 30% of dietary TAG to diacylglycerol (DAG) and free fatty acids (FFA), with a higher affinity for MC-TAG than LC-TAG (Mu & Hoy, 2004). Lipids enter the small intestine as fine droplets, less than 0.5μm in diameter. Emulsifying bile acids, released from the gall bladder or directly from the liver in hormonal response to cholecystokinin (CCK), contain phospholipids such as phosphatidylcholine (which are hydrolysied by pancreatic phospholipase A2) and cholesterol esters (which are hydrolysed by pancreatic acinar cholesterol esterases) (Tso & Liu, 2004). Pancreatic lipases, activated by colipase, calcium ions and bile salts, hydrolyse TAG to sn2-monoacylglycerol (MAG) and FFA. A rearrangement of sn2-MAG to sn1- or sn3-MAG results in complete degradation to glycerol and FFA. FFA diffuse along a concentration gradient from intestinal micelles into enterocytes, but have elsewhere been reported to be taken up by intestinal enterocytes by carrier-mediated processes. A number of proteins associated with the apical brush border membrane have been demonstrated to bind lipids, including GP330 (megalin), CD36, caveolin and fatty acid transporter proteins such as FATP4 (Stahl et al. 1999; Tso & Liu, 2004). Lipids are resynthesised and accumulate within the smooth endoplasmic reticulum. In the fed state, TAGs are assembled into chylomicron (CM) lipoprotein structures for aqueous transport. Miller & Small (1983) found the surface of CM to contain 2-4% TAG, 5-8% cholesterol and 1% cholesteryl ester (CE). Fat-soluble vitamins and carotenoids reside in the core. TAGs in the core of CM are reported to reflect the fatty acid composition of dietary lipids. In the fasted state, TAGs are assembled into VLDL lipoprotein structures that are more dense than CM, with greater protein and lesser lipid content (Mu & Hoy, 2004). The apolipoprotein content of enterocyte-derived lipoproteins is low and features apoB48, apoB100, apoA-I, apoA-II, apoA-IV, apoC and apoE. It is generally held that CM contains apoB48 whereas VLDL contains apoB100. CM are exocytosed from the enterocyte into lymphatic system appendices of the intestinal microvilli. At the thoracic duct, CM enter the subclavian vein and undergo intravascular conversion to CM remnants. At least 39% of protein is transferred to HDL, with a selective loss of apoA, leaving apoB, apoE and apoC. CM TAGs are hydrolysed by extra-hepatic lipoprotein lipase (LPL) in the vicinity of capillary endothelium, particularly within adipose and muscle tissue generating non-esterified fatty acids (NEFA). The relative content of CE
in the CM remnant increases. The CM remnant is assimilated by the liver by recognition of apoE. CM remnant lipids and de novo lipids synthesised from non-lipid precursors are packaged by the liver into VLDL and HDL and enter the circulation. LDL particles are principally derived from the intravascular catabolism of VLDL (Esteve et al. 2005). LDL is the major cholesterol transporting lipoprotein in the plasma and is responsible for the regulated delivery of sterol to cells for growth and hormone or bile acid production (Mu & Hoy, 2004). LDL interacts with LDL receptors, via apoB100 recognition, and is internalised and degraded by lysosomal enzymes of the target cell. Uptake of LDL is also facilitated by non-receptor mediated pathways. HDL removes unesterified cholesterol from cells and other lipoproteins and returns it to the liver for excretion in bile. The LDL receptor recognises apoE expression on HDL, indicating that HDL may complete with LDL for ligation. The apoAI component of the HDL molecule stimulates the activity of lecithin:cholesterol acyltransferase (LCAT) which forms cholesterol esters from free cholesterol. Free cholesterol may readily exchange among plasma lipoproteins by CE transfer protein (CEPT) and is ultimately transported to the liver for hydrolysis and excreted in bile (Groff & Gropper, 2000).

1.7.4 Lipoproteins and atherosclerosis
Altered lipoprotein metabolism is observed in the acute phase response. Cytokine mediation redirects lipoproteins towards sites of injury, initially in a protective capacity. The increased delivery of lipoproteins to activated immune cells helps bind microbial products such as the lipid A moiety of LPS, binds viruses and urate crystals and blocks their cytotoxic effects, and induces lysis of parasites such as Trypanosoma brucei (Esteve et al. 2005). With chronic inflammation these functions may become exaggerated and pathogenic, ultimately compounding disease progression.

CM and VLDL are hydrolysed by LPL, yielding remnant particles smaller in size, relatively reduced in TAG and phospholipid and relatively enriched in free and esterified cholesterol. The TAG is removed to tissue or other lipoproteins. The remnants are generally removed by receptor-mediated endocytosis in the liver and other tissues, including arteries (Redgrave et al. 2001). The rapid clearance of remnant particles is a vital physiological function mediated by the presence of heparan sulfate proteoglycans, apoE, LPL, hepatic lipase, LDLR and related protein and potentially class B scavenger receptors (Martins & Redgrave, 2004).
Dysregulation of this system is pro-atherogenic. TAG-rich lipoproteins (TRL) have been associated with CVD (Phillips et al. 1993; Tornvall et al. 1993) and defective remnant clearance increases the risk of atherosclerosis (Bjorkegren et al. 1998; Redgrave et al. 2001). Small quantities of lipoproteins may penetrate the arterial endothelium by the unregulated and non-specific mechanism of transcytosis. This aberrant exposure of endothelium to lipoproteins is limited by rapid TRL clearance (Proctor & Mamo, 2003). Compromised endothelial integrity or compromised TRL clearance increases exposure and may allow arterial passage of CM and VLDL, potentiating the accumulation of lipids within an arterial lesion. During inflammation, increased lipolysis results in hepatic NEFA flux and promotes VLDL secretion. Pro-inflammatory cytokines correlate positively with plasma TAG concentrations in subjects with AIDS, SLE, hypertension, type 2 diabetes and those with increased prevalence of CVD, whereas IL-10 correlates negatively with TAG (Esteve et al. 2005). IL-1, IL-2, IL-6, IFNα, TNFα and LPS stimulate hepatic VLDL synthesis (Hardardottir et al. 1994; Nonogaki et al. 1995). IL-1, IL-6, IFNα, TNFα and LPS reduce LPL activity in vivo and in vitro. IL-1 and TNFα reduce hepatic and extra-hepatic apoE mRNA expression, decreasing this recognition molecule on VLDL. IL-1 and LPS decrease hepatic lipase, which facilitates the delipidation and clearance of CM and VLDL (Esteve et al. 2005). Pro-inflammatory cytokines thus reduce the clearance of TRL.

Elevated LDL concentration is considered one of the major risk factors for cardiovascular disease. LDL modified in the circulation, principally by oxidation, has been addressed earlier as an antigenic stimulus in the atherogenic process. In humans, plasma LDL concentrations decrease during sepsis. In hepatic cell lines, administration of IL-6 and TNFα decreased apoB secretion and IL-1 inhibited cholesterol synthesis. IL-1 and TNFα override the suppression of LDLR normally induced by a high intracellular concentration of cholesterol. Inflammation also induces expression of scavenger receptors and decreases ATP-binding cassette A1 (ABCA1)-mediated efflux of cholesterol. It is speculated that the decrease observed in plasma LDL during inflammation is due to a concurrent increase in cellular cholesterol accumulation. High TNFα levels and LPS administration are associated with smaller and more dense LDL which displays a higher capacity for oxidation, decreased affinity for LDLR, a higher ability for transcytosis and uptake by foam cells and, thus, increased atherogenicity (Esteve et al. 2005).
Plasma concentrations of HDL related inversely to the mean percentage increase in coronary artery stenosis in subjects with established coronary artery disease (Phillips et al. 1993). HDL is considered atheroprotective in a number of capacities. Principally, it promotes the efflux of cholesterol from cells (Stein & Stein, 1999; Barter et al. 2004). It acts as an antioxidant by its content of apoAI, apoAII and associated enzyme paraoxonase (Stein & Stein, 1999; Barter et al. 2004). HDL inhibits the expression of adhesion molecules in arterial cells (Barter et al. 2004). HDL binds lysolecithin, an inhibitor or vasorelaxation contained in oxidised LDL. However, in transgenic rabbits expressing human apoAI, vasorelaxation was impaired despite a 60% increase in HDL (Lebuffe et al. 1997). Inflammation impairs the atheroprotective capacity of HDL. HDL concentrations correlate inversely with IL-6 and sTNFR in healthy and atherosclerotic subjects, and positively with IL-10. LPS and pro-inflammatory cytokines antagonise the functions of HDL by reducing the expression of the ABCA1, PPAR and liver X receptor (LXR) α genes involved in absorption, transport and elimination of cholesterol, thereby increasing intracellular cholesterol accumulation. Stimulation of TLRs, detected in human atherosclerosis, further antagonises LXR and inhibits macrophage expression of ABCA1 and, thereby, cholesterol transport to HDL. IL-1, TNFα and LPS have induced down-regulation of apoAI, the main structural apolipoprotein of HDL necessary for reverse cholesterol transport, in animal models and cultured adipocytes. Inflammation reduces LCAT and CETP activity. In addition, the antioxidant capacity of HDL is lessened during inflammation by reduced paraoxonase levels (Esteve et al. 2005).

### 1.7.5 Non-esterified fatty acids

Circulating concentrations and composition of NEFA represent a discrete paradigm in lipid metabolism, inflammation and the development of atherosclerosis. NEFA display unique physiological and pathological properties and emerging concepts of their selective deposition in and mobilisation from adipose tissue indicates that they are more than incidental to TRL metabolism.

#### 1.7.5a Source of non-esterified fatty acids

During fasting or after prolonged exercise the primary origin of circulating NEFA is adipose tissue (Chung et al. 1998), where stored TAGs are mobilised and hydrolysed by hormone sensitive lipase (HSL). In the fed state, endothelial-bound LPL hydrolysis of
TRL is the primary source of NEFA (Chung et al. 1995 and 1998) and adipose release of NEFA is inhibited by the negative control of insulin on HSL (Roche et al. 1998). The profile of NEFA in post-prandial serum resembles the fatty acid composition of test meals (Yli-Jama et al. 2001; Fernandez-Real et al. 2003) but correlations between dietary fatty acid intake and serum NEFA are weaker than those between adipose tissue TAG content and serum NEFA (Yli-Jama et al. 2001), indicating that adipose tissue is a more likely direct source of serum NEFA than diet.

1.7.5b Selective deposition and mobilisation of NEFA

Although the fatty acid content of adipose TAGs largely reflects the dietary intake of fatty acid, selective deposition has been argued. Preferential incorporation into rat adipose of EPA, C18:4n-3, DHA and docosapentanoic acid (DPA), in this order, has been reported (Raclot et al. 1997). Connor et al. (1996) demonstrated that the differential deposition of different dietary fatty acids into adipose tissue is based on their structure rather than presence in the diet.

The release of NEFA from their principal source, adipose tissue, appears to be specific. It has been increasingly demonstrated that NEFA are selectively mobilised from adipose tissue not by their content but by their chain length and degree of unsaturation. Halliwell et al. (1996) suggested a selective mobilisation of fatty acids from adipose tissue based on differences between the composition of veno-arterial NEFA and human adipose tissue TAG. ACTH-induced lipolysis of weanling rabbits, dietarily replete with essential and non-essential fatty acids, showed a relationship between the structure of the fatty acids and their mobilisation rather than their content in adipose tissue. SFA were mobilised the least and PUFA, particularly arachidonic acid and EPA, were mobilised the most. MUFA mobilisation was intermediate. Surprisingly, DHA was among the least mobilised fatty acids. DHA is the most abundant fatty acid in CNS phospholipid membranes and is not generally metabolised for energy or prostaglandin purposes. Its slow turnover from adipose tissue may reflect sparing for more specific purposes (Connor et al. 1996). Following induced lipolysis in humans, serum NEFA were more enriched with some PUFA (C18:3n-3, arachidonic acid and EPA) than adipose tissue triglycerides, indicating their ready mobilisation from adipose tissue. Adipose tissue TAGs were about two-fold more enriched with very long chain SFA and MUFA than serum NEFA, indicating their limited release into the circulation (Raclot et al. 1997). PUFA have been shown to be relatively more abundant in serum
NEFA than in adipose tissue TAGs, whereas MUFA were relatively more abundant in adipose tissue TAGs than in serum NEFA, indicating selective and preferential mobilisation of PUFA at the expense of MUFA (Yli-Jama et al. 2001). The mobilisation of fatty acids from adipose tissue correlates positively with unsaturation and negatively with chain length of the fatty acid (Connor et al. 1996; Chung et al. 1998), with preferential release of non-esterified PUFA of a limited chain length. Hormone stimulated lipolysis takes place at the water-lipid interface. The selective mobilisation of NEFA may result from differential accessibility of fatty acids to HSL, with partitioning of fatty acids based on their physiochemical properties, at the lipid-water interface (Raclot et al. 1997). Selective mobilisation could also originate from differential binding of fatty acids to fatty acid binding proteins and albumin during transport and transfer (Raclot et al. 1997). It is postulated that more polar, shorter chain and UFAs are more accessible to hormone-sensitive lipase, for hydrolysis, and membrane fatty acid binding protein, for transport, and are thus selectively mobilised from adipose tissue TAGs (Connor et al. 1996).

1.7.5c Physiological functions of NEFA
NEFA have important physiological functions. Their oxidation provides vital energy for heart and skeletal muscle in fasting conditions (Yli-Jama et al. 2001; Yli-Jama et al. 2002b). They are non-oxidatively processed in lipid, membrane and eicosanoid biosynthesis (Chung et al. 1995 and 1998, Raclot et al. 1997). Selective mobilisation of fatty acids from adipose tissue can spare essential fatty acids for specific utilisation (Connor et al. 1996), such as the potential shunting of DHA to CNS. Serum NEFA are the primary substrate for hepatic VLDL synthesis (Roche et al. 1998). Physiologically, NEFA support 30 to 50% of basal insulin secretion and induce glucose-stimulated insulin secretion. NEFA also induce insulin resistance and are elevated in the obese. However, the insulin stimulatory effect of NEFA is compensatory and responsible for the fact that the majority of obese insulin resistant people do not develop Type 2 diabetes (Boden et al. 2003). CE, which prevent excess cholesterol accumulation and regulate membrane fluidity, are synthesised from fatty acyl Co A by its cholesterol acyltransferase (ACAT). NEFA are a substrate for fatty acyl Co A, and thus CE, and can regulate ACAT mRNA expression (Seo et al. 2001).

However, NEFA are associated with adverse health effects in the majority of published research. Their elevation has been associated with abdominal obesity, insulin
resistance (Pankow et al. 2004) and increased risk of CVD (Yli-Jama et al. 2002b; Tripathy, 2003). The pathological effects of elevated and plasma composition of NEFA are discussed in Chapter 4, particularly with respect to inflammatory components of atherosclerosis.

1.8 SUMMARY OF ELEMENTS INVOLVED IN ATHEROSCLEROSIS AND THERAPEUTICS

The development of the atherosclerotic plaque has been described in this introduction by gross lesional features and the principal cells involved. Atherosclerosis is not just a disease of lipid deposition. It has a very strong inflammatory component. Inflammatory proteins that mediate atherosclerosis, investigated in the experimental chapters of this thesis, were detailed along with the NF-κB signalling pathway involved in cytokine transcription. Lipoprotein and NEFA metabolism in atherosclerosis have also been described.

Lipid and inflammatory elements are well recognised in the aetiology of atherosclerosis, but are often addressed dichotomously. However, lipid and inflammatory mediators interact in a common and comprehensive model of disease initiation and progression. Indeed, a number of drugs thought to have discrete anti-inflammatory or lipid-lowering effects have recently been shown to possess both anti-inflammatory and lipid-lowering properties.

Statins inhibit HMG-CoA-reductase, the rate-limiting hepatic enzyme required for endogenous cholesterol biosynthesis, activate SREBP, upregulate LDLR and enhance clearance of LDL from plasma (Glass & Witztum, 2001). In addition, statins have been found to reduce circulating IL-6, CRP and TNF (Wang et al. 2003; Luo et al. 2004). They inhibited phytohaemagluttinin (PHA)-activated secretion of IFNγ and IL-2 from lymphocytes and slightly reduced plasma IL-2 concentrations independently of LDL metabolism (Okopien et al. 2004; Pillarisetti et al. 2004). Fluvastatin and Simvastatin reduced MMP-9 secretion by human and murine macrophages (Bellosta et al. 1998). Fibrates, such as Gemfibrozil, are prescribed as lipid-lowering agents. TNFα release from PBMC has been shown to decrease in a dose-dependent manner to Gemfibrizol treatment (Zhao et al. 2003). PPARα activator fibrates, such as ciprofibrate and fenofibrate, inhibited the release of PHA-activated lymphocyte IL-2 and IFNγ in a lipid-lowering dependent manner, although with less capacity than the statins (Okopien et al. 2004). Salicylates and aspirin, besides inhibiting COX1, have also been described
as inhibitors of the activation of the NF-κB as well as NF-κB-dependent gene expression (Iñiguez et al. 1999).

Fatty acids, particularly PUFA, have reported beneficial effects on lipid and inflammatory mediators of atherosclerosis. Two main classes of PUFA are investigated in this thesis, the conjugated isomers of linoleic acid and the n-3 LC-PUFA.

1.9 CONJUGATED LINOLEIC ACID
Conjugated linoleic acid (CLA) refers to the positional and stereo-isomers of linoleic acid (Sebedio et al. 1999; Belury et al. 2002). The biologically active forms, $c9, t11$-CLA and $t10, c12$-CLA isomers, are represented in Figure 1.11.

1.9.1 Dietary sources of CLA
CLA is naturally found in ruminant animal fat and dairy products with 85 to 90% prevalence as $c9, t11$-CLA, with lesser amounts of $c7, t9$-CLA, $c11, t13$-CLA, $c8, t10$-CLA and $t10, c12$-CLA isomers. Temperature, protein quality, starter culture and period of aging may affect CLA content in meat and dairy products. Feeding regime, animal breed and season affect the content of CLA in cow’s milk (Sebedio et al. 1999). Typical CLA content in total fat of meat, dairy foods and oils are presented in the Table 1.7.

<table>
<thead>
<tr>
<th>Food Source</th>
<th>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>
A synthetic mix of CLA may also be found in nutritional supplements and is composed primarily of c9, t11-CLA and t10, c12-CLA isomers (Belury et al. 2002).

1.9.2 Biosynthesis of CLA

CLA has been reported to originate from rumen fermentation during microbial biohydrogenation of linoleic and linolenic acid (Kepler et al. 1966). Recent evidence indicates that other fatty acids, including oleic acid, produce the intermediates of CLA biosynthesis. Microbes such as Butyrivibrio fibrisolvens are not the only synthesisers of CLA and tissue, such as the mammary gland and liver microsome, is involved in non-microbial CLA biosynthesis. As a consequence non-ruminant, as well as ruminant, animals may synthesise CLA.

During biohydrogenation, linoleic and linolenic acids are converted to t11-containing monoene and diene intermediates (Mosley et al. 2002). Trans-monoenoic acid, such as t11-C18:1 (trans vaccenic acid, alternatively known as t11-octadecenoic acid or TVA) intermediates have also been observed in the metabolism of oleic to stearic acid. A radioactive trans-monoenoic acid fraction was recovered when radiolabelled oleic acid was hydrogenated by ruminal microbes to stearic acid (Ward et al. 1964). A multitude of t11-C18:1 isomers were identified upon oleic acid biohydrogenation by ruminal microbes (Mosley et al. 2002) and a significant increase in t11-C18:1 was demonstrated in the milk fat of cows fed oleic acid (Seiner & Schultz, 1980). Single bacterial species cannot carry out all steps of biohyrdrogenation of fatty acids and ruminal microbes are grouped according to their specific substrate. Group A ruminal bacteria hydrogenate linoleic acid and α-linolenic acid to t11-C18:1. Group B ruminal bacteria hydrogneate oleic, t11-C18:1 and linoleic acid to stearic acid (Mosley et al. 2002). c9, t11-CLA formed in the rumen may be directly absorbed to contribute to milk fat (Peterson et al. 2002) or is further metabolised to t11-C18:1 acid by rumen microorganisms (Pariza et al. 2001).

The main product of rumen biohydrogenation of fatty acids appears to be t11-C18:1. This may be reduced in the rumen to stearic acid (Mosley et al. 2002). Absorbed t11-C18:1 is converted to c9, t11-CLA by Δ9 desaturase in mammary tissue (Loor & Herbein 1998; Calder, 2002; Mosley et al. 2002; Peterson et al. 2002) and this may represent the major biosynthesis of c9, t11-CLA. The biosynthetic pathways of CLA formation are presented in Figure 1.13.
Mammary biosynthesis of \( c_9, t_{11} \)-CLA reduces the likelihood that CLA is produced only in ruminant animals. CLA is produced in rats by the biohydrogenation of linoleic acid by intestinal flora or the \( \Delta 9 \) desaturation of \( t_{11} \)-C18:1 in liver microsomes. Serum CLA content was increased in humans fed a high \textit{trans} fatty acid diet, rich in \( t_{11} \)-C18:1. It has been hypothesised that CLA may be synthesised in humans through \( \Delta 9 \) desaturation of \( t_{11} \)-C18:1, as demonstrated in the rat (Salminen et al. 1998).

### 1.9.3 Industrial synthesis of CLA

CLA isomers are commonly created by the alkaline isomerisation of linoleic acid (Banni & Martin, 1998). Commerically, safflower or safflower oil is used as the starting material. Linoleic acid is progressively converted to CLA, without affecting the SFA or MUFA present in the substrate oil, by migration of double-bonds to form four main isomers \( t_8, c_{10} \)-CLA, \( t_{10}, c_9 \)-CLA, \( t_{11} \)-CLA, \( t_{10}, c_{12} \)-CLA and \( c_{11}, t_{13} \)-CLA (Gaullier et al. 2002). The percentage isomeric composition of industrially produced CLA supplements commonly used in human supplementation trials are presented in Table 1.8.
<table>
<thead>
<tr>
<th>Product</th>
<th>Manufacturer</th>
<th>$c9, t11$-CLA</th>
<th>$t10, c12$-CLA</th>
<th>$c, c$-CLA</th>
<th>$t, t$-CLA</th>
<th>$c11, t13$-CLA</th>
<th>$t8, c10$-CLA</th>
<th>Other CLA</th>
<th>Total CLA (form)</th>
<th>Other FA &amp; impurities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tonalin-FFA</td>
<td>Natural, Norway</td>
<td>38.73</td>
<td>41</td>
<td>2.42</td>
<td>1.58</td>
<td>0.24</td>
<td>0.2</td>
<td>1.58</td>
<td>84.37 (FFA)</td>
<td>15.63 (FFA)</td>
</tr>
<tr>
<td>Tonalin-TG</td>
<td>Natural, Norway</td>
<td>37.49</td>
<td>38.02</td>
<td>1.88</td>
<td>1.95</td>
<td>0.6</td>
<td>0.39</td>
<td>0.44</td>
<td>80.77 (TAG)</td>
<td>19.23 (TAG)</td>
</tr>
<tr>
<td>Tonalin</td>
<td>PharmaNutrients, IL, USA</td>
<td>11.44</td>
<td>14.69</td>
<td>6.74</td>
<td>6</td>
<td>15.34</td>
<td>10.79</td>
<td>-</td>
<td>65</td>
<td>35 (FFA)</td>
</tr>
<tr>
<td>Clarinol</td>
<td>Loders Croklaan, NL, EU</td>
<td>39.36</td>
<td>38.16</td>
<td>0.75</td>
<td>0.38</td>
<td>0.28</td>
<td>Unknown</td>
<td>80.48</td>
<td>80.48 (TAG)</td>
<td>19.52 (TAG)</td>
</tr>
<tr>
<td>Jahreis preparation</td>
<td>non-commercial</td>
<td>8.34</td>
<td>7.86</td>
<td>4.71</td>
<td>17.72</td>
<td>7.1</td>
<td>5.96</td>
<td>2.51</td>
<td>54.2 (TAG)</td>
<td>45.8 (TAG)</td>
</tr>
<tr>
<td>CLA capsules</td>
<td>Fitness Pharma, Norway</td>
<td>21.7 (with $t8, c10$)</td>
<td>19.1</td>
<td>4.2</td>
<td>3.1</td>
<td>8.5</td>
<td>21.7 (with $t10, c12$)</td>
<td>-</td>
<td>56.6 (FFA)</td>
<td>43.4 (FFA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.9.4 Therapeutic potential of CLA

The number of articles published on CLA has until recently been limited. Up to 1994, 5 to 10 papers appeared each year (Sebedio et al. 1999). Interest in CLA has grown from reports of beneficial and therapeutic effects in a number of pathological states. The number of papers published on CLA has increased each year since the mid 1990’s, with 155 related publications listed on Pubmed in 2004. The anti-inflammatory effects of CLA are discussed in Chapter 3.

1.9.4a CLA and lipid mediators of atherosclerosis

Animal feeding studies initially indicated an anti-atherogenic effect of CLA feeding. TAG, total and LDL cholesterol concentrations were markedly reduced in rabbits fed an atherogenic diet supplemented with CLA compared to controls (Lee et al. 1994). Hamsters fed a CLA-enriched atherogenic diet had significantly reduced TAG, total and non-HDL cholesterol concentrations and histological atherosclerosis compared to linoleic acid fed controls (Nicolosi et al. 1997). CLA-feeding induced regression of established atherosclerosis in New Zealand white rabbits (Kritchevsky et al. 2000). A CLA-enriched atherogenic diet retarded development and induced regression of aortic lesions in apoE -/- mice with established atherosclerosis (Toomey et al. 2003). Dietary CLA decreased TAG concentration, increased HDL cholesterol and increased aortic fatty streak formation in C57BL/6 mice (Munday et al. 1999). Increased formation of fatty streaks, which are defined as clinically inert, occurred independently of serum lipoprotein profile, TAG or body weight. Furthermore, this effect occurred in mice receiving a lower dose of CLA (2.5g/kg/day) and lesion formation was ameliorated in mice receiving 5g/kg CLA per day. Smedman & Vessby (2001) and Benito et al. (2001) showed that CLA supplementation had no effect on plasma lipid concentration in human subjects. Supplementation with a 50:50 isomeric blend of c9, t11-CLA and t10, c12-CLA, but not an 80:20 blend, significantly reduced plasma TAG in healthy volunteers (Noone et al. 2002). The t10, c12-isomer appears more effective at reducing TRIG and apoB secretion from HepG2 cells (Yotsumoto et al. 1999; Lin et al. 2001). The 80:20 c9, t11-CLA and t10, c12-CLA supplement significantly lowered VLDL-cholesterol concentrations (Noone et al. 2002).
1.9.4h CLA and cancer

A mix of CLA geometric isomers from fried ground beef topically applied to the dorsal area of mouse skin inhibited 7,12-dimethyl-benz(a)anthracene (DMBA)-induced tumour incidence by approximately 20% and tumour yield by approximately 50%, apparently through inhibition of cytochrome p450 activity (Ha et al. 1987). DMBA-induced mammary tumours were inhibited in rodents by feeding CLA at 0.5 to 1.5% of body weight, with a decrease of lipid peroxidation in the mammary gland (Ip et al. 1991). Elsewhere, no association between CLA levels in breast adipose tissue or prognonsis factors of metastatic occurrence was found in humans (Chajes et al. 2003). Physiological concentrations of CLA are cytostatic and cytotoxic to human malignant melanoma, colorectal and breast cancer cells in vitro (Shultz et al. 1992). CLA preparations inhibited colorectal cancer cell proliferation, with a 50:50 blend of c9, t11-CLA and t10, c12-CLA more potent than a preparation predominantly of c9, t11-CLA. In addition, CLA preparations were not effective in inhibiting prostate cancer cell proliferation (Palombo et al. 2002).

1.9.5 Potential modes of CLA action

The mechanisms through which CLA exerts therapeutic potential in atherosclerosis and cancer remain obscure. CLA has demonstrated anti- and pro-oxidant effects, anti- and pro-thrombotic effects and anti- and pro-inflammatory effects. These discrepancies appear to be due, in some circumstances, to the specific effects of the bioactive CLA isomers, the doses at which they are used and the species in which they are tested.

As a PUFA, CLA may be readily oxidised and act, in theory, as an antioxidant. Ip et al. (1991) demonstrated a decrease of lipid peroxidation in the mammary gland with tumour inhibition. However, CLA did not exhibit anti-oxidant potential in model membranes or in breast cancer cells (Van den Berg, 1995; Cunningham, 1997). Supplementation of healthy subjects with CLA significantly increased urinary 8-iso-PGF(2α) and 15-keto-dihydro-PGF(2α), biomarkers of non-enzymatic and enzymatic lipid peroxidation, respectively (Basu et al. 2000). In a gas chromatographic model of antioxidant activity t10, c12-CLA was more effective an anti-oxidant than c9, t11-CLA. c9, t11-CLA acted as an anti-oxidant at concentrations below 20μM; however at 200μM it acted as a strong pro-oxidant (Leung & Liu, 2000).

It is proposed that CLA modulates arachidonic acid metabolism, competing for COX and LOX, alters eicosanoid synthesis and, thereby, affects tumourigenesis
CLA has been shown to reduce renal production of PGE$_2$ without reduced availability of arachidonic acid (Ogborn et al. 2003). CLA displaced arachidonic acid and suppressed linoleic acid metabolites in mammary tissue in a rodent feeding study (Banni et al. 1999). CLA inhibited arachidonic acid and collagen-induced platelet aggregation (Truitt et al. 1999). Formation of 6-ketoPGF(1α), the inactive metabolite of the vasodilator and inhibitor of platelet function PGI$_2$, was increased by pre-incubation of IL-1β-exposed HUVECs with c9, t11-CLA and t10, c12-CLA (Torres-Duarte et al. 2003). However, in the same study, formation of 6-ketoPGF(1α) was inhibited by pre-incubation of thrombin-exposed HUVECs with the CLA isomers. This indicates both anti- and pro-thrombotic potential of CLA. Treatment of HSVECs with a 50:50 isomeric blend of c9, t11-CLA and t10, c12-CLA inhibited the production of all 2-series eicosanoids derived from arachidonic acid (PGI2, TXB2, PGF2α, PGE2 and PGD2). However, when HSVECs were stimulated by calcium ionophore A23187 treatment with c9, t11-CLA followed the general trend of inhibition but the t10, c12-CLA isomer induced significant stimulation of the 2-series eicosanoids (Urquhart et al. 2002), indicating the different biological activity of the two isomers.

CLA isomers are ligands for peroxisome-proliferator activated receptors (PPARs) α and γ (Moya-Camarena et al. 1999; Belury, 2002; Yu et al. 2002). CLA activated PPARγ, decreased IFNγ-induced COX-2, iNOS and TNFα mRNA expression and reduced PGE$_2$, NO, TNFα, IL-1β and IL-6 secretion. Transfecting dominant negative PPARγ into the RAW cells ablated the ability of various CLA isomers to regulate the iNOS reporter construct (Yu et al. 2002). CLA has also been shown to inhibit NF-κB signal transduction (Nugent, 2003) and NF-κB target genes such as p53 and Bcl-XS (Pahl, 1999; Majumder et al. 2002).

1.10 n-3 LC-PUFA EICOSAPENTANOIC ACID AND DOCOSAHEXANOIC ACID

The main dietary source of n-3 LC-PUFA is oil from “fatty” fish such as mackerel, herring and salmon. The potential for their endogenous biosynthesis in mammals from α-linolenic acid has been discussed.
1.10.1 Therapeutic effects of n-3 LC-PUFA

The n-3 LC-PUFA have displayed therapeutic benefit in conditions such as inflammatory bowel disease, asthma, cystic fibrosis and rheumatoid arthritis (Ruxton et al. 2004). n-3 LC-PUFA enable fluidity in neuronal membranes and deficiency has been associated with impaired brain development (Reisbick et al. 1997; Yehuda et al. 1999). A relationship is hypothesised to exist between poor n-3 LC-PUFA status and depression, attention-deficit hyperactivity disorder (ADHD), dyslexia and autism (Freeman, 2000; Mischoulon & Fava, 2000; Richardson & Ross, 2000). Therapeutic effects of n-3 LC-PUFA have been investigated and indicated in CVD and atherosclerosis.

1.10.1a n-3 LC-PUFA and mediators of atherosclerosis

Epidemiological studies indicate a reduced prevalence of cardiovascular-related mortality among populations traditionally consuming a diet rich in oily fish, such as the Inuit Eskimos of Greenland, Canada and Alaska (Calder, 2004). Traditionally, fish is an important component of the Japanese diet and cardiovascular-related mortality is low in the Japanese population. However, with Westernisation of dietary habits, population average serum total cholesterol has increased, and in young Japanese adults is similar to that of age-matched adults in the US (Okuda et al. 2005). Feeding and supplementation studies have indicated that consumption of fish oils reduces the risk of cardiovascular-related mortality in Western populations. In view of this, in 2004 the UK Food Standards Agency recommended a weekly intake of 1 to 4 portions of oily fish for the general population, providing 0.45 to 0.9g/day n-3 LC-PUFA. This is a significant increase from the 1995 recommendation of 0.2g/day n-3 LC-PUFA from the UK Committee of Medical Aspects of Food Policy (COMA) (Ruxton et al. 2004). The AHA recommends 1g/day of EPA and DHA for patients with existing CVD (Lee & Wander, 2005).

Of 25 human feeding and supplementation studies reviewed by Calder (2004), 23 showed an inverse association between n-3 LC-PUFA and CVD, and two showed no effect. In healthy subjects increased consumption of EPA and DHA is associated with falls in serum TAG and VLDL, and at high doses both plasma cholesterol and apoB concentrations are decreased (Das, 2000). Moderate supplementation with fish oil decreases serum TAG concentrations with some increase in HDL in various dyslipidemias (Prichard et al. 1995). However, evidence indicating limited or negative
effects of n-3 LC-PUFA on lipid and lipoprotein mediators pertaining to atherosclerosis persists. LDL concentrations showed a tendency to increase in diabetic patients when supplemented with fish oil (Haines et al. 1986; Schectman et al. 1989). In hypertriglyceridemic patients long-term supplementation with n-3 LC-PUFA decreased plasma TAG and VLDL concentrations but increased LDL (Putadechakum et al. 2005). Supplementation with EPA & DHA had no effect on total cholesterol, HDL, LDL, VLDL and TAG in spinal cord injury patients (Javierre et al. 2005).

Reported effects of n-3 LC-PUFA on clotting and coagulation factors have been inconsistent. Hostmark et al. (1988) and Radack et al. (1989) reported a fall in plasma fibrinogen while Brown et al. (1991) found no significant change. Finnegan et al. (2003) conclude that n-3 LC-PUFA generally have been shown not to affect coagulation and fibrinolysis variables (Sanders et al. 1981; Brox & Nordoy, 1983; Eritsland, 1995), although changes consistent with Factor VII activation have been reported (Hornstra, 1982).

n-3 LC-PUFA have a small, but significant hypotensive effect in both normotensive and hypertensive individuals. They also cause endothelial relaxation and promote arterial compliance, which might relate to altered NO production (Calder, 2004).

EPA and DHA are readily oxidised due to their extensive unsaturation, and this anti-oxidant effect has been speculated to induce susceptibility of LDL to oxidation. EPA, but not DHA, was demonstrated to promote LDL oxidation (Mesa et al. 2004). Firbank et al. (2002) found that neither EPA nor DHA were independent determinants of LDL oxidisability, although total n-3 PUFA increased susceptibility of LDL to oxidation. Elsewhere, DHA was shown to have a relative protective effect against the oxidation of LDL (de Ruiz et al. 2002). Interestingly, oxidised, but not native, EPA has been shown to inhibit cytokine-induced leukocyte adhesion on, leukocyte transmigration across, and expression of MCP-1 from glomerular endothelial cells. In addition, oxidised, but not native, EPA inhibited cytokine-induced NF-κB in glomerular endothelial and mesangial cells (Chaudhary et al. 2004). Elsewhere, oxidised LDL rich in EPA and DHA induces less apoptosis in human pro-monocytes relative to oxidised LDL enriched with linoleic acid (Wu et al. 2002). Oxidised LDL and 4-hydroxyhexenal from post-menopausal women supplemented with fish oil reduced apoptosis in in vitro pro-monocytes (Lee & Wander, 2005).
Discrepancies lie in reported effects of EPA and DHA on atherogenic lipid and lipoprotein mediators, clotting and coagulation mediators and on their oxidative/anti-oxidative properties. It seems likely that the n-3 LC-PUFA have other effects that contribute to their cardioprotective potential. The anti-inflammatory effects of EPA and DHA are discussed in Chapter 5.

1.10.2 Potential modes of n-3 LC-PUFA action

The principal mechanism by which EPA and DHA exert their therapeutic effects is reported to involve displacement of arachidonic acid as an eicosanoid precursor, yielding a less inflammatory, aggregatory and thrombotic selection of prostaglandins. This mechanism is discussed in Chapter 5. Additionally, the effects of EPA and DHA on the NF-κB signalling pathway are discussed in Chapter 5.

The effects of EPA and DHA may relate to their unique structure. Although they are LC-PUFA, it has been established using ²H-NMR and molecular dynamics simulations that, in membranes, DHA is relatively shorter or more compact than more saturated chains (8.2 Å relative to 14.2 Å of oleic at 41°C). DHA in membranes is conformationally characterised by pronounced twists of the chain, a “back-bent” shape in which the methyl groups add bulk to the interior region, and separation between the ends of the molecule are diminished. The structure of n-3 LC-PUFA lends fluidity to membranes and may influence permeability properties of the bilayer. Valentine & Valentine (2004) describe membrane fluidity as a unifying concept between bacteria, plants and animals. EPA and DHA may block or disrupt formation of islands of gel-phase lipids in the membrane, analogous to anti-freeze. Bacteria can continue to grow and replicate, albeit slowly, at temperatures where 10 to 15% of their total lipid remains fluid. Growth ceases when about 90% of the membrane is in the gel phase. n-3 LC-PUFA facilitate survival and growth of bacteria at low temperatures. In chloroplasts, high fluidity is believed to play a role in long-distance electron transport. It is hypothesised that DHA phospholipids in mitochondrial membranes, even in endothermic animals, work by a similar mechanism as described for bacteria above. The purpose, in this case, might be to maximise or sustain energy production (Valentine & Valentine, 2004).
1.11 RESEARCH OBJECTIVES

The objective of this thesis was to investigate the effects of PUFA on mediators of inflammation associated with atherosclerosis. Within this were the following aims:

a. To represent the major immune cells of the atherosclerotic process, activated T lymphocytes, monocytes and macrophages

b. To investigate the effects of PUFA on secretory phenotype of these cells; the Th1/Th2 pattern of cytokine secretion by T lymphocytes, pro-inflammatory cytokines secreted by monocytes, pro-inflammatory cytokines and matrix metalloproteinase secreted by macrophages

c. To investigate the effects of PUFA on the transcription of pro-inflammatory cytokines in these immune cells

d. To investigate the effects of PUFA on the NF-κB signalling pathway in activated macrophages

Studies specifically aimed:

e. To investigate the effects of CLA supplementation on cytokine secretion by ex vivo Con A stimulated PBMC

f. To investigate the effects of CLA supplementation on systemic mediators of inflammation, lipid metabolism and fasting glucose

g. To investigate the isomer-specific effects of CLA on pro-inflammatory cytokine production in activated monocytes in vitro

h. To investigate the effects of altering the SFA, MUFA and n-3 PUFA composition of a blend of fatty acids representative of serum NEFA on pro-inflammatory mediator production in activated macrophages in vitro

i. To investigate the effects of the n-3 LC-PUFA EPA and DHA on pro-inflammatory mediator production in activated macrophages in vitro and to elucidate the effects of these fatty acids on NF-κB cytoplasmic liberation, nuclear translocation and DNA transactivational binding
2.1 COLLECTION OF BLOOD SAMPLES
Eight weeks of phlebotomy training was completed in St. James' Hospital, Dublin. Blood samples were drawn in a clinical setting with the volunteer in an upright seated position using the Vacutainer blood collection system (Becton Dickinson, Oxford, UK). A tourniquet was applied about 10cm above the site of venipuncture. The veins of the antecubital fossa found at the inner elbow are preferentially used for venipuncture. The sterile needle, in line with the vein, with bevel facing upwards was introduced at a 15 to 30° angle and blood was drawn by vacuum into the blood collection system. An order of draw was maintained to minimise contamination between samples when multiple tubes with different additives were used in sampling. The order was 1) serum tube, 2) citrate-, 3) lithium heparin- and 4) EDTA- coated tubes. Serum tubes contain no preservative or anticoagulant and blood clots within these tubes, separating serum. EDTA tubes contain potassium salts of ethylendiaminetetraacetic acid which chelate calcium salts and prevents coagulation of blood. When centrifuged, plasma separates from the rest of the blood. Sodium citrate, also a calcium-chelating anticoagulant, is used as a preservative of whole blood samples for coagulation studies. Lithium heparin inactivates thrombin and thromboplastin, causes least interference in clinical chemistry tests than other anticoagulants and is osmotically sensitive to red blood cells. Circulating cytokines were measured in serum, acute phase reactants in citrated plasma, peripheral blood mononuclear cells were isolated from heparinised blood, and lipids and glucose were analysed in EDTA-derived plasma.

2.2 ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS
Density gradient solutions exploit physical laws of density and osmolality in separating cells from a heterogenous suspension, such as mononuclear cells from whole blood. When centrifuged with a solution of density 1.077g/ml blood cells separated into two major fractions. Monocytes and lymphocytes are isolated at the top of the separation fluid whereas erythrocytes and granulocytes are sedimented to the bottom. In addition, cell volume (and hence density) is a function of the osmolality of the medium. Membrane potassium channels were recently demonstrated to be activated in monocytes from citrated blood, reducing their volume, increasing their density and sedimentation rate, when centrifuged with NycoPrep™ (Nycomed Pharma, Oslo, Norway) yielding a relatively pure suspension of lymphocytes (Boyum et al. 2002). Mixed mononuclear cell suspensions isolated by centrifugation with density gradient solutions such as
Lymphoprep™ (AXIS-SHIELD, Oslo, Norway) typically comprise of 70 to 85% lymphocytes and 15 to 30% monocytes (Boyum et al. 2002).

PBMC were isolated from freshly collected heparinised blood. Whole blood diluted 1:1 with 25mM Hepes-buffered Hanks Balanced Salt Solution (HBSS; Gibco, Grand Island, New York) was carefully layered, 4:1, onto Lymphoprep density gradient solution. Lymphoprep contains sodium diatrizoate (9.1% w/v) and polysaccharide (5.7% w/v), has a density of 1.077g/ml and an osmolality of 280mOsm. The layered blood and Lymphoprep was centrifuged at 1200rpm for 30 minutes and erythrocytes were sedimented (contamination of mononuclear cell isolates with erythrocytes is estimated at 1 to 5%) The ‘buffy coat’ interphase containing the PBMC was collected using a sterile pasteur pipette, diluted approx. 1:5 in Hepes-buffered HBSS and centrifuged for a further 10 minutes at 1200rpm. The supernatant was decanted, the cells gently washed in 10ml fresh Hepes-buffered HBSS and centrifuged again at 1200rpm for 5 minutes. Cells were re-suspended in Royal Park Memorial Institute (RPMI; Gibco, Grand Island, New York) medium supplemented with 2mmol L-glutamine/L, 100mg streptomycin/ml and 100μg penicillin/ml (Sigma, Saint Louis, MI, USA). After enumeration (Section 2.4), cells were seeded at the appropriate concentration in RPMI medium supplemented with antibiotics and glutamine as above and 2.5% v/v autologous serum from the blood donor.

2.3 THP-1 CELL LINE

The THP-1 cell line was derived from a 1 year-old suffering from acute monocytic leukaemia. It resembles the human monocyte with respect to morphology, secretory products, expression of oncogenes, membrane antigens and genes involved in lipid metabolism (Auwerx, 1991). It retains its monocytic phenotype for 14 months (Tsuchiya et al. 1980). The cell line was purchased from the European Collection of Animal Cell Cultures (ECACC No. 88081201, Salisbury, UK).

2.3.1 Establishing culture

Cryopreserved THP-1 monocytes were rapidly thawed in the hand and resuspended 1:10 in fresh pre-warmed RPMI media supplemented with 2mmolL-glutamine/L, 100mg streptomycin/ml and 100μg penicillin/ml, as above, and 20% heat-inactivated (56°C for 30 minutes) foetal calf serum (FCS; Gibco, Grand Island, New York). Cells were incubated at 37°C, 5% CO₂ and 95% air in a humidified incubator. Culture stability
and growth were assessed by light microscopy and good proliferation was generally observed after 5 to 7 days.

2.3.2 Maintaining culture

The THP-1 cell line was routinely maintained in RPMI media supplemented with 2mmol L-glutamine/L, 100mg streptomycin/ml and 100μg penicillin/ml, as above, and 10% heat-inactivated FCS. Cell proliferation and health of culture was assessed every 3 to 4 days by light microscopy. Culture was passaged 1:4 with fresh media and incubated at 37°C, 5% CO₂ and 95% air in a humidified incubator. Cells were not used after the 25th passage.

2.3.3 Cryopreserving culture

THP-1 suspensions of relatively low passage were routinely frozen and stored. Dense culture (3 to 4 days after passaging) was centrifuged at 1000rpm for 10 minutes, enumerated (Section 2.4) and resuspended to 4 x 10⁶ cells/ml in RPMI media supplemented with 2mmol L-glutamine/L, 100mg streptomycin/ml and 100μg penicillin/ml. A cryopreservation solution of 45% heat-inactivated FCS, 5% filter-sterilised dimethyl sulfoxide (DMSO; Sigma, Saint Louis, MI, USA) and 50% cell suspension was prepared and immediately aliquoted at 1ml/sterile cryotube (Nunc, Roskilde, Denmark). Cyrotubes were placed immediately on ice to chill, frozen at -20°C overnight and subsequently transferred to -80°C for long-term storage.

2.4 CELL ENUMERATION AND VIABILITY

THP-1 suspensions were centrifuged at 1000rpm for 10 minutes. Media was decanted and cells suspended in a fixed volume of media. Cell suspension (10μl) was diluted 1:10 with ethidium bromide/acridine orange (EB/AO; 90μl) and 10μl of this pipetted under a cover slip on a haemocytometer. Cells were counted under fluorescent light. Live viable cells fluoresced green whereas nonviable cells stained orange.

2.5 MYCOPLASMA TESTING

Mycoplasma are wall-less bacteria of an unusually small genome size and limited biosynthetic capabilities. They are common commensal parasites in humans (Rawadi et al. 1998) but are involved in the pathogenesis of a wide range of autoimmune and immune-related disorders. Mycoplasma act as mitogens to activate lymphocytes,
inducing T and B cell proliferation, formation of cytotoxic T lymphocytes and subsequent secretion of immunoglobulins and cytokines (Ruuth & Praz, 1989). Mycoplasma fermentans and derived materials activate peripheral blood monocytes and macrophages to secrete high levels of IL-1, IL-6, TNFα and prostaglandins (Muhlradt & Schade, 1991; Gallily et al. 1992). Synthetic analogues of mycoplasmal lipopeptides activated NFkB in a murine macrophage cell line (Sacht et al. 1998). Rawadi et al. (1998) also reported the heightened production of cytokine activation of the MAPK ERK1/2, JNK and p38 by mycoplasma in cultured murine macrophages. Mycoplasma are a common contaminant of cell lines in continuous culture. They may be unnoticed for months, inducing a reduction in the rate of cell proliferation and altered gene expression, such as a heightened pro-inflammatory cytokine production.

The MycoAlert™ Mycoplasma Detection Kit (Cambrex, Rockland, ME, USA) was routinely used to test for the presence of mycoplasma in cell culture. This assay exploits the activity of specific mycoplasma enzymes involved in catalysing a conversion of ADP to ATP. The level of ATP, created by mycoplasma lysing in a sample, is measured linearly by luminescence. A ratio of post-lysis to pre-lysis readings indicates mycoplasma absence or presence.

\[
\text{ATP} + \text{Luciferin} + O_2 \xrightarrow{\text{Luciferase} + \text{Mg}^{++}} \text{Oxyluciferin} + \text{AMP} + \text{PP}_i + \text{CO}_2 + \text{LIGHT}
\]

Figure 2.1 The chemiluminescent reaction of the MycoAlert™ Mycoplasma Detection Kit

Cells were passaged at least twice in antibiotic-free media prior to mycoplasma testing. The MycoAlert Mycoplasma Detection kit was stored at 4°C and the assay conducted at room temperature, optimal for luciferase activity, according to manufacturer’s instructions. Lyophilised pellets of MycoAlert reagent and MycoAlert substrate were reconstituted in assay buffer and equilibrated at room temperature for 15 minutes. 2mls of cell suspension were centrifuged in sterile 10ml tubes at 1500rpm for 5 minutes. 100μl of clear supernatant were transferred per well of a 96-well white-walled luminometer-compatible microplate. 100μl MycoAlert reagent was added per well and incubated at room temperature for 5 minutes. A 1 second integrated reading was performed on the wells by the luminometer (SPECTRAFluor Plus and XFLUOR Version 3.21, Reading UK). This was designated “Reading A”. 100μl MycoAlert
substrate solution was added per well and incubated at room temperature for 10 minutes. Another reading was performed by the luminometer and this was designated “Reading B”. The ratio of Reading B/Reading A was calculated, indicating ATP presence in media before and after a mycoplasma-specific lysis. A negative reading is a ratio <1, ratios from 1 to 1.3 are inconclusive and higher readings indicate infection. Sample output from a mycoplasma test performed in duplicate on positive controls, negative controls and THP-1 monocytes is presented in Table 2.1.

Table 2.1 Mycoplasma testing of THP-1 monocyte cell line in duplicate with positive and negative controls. Testing of cell lines was conducted every 3 months

<table>
<thead>
<tr>
<th>Test</th>
<th>Reading A</th>
<th>Reading B</th>
<th>Ratio B/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>55</td>
<td>713</td>
<td>12.96</td>
</tr>
<tr>
<td>Positive control</td>
<td>62</td>
<td>407</td>
<td>7.83</td>
</tr>
<tr>
<td>Negative control</td>
<td>71</td>
<td>44</td>
<td>0.62</td>
</tr>
<tr>
<td>Negative control</td>
<td>81</td>
<td>46</td>
<td>0.57</td>
</tr>
<tr>
<td>THP-1</td>
<td>40</td>
<td>30</td>
<td>0.75</td>
</tr>
<tr>
<td>THP-1</td>
<td>44</td>
<td>46</td>
<td>1.05</td>
</tr>
</tbody>
</table>

2.6 MITOGEN PREPARATION

2.6.1 Concanavalin A

The plant lectin Con A stimulates human T lymphocyte proliferation and synthesis of cellular products. It was purchased as 5mg of lyophilised powder (Sigma, Saint Louis, MI, USA). Stock solutions of 10mg/ml were aseptically prepared by dissolution in 500μl RPMI. 50μl aliquots were stored at -20°C. Final concentrations of 10μg/ml in PBMC culture were obtained by adding 1μl stock per ml of media.

2.6.2 Lipopolysaccharide

LPS, bacterial endotoxin, is a major constituent of the cell wall of most gram-negative bacteria, including E.coli. It is highly immunogenic and stimulates macrophage proliferation. It was purchased as a 1mg lyophilised powder (Sigma, Saint Louis, MI, USA). Stock solutions of 1mg/ml were aseptically prepared by dissolution in 1ml RPMI. 50μl aliquots were stored at -20°C. Final concentrations of 0.1 to 1μg/ml in THP-1 monocyte-derived macrophage culture were obtained by adding 0.1 to 1μl stock per ml of media.
2.6.3 Phorbol 12-myristate 13-acetate

PMA inhibits proliferation of the THP-1 monocytic cell line and induces differentiation to functional macrophages (Traore et al. 2005). Differentiated macrophages adhere to the plastic surfaces of culture plates. PMA was purchased as a 1mg lyophilised powder (Sigma, Saint Louis, MI, USA). Stock solutions of 1mg/ml were aseptically prepared by dissolution in 1ml RPMI. 50μl aliquots were stored at -20°C. Final concentrations of 0.1μg/ml in THP-1 monocyte culture were obtained by adding 0.1μl stock per ml of media.

2.7 PREPARATION OF FATTY ACIDS

DMSO has a highly polar domain and two apolar groups, making it soluble in both aqueous and organic media. DMSO scavenges reactive oxygen species and displays a number of anti-inflammatory effects including inhibition of IL-8, inhibition of platelet aggregation, stimulation of PGE₂, inhibition of NFkB activation and potentiation of TNFα-induced cytotoxicity (Santos et al. 2003). DMSO (filter sterilised; Sigma, Saint Louis, MI, USA) was used in preference to albumin as the vehicle for fatty acids in the in vitro experiments of this thesis. Fatty acid stocks of 100mmol/L were prepared (Table 2.2) and 50μl aliquots stored at -20°C prior to use. Fresh fatty acids were prepared every 6 months. The concentration of DMSO in cultured cells was always ≤0.1%. The CLA isomers were supplied by Cayman Chemicals (Ann Arbor, MI, US). All other fatty acids were obtained from Sigma (Saint Louis, MI, USA).

Table 2.2 Preparation of fatty acids used in this thesis.
Fatty acids were dissolved in filter-sterilised DMSO to 100mmol/L

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Mw</th>
<th>100mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic acid</td>
<td>256.4</td>
<td>25.64mg/ml</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>284.5</td>
<td>28.45mg/ml</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>282.4</td>
<td>282.4mg/ml</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>280.4</td>
<td>28.04mg/ml</td>
</tr>
<tr>
<td>Conjugated linoleic acid</td>
<td>280.5</td>
<td>28.05mg/ml</td>
</tr>
<tr>
<td>Eicosapentanoic acid</td>
<td>302.5</td>
<td>30.25mg/ml</td>
</tr>
<tr>
<td>Docosahexanoic acid</td>
<td>328.5</td>
<td>32.85mg/ml</td>
</tr>
</tbody>
</table>
2.8 IN VITRO EXPERIMENTAL CONDITIONS

PBMC were isolated as previously described and cultured at $1 \times 10^6$ cells/ml with or without Con A at $10\mu$g/ml for 4 and 24 hours depending on the assay of interest (Chapter 3A).

THP-1 monocytes were cultured at $1 \times 10^6$ cells/ml and pre-treated with fatty acids for 48 hours. Cells were reEnumerated because of ongoing proliferation and plated at $1 \times 10^6$ cells/ml. Monocytes were activated with $1\mu$g/ml LPS for 6 and 24 hours depending on the assay of interest (Chapter 3B).

THP-1 monocytes were differentiated to macrophages by the addition of PMA at $0.1\mu$g/ml of culture media for 72 hours at 5% CO$_2$, 95% humidity and 37$^0$C. They were washed twice with pre-warmed HBSS and incubated in serum-free RPMI media supplemented with 2mmol L-glutamine/L, 100mg streptomycin/ml and 100$\mu$g penicillin/ml, and 1% Nutridoma (Roche Molecular Biochemicals, IN, USA) for 24 hours. Nutridoma is a biochemically defined serum-free media supplement composed of albumin, insulin, transferrin and other defined organic and inorganic compounds. Macrophages were treated with fatty acids for 48 hours and then activated with 0.1 to $1\mu$g/ml LPS for $\frac{1}{2}$, 1, 2, 5, 6 and 24 hours depending on the assay of interest (Chapters 4 and 5).

2.9 MTS CELL VIABILITY ASSAY

Cell viability was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). This assay contains [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, Owen’s reagent, the MTS tetrazolium compound exclusively licensed to Promega Corporation. Metabolically active cells convert the yellow MTS salt to its water-soluble dark blue formazan by reductive cleavage of the tetrazolium ring. Tetrazolium compounds such as MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], used in some cell viability kits, yield insoluble formazan and requires the additional chemical and mechanical dissolution of crystals with isopropanol. The quantity of formazan produced from MTS or MTT, measured by absorbance of 490nm, is directly proportional to the number of living cells in culture. The reduction of formazan by metabolically active cells is not completely understood. It is catalysed principally by mitochondrial succinate dehydrogenase, but also by non-mitochondrial dehydrogenases and flavin oxidases (Bruggisser et al. 2002). The antioxidant potential
of screening and test substances should be taken into account when employing the reduction of MTS or MTT as a measure of cell viability. The advantages of the assay are that it is non-radioactive, fast and economical.

THP-1 derived macrophages were cultured in 96-well tissue culture plates (Nunc, Roskilde, Denmark) at 1 x 10^6 cells/ml with 100μl per well. Cells were treated with test concentrations of fatty acids and DMSO or were untreated. Phenol red-free media (Gibco, Grand Island, New York) may be used to eliminate contribution from media to absorbance values. However it was not deemed necessary to use as similar results were obtained with phenol red-free and regular media when test optical densities were normalised to those of untreated cells.

The MTS stock reagent was stored at −20°C and working solutions at 4°C, protected from light. 20μl of the MTS reagent was pipetted into each well and the plate incubated at 37°C in a 5% CO₂, 95% air atmosphere for 4 hours. The wells were measured spectrophotometrically at 490nm after 4 hours. Optical densities from DMSO and fatty acid treated wells were expressed as a percentage of those of untreated cells. The formazan production of untreated cells is presumed to represent 100% viability.

2.10 PLASMA FATTY ACID ANALYSIS

2.10.1 Lipid extraction

Total lipids were isolated from plasma using a modified protocol of Folch et al. (1957). Blood collected in citrated tubes was centrifuged at 200g for 20 minutes yielding platelet-rich-plasma (PRP) which was removed from the blood collection tube to a 15ml conical tube. PRP was centrifuged at 670g for 15 minutes to pellet platelets and yield platelet-poor-plasma (PPP). 400μl PPP was mixed with 1ml TRIS:EDTA. This was transferred to a 16 x 100mm Boroscillate tube. 2.5mls methanol:chloroform (1:2) with 0.01% w/v butylated hydroxy toluene (BHT) was added and vortexed for 1 minute to form a milky solution. This was centrifuged at 2500rpm for 10 minutes yielding a triphasic solution with an upper aqueous phase, a web-like interphase and a lower organic infranatant.

The organic infranatant was removed by glass Pasteur pipette, without disturbing the interphase, and placed in a smaller 12 x 75mm Boroscillate tube. 2mls chloroform with 0.01% BHT were added to the remaining aqueous phase/interphase solution in the 16 x 100mm boroscillate tube, vortexed for 1 minute, centrifuged at 2500rpm for 5 minutes and infranant removed to the 12 x 75mm boroscillate tube. The
pooled organic infranatants were dried overnight in a vortex evaporator evaporator (AGB Scientific Ltd., Dublin, Ireland). The dried lipid samples were flushed with nitrogen (N₂), sealed to prevent lipid oxidation and stored at -20° C.

2.10.2 Transesterification of fatty acids
Methyl esters of the extracted lipids 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 N₂ and placed on a heating block at 60° C for 15 minutes. Boron Trifluoride Methanol (14% BF₃, Sigma, Saint Louis, MI, USA; 750μl) was then added, samples vortexed for 30 seconds and incubated at 60° C for 15 minutes. Lipids were extracted three times using 0.5 ml hexane, samples dried in a vortex evaporator and stored under N₂ at -20°C until analysis.

2.10.3 Standard for gas chromatography
A fatty acid methyl ester (FAME) standard was prepared containing 19 fatty acid esterified derivatives (Table 2.3).

Palmitic acid was present in two products added to the mix; its concentration was twice that of the other FAME and it produced a markedly greater, easily identifiable peak on the chromatogram. Unesterified c9, t11-CLA and t10, c12-CLA isomers (Cayman Chemicals, Ann Arbor, MI, USA) were dried overnight in a vortex evaporator and transesterified with NaOH in dry methanol and BF₃ as outlined earlier for plasma lipids. A chromatogram was prepared for each isomer separately to evaluate concentration and retention time. The 19-FAME was spiked with each methyl-esterified CLA isomer to produce a 21-FAME standard.

2.10.4 Gas chromatography and integration of chromatogram
The fatty acid methyl ester composition of plasma lipids were analysed for incorporation of CLA isomers (Chapter 3A) using a Shimadzu GC-14A gas liquid chromatograph (Mason Technologies, Dublin) fitted with a Shimadzu C-16A integrator. A CP Sil 88 fused Silica Column (50m x 0.22m file thickness; Chrompack Ltd, Middleburg, NL) was installed. Nitrogen was used as the carrier gas. Oven temperature conditions for each run were an initial column temperature of 180° C, increasing to 195° C at a rate of 5° C per minute. Column temperature was held at 195° C for 40 minutes, subsequently increased to 220° C at a rate of 2° C per minute and held at 220° C for 20
minutes. Before and after use of the GC, and between standards and samples, hexane was run through the column. 21-FAME standards were run between every 6 to 10 samples. Fatty acids were identified in sample runs by comparison of retention times to those of standards. Fatty acid compositions of samples were calculated as a percentage of the total fatty acids.

Table 2.3 Fatty acids of the 19-FAME standards used for identification of sample peaks in gas chromatography

<table>
<thead>
<tr>
<th>Product (supplier)</th>
<th>FAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLC-30 (Supelco, Bellefonte, PA, USA)</td>
<td>C8:0 Caprylic acid</td>
</tr>
<tr>
<td></td>
<td>C10:0 Capric acid</td>
</tr>
<tr>
<td></td>
<td>C12:0 Lauric acid</td>
</tr>
<tr>
<td></td>
<td>C14:0 Myristic acid</td>
</tr>
<tr>
<td></td>
<td>C16:0 Palmitic acid</td>
</tr>
<tr>
<td></td>
<td>C16:0 Palmitic acid</td>
</tr>
<tr>
<td></td>
<td>C18:0 Stearic acid</td>
</tr>
<tr>
<td>GLC-10 (Supelco)</td>
<td>C18:1 Oleic acid</td>
</tr>
<tr>
<td></td>
<td>C18:2 Linoleic acid</td>
</tr>
<tr>
<td></td>
<td>C18:3 Linolenic acid</td>
</tr>
<tr>
<td>189-13 (Supelco)</td>
<td>C20:1 Eicosanoic acid</td>
</tr>
<tr>
<td></td>
<td>C20:2 Eicosadienic acid</td>
</tr>
<tr>
<td></td>
<td>C20:3:6 Eicosatrienoic acid</td>
</tr>
<tr>
<td></td>
<td>C20:4:6 Eicosatetraenoic acid</td>
</tr>
<tr>
<td></td>
<td>C20:5:3 Eicosapentanoic acid</td>
</tr>
<tr>
<td>P-9667 (Sigma)</td>
<td>C16:1 Palmitoleic acid</td>
</tr>
<tr>
<td>L-6503 (Sigma)</td>
<td>C18:4:3 ω-linoleic acid</td>
</tr>
<tr>
<td>E-3511 (Sigma)</td>
<td>C20:3:3 Eicosatrienoic acid</td>
</tr>
<tr>
<td>D-3534 (Sigma)</td>
<td>C22:4:6 Docosatetraenoic acid</td>
</tr>
<tr>
<td>D-2659 (Sigma)</td>
<td>C22:6:3 Docosahexanoic acid</td>
</tr>
</tbody>
</table>
2.11 PLASMA LIPID AND GLUCOSE ANALYSIS

Colorimetric analysis of plasma total cholesterol, LDL, HDL, TAG and glucose was performed with the ILAB 600 Clinical Chemistry Analyser (Instrumentation Laboratory, Warrington, UK). All reagents used in these assays, including calibrators and control sera, were supplied by Instrumentation Laboratory, Warrington, UK.

2.11.1 Total Cholesterol Concentration

The concentration of cholesterol in plasma and in the lipoprotein fractions was determined by endpoint biochromatic analysis using a colorimetric assay. The assay is based on the principle that cholesterol ester is converted to free cholesterol and fatty acids by the action of cholesterol esterase. The free cholesterol is then oxidised by the action of cholesterol oxidase, forming cholest-4-en-3-one and H$_2$O$_2$. H$_2$O$_2$ in combination with 4-aminoantipyrine and phenol, in the presence of peroxidase, yields the chromagen quinoneimine and water. Quinoneimine production is proportional to the cholesterol concentration in the sample, and is quantified spectrophotometrically at 510nm, with a blanking wavelength of 700nm.

2.11.2 LDL cholesterol

LDL was isolated using an LDL Precipitation Reagent (Randox, Antrim, Ireland). The reagent contains heparin, which causes LDL to be precipitated at its isoelectric point (pH 5.04). LDL precipitating reagent was added to fresh serum (1ml/100µl serum) vortexed, and incubated at room temperature for 10 minutes. Samples were then centrifuged for 15 minutes at 2500g. After centrifugation, HDL and VLDL remain in the supernatant while LDL forms a pellet at the bottom of the tube. The supernatant was removed and stored at -20°C until cholesterol analysis was preformed (Section 2.11.1). The concentration of plasma LDL-cholesterol was calculated as the difference between the total cholesterol concentration of the plasma sample and the cholesterol concentration of the supernatant.

2.11.3 HDL cholesterol

Plasma HDL was isolated using the Quantolip HDL Cholesterol Precipitation Reagent A (Immuno Ag, Vienna, Austria). Reagent A precipitates the VLDL, CM and LDL fractions, leaving HDL in the supernatant. The precipitation reagent was added to fresh
plasma samples in a ratio of 2:1. The mixture was vortexed, incubated at room temperature for 10 minutes and centrifuged for 15 minutes at 2500g. The supernatants was removed and frozen at -20°C for subsequent analysis (Section 2.11.1).

2.11.4 TAG
The concentration of TAG in plasma was measured by enzymatic, endpoint analysis using a colorimetric assay. In this assay TAG is hydrolysed by lipoprotein lipase forming glycerol and fatty acids. In the presence of ATP, glycerol is converted to glycerol-3-phosphate and ADP by glycerol kinase. Glycerol-3-phosphate is then oxidised by glycerophosphate oxidase to form dihydroxyacetone phosphate and H$_2$O$_2$. H$_2$O$_2$ reacts with 4-aminoantipyrine and phenol in the presence of peroxidase, yielding quinoneimine and water. Quinoneimine production is proportional to the concentration of TAG in the sample, and is quantified spectrophotometrically at 510nm, with a blanking wavelength of 700nm.

2.11.5 Glucose
Plasma glucose was determined by endpoint, bichromatic analysis with coupled hexokinase methodology using a colorimetric assay. In the presence of ATP, glucose is converted to glucose-6-phosphate by the action of hexokinase. Glucose-6-phosphate dehydrogenase converts glucose-6-phosphate and NAD$^+$ to 6-phosphogluconate and NADH. Although hexokinase catalyses the phosphorylation of hexoses other than glucose, the glucose-6-phosphate dehydrogenase is specific for glucose-6-phosphate, and therefore glucose. The increased absorbance due to the conversion of NAD$^+$ to NADH is directly proportional to the glucose present in the sample. Absorbance is measured at a primary wavelength of 340nm and a blanking wavelength of 375nm.

2.12 FIBRINOGEN CLOTTING ASSAY
When thrombin is added to plasma, fibrinogen is converted enzymatically to fibrin. Fibrin undergoes polymerisation to form a fibrin network. Factor XIII, activated by an added excess of thrombin, catalyses the formation of stabilising cross-links to produce a visible clot. The elapsed time from addition of thrombin to the formation of a clot is inversely proportional to fibrinogen concentration.

Fibrinogen concentration was determined by the method of Clauss (1957) using an automated clotting assay (Fibriquik, Organon Teknika Corporation, North Carolina,
USA) using a Coag-A-Mate MTX (Organon Teknika Corporation, North Carolina, USA). Verify Reference Plasma (Bio Mérieux, North Carolina, USA) was used to calibrate the assay prior to sample analysis. A normal and abnormal sera (Universal Coagulation Reference Plasma and Abnormal Coagulation Reference Plasma, respectively, Pacific Haemostasis, Fischer Diagnostics, Middleton, VA, USA) were used as coagulation references to test assay performance. Measurements were taken in duplicate and readings averaged.

2.13 INFLAMMATORY MEDIATOR SECRETION BY ELISA
Enzyme-linked Immunosorbent Assays (ELISA) combine the specificity of antibodies with the sensitivity of simple enzyme assays to detect and colorimetrically quantitate the presence of antigen. Sandwich ELISA involves the layering of antigen between two antibodies, each of which has the capacity to bind to the antigen. The primary (capture) antibody specific to the antigen is coated to a solid phase such as the walls of a microtiter plate. Antigen becomes immobilised by the capture antibody. The secondary (detection) antibody is conjugated with an enzyme such as horseradish peroxidase (HRP) that acts upon a substrate, such as the chromagen tetramethylbenzidine (TMB), to produce blue colour in direct proportion to antigen presence. An acidic solution, such as H₂SO₄, stops the oxidation of TMB and changes the reaction colour to yellow. The main steps in the ELISA procedure are presented in Figure 2.2.

Figure 2.2 Principle of sandwich ELISA
A number of commercially available ELISA kits were purchased for the capture of antigen in samples where high-sensitivity detection was required. These kits include 96-well microtiter plates pre-coated with primary antibody, standards, conjugated secondary antibody, HRP, TMB, stop solution, sample and standard diluents and wash buffer. In this thesis, high and ultra sensitivity ELISA kits were used for the detection of IL-6 in serum (Biosource Europe, Nivelles, Belgium), CRP in plasma (BioCheck Inc., Burlingame CA, USA) and MMP-9 in macrophages supernatant (RnD systems, Oxon, UK).

Commercially available kits for the measure of all other antigens measured in PBMC, monocyte or macrophage supernatants (IL-1β, IL-2, IL-6, IL-10 and TNFα) were of routine sensitivity. All were purchased from RnD Systems (Oxon, UK) and the protocol was identical for all assays. Kits contain antibodies, standards and HRP and are stored at 4°C. Capture antibody, detection antibody and standards supplied as lyophilised powders were reconstituted, aliquoted into stock solutions and stored at -20°C until use. Capture antibody was diluted to a working concentration, as per manufacturer’s instruction, with phosphate buffered saline (PBS; pH 7.2 to 7.4) and coated onto high affinity microtiter plates (Nunc, Roskilde, Denmark). The plates were sealed and left overnight at room temperature. Wells were then washed by submerging the plate in 0.05% Tween 20® (Sigma, Saint Louis, MI, USA) in PBS, gently agitating and flicking out the wash buffer. This was performed three times. Plates were then tapped dry against tissue. Wells were blocked with 1% bovine serum albumin (BSA), 5% sucrose in PBS with 0.05% NaN₃ for 1 hour. Standards were reconstituted to working concentrations by serial 1:2 dilutions in reagent diluent composed of 1% BSA in PBS. Samples were diluted to optimised concentrations (detailed later) in reagent diluent. After blocking, wells were washed as before and loaded with standards and samples and blank wells of reagent diluent only. Plates were sealed and incubated at room temperature for 2 hours. Alternatively plates may be incubated overnight at 4°C. Three washes were performed and detection antibody plated for 2 hours. After washing the Streptavidin-HRP enzyme was added to each well for 20 minutes in the dark to conjugated to secondary antibody. Plates were washed again. Substrate solution tetramethyl benzidine (TMB; Sigma, Saint Louis, MI, USA) was added to each well and its oxidation occurred over 20 minutes incubation in the dark. 2N H₂SO₄ stopped the reaction and changed the colour in the wells from blue to yellow. Absorbance in the
wells was read in the microplate reader (SPECTRAFluor Plus and XFLUOR Version 3.21, TECAN, Reading, UK) at 450nm with a wavelength correction of 570nm. A standard curve was constructed for each assay by plotting mean absorbance values (optical density, OD) from the standard solutions versus known cytokine concentrations. Sample concentrations were calculated through the equation of the standard curve.

Prior to ELISA of complete sample sets, for each cytokine wells were prepared optimising the dilution of samples to fit within the sensitivity of the kits. Typical cytokine concentrations published from similar cell models (cell type, seeding density and stimulus) were reviewed. Supernatants from a number of resting and activated cell samples were diluted according to expected cytokine yields and the detection capabilities of the kit. These preparatory ELISA established optimal dilution factors. In the main assay, however, samples with a concentration greater than the standard curve were diluted and reassayed to obtain a precise concentration.

2.14 GELATIN ZYMOGRAPHY

Zymography was used in the present study to investigate the gelatinolytic activity of MMPs. MMPs are secreted as inactive pro-forms, with the Zn$^{2+}$ of the catalytic domain bound to a Cys$^{73}$ residue of the pro-peptide domain, yielding a latent zymogram 10kDA larger than the active enzyme. Disruption of the Cys$^{73}$ – Zn$^{2+}$ bond by hydrolysis, referred to as the “cysteine switch” (Snoek-van Beurden & Von den Hoff, 2005), activates the catalytic Zn$^{2+}$ to form an intermediate active enzyme. Additional cleavage of the catalytic domain from the pro-peptide domain, by autolysis or other proteases, releases the truncated, reduced molecular weight and fully activated enzyme (Figure 2.4).
MMP pro-forms are separated by electrophoresis through a polyacrylamide gel in which sodium dodecyl sulphate (SDS) is copolymerised with gelatin, the enzyme substrate. SDS denatures and inactivates the MMP. Upon removal of SDS by Triton X 100, the enzymes partially renature and digest their substrate. Coomassie Blue staining and subsequent destaining of the gel allow clear bands of proteolysis to be visualised against a blue background of undegraded substrate. Computer-supported imaging and densitometry allows for semi-quantification of enzyme activity. Absolute quantification remains difficult as limited numbers of wells do not allow a full standard curve and samples to be run on the same gel (Leber & Balkwill, 1997). *In vitro* MMP activation can occur by chemical and physical agents, including a low pH and heat treatment. Although gelatin is not the preferred substrate of MMP-1, MMP-8 and MMP-13 their gelatinolytic activity has been demonstrated and may contribute to protein digestion in gelatin zymography. Samples should not be boiled as this denatures MMP nor should B-mercaptoethanol (BME) be added to sample buffer as it destroys the disulphide bonds of the MMP. Zymography has a number of advantages over techniques such as ELISA in the investigation of the gelatinases, though the techniques should be considered...
complementary rather than alternative. Zymography is relatively inexpensive to perform and several proteases can be separated and quantified on a single gel, such as MMP-2 and MMP-9 gelatinases. Both the pro-form and active enzyme may be detected on the gel after electrophoresis. SDS causes dissociation of the MMP from the tissue inhibitors of matrix metalloproteinases (TIMP), inducing MMP disinhibition and allowing pure activity to be investigated.

2.14.1 Preparation of gels
Gelatin 5mg/ml, Tris HCl 1M pH 6.8 and Tris HCl 1.5M pH 8.8 stocks were stored at 4°C and brought to room temperature before use. SDS 10% solution (Sigma, Saint Louis, MI, USA) was stored at room temperature. Acrylamide/bis-Acrylamide 30% (Sigma, Saint Louis, MI, USA) was stored at 4°C. TEMED (Sigma, Saint Louis, MI, USA) was stored at room temperature and opened in a fume hood. Ammonium persulphate (APS; Sigma, Saint Louis, MI, USA) 100mg/ml was prepared fresh with distilled H$_2$O prior to use.

Gel-rig glass plates were inspected, cleaned with ethanol and set in their casing using spring clamps. An 8% acrylamide separating gel (Table 2.4) was poured between the glass plates, up to approximately 1cm below the top of the plates and overlaid with butanol to smooth the surface and eliminate air bubble. When the gel was set the butanol was removed and a stacking gel (Table 2.4) poured on top. Combs were placed between the glass plates, around which the stacking gel set.

Table 2.4 Composition of separating and stacking gels

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock</th>
<th>Separating gel (mls/gel)</th>
<th>Stacking gel (mls/gel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled H$_2$O</td>
<td>-</td>
<td>1.3</td>
<td>1.35</td>
</tr>
<tr>
<td>Gelatin</td>
<td>5mg/ml</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Tris Ph 8.8</td>
<td>1.5M</td>
<td>1.25</td>
<td>-</td>
</tr>
<tr>
<td>Tris pH 6.7</td>
<td>1.0M</td>
<td>-</td>
<td>0.25</td>
</tr>
<tr>
<td>SDS</td>
<td>10%</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>30%</td>
<td>1.35</td>
<td>0.335</td>
</tr>
<tr>
<td>TEMED</td>
<td>-</td>
<td>0.003</td>
<td>0.002</td>
</tr>
<tr>
<td>APS</td>
<td>100mg/ml</td>
<td>0.05</td>
<td>0.02</td>
</tr>
</tbody>
</table>

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2.14.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Stock running buffer contained 3g trizma base, 14.4g glycine and 1g SDS (all from Sigma, Saint Louis, MI, USA) per litre, made up with distilled H₂O. Stock sample buffer (2X) contained 1.2g SDS, 4ml glycerol, 2ml 1M Tris-HCL (pH 6.8), 40mg bromophenol blue and 14ml H₂O. Triton X was diluted to a 1X solution with distilled H₂O. Stock incubation buffer contained 6.06g trizma base, 1.47g CaCl₂ and 2.92g NaCl per litre, pH adjusted to 7.6 with HCl, and made up with distilled H₂O. Coomassie Blue 0.25% w/v was made up in destain solution, mixed overnight and passed through filter paper in a Buchner flask aided by a mini-pump system. Destain solution comprised 10% glacial acetic acid, 30% methanol and 60% distilled H₂O.

When the stacking gel was set combs were removed and the gel rig filled with running buffer (approximately 300ml running buffer per rig). MMP-2 and MMP-9 molecular markers (Merck, Darmstadt, Germany) were loaded into the first well. 12.5μl of sample supernatant and 7.5μl sample buffer were vortexed in ependorff tubes and 15μl of each sample was loaded per well. Electrophoresis was performed at 150V for approximately 30 minutes until sample buffer was seen to run to the end of the glass plates.

2.14.3 Gelatinase degradation, staining and imaging

The gels were removed from the glass plates, marked for orientation by cutting a corner and floated in Triton X on an orbital shaker for 30 minutes. The gels were then incubated for 18 hours at 37°C in incubation buffer after which they were stained with Coomassie Blue for 30 to 60 minutes. The gels were placed in destaining solution for approximately 30 minutes and imaged under white light using GeneGenius and GeneSnap (Syngene, Cambridge UK). The image was then transferred to GeneTools Analysis Software (Syngene, Cambridge UK) for densitometric analysis.

2.15 REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

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. 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 (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 2.5 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 for all experiments.

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. Analysis is based on the parameter threshold cycle time (Ct). Ct is the fractional cycle number at which the fluorescence passes a fixed threshold 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-1β, IL-2, IL-6 and TNFα) and 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.)
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.

Figure 2.5 Representation of the structure-dependent polymerisation association 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)
2.15.1 RNA extraction

Total ribonucleic (RNA) was isolated from cultured cells using Trizol® Reagent (GIBCOBRL, Life Technologies™), a mono-phasic solution of phenol and guanidine isothiocyanate. It maintains the integrity of RNA while disrupting cells and dissolving cell components. PBMC and monocytes culture suspensions were removed to 30ml sterilin tubes and centrifuged at 3000rpm for 10 minutes. The culture wells were meanwhile rinsed with 1ml Trizol/well. The medium from the centrifuged culture was removed and pelleted cells were cells lysed by repetitive pipetting with the Trizol from their respective culture well. Samples were transferred to 1.5ml eppendorff tubes (Sarstedt, Nümbrecht, Germany) and incubated at room temperature for 15 minutes to allow complete dissociation of nucleoprotein complexes. The supernatant of cultured macrophages was removed and Trizol added directly to the adherent cells in the well. The well surface was scraped and washed using sterile plastic transfer pipettes. The Trizol homogenate was transferred to 1.5ml eppendorff tubes and incubated at room temperature for 15 minutes as above. 200µl of chloroform was added per initial 1ml Trizol, tubes shaken vigorously by hand for 15 seconds and incubated at room temperature for 2 to 3 minutes. Samples were then centrifuged at 12,000rpm for 15 minutes yielding a biphasic solution. The upper aqueous phase containing the RNA was transferred to a fresh tube and RNA was precipitated by adding 100% isopropanol (500µl per ml Trizol used initially). Samples were incubated at room temperature for 10 minutes and then centrifuged at 12,000rpm for 10 minutes. The supernatant was removed and the RNA containing pellet washed with 75% ethanol (1ml per ml Trizol used initially). 75% ethanol was prepared in 0.1% diethyl pyrocarbonate (DEPC) treated water, which is RNase free. Samples were vortexed, centrifuged at 7,500rpm for 5 minutes, supernatants removed and samples left to air-dry. Care was taken not to over-dry the RNA as this decreases the solubility of the RNA. RNA pellets were dissolved in 20µl DEPC-treated water and stored at -80°C until quantified.

2.15.2 Quantitation of RNA

The quantity and purity of the eluted RNA was determined spectrophotometrically (Eppendorff BioPhotometer, Hamburg, Germany) by measuring absorbance at 260nm and 280nm. Readings were performed using a 1:25 dilution in a cuvette. RNase free water was used as the diluent control. RNA purity can be assessed from the OD at
230nm, 260nm and 280nm. An OD$_{260/280}$ < 1.6 implies partially dissolved RNA; an OD$_{260/280}$ of 1.7 to 2.0 indicates good purity while an OD$_{260/280}$ of greater than 2 implies purity and no residual guanidinium contamination.

2.15.3 RNA agarose gel electrophoresis
To check the integrity of total RNA, 1 to 5µg RNA was separated electrophoretically through an agarose gel. The gel was prepared in a horizontal rig. 0.56g agarose powder was dissolved in 75ml DEPC-treated water in a microwave oven. 4µl ethidium bromide was added to the agarose solution and swirled to mix. The agarose solution was poured into the sealed rig in a fume hood, combs place and the gel allowed set for about 1 hour prior to electrophoresis. A buffer of tris-acetate-EDTA buffer (TAE; Promega Corporation, WI, USA) was run through the gel for about 10 minutes at constant voltage (70V) prior to loading with sample. Samples containing 1 to 5µg RNA were aliquoted from stocks and diluted up to a volume of 15µl with RNA loading dye (50% glycerol, 1mmol/L EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol). Samples were loaded into wells and electrophoresis performed at 70V using TAE running buffer as before. Electrophoresis was stopped after 30 to 40 minutes, when the bromophenol blue tracing dye had run three-quarters of the length of the gel. The RNA was visualised using the UV transilluminator of GeneGenius and the GeneSnap programme (Syngene, Cambridge, UK). Two clear bands representing 28S (upper band) and 18S (lower band) RNA indicate its integrity.

2.15.4 DNase treatment of RNA
2µg RNA was diluted to a volume of 13µl with DEPC-treated water in 500µl ependorff tubes (Sarstedt, Nümbrecht, Germany) on ice. An extra sample, to be later included as a negative control for reverse transcription, was prepared. 1µl 10X DNase reaction buffer and 1µl DNase I, Amp Grade, 1U/µl enzyme (Invitrogen, Carlsbad, CA, USA) were added to each tube and incubated at room temperature for 15 minutes. DNase I, Amp Grade, purified from bovine pancreas, digests single- and double- stranded DNA to oligodeoxy-ribonucleotides and eliminates and possible contamination of the RNA by genomic DNA. The reaction was inactivated by the addition of one-tenth the volume of the reaction mix (ie: 1.5µl) 25mmol/L EDTA (ph 8.0). The final volume in each tube was 16.5µl. Samples were heated at 65°C for 10 minutes.
2.15.5 Reverse transcription

2μg of DNase treated RNA was reverse transcribed (RT) using the enzyme Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Promega Corporation, WI, USA). This enzyme is purified from an E.coli strain expressing a recombinant clone (Roth et al. 1985). 1μl of random primers at 500μg/ml (Promega Corporation, WI, USA) were added to the 2μg of DNase treated RNA 16.5μl samples which were then heated to 70°C for 5 minutes to melt secondary structure within the template. After this samples were immediately placed on ice to prevent secondary structure reforming. To each sample 5μl M-MLVRT 5X reaction buffer and 1μl 10X dNTPs (containing 10mmol/L each dATP, dCTP, cGTP and dTTP) (Promega Corporation, WI, USA) were added. Tubes were centrifuged briefly to bring all reagents together. 1.5μl M-MLVRT enzyme was added on ice to each tube except the negative control (-RT) to which 1.5μl DEPC-treated water was added instead. The final volume in each tube was 25μl, giving a concentration of 80ng/μl. Samples were reverse transcribed, yielding cDNA, by incubating at 37°C for 60 minutes followed by 70°C for 10 minutes to inactive the enzyme. Heating of samples was performed with programmes of a PCR machine (TouchGene, Techne, Staffordshire, UK).

2.15.6 Standard and sample dilution

5μl from each of 5 samples above were pooled giving 25μl of an 80ng/μl cDNA solution (upper standard). The remaining 20μl (1600ng) of each of these samples was diluted to 160μl with DEPC-treated water to give a 10ng/μl sample. All other samples from the RT step were diluted to 200μl, giving a concentration of 10ng/μl. Serial 1:2 dilutions of the upper standard of 80ng/μl yielded lower standards of 40ng/μl, 20ng/μl, 10ng/μl and 5ng/μl.

2.15.7 Polymerase chain reaction

PCR was preformed for the cytokines IL-1β, IL-2, IL-6 and TNFα using the Assays-on-Demand™ gene expression products (Applied Biosystems, Warrington, 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. Each reaction had a volume of 25μl. For all primer/probe sets (IL-1β, IL-2, IL-6, TNFα and GAPDH) a separate mastermix was prepared consisting of:
- 12.5μl 2X Taqman® Universal PCR Master mix (Applied Biosystems, Warrington, 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.
- 1.25μl 20X Target Primers and probes (human IL-1β, IL-2, IL-6, TNFα and GAPDH Assays-on-Demand kits (Applied Biosystems, Warrington, UK).
- 9.25μl DEPC-treated water.

This Primer-probe-mastermix was mixed well prior to addition of 23μl per well to a MicroAmp® Optical 96-well reaction plate (Applied Biosystems, Warrington, UK). 2μl of DEPC-treated water (PCR negative control), -RT sample, standard or +RT-ed samples (equivalent to 20ng RNA), was added to the respective wells and the plate sealed with a MicroAmp® Optical caps (Applied Biosystems, Warrington, 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, UK). PCR reaction thermal cycle conditions involved an initial step of 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The 2 minute 50°C step was necessary for optimal AmpErase uracil-N-glycolyase enzyme activity, while the 10 minute 95°C step was required to activate AmpliTaq Gold DNA polymerase. Analysis was conducted using the ABI prism 7700 sequence detection system 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 was calculated for both target genes and GAPDH using these standard curves. Samples are expressed normalised to GAPDH and relative to a control experiment.
2.16 WESTERN IMMUNOBLOTTING
To harvest sufficient protein to perform SDS-PAGE and Western Immunoblotting THP-1 monocytes were plated at $4 \times 10^6$ cells/well of a 6-well plate (Nunc, Roskilde, Denmark). Monocytes were differentiated, treated with fatty acids and stimulated with LPS (Section 2.8).

2.16.1 Extraction of nuclear and cytoplasmic proteins
Nuclear and cytoplasmic proteins were extracted from macrophages using a method based on that of Osborn et al. (1989). Three stock buffers (A, C and D), of varying salt concentration, were prepared directly in advance of use. Sterile stock solutions of their components were maintained. Hepes, MgCl$_2$, KCl, PMSF, NaCl, EDTA and PMSF stocks are stored at 4°C. DTT is stored at -20°C. The composition of the buffers and the concentration of stock components are presented in Table 2.5. A fourth buffer, B, is composed of Buffer A with 0.1% NP-40 (IGEPAL; Sigma, Saint Louis, MI, USA) and made directly before use. All buffers and PBS were stored on ice during the extraction.

Media was discarded from adherent macrophages and cells were washed twice with PBS. 1ml Buffer A was added per well ($4 \times 10^6$ cells). Cells were scraped by sterile transfer pipette from the base and sides of the well and thus homogenised in Buffer A. The homogenate was removed to sterile 1.5ml ependorff tubes (Sarstedt, Nümbrecht, Germany) and centrifuged at 10,000rpm for 10 minutes at 4°C. The supernatant was discarded and pellets resuspended in 50μl Buffer B. Samples were mixed by pipetting, incubated on ice for 20 minutes and vortexed every 5 minutes of this incubation. Samples were then centrifuged at 10,000rpm for 10 minutes at 4°C. The supernatant, which contains only cytoplasmic proteins, was removed to labelled tubes and stored at -20°C until quantified. The pellet was resuspended in 30μl Buffer C, mixed by pipetting and incubated on ice for 15 minutes. Samples were then centrifuged at 10,000rpm for 10 minutes at 4°C. The supernatant, which contains nuclear proteins, was removed to labelled tubes. 75μl Buffer D was added to each nuclear sample, which were then stored at -80°C until quantified.
Table 2.5 Components of and their concentration within Buffers A, C and D for nuclear and cytoplasmic protein extractions.
The concentration of stock solutions is presented. The concentration of stock solutions within the buffers, and the equivalent volume of stock to yield 10mls of buffer are presented. * indicates compounds to be added last in the fume hood

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock Concentration</th>
<th>Buffer A</th>
<th>Buffer C</th>
<th>Buffer D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile H₂O</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hepes (pH 7.9)</td>
<td>1M</td>
<td>10 mM</td>
<td>20 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1M</td>
<td>1.5 mM</td>
<td>1.5 mM</td>
<td>-</td>
</tr>
<tr>
<td>KCl</td>
<td>1M</td>
<td>10 mM</td>
<td>-</td>
<td>50 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>1M</td>
<td>-</td>
<td>120 mM</td>
<td>-</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.5M</td>
<td>-</td>
<td>0.2 mM</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2500</td>
</tr>
<tr>
<td>PMSF</td>
<td>0.5M</td>
<td>0.5 mM</td>
<td>0.5 mM</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>DTT</td>
<td>1M</td>
<td>0.5 mM</td>
<td>-</td>
<td>0.5 mM</td>
</tr>
</tbody>
</table>

Buffer Concentration (μl) for 10mls:
- Buffer A: 9770
- Buffer C: 3070
- Buffer D: 7380
2.16.2 Determination of nuclear and cytoplasmic protein concentrations by Bradford assay

Determination of protein concentration in samples was performed according to the method originally described by Bradford (1976) in a 96-well plate format. For nuclear extracts, samples were diluted 1:60 in Buffer D (3μl sample, 177μl Buffer D) per well. For cytoplasmic fractions, samples were diluted 1:300 in PBS (0.6μl sample, 179.4μl PBS) per well. Standards were prepared from a stock solution of 1mg/ml bovine serum albumin (BSA) (Sigma, Saint Louis, MI, USA) and ranged from 0μg/ml to 25μg/ml in Buffer D for nuclear samples and PBS for cytoplasmic samples. 180μl of each standard and sample was incubated with 40μl of protein assay dye (Bio-Rad, Hercules, CA, USA). Colour was allowed to develop for 10 minutes and absorbance was measured at 590 nm using a spectrophotometer (SPECTRAFluor Plus and XFLUOR Version 3.21, TECAN, Reading, UK). The concentration of protein in the samples was calculated from the standard curve generated with the BSA standards.

2.16.3 Preparation of samples for gel electrophoresis and Western blotting

5μg of nuclear protein or 25μg of cytoplasmic protein was acetone precipitated in five times its volume of ice-cold acetone for at least 1 hour at -20°C. After this incubation period, the samples were centrifuged at 12,000 rpm for 5 minutes, the acetone aspirated and the pellet allowed to air-dry. The pellet was resuspended in 20μl of sample buffer (62.5 mM Tris-HCL pH 6.8, 10% v/v glycerol, 2% w/v SDS, 5% v/v b-mercaptoethanol, 0.05% w/v bromophenol blue) and boiled at 100°C for 5 minutes to denature the proteins. The samples were centrifuged briefly and placed on ice prior to immediate use for SDS-PAGE electrophoresis.

2.16.4 SDS PAGE

Cytoplasmic and nuclear proteins were separated on 10% acrylamide gels. The composition of separating and stacking gels is presented in Table 2.6.
Table 2.6 Composition of separating and stacking gels

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock</th>
<th>Separating gel (µl/gel)</th>
<th>Stacking gel (µl/gel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled H₂O</td>
<td>-</td>
<td>2000</td>
<td>0.76</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>30%</td>
<td>1675</td>
<td>0.16</td>
</tr>
<tr>
<td>APS</td>
<td>100mg/ml</td>
<td>50</td>
<td>12.5</td>
</tr>
<tr>
<td>SDS</td>
<td>10%</td>
<td>50</td>
<td>12.5</td>
</tr>
<tr>
<td>Tris pH 8.8</td>
<td>1.5M</td>
<td>1250</td>
<td>-</td>
</tr>
<tr>
<td>Tris pH 6.8</td>
<td>0.5M</td>
<td>-</td>
<td>312.5</td>
</tr>
<tr>
<td>TEMED</td>
<td>-</td>
<td>15</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Acrylamide gels were cast between two glass plates and affixed to the electrophoresis unit using spring clamps. Electrode running buffer (5mM Tris Base, 200mM glycine, 17mM SDS) was added to the upper and lower reservoirs. After standard (Precision Plus Protein™ Standards Dual Colour, Bio-Rad Laboratories Inc.) and sample loading, electrophoresis was carried out at 30 mA per gel for 45 to 60 minutes until the migration dye front had reached the gel base.

2.16.5 Western blotting

The semi-dry method for transfer of electrophoresed proteins to immobilizing membranes as described by Towbin (1979) was preformed using a semi-dry blot system (Model B2529; Sigma, Saint Louis, MO, USA). 0.45μm Biotrace™ polyvinylidene fluoride (PVDF) transfer membrane (Pall Corporation, Pensacola, Florida) was soaked in methanol for 20 seconds and then saturated in transfer buffer (25 mM Tris Base, 192 mM glycine, 20% v/v methanol, 0.05% w/v SDS) for 1 hour prior to use. Whatman 3mm CHR filter paper was cut to size and saturated in transfer buffer immediately prior to transfer. The blot sandwich was prepared as follows: 2 sheets of pre-soaked filter paper, the soaked PVDF membrane, the gel, and 2 sheets of pre-soaked filter paper. Care was taken to ensure the absence of air bubbles and to note the orientation of the gel. Electrophoresis transfer was performed at 225 mA per gel for 75 minutes.
2.16.6 Detection of NFκB p65 and p50 subunits, IκBa and IκK

Blots were blocked overnight at 4°C with a solution of PBS/Tween 20 (0.05%) containing 10% w/v Marvel non-fat dried milk and then washed for 30 minutes (6 x 5 minutes) with PBS/Tween. Blots were then incubated with primary antibody. p65 (sc-372), IκBa (sc-372), p50(sc-1190X) or IKK (sc-535) antibodies were diluted 1:2000 to 1:1000 in 5% w/v Marvel-PBS/Tween 20 for nuclear and cytoplasmic blots (Santa-Cruz Biotechnology Inc.). Blots were incubated in primary antibody for 1 hour at room temperature or overnight at 4°C with agitation. After incubation with primary antibody, the blot was washed for 30 minutes (6 x 5 minutes) with PBS/Tween. The membrane was incubated with peroxidase-conjugated secondary antibody (polyclonal goat anti-mouse IgG-peroxidase conjugated immunoglobulins; Sigma, Saint Louis, MI, USA) diluted in 5% w/v Marvel-PBS/Tween for 1 hour at room temperature with agitation. The membrane was washed with PBS/Tween for 50 minutes (10 x 5 minutes).

Antigens were detected by incubating the membrane in a 1:1 solution of Luminol enhancer solution: stable peroxidase buffer (Supersignal® West Dura, Pierce) for 5 minutes at room temperature. Excess reagent was drained from the membrane, which was then placed between two sheets of transparent acetate in an autoradiography cassette (Sigma-Aldrich). High performance chemiluminescence film (Amersham Biosciences, Buckinghamshire, UK) was applied to the blots in the dark-room, folded in the cassette and exposed for 1 minute to 24 hours, as required. The films were developed using an AGFA CURIX 60 (AGFA-Gevaert, AG Munich, Germany).

2.16.7 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/Tween for 30 minutes (6 x 5 minutes) the blot was incubated for 30 minutes at 50°C in stripping buffer (31.25 mM Tris pH 6.8, 100 mM β-mercaptoethanol, 2% w/v SDS). After washing for 1 hour in PBS/Tween 20, the blot was ready to use again commencing with the blocking stage in western blot protocol.

2.16.8 Quantification of protein bands

Imaging of bands was performed using GeneGenius and GeneSnap (Syngene, Cambridge, UK). Quantification of protein bands was performed using GeneTools Analysis Software (Syngene, Cambridge, UK).
2.17 NFκB NUCLEAR BINDING

The TransAM™ NFκB p65 Transcription Factor Assay (Active Motif, Rixensart, Belgium) is an ELISA-based assay used to detect and quantify nuclear p65 activation. A white-walled chemiluminescent-compatible 96-well microplate is pre-coated with oligonucleotide containing a discrete nucleotide sequence (5′-GGGACCTTCC-3′) (Figure 2.6). The Rel-homology domain of the NFκB family binds to this consensus site and NFκB is thus immobilised to the plate. Primary antibody is directed against an epitope on p65 that is accessible only when NFκB is activated and bound to its target DNA. Secondary antibody is conjugated with horseradish peroxidase that exerts chemiluminescence in proportion to the presence of active p65. The TransAM NFκB Transcription Factor Assay also includes primary antibody for p50. The kit is sensitive, providing quantitative results for 0.039μg to 2.5μg of nuclear or whole cell extract per sample. The assay takes about 3.5 hours to perform and is faster than alternative methods such as Western blotting and electrophoretic mobility shift assay (EMSA). The technique can use less protein than either immunoblotting and EMSA. In addition, chemiluminescent detection may be preferable to autoradiography used in EMSA. The ELISA-style protocol is relatively straightforward and sample treatment is minimal. The 96-well format offers high-throughput capabilities (Rosenau et al. 2004).

Figure 2.6 Principle of ELSA-style TransAM NFκB nuclear binding kit
The kit contains a 96-well frame with twelve 8-well removable strips. Once removed from -20°C storage strips may be stored at 4°C for 6 months sealed in a foil pouch. Complete Binding Buffer (CBB) and Complete Lysis Buffer (CLB) are prepared from kit components fresh for each assay (Table 2.7). Antibody Binding Buffer (ABB) and wash buffer are provided in concentrate. BSA and Tween 20 may form clumps in ABB and wash buffer stocks, respectively, and concentrates should be brought to room temperature and vortexed for homogenisation prior to use. ABB working solution (≈1.5mls needed for 10 wells) is prepared by a 1:10 dilution of stock in distilled H₂O. Working wash buffer (≈30mls needed for 10 wells) is prepared by a 1:10 dilution of stock in distilled H₂O. ABB working solution should be discarded after use; wash buffer may be stored at 4°C for one week after dilution. Chemiluminescent Reagent and Reaction Buffer may be stored at -20°C or at 4°C, protected from light and should be brought to room temperature before use. Stocks of DTT (1M), protease inhibitor cocktail, Herring sperm DNA (1μg/μl), NFκB p65 antibody (0.2μg/ml), anti-rabbit HRP-conjugated IgG (0.2μg/μl) and positive Jurkat nuclear extract control (2.5ng/μl) are provided in the kit and stored at -20°C. The secondary antibody may be partially diluted (eg: 1:500) in PBS and stored at 4°C for convenience; its assay dilution factor is 1:10,000 and would require an unnecessarily large volume of ABB working solution to be made up immediately prior to use to obtain this dilution.

Table 2.7 Composition of Complete Binding and Complete Lysis buffers.
Constituents are supplied with TransAM NFκB nuclear binding kit

<table>
<thead>
<tr>
<th>Complete Binding Buffer (≈ 10 wells)</th>
<th>Complete Lysis Buffer (≈10 wells)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>μl</strong></td>
<td><strong>μl</strong></td>
</tr>
<tr>
<td>DTT</td>
<td>0.7</td>
</tr>
<tr>
<td>Herring sperm DNA</td>
<td>3.4</td>
</tr>
<tr>
<td>Binding Buffer AM2</td>
<td>334</td>
</tr>
</tbody>
</table>

Nuclear protein extracts were isolated as previously described (Section 2.15.1). CBB, CLB, ABB and wash buffer were made up prior to immediate use. Sufficient 8-well strips were placed in the 96-well frame. 30μl CBB was added to each well. 1.25μg nuclear protein was diluted up to 20μl in CLB in each well. A positive control well of 1.25μg Jurkat nuclear extract in CLB and a blank well containing only CLB were included on the plate. When control, blank and samples were loaded the plate was
sealed and incubated at room temperature for 1 hour with gentle orbital agitation. Wells were then washed by pipetting 200 µl of wash buffer into each well, tapping the plate gently and flicking the liquid out. This was done three times. After the third wash the plate was inverted and tapped gently against tissue to remove remaining wash buffer. 50 µl of primary p65 antibody solution (1:1000 dilution of antibody in ABB working solution) was added per well, the plate was sealed and incubated without agitation at room temperature for 1 hour. The plate was then washed as before. 50 µl HRP-conjugated secondary antibody solution (1:10,000 dilution of antibody, obtained by a 1:20 dilution in ABB of pre-diluted 1:500 stock) was added per well, the plate was sealed and incubated without agitation at room temperature for 1 hour. During this incubation the Chemiluminescent Reagent (CR) and Reaction Buffer (RB) were incubated at room temperature. After secondary antibody incubation the wells were washed four times as before. 50 µl chemiluminescent working solution was added per well, made up of CR and RB at 1:2 (200 µl and 400 µl, respectively, for ≈10 wells). The plate was read immediately with maximal integration at 225 manual gains on a chemiluminometer (SPECTRAFluor Plus and XFLUOR Version 3.21, TECAN, Reading, UK). Readings can be taken over 1 hour with minimal change in signal intensity. Results were expressed as relative luminescence units (RLU).

2.18 HIGH CONTENT SCREENING OF NFκB NUCLEAR TRANSLOCATION USING CELLOMICS INC. TECHNOLOGY

Techniques commonly used to investigate the effects of drugs and nutrients on target cells require isolation of cellular or extra-cellular components. Cells are denatured for nucleic acid isolation in PCR, cytoplasmic and nucleic protein isolation in immunoblotting and are discarded from culture media for ELISA. Furthermore, large masses of cells, presumed to be completely homogenous, are required in these techniques (ranging, in this thesis, from 1 to 4 x 10^6 cells per sample). Such genomic and proteomic assays are vital, but lend a degree of artificiality in terms of the whole cell response. The living, entire cell is the target of drug and nutrient treatments. Cellomics, the knowledge of cellular phenotype and function, relates cellular components in concert and in context. The elegance of a cellomics approach to drug and nutrient treatments sees scientific and medical investigation mature, with “systems biology” as the prime focus rather than isolated cellular biomolecules. Physiological and therapeutic processes are often accompanied by altered cellular morphology, shape,
area, motility, organelle and cytoskeletal location and proximity between cells (Ghosh & Haskins, 2004). Investigation and quantification of morphological cellular responses lends greater bearing to the effects of drug and nutrient treatments. The requirements of the “systems biology” approach, integrating biomolecules and their higher order assemblies in native configuration to fulfil life functions (Abraham et al. 2004) are increasingly met by High Content Screening (HCS). HCS is defined as the automation of high-content investigation of arrayed cells including key operations of experimental design, sample preparation, image acquisition, processing and analysis and cellular knowledge mining. Processes are defined spatially and temporally in the context of the structural and functional integrity of each individual cell within an array of cells (Ghosh & Haskins, 2004). Cellomics Inc. (Pittsburgh, PA) provides a completely integrated system for HCS, either in live-cell kinetic or fixed-endpoint assays for a range of BioApplications. For the purposes of this thesis, the Cellomics Inc. tools of only fixed-endpoint nuclear translocation assays will be discussed.

2.18.1 Components of Cellomics technology

2.18.1a Cellomics HitKit® Reagent Kits

The HitKit comprises of reagents and optimised protocols for performance of specific high content cell-based assays (Abraham et al. 2004) investigating cell signalling targets, cell motility and morphology, toxicology and cell cycle. In the area of cell signalling, Cellomics HitKits are currently available for tracing ATF-2, c-Jun, ERK MAPK, NFkB, NFAT-1, p38 MAPK, PKCa, STAT1, STAT2 and STAT3 activation indicated by cellular compartmentalisation or translocation. Cell signalling assays are based on indirect immunofluorescence. The primary antibody recognises the target biomolecule. The secondary antibody is conjugated with a fluorophore. Thus, a fluorescently labelled protein, termed a “fluorescent-protein biosensor” (Giuliano and Taylor, 1998) is created. Activation of the target protein is measured by an increase in nuclear relative to cytoplasmic fluorescence. In the present study, the Cellomics NFkB HitKit (K01-0001-1) was employed. The primary antibody supplied is specific for NFkB. The secondary antibody is conjugated to Alexa® Fluor 488. This fluorophore, licenced to Cellomics Inc. exclusively for HCS by Molecular Probes Inc., is reported to be superior to other similar fluorophores, such as fluorescein isothiocyanate (FITC) or carboxyfluorescein (FAM). Alexa Fluor 488 excites at 495nm and emits at 519nm; its absorption spectra cover the entire spectrum and match the principle output wavelengths.
of common excitation sources. It is reported to have superior fluorescent intensity and photostability. It is pH insensitive between values of 4 and 10. In addition, the hydrophilicity of Alexa Fluor 488 allows its conjugates good water solubility and minimises their precipitation during long-term storage. Hoechst 34442 (H342) dye is used in the HitKit to identify nuclei. H342 binds to AT rich regions of DNA and absorbs in the UV range (ex/em maxima 355/460nm) (Parish, 1999).

2.18.1b Cellomics compartmental analysis BioApplication
The nuclear translocation of NFkB, indicating NFkB activation, was investigated in the present work using the compartmental analysis BioApplication. Translocation events are measured by the relative distribution of target fluorescent intensities between the cytoplasmic and nuclear regions of a cell. Two channels of visualisation were used. Channel 1 allows identification of the object by nuclear staining with H342. The BioApplication defines a nuclear and a cytoplasmic region based on user-defined parameter inputs in Channel 2. The difference in fluorescent intensities between the defined nuclear and cytoplasmic regions is analysed and indicates the magnitude of translocation from the cytoplasm to the nucleus. Analysis of NFkB nuclear translocation in IL-1-stimulated human DC was comparable between EMSA and Cellomics BioApplication, but imaging analysis saved resources, required fewer cells and provided single cell based data (Vakkila et al. 2004).

2.18.1c Cellomics KineticScan® HCS Reader
The KineticScan HCS reader is an automated imaging system for live-cell kinetic or fixed endpoint assays. Its general design is analogous to an inverted fluorescent microscope. The components of KineticScan Reader imaging are presented in Figure 2.5 and Section 2.17.1d. The system scans multiple fields in individual wells, finds and focuses cells, acquires information about the spatial distribution of fluorescent-protein biosensors in cells and analyses cell images according to defined algorithms (Vakkila et al. 2004). Cellular imaging allows the homogeneity of the cell population to be assessed. The algorithm can be designed to omit cells of unusual morphology and to include only cells uniform to a selected definition. The system is equipped with emission and excitation filters (XP93) for selectively imaging fluorescent signals emitted by H342 and Alexa Fluor 488. The capacity of the KineticScan HCS reader for sample preparation, by means of a work platform and robotic pipettor aim, was not used.
in the present study. In addition, the onboard environmental control, which allows maintenance of precise physiological conditions throughout time course studies, was not necessary to use.

2.18. Id Cellomics KineticScan HCS Reader Imaging (adapted from user manual)
Several optical elements housed in the HCS KineticScan Reader determine the wavelength of the light reaching the camera. The main components, which may be selected or altered depending on the experimental protocol, involved in the imaging system are presented in Figure 2.7.

![Figure 2.7 Imaging components of the Cellomics KineticScan HCS Reader](image)

Light is emitted from a high-pressure mercury discharge lamp (1) located externally to the HCS KineticScan Reader. The light beam is guided (2) towards the Reader's illumination port (3). The light passes through an excitation filter (4), aperture diaphragm (5) and an illuminated field diaphragm (6) before being directed towards the 96-well plate (13) by the dichroic reflector module (10) via a laser autofocus dichroic mirror (11) and objective (12). In addition, the beam from the laser diode (9), which is used in the system's optical autofocus function, may be coupled into the objective (12)
via the mirror (11). Fluorescent light emitted from the well passes through the objective 
(12), mirror (11), reflector module (10) and emission filter (8) to the CCD element in 
the camera (7).

2.18.1e Cellomics Software

The KineticScan Reader system includes a Dell 530 Workstation with Cellomics 
software. KineticScan version 1.0 develops protocols, runs screens and operates the 
KineticScan system. Cellomics Data Viewer combines extensive graphing and reporting 
tools to aid in analysing all of the spatial and temporal data collected for a plate. 
Software for chosen BioApplications is installed. Cellomics Store allows storage and 
access to data and images produced by HCS (adapted from Cellomics KineticScan HCS 
Reader System manual).

2.18.2 Implementation of Cellomics technology

2.18.2a Cellomics HitKit® Reagent Kit

Samples were prepared according to the manufacturer’s instructions. Wash buffer, 
permeabilisation solution, detergent buffer, primary antibody, conjugated secondary 
antibody and H342 were supplied with the HitKit and stored at 4°C. All treatments and 
washes were performed at room temperature using a multi-channel pipette, taking care 
not to scrape the bottom of the wells. After activation of adherent cells cultured in 
recommended 96-well high-density black plates (Parkard ViewPlate®, Foss, Dublin) 
culture media was aspirated and replaced with 100μl pre-warmed fixation solution per 
well for 10 minutes (37% formaldehyde diluted 1:10 with 1X wash buffer) in a fume 
hood. Fixation solution was aspirated and cells were washed once with 1X wash buffer. 
100μl 1X permeabilisation solution was added for 90 seconds and wells were washed 
again with 1X wash buffer. 50μl primary antibody was added per well for 1 hour. 
Primary antibody was aspirated and 100μl 1X detergent buffer was added for 15 
minutes. Wells were washed twice with wash buffer. 50μl staining solution containing 
secondary antibody conjugated with Alexa Fluor 488 and H342 was added to each well 
and the plate was incubated for one hour in the dark. Staining solution was aspirated 
and 100μl detergent buffer was added per well for 10 minutes. The wells were washed 
once more. Finally, 200μl 1X Wash Buffer was added to each well, the plate was sealed 
and stored at 4°C protected from light.
2.18.2b Cellomics KineticScan® HCS Reader

The external high-pressure mercury gas discharge lamp and the Cellomics KineticScan HCS Reader were switched on in advance of use. The lamp was configured for desktop control and switched on from the desktop. When the lamp was registered as stable (after approximately 400 seconds) the shutter was opened from desktop controls. The 96-well ViewPlate was placed on the KineticScan balcony without penetrating the light beam that shields the systems from manual interference. Upon software command the 96-well plate was loaded into the sample compartment for imaging.

2.18.2c Cellomics compartmental analysis BioApplication

An assay, kinetic and plate protocol were established and saved for the experimental model. In the present study a 10X objective viewing 5 fields per well was generally employed. An image set was acquired for a sample well in Channel 1 and Channel 2. A number of representative cells were chosen and an algorithm, defining the spatial and temporal parameters of the representative cells, was run and object inclusion assessed. When satisfied with the algorithm, the plate was scanned by the chosen protocol. During the scan Channel 1, Channel 2 and composite images per field are displayed. Furthermore, the BioApplication registers if sufficient cell numbers fitting the algorithm are present per well. This allows the scan to be monitored and it may be stopped and the protocol and/or algorithm altered at any time. After the scan the lamp shutter was closed and the lamp turned off.

2.18.2d Cellomics Software

After the scan, results were presented in Data Viewer. Data is available for a wide variety of morphological and fluorescent parameters. In the present study the results chosen for analysis were related only to the nuclear translocation of NFkB. Also, results were not analysed in terms of individual cells, although possible, as it generates vast amounts of data. Well analysis, with the average of results from cells selected by algorithm, was the more appropriate available bioinformatic tool. Graphing functions of Data Viewer allowed immediate assessment of results. However for ease of data management, results were transferred to Microsoft Excel for storage and to DataDesk 6.0 for statistical analysis.
Chapter 3

This chapter is divided into two studies. The first (3A), a human intervention trial, investigated the effects of an isomeric conjugated linoleic acid supplement on mediators of inflammation and plasma lipids. The second study (3B) was an *in vitro* investigation of the isomer-specific effects of conjugated linoleic acid on pro-inflammatory cytokines.
3A. EFFECTS OF CONJUGATED LINOLEIC ACID SUPPLEMENTATION ON INFLAMMATORY AND LIPID MEDIATORS ASSOCIATED WITH CARDIOVASCULAR DISEASE AND ATHEROSCLEROSIS. Plasma cholesterol, TAG, glucose and fibrinogen analysis was performed by Fiona Moloney, Nutrigenomics Research Group, Trinity College Dublin.

3A.1 INTRODUCTION

CLA refers to the geometric and positional conjugated dienoic isomers of linoleic acid. It is naturally found in ruminant meat and dairy products mainly as the c9, t11-CLA isomer (Chin et al. 1992; Kramer et al. 1997; Parodi, 1997). CLA is produced synthetically by base-catalysed isomerisation of linoleic acid (Banni & Martin, 1998) resulting in a mixture of c9, t11-CLA and t10, c12-CLA isomers. Numerous in vitro and animal studies suggest that CLA may have therapeutic effects with respect to cancer (Ip et al. 1991; Banni et al. 1999; Field & Schley, 2004), diabetes (Houseknecht et al. 1998; Roche et al. 2002) and atherosclerosis (Kritchesvky et al. 2000; Toomey et al. 2003). CLA has been reported to alter plasma cholesterol and TAG concentrations in animal feeding (Lee et al. 1994; Nicolosi et al. 1997; Munday et al. 1999) and human supplementation (Noone et al. 2002) studies. CLA also appears to have intrinsic immune-modulatory properties, particularly with respect to cell-mediated immune function. It has been reported to decrease in vitro porcine lymphocyte (Chew et al. 1997) and increase ex vivo murine splenocyte IL-2 secretion (Hayek et al. 1999), inhibit macrophage phagocytosis (Cook et al. 1993; Miller et al. 1994) and reduce macrophage TNFα and IL-6 production (Turek et al. 1998). Dietary CLA supplementation alleviated growth depression and the elevation of TNFα and IL-6 production induced by LPS challenge in weaned pigs (Changhua et al. 2005). Several recent reviews have documented the potential immuno-modulatory effects of CLA in detail (Roche et al. 2001; Field & Schley, 2004; O’Shea et al. 2004; Wahle et al. 2004).

To date, relatively few studies have investigated the effects of CLA supplementation on inflammatory mediators in man. Kelley et al. (2000 & 2001) showed that CLA supplementation had no effect on lymphocyte proliferation, serum antibody titer, delayed type hypersensitivity or ex vivo PBMC cytokine production in healthy women aged 20 to 41 years. Volunteers were supplemented with 3.9g CLA daily for 63 days, receiving 0.88g t10, c12-CLA, 0.69g c9, t11-CLA and 2.3g of other CLA isomers. Albers et al. (2003) showed that CLA beneficially affected the initiation
of an immune response to hepatitis B vaccination but had no effect on *ex vivo* PBMC cytokine and eicosanoid secretion in a group of healthy men aged 31-69 years. CLA supplemented volunteers received either a 50:50 blend (containing 1.7g CLA fatty acids) or an 80:20 blend (containing 1.6g CLA glycerides) of the *c*9, *t*11-CLA and *t*10, *c*12-CLA isomers daily for 12 weeks. Tricon *et al.* (2004) showed that *c*9, *t*11-CLA and *t*10, *c*12-CLA single-isomer supplementation resulted in a dose-dependent reduction in the mitogen-induced activation of T lymphocytes but had little effect on cytokine production. Our group recently reported that CLA supplementation had little effect on immune function in young healthy subjects, males and females (31.5 ± 9.9 years), receiving 1.9g 50:50 or 1.7g 80:20 *c*9, *t*11-CLA and *t*10, *c*12-CLA supplements daily for 8 weeks (Nugent *et al.* 2005). Differences in the amount and composition of CLA consumed, the age and gender of volunteers and length of supplementation must be considered when interpreting the results of these studies.

The aim of the present study was to investigate the effects of CLA supplementation on inflammatory and lipid mediators associated with CVD in a healthy, but susceptible cohort. *Ex vivo* PBMC stimulated with Con A were used to investigate the effects of CLA supplementation on cytokine mediators of inflammation, particularly those produced by T helper lymphocytes. The effects of CLA supplementation on acute phase reactants was investigated from serum and plasma samples. In addition, the effect of CLA supplementation on fasting plasma cholesterol, triglyceride and glucose concentrations was investigated.

### 3A.2 METHODS

#### 3A.2.1 Subjects & Intervention Details

This double-blind placebo-controlled study was approved by the ethics committee of the Federated Dublin Voluntary Hospitals, Ireland. Thirty middle-aged healthy male volunteers, recruited to typify a cohort at risk of CVD, gave written, informed consent and completed the trial. Volunteers had no history of inflammatory disorders or prescribed medications. All, but one, of the volunteers were non-smokers. No volunteer was vegetarian, adhering to a special diet or consumed dietary supplements prior to or throughout the study period. All volunteers habitually consumed meat and dairy products and, on the basis of dietary interviews, intakes of CLA were estimated to be 197 ± 94mg per day, comparable to intakes reported in other studies (Ens *et al.* 2001; Ritzenthaler *et al.* 2001; Fremann *et al.* 2002). Subjects were randomly assigned to
receive 2.2g of CLA or placebo daily for eight weeks, presented as six 0.5g capsules per day. The composition of the CLA and placebo supplements is presented in Table 3A.1. Supplements were provided by Loders Croklaan (Wormeveer, The Netherlands). The placebo contained a blend of fatty acids typical of the fatty acid composition of the Irish and UK diet.

<table>
<thead>
<tr>
<th>Fatty acid composition (w/w%) of CLA and placebo supplements</th>
</tr>
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<td></td>
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<tr>
<td>C18:0</td>
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<td>C18:1</td>
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<tr>
<td>C18:2</td>
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<tr>
<td>C20:1</td>
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<td>Other saturated fatty acids</td>
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<thead>
<tr>
<th>CLA isomers (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CLA</td>
</tr>
<tr>
<td>c9, t11-CLA</td>
</tr>
<tr>
<td>t10, c12-CLA</td>
</tr>
</tbody>
</table>

3A.2.2 Blood Sampling and PBMC preparation

Fasting blood samples were drawn from volunteers (Vacutainer™, Becton Dickinson, Oxford, UK) at the beginning and end of the supplementary period (Chapter 2.1). Citrated plasma and serum were harvested from blood by centrifugation, snap frozen and stored (-80°C) until subsequent analysis of fibrinogen, CRP and IL-6. PBMC from heparinised blood were isolated by density gradient centrifugation (Chapter 2.2). Briefly, blood diluted (1:1) in Hepes-buffered HBSS (Gibco, Grand Island, New York) was gently layered onto Lymphoprep™ (density 1.077 g/ml, Nycomed Pharma, Oslo), centrifuged slowly and PBMC were removed from the buffy interface. Cells were washed twice with HBSS and resuspended in RPMI-1640 medium (Gibco, Grand Island, New York) supplemented with 2mmol L-glutamine/L, 100mg streptomycin/ml and 100μg penicillin/ml (Sigma, MO, USA). Cell viability and concentration were assessed by fluorescent microscopy using ethidium bromide/acridine orange stain. Cells were plated and incubated at a concentration of 1 x 10⁶ cells/ml in RPMI medium.
supplemented with 2.5% v/v autologous serum (Yaqoob et al. 2000; Thies et al. 2001; Nugent et al. 2005), with or without the lectin Con A at a concentration of 10μg/ml culture. Con A (12.5 to 25μg/ml) has been used to stimulate 1x10^6 PBMC in other similar studies (Yaqoob et al. 1999; Kew et al. 2003; Tricon et al. 2004).

3A.2.3 Protein Assays

IL-2, IL-10 and TNFα concentrations from PBMC supernatant were measured using commercial ELISA kits (R&D Systems, Oxon, UK) after 24 hours incubation at 37°C and 5% CO₂ (Chapter 2.8 and 2.12). This time point was deemed appropriate for optimal investigation of cytokine secretion from PBMC (Yaqoob et al. 1999; Dooper et al. 2002). Serum IL-6 and plasma CRP assays were performed using high sensitivity ELISA kits (Biosource, California and BioCheck Inc, California, respectively) following manufacturers instructions. Fibrinogen clotting activity was measured using an automated assay (Chapter 2.13).

3A.2.4 Gene Expression Analysis

Total RNA was extracted from PBMC after 4 hours incubation using TRIzol® reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. RNA yields were quantified spectrophotometrically and integrity was confirmed by the visualisation of the 18s and 28s bands following formaldehyde denaturing agarose gel electrophoresis (GeneSnap from Syngene, Cambridge, UK). RNA was treated with Deoxyribonuclease I, Amplification Grade as per manufacturer’s protocol to remove contaminating genomic DNA. Reactions containing 2 μg of RNA were reversed transcribed and 1:2 serial dilutions performed to generate a standard curve for the subsequent PCR analysis (ABI Prism™ 7700 Sequence Detector, Applied Biosystems, Warrington, UK) with glyceraldehyde dehydrogenase as an endogenous control (Chapter 2.15).

3A.2.5 Fatty Acid Analysis

Total plasma lipid fatty acid composition was determined using a Shimadzu GC-14A gas liquid chromatograph (Mason Technologies, Dublin) and a Shimadzu CR6A integrator, fitted with a CP Sil 88 fused Silica Column (50 m x 0.22 m file thickness; Chrompack Ltd, Middleburg, The Netherlands). The method used was specifically designed and validated wherein it detected both the c9, t11-CLA and t10, c12-CLA
isomers (Noone et al. 2002) (Chapter 2.10). Briefly, total plasma lipids were isolated using a method derived from that of Folch et al. (1957). Methyl esters of total plasma lipid were prepared by adding 0.5ml 0.01M-NaOH in dry methanol followed by boron trifluoride (0.5ml). Nitrogen was used as the carrier gas. The column initial temperature (180°C) was increased (5°C/minute) to 195°C, held for 40 minutes and then increased (2°C/minute) to 220°C, and held for 20 minutes. Peaks were identified using a fatty acid methyl ester standard spiked with known concentrations of the $c_9$, $t11$-CLA and $t10$, $c12$-CLA isomers (Cayman Chemical, Michigan, USA). Fatty acids were identified by comparison with the retention times of the standard. Plasma fatty acid compositions were calculated as a percentage of the total fatty acids.

3A.2.6 Plasma lipid and glucose analysis
The colorimetric analyses of plasma total cholesterol, LDL, HDL, TAG and glucose, performed with the ILAB 600 Clinical Chemistry Analyser (Instrumentation Laboratory, Warrington, UK), are detailed in Chapter 2.11. By centrifugation with density solutions, precipitated LDL and suspended HDL fractions were isolated from plasma. Cholesterol concentrations of these and plasma samples were determined by a common colorimetric protocol. TAG and glucose were measured in plasma by enzyme-linked colorimetric assays described in Chapter 2.11.4 and 2.11.5, respectively.

3A.2.7 Statistical Analysis
Statistical analyses were performed using the two-way ANOVA (subject and time were the independent variables; results confirmed by repeated measure ANOVA) and pooled T-test from DataDesk® 6.0 (Data Description Inc., Ithaca, USA). Data was normalised and transformed where necessary. Results are expressed as mean ± SEM. A P value < 0.05 was considered statistically significant.

3A.3 RESULTS
3A.3.1 Subject details and study compliance
Thirty volunteers participated in and completed the study, with fifteen subjects in each supplementation group. Both groups were similar in respect of all screening markers (age, body mass index, total serum cholesterol, serum TAG, haemoglobin, urinary glucose) except the hepatic enzyme gamma-glutamyltransferase (GGT), measured in
serum, which was significantly higher in the CLA group (P=0.02). Volunteer characteristics at screening are presented in Table 3A.2.

Table 3A.2 Screening characteristics of CLA and placebo supplemented groups. Values are expressed as mean with SEM in parenthesis; *indicates P≤0.05 significant difference from placebo group

<table>
<thead>
<tr>
<th></th>
<th>CLA</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>51.0(2.01)</td>
<td>47.8(1.59)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>83.4(2.37)</td>
<td>84.5(3.19)</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.78(0.02)</td>
<td>1.8(0.01)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.2(0.58)</td>
<td>26.0(0.85)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.31(0.17)</td>
<td>5.20(0.22)</td>
</tr>
<tr>
<td>Serum TAG (mmol/L)</td>
<td>1.31(0.14)</td>
<td>1.27(0.14)</td>
</tr>
<tr>
<td>Serum GGT (IU)</td>
<td>35.0*(4.76)</td>
<td>20.7(2.17)</td>
</tr>
<tr>
<td>Plasma Haemoglobin (g/day L)</td>
<td>14.5(0.09)</td>
<td>14.4(0.26)</td>
</tr>
<tr>
<td>Urinary Glucose</td>
<td>negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Study compliance was assessed by capsule count and total plasma fatty acid composition. 96.5% and 95.9% of supplements were consumed by the control and CLA groups, respectively. Total plasma fatty acid c9, t11-CLA levels were significantly increased after CLA supplementation (2.3 ± 0.6 and 7.1 ± 0.6 mg/g total plasma fatty acids pre- and post-supplementation, respectively; P<0.001). In contrast, the t10, c12-CLA levels were negligible following CLA supplementation. The placebo group showed no significant levels of c9, t11-CLA or t10, c12-CLA pre-and post-intervention.

3A.3.2 PBMC markers of inflammation (Figure 3A.1 & Table 3A.3)

Secretion of IL-2 from unstimulated PBMC was not significantly affected by CLA or placebo supplementation (data not shown). Con A-stimulated IL-2 secretion was significantly lower (37.1%) following CLA supplementation (P=0.02) whereas the placebo had no effect (Figure 3A.1a). This result was confirmed by repeated measures ANOVA (P=0.03). Con A-stimulated PBMC IL-2 transcription was reduced (14.4%)
after CLA supplementation (Figure 3A.1b). However, IL-2 mRNA expression was variable between subjects and this change was not statistically significant.

Figure 3A.1 The effect of CLA supplementation on the increase in Con A-stimulated IL-2 cytokine secretion from (a) and transcription in (b) PBMC. PBMC were stimulated with 10μg/ml Con A for 4 and 24 hours. Secretion was quantified by ELISA at 24 hours and transcription was semi-quantified by RT-PCR at 4 hours. (a) Results are expressed as IL-2 concentration (pg/ml) in supernatant at weeks 0 and 8. (b) The values expressed are normalised to GAPDH and relative to baseline IL-2 expression. * indicates P≤0.05 significant difference within group; † indicates P≤0.05 significant difference between groups.

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Unstimulated PBMC TNFα secretion was not changed by CLA or placebo supplementation and IL-10 was undetectable by ELISA in these samples. Con A-stimulated PBMC TNFα and IL-10 secretion were not directly altered by either the placebo or CLA supplements. The concentrations of these inflammatory mediators before and after supplementation are presented in Table 3A.3. Observations of significant changes in TNFα secretion following volunteer ranking by baseline plasma CRP concentrations are presented in the discussion.

### 3.3.3 Systemic markers of inflammation (Table 3A.3)

Serum IL-6, plasma CRP and fibrinogen were not significantly altered by either the placebo or CLA supplements. The concentrations of these inflammatory mediators before and after supplementation are presented in Table 3A.3.

### 3.3.4 Plasma lipid and glucose analysis (Table 3A.4)

Total cholesterol was higher in the placebo than the CLA group at baseline (approaching significance at P=0.07) and at week 8 (P=0.04). This was explained by a significantly higher LDL concentration in the placebo group at baseline relative to the CLA group (P=0.05). The CLA treated group had significantly higher baseline HDL concentrations than the placebo group (P=0.003) and again at week 8 (P=0.03). Neither CLA nor placebo supplement exerted an effect on total, LDL or HDL cholesterol concentration. The placebo supplement decreased TAG concentrations over the study (P=0.03). CLA had no effect on plasma TAG. Neither CLA nor placebo supplement had an effect on fasting glucose concentrations. These data are presented in Table 3A.4.
Table 3A.3 Mean concentrations of inflammatory mediators in CLA and placebo supplemented groups.
Values are expressed as mean with SEM in parenthesis

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Units</th>
<th>Week 0</th>
<th>Week 8</th>
<th>Week 0</th>
<th>Week 8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CLA</td>
<td>Placebo</td>
<td>CLA</td>
<td>Placebo</td>
</tr>
<tr>
<td>IL-10 (Con A)</td>
<td>pg/ml</td>
<td>587.43 (177.88)</td>
<td>384.83 (123.46)</td>
<td>517.90 (115.51)</td>
<td>487.72 (116.32)</td>
</tr>
<tr>
<td>TNFα</td>
<td>pg/ml</td>
<td>226.17 (77.37)</td>
<td>218.06 (61.63)</td>
<td>182.19 (59.52)</td>
<td>213.01 (91.01)</td>
</tr>
<tr>
<td>TNFα (Con A)</td>
<td>pg/ml</td>
<td>2041.49 (309.52)</td>
<td>2200.70 (401.33)</td>
<td>2015.76 (212.93)</td>
<td>1920.42 (243.43)</td>
</tr>
<tr>
<td>IL-6</td>
<td>pg/ml</td>
<td>2.75 (2.12)</td>
<td>2.10 (1.47)</td>
<td>3.48 (2.28)</td>
<td>3.94 (2.49)</td>
</tr>
<tr>
<td>CRP</td>
<td>mg/l</td>
<td>1.10 (0.27)</td>
<td>1.17 (0.30)</td>
<td>1.75 (0.54)</td>
<td>0.97 (0.25)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>mg/dl</td>
<td>279.85 (7.85)</td>
<td>278.43 (5.34)</td>
<td>281.99 (8.66)</td>
<td>284.15 (6.85)</td>
</tr>
</tbody>
</table>
Table 3A.4 Fasting plasma total cholesterol, LDL, HDL, TAG and glucose of CLA and placebo supplemented groups.
Values are expressed as mean with SEM in parenthesis. *indicates P<0.05 within group

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Units</th>
<th>CLA</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Week 0</td>
<td>Week 8</td>
</tr>
<tr>
<td>Total Cholesterol</td>
<td>mmol/L</td>
<td>5.64 (0.33)</td>
<td>5.59 (0.33)</td>
</tr>
<tr>
<td>LDL Cholesterol</td>
<td>mmol/L</td>
<td>4.01 (0.27)</td>
<td>3.87 (0.25)</td>
</tr>
<tr>
<td>HDL Cholesterol</td>
<td>mmol/L</td>
<td>1.17 (0.07)</td>
<td>1.16 (0.07)</td>
</tr>
<tr>
<td>TAG</td>
<td>mmol/L</td>
<td>1.61 (0.17)</td>
<td>1.56 (0.18)</td>
</tr>
<tr>
<td>Glucose</td>
<td>mmol/L</td>
<td>5.12 (0.13)</td>
<td>5.24 (0.15)</td>
</tr>
</tbody>
</table>
3A.4 DISCUSSION

In the present study *ex vivo* PBMC cytokine secretion was measured to investigate the effects of CLA supplementation on markers of the T helper lymphocyte (Th)1/Th2 inflammatory responses. CLA significantly reduced Con A-stimulated PBMC IL-2 secretion by 37.1% (P=0.02). IL-2 mRNA was also reduced by 14.4% following CLA supplementation but due to a large degree of subject variability at the transcriptional level this change was not statistically significant. These results indicate that CLA may modulate the IL-2 component of the Th1 inflammatory response. IL-2 is a pro-inflammatory cytokine. Elevated IL-2 levels are associated with chronic pro-inflammatory states and have been observed in patients with unstable angina (Mazzone *et al.* 1999). Interestingly, recent research has demonstrated that the common hypolipidemic drugs, statins and fibrates, inhibit the release of the Th1 cytokines IL-2 and IFNγ from lymphocytes of patients with primary type II dyslipidemia (Okopien *et al.* 2004). It has been demonstrated that injection of recombinant IL-2 induces a greater pro-atherogenic burden and injection of anti-IL-2 has a profound anti-atherogenic effect in apo-E knockout mice (Upadhya *et al.* 2004). There is increasing evidence that IL-2, an important classical Th1 inflammatory cytokine, may potentate atherosclerosis. Therefore nutritional interventions, which attenuate IL-2 secretion, as observed in the present CLA supplementation trial, may be considered beneficial.

IL-10 is a Th2 cytokine that inhibits Th1 proliferation, chemotaxis and expression of IL-2 and IFNγ (Mocellin *et al.* 2004). It is considered anti-inflammatory and protective against atherosclerosis (De Palma *et al.* 2003). In a model of porcine colitis CLA supplementation suppressed lymph node IL-10 expression, maintaining it at non-infected state levels (Hontecillas *et al.* 2002). Our study demonstrated that CLA had no effect on Con A-induced PBMC IL-10 secretion. This finding agrees with a recent publication which showed that neither c9, t11-CLA nor t10, c12-CLA had a significant effect on PBMC IL-10 production (Tricon *et al.* 2004).

TNFα promotes pro-inflammatory cytokine expression, activates monocytes and macrophages and increases host defences (Tracey & Cerami, 1994). In accordance with data from other human trials (Kelley *et al.* 2001; Albers *et al.* 2003; Tricon *et al.* 2004) our study showed that CLA did not directly affect PBMC TNFα secretion. However upon further analysis, when volunteers were ranked according to baseline plasma CRP concentrations, CLA significantly modulated TNFα secretion. CRP is a predictive risk
factor for CVD (Rerkasem et al. 2002; Albert et al. 2003). CLA supplementation had no effect on plasma CRP concentrations in this healthy male cohort not suffering from any pro-inflammatory diseases. Nevertheless when volunteers were ranked into “low” and “high” CRP groups according to baseline CRP concentrations (CRP<0.8mg/L and CRP≥0.8mg/L, respectively) TNFα secretion was significantly reduced (36.5%; P=0.01) after CLA supplementation in the high CRP group but significantly increased (40.5%; P=0.03) by CLA supplementation in the low CRP group (Figure 3A.2). This effect of CLA on TNFα according to baseline CRP levels was not evident in the placebo-supplemented groups. Interpretation of these trends may be limited by the number of volunteers in the high and low CRP groups (n=16 and n=12, respectively). Nevertheless this analysis indicates that subtleties in the anti-inflammatory effects of CLA, or any nutrient based immune regulator, may be affected by a background inflammatory profile that reflects an individual’s genotype and/or other environmental exposures.

Figure 3A.2 The effect of supplementation on Con A-stimulated PBMC TNFα secretion in the CLA group on the basis of high and low plasma CRP concentrations. CLA supplemented volunteers were ranked by their baseline plasma CRP concentrations as “high” (CRP≥0.8mg/L) or “low” (CRP<0.8mg/L) for comparison. Results are expressed as TNFα concentration (pg/ml) in supernatant at weeks 0 and 8. * indicates P≤0.05 significant difference within “high” or “low” group.
To date, relatively few studies have investigated the effects of CLA supplementation on markers of human immunity and all have been limited to relatively young subjects. The first showed no significant effect of CLA supplementation on PBMC TNFα, IL-1β, prostaglandin E₂ (PGE₂) and IL-2 production (Kelley et al. 2001). Albers et al. (2003) demonstrated that CLA may beneficially affect the initiation of an immune response to the hepatitis B vaccination but had no effects on ex vivo PBMC secretion of TNFα, IL-1β, IL-6, IFNγ, IL-2, IL-4 and PGE₂. More recently, Tricon et al. (2004) showed that c9, t11-CLA and t10, c12-CLA single-isomer supplementation resulted in a dose-dependent reduction in the mitogen-induced activation of T lymphocytes but had little effect on a range of cytokines. The minimal effects of CLA on immune function were observed despite significant increase in the CLA content of PBMC (Kelley et al. 2001; Albers et al. 2003; Burdge et al. 2004) following 8 to 12 weeks CLA supplementation. The present study demonstrated a significant increase in the c9, t11-CLA composition of plasma fatty acids following 8 weeks supplementation, indicating that an 8 week supplementary period is sufficient to achieve CLA incorporation into plasma and immune cells, with subsequent possible effects on markers of inflammation.

The difference in the reported effects of CLA supplementation on IL-2 production between the present and other human studies may relate to a number of experimental differences, including the nature of the pro-inflammatory mitogen, age profile of the cohort, etc. Con A was employed in the present study; other studies used PHA or LPS (Kelley et al. 2001; Albers et al. 2003; Nugent et al. 2005). Both Con A and PHA bind to the T idiotype portion of the TCR. However the effect of mitogens on CD3, a complex of five invariant polypeptide chains that form the non-antigenic-related portion of the TCR, is controversial (Licastro et al. 1993). While Con A binds to CD3 there is some debate as to whether PHA interacts with CD3. Also, Con A binds more effectively with CD2, another T lymphocyte surface glycoprotein, than PHA (Jason & Inge, 1996). This suggests different affinities for the TCR and other T cell surface molecules and, thus, mitogenic potencies. Indeed our group have shown that PHA-induced cytokine secretion was not significantly altered by CLA supplementation (Nugent et al. 2005). But in the present study Con A-induced IL-2 secretion was affected by CLA supplementation. It is also worthy to note that the mean age (31.5 ± 1.3 years) of the subjects in our latter study was considerably younger that the present group (49.4 ± 1.3 years). As age affects immune function and reactivity this could also account for differences between studies (Hamer & Meydani, 2001; Wu et al. 2003).
CLA had no significant effects on the systemic inflammatory mediators associated with CVD investigated in this study. IL-6 is produced by a variety of cell types including fibroblasts, endothelial cells, monocytes, T and B lymphocytes. It increases basal glucose uptake, alters insulin sensitivity, increases the release of adhesion molecules from endothelium, increases hepatic release of fibrinogen and regulates hepatic synthesis of CRP (Yudkin et al. 2000). It has been shown that CLA suppressed IFNγ-induced IL-6 production in RAW macrophages (Yu et al. 2002). The present study found no effect of CLA supplementation on serum IL-6 concentration. This finding agrees with the result of a recent study which showed that t10, c12-CLA supplementation had no effect on plasma IL-6 concentration in men with the metabolic syndrome (Riserus et al. 2002). That study also showed that t10, c12-CLA markedly increased CRP. Tricon et al. (2004) found neither the c9, t11-CLA nor t10, c12-CLA isomer affected serum CRP in healthy volunteers. There was no effect of the CLA mix on CRP in the present study, indicating that potential adverse effects of t10, c12-CLA may be limited to subjects with the metabolic syndrome. Also the lack of effect of CLA on CRP and fibrinogen may have been in consequence of the regulatory role of IL-6 on their hepatic synthesis and release.

CLA supplementation had no effect on plasma total, LDL or HDL cholesterol, TAG or glucose concentrations. Animal feeding studies have yielded inconsistencies in the effects of CLA on plasma lipids. A pro-atherogenic increase in the LDL:HDL ratio, a trend towards increased TAG, cholesterol and phosphatidlycholine in VLDL and LDL, without distinct changes in the HDL fraction, were observed in pigs fed a 1% energy multi-isomeric CLA diet for 6 weeks (Stangl et al. 1999). During an induced acute phase response, CLA exaggerated the rise in plasma and LDL cholesterol in hamsters not pre-fed a 0.3% cholesterol diet whereas plasma and liver cholesterol were reduced up to 40% in cholesterol-fed animals (Sher et al. 2003). However, hamsters fed a 0.06 – 1.1% energy CLA diet had significantly reduced concentrations of total cholesterol, non-HDL cholesterol and TAG compared to controls, with no effect on HDL cholesterol (Nicolosi et al. 1997). Lee et al. (1994) demonstrated a significantly reduced LDL cholesterol concentration in CLA-fed rabbits relative to control, also with no significant difference in HDL. C57BL/6 mice had significantly increased serum HDL:total cholesterol ratio following CLA feeding relative to linoleic acid-fed controls (Munday et al. 1999). Single isomer studies highlight the specificity of c9, t11-CLA and t10, c12-CLA effects on plasma lipid concentrations. t10, c12-CLA isomer has been
effective in lowering plasma TAG, total cholesterol and LDL cholesterol in hamster feeding studies relative to linoleic acid-fed control, whereas the c9, t11-CLA has had no effect (Gavino et al. 2000; Navarro et al. 2003). De Deckere et al. (1999) demonstrated that hamsters fed an isomeric CLA mix or t10, c12 CLA supplemented diet had decreased fasting LDL and HDL and increased VLDL-TAG relative to controls and c9, t11-CLA fed animals, indicating that t10, c12-CLA is the active isomer affecting lipid levels. However, Ob/Ob mice fed a c9, t11-CLA diet for five weeks showed significantly lower plasma TAG concentration than either t10, c12-CLA or linoleic acid-fed control mice (Noone et al. 2001). Supplementation studies in healthy humans have also produced mixed and isomer specific results. Supplementation with 3.9g/day of a multi-isomeric blend of CLA for 93 days had no effect on plasma cholesterol, LDL, HDL or TAG in a group of healthy female volunteers (Benito et al. 2001). Smedman & Vessby (2001) also demonstrated no effect of 4.2g/day 50:50 c9, t11-CLA and t10, c12-CLA supplementation for 12 weeks on plasma lipid concentrations.

Supplementation with 3g/day of 50:50 c9, t11-CLA and t10, c12-CLA isomers for 8 weeks significantly reduced plasma TAG concentrations (Noone et al. 2002). Noone et al. (2002) demonstrated that this effect was lost by an 80:20 c9, t11-CLA and t10, c12-CLA supplement and indicate that t10, c12-CLA is effective in lipid lowering. In agreement with the former studies, the present study found no effects of 50:50 c9, t11-CLA and t10, c12-CLA supplementation on fasting plasma lipids and glucose.

It is important to note that CLA has been associated with potentially adverse effects, particularly with respect to insulin sensitivity, lipid peroxidation and inflammatory markers in obese and type 2 diabetic subjects (Moloney et al. 2004; Riserus et al. 2004). In the present study none of the classical lipid related factors were adversely affected by CLA, an effect that probably reflects this cohort’s normoglycaemic state and low risk of impaired glucose tolerance and insulin resistance. On the basis of the parameters investigated in the present study, there were no indications of potential adverse health effects of supplementation with 2.2g CLA mix in a cohort of middle-aged healthy men.

In conclusion, this study showed that CLA decreased PBMC IL-2 production in Con A-stimulated PBMC. This suggests that CLA may modulate components of the Th1 inflammatory response. The potential benefits of an immuno-modulatory agent represent a fine balance between attenuating a chronic pro-inflammatory response, whilst not adversely affecting immune defences. A potential limitation of the present
study is the number of subjects. Ideally a study measuring the effect of the immune response should include up to 25 to 50 subjects to demonstrate statistical differences following CLA supplementation. More work is required to determine if CLA could have beneficial effects in models of Th1-mediated inflammatory conditions. Also the isomer-specific, and possibly age dependent, effects of CLA on inflammatory mediators associated with human disease need to be investigated in vivo.
3B. THE ISOMER-SPECIFIC EFFECTS OF CONJUGATED LINOLEIC ACID ON PRO-INFLAMMATORY CYTOKINE PRODUCTION FROM THP-1 MONOCYTES. This section presents experimental work contributed to an investigation of the effects of the CLA isomers on the NF-κB signalling pathway in LPS-activated monocytes (Nugent, 2003).

3B.1 INTRODUCTION

Current literature addressing the effects of CLA on cytokine expression has been reviewed in Chapter 3A. Published effects of CLA on the pro-inflammatory cytokines IL-1β, IL-6 and TNFα are limited and contrary. Mice fed a 1% isomer-unspecified CLA enriched diet for 8 weeks demonstrated significantly reduced concentrations of serum TNFα compared to linoleic acid controls (Akahoshi et al. 2002). CLA feeding reduced rat peritoneal macrophage TNFα and IL-6 production (Turek et al. 1998). c9, t10-CLA and t10, c12-CLA isomers were shown to suppress the in vitro mRNA and protein expression of IL-1β, IL-6 and TNFα from porcine PBMC (Changhua et al. 2005). Elsewhere, isomer-specific feeding of c9, t11-CLA and t10, c12-CLA significantly increased in vitro murine splenocyte TNFα and IL-6 (Kelley et al. 2002).

The human intervention trial, Chapter 3A, investigated the effects of an isomeric blend of CLA on mediators of inflammation associated with PBMC, specifically Con A-stimulated T lymphocytes. The present study investigates the specific effects of the two main bioactive CLA isomers, their non-conjugated parent linoleic acid and a saturated fatty acid, palmitic acid, on mediators of inflammation associated with human mononuclear cells. Monocytes comprise approximately 5 to 10% of PBMC (De Palma et al. 2003) and large volumes of blood are required for their isolation. In addition, variation in cytokine mRNA expression and secretion is great between individuals (Yaqoob et al. 1999; Grimble et al. 2002), as demonstrated in Chapter 3A. Therefore, the THP-1 cell line was employed to provide sufficient and uniform monocytes for the in vitro treatment of human mononuclear cells with the c9, t11-CLA and t10, c12-CLA isomers.
3B.2 METHODS

3B.2.1 Experimental Model
The human monocytic cell line, THP-1, was maintained as outlined in Chapter 2.3. Viable cells were enumerated by EBAO staining and seeded at a density of $1 \times 10^6$ cells/ml in routine media in 25ml tissue culture flask.

3B.2.2 Fatty acid treatments
Monocytes were treated with 100μmol/L c9, t11-CLA, t10, c12-CLA, linoleic acid, palmitic acid or DMSO for 48 hours in 25ml tissue culture flasks. 100μmol/L was previously determined as optimal by MTS cell viability assay (Nugent, 2003) (Chapter 2.9). After 48 hours, cells were enumerated and re-seeded at a density of $1 \times 10^6$ cells/ml and plated at 1ml/well in 24-well tissue culture plates.

3B.2.3 Cytokine secretion
Following activation of treated monocytes with 1μg/ml LPS for 6 and 24 hours, culture media was removed and centrifuged at 14,000g for 10 minutes in 1.5ml tubes. Supernatants were removed from the resulting monocyte pellets and stored at −80°C. Secretion of IL-6, IL-1β and TNFα into supernatant was quantified by ELISA (Chapter 2.13).

3B.2.4 Cytokine expression
RNA was extracted from the monocytes (Chapter 2.15). Briefly, 1ml TRIZOL® reagent (Invitrogen, Carlsbad, CA) was added to each well after cell culture media was removed, on ice, for 10 minutes. This homogenate was then transferred to the respective sample tube containing pelleted monocytes after supernatants were removed for ELISA analysis. RNA was extracted from the monocytes following manufacturer’s instructions. RNA yields were quantified spectrophotometrically and integrity assessed by visualisation of the 18s and 28s bands following denaturing agarose gel electrophoresis. RNA was DNase-treated and first strand cDNA synthesised by reverse transcription. TaqMan RT-PCR was performed for TNFα, IL-1β and IL-6 using pre-developed assay reagent kits (ABI Prism™ 7700 Sequence Detector, Applied Biosystems, Warrington, UK). After PCR, standard curves were constructed and the Ct readings for each of the unknown samples were used to calculate the amount of target gene or GAPDH relative
to the standard. For each sample, results were normalised by dividing the amount of target gene by the amount of GAPDH and expressed relative to DMSO vehicle control.

3B.2.5 Statistical analysis
Statistical analysis was performed with DataDesk 6.0 (Data Description Inc. NY). The distribution of data for each variable was assessed and variables transformed to normalise the distribution of data if necessary. Multiple comparisons were performed by one-way ANOVA. Individual differences were subsequently tested by Fisher’s least significant difference (LSD) test after demonstration of significant inter-group differences by ANOVA. A probability of $P \leq 0.05$ was considered statistically significant.

3B.3 RESULTS
3B.3.1 IL-1β secretion and transcription (Figure 3B.1)
IL-1β was not detected in the supernatants of unstimulated monocytes. IL-1β was not detected in the supernatants of LPS-stimulated monocytes treated with linoleic acid at 6 hours. IL-1β was detectable in all other supernatants at 6 and 24 hours activation with LPS. Palmitic acid significantly increased IL-1β secretion relative to DMSO at 6 hours (>10 fold, $P<0.000001$) and 24 hours (>2.5 fold, $P<0.000001$). Neither $c9$, $t11$-CLA nor $t10$, $c12$-CLA affected IL-1β secretion at 6 hours relative to DMSO. At 24 hours, IL-1β secretion was decreased relative to DMSO by $c9$, $t11$-CLA (72.8%, $P=0.00006$), $t10$, $c12$-CLA (39.8%, $P=0.02$) and linoleic acid (88.3%, $P<0.000001$). There was no significant difference in the potency of $c9$, $t11$-CLA and linoleic acid (Figure 3B.1a; 24 hour results).

Transcription of IL-1β was decreased relative to DMSO treated cells at 6 hours by $c9$, $t11$-CLA (28.6%) and significantly by $t10$, $c12$-CLA (38.9%, $P=0.01$). Neither linoleic nor palmitic acids had a significant effect on IL-1β mRNA expression relative to DMSO treated cells, although they were significantly higher relative to $t10$, $c12$-CLA treated cells ($P=0.02$ and $P=0.03$, respectively) (Figure 3B.1b).
Figure 3B.1 IL-1β secretion from (a) and transcription in (b) THP-1 monocytes treated with 100μmol/L DMSO □, c9, t11-CLA □, t10, c12-CLA □, linoleic acid (LA) □ and palmitic acid (PA) □. Monocytes were stimulated with 1μg/ml LPS for 6 and 24 hours. Secretion was quantified by ELISA at 24 hours and transcription semi-quantified by RT-PCR at 6 hours. (a) Results are expressed as IL-1β concentration (pg/ml) in supernatant. (b) The values expressed are normalised to GAPDH and relative to DMSO. Results represent the mean of 5-6 independent experiments.

* indicates P≤0.05, ** indicates P≤0.0001 relative to DMSO.

3B.3.2 IL-6 secretion and transcription (Figure 3B.2)
IL-6 was not detectable in the supernatants of unstimulated monocytes. IL-6 was detectable in the supernatants of LPS stimulated monocytes at 6 and 24 hours for all fatty acid treatments. At 6 and 24 hours only the t10, c12-CLA isomer treated monocytes secretion of IL-6 remained non-significantly different from DMSO cells. c9, t11-CLA treated monocytes secreted significantly less IL-6 relative to DMSO at 6 (37.5%, P=0.0001) and 24 (43.3%, P<0.000001) hours. Linoleic acid treated cells secreted significantly less IL-6 than DMSO cells at 6 (81.2%, P<0.000001) and 24

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(79%, P<0.000001) hours. Linoleic acid was significantly more potent than c9, t11-CLA at decreasing IL-6 at both time points. Palmitic acid significantly decreased IL-6 secretion relative to DMSO at 6 hours (75.3%, P<0.000001) with a potency not significantly different from linoleic and at 24 hours (41.5%, P<0.000001) with a potency not significantly different from c9, t11-CLA (Figure 3B.2a; 24 hour results).

Transcription of IL-6 was depressed relative to DMSO by c9, t11-CLA (27.7%, P=0.0008), t10, c12-CLA (38.5%, P=0.0001) and linoleic acid (33.3%, P=0.0003). IL-6 mRNA expression was significantly increased by palmitic acid relative to DMSO (38%, P=0.0003). There were no significant differences in the potencies of c9, t11-CLA, t10, c12-CLA and linoleic acid in their reduction of IL-6 mRNA expression relative to DMSO (Figure 3B.2b).

![Figure 3B.2a](image)

**Figure 3B.2a** IL-6 secretion from (a) and transcription in (b) THP-1 monocytes treated with 100μmol/L DMSO □, c9, t11-CLA □, t10, c12-CLA □, linoleic acid (LA) □ and palmitic acid (PA) ■. Monocytes were stimulated with 1μg/ml LPS for 6 and 24 hours. Secretion was quantified by ELISA at 24 hours and transcription semi-quantified by RT-PCR at 6 hours. (a) Results are expressed as IL-6 concentration (pg/ml) in supernatant. (b) The values expressed are normalised to GAPDH and relative to DMSO. Results represent the mean of 4-6 independent experiments.

* indicates P≤0.05, ** indicates P≤0.0001 relative to DMSO
3B.3.3 TNFα secretion (Figure 3B.3)

TNFα was not detected in the supernatants of unstimulated monocytes. TNFα was detected in the supernatants of LPS stimulated monocytes at 6 and 24 hours for all fatty acid treatments. At 6 hours TNFα secretion was significantly higher relative to DMSO controls from monocytes treated with \(c9, t11\)-CLA (32.7%, \(P=0.008\)), \(t10, c12\)-CLA (32.9%, \(P=0.008\)) and palmitic acid (89.9%, \(P<0.000001\)). There was no significant difference in potencies between the two CLA isomers, or between linoleic acid and DMSO treated cells. At 24 hours, there was no significant difference between secretion of TNFα from cells treated with \(c9, t11\)-CLA, \(t10, c12\)-CLA, linoleic or palmitic acids relative to DMSO treated controls. Palmitic acid lowered TNFα secretion significantly more than linoleic acid (\(P=0.002\)) (Figure 3B.3; 24 hour results). Because there was no extreme effect of fatty acid treatments on TNFα secretion, analysis of cDNA samples for TNFα gene transcription was not performed.

![Figure 3B.3 TNFα secretion from THP-1 monocytes treated with 100μmol/L DMSO □, \(c9, t11\)-CLA □, \(t10, c12\)-CLA □, linoleic acid (LA) □ and palmitic acid (PA) □. Monocytes were stimulated with 1μg/ml LPS for 6 and 24 hours. Secretion was quantified by ELISA at 24 hours. Results are expressed as TNFα concentration (pg/ml) in supernatant. Results represent the mean of 6 independent experiments.](image)

3B.4 DISCUSSION

Studies investigating the effects of CLA on inflammatory phenotypes tend to use preparations of mixed isomers, making it impossible to determine which isomer is responsible for observed effects (Field & Schley, 2004). In the present study, leukaemic monocytes were treated with \(c9, t11\)-CLA, \(t10, c12\)-CLA, linoleic and palmitic acids...
prior to activation with LPS. IL-1β secretion was significantly decreased at 24 hours of LPS activation by both CLA isomers and linoleic acid and IL-1β mRNA expression was significantly reduced by the \( t_{10}, c_{12} \)-CLA isomer. The \( c_{9}, t_{11} \)-CLA isomer had a non-significant suppressive effect on IL-1β transcription. Palmitic acid significantly increased secretion, but not transcription, of IL-1β relative to DMSO. The \( t_{10}, c_{12} \)-CLA isomer had no effect on IL-6 secretion. The \( c_{9}, t_{11} \)-CLA isomer, linoleic and palmitic acids significantly decreased IL-6 secretion, with linoleic acid being the most potent. Transcription of IL-6 was significantly suppressed by both CLA isomers and linoleic acid but was increased by palmitic acid. TNFα secretion was initially increased by the CLA isomers and palmitic acid but over the course of LPS activation there was no significant effect of any fatty acid treatment on monocyte production of TNFα.

Anti-inflammatory effects of the CLA isomers were observed for IL-1β and IL-6 secretion and transcription relative to DMSO control. Anti-inflammatory effects of linoleic acid were generally more potent than those of the CLA isomers. Linoleic acid is commonly used as a control for CLA and its isomers. The present findings indicate that linoleic acid may obscure the anti-inflammatory effects of the CLA isomers by its own capacity, if used as a control. Generally, the saturated fatty acid, palmitic acid, induced a pro-inflammatory phenotype in the monocyte.

The reported effects of CLA and linoleic acid on the production of pro-inflammatory cytokines are mixed. In accordance with the present findings Changhua et al. (2005) reported a suppressive effect of both bioactive CLA isomers on transcription and secretion of IL-1β in cultured porcine PBMC. CLA isomers inhibited IFNγ-induced IL-1β secretion from RAW 264.7 murine macrophages (Yu et al. 2002). CLA has been shown to decrease IL-6 secretion from rat peritoneal macrophages (Turek et al. 1998) and the bioactive isomers to each suppress IL-6 transcription and secretion in cultured porcine PBMC (Changhua et al. 2005). IL-6 secretion from RAW 264.7 murine macrophages was limited by CLA isomers (Yu et al. 2002). Linoleic acid inhibited IL-6 mRNA expression and protein secretion induced by palmitic acid in the myotubes of human skeletal muscle (Weigert et al. 2004). Elsewhere, IL-6 production from rat peritoneal macrophages has been correlated positively with intake of linoleic acid (Grimble & Tappia, 1998). Production of TNFα has been diminished in rat peritoneal macrophages by linoleic acid (Tappia et al. 1995), in murine serum by CLA feeding (Akahoshi et al. 2002), in cultured porcine PBMC treated with \( c_{9}, t_{11} \)-CLA and \( t_{10}, c_{12} \)-CLA (Changhua et al. 2005) and in CLA treated murine macrophages activated by
IFNγ and LPS (Yu et al. 2002; Yang & Cook, 2003). In contrast, Kelley et al. 2002, found the bioactive isomers of CLA to increase murine splenocyte TNFα secretion.

The present findings contributed to an investigation of the effects of the CLA isomers on the NF-κB signalling pathway in LPS-activated monocytes (Nugent, 2003). It has been demonstrated that c9, t11-CLA decreased LPS-induced NF-κB nuclear binding in THP-1 monocytes. In addition, fatty acids down-regulated NF-κB:DNA binding in the nucleus of PBMC with c9, t11-CLA>linoleic acid>t10, c12-CLA>palmitic acid in order of efficacy (Nugent, 2003). Pre-treatment of RAW 264.7 macrophages with CLA significantly reduced LPS-induced phosphorylated IkBα expression, NF-κB p65 nuclear expression and NF-κB nuclear binding activity (Cheng et al. 2004). Limited nuclear translocation of the p65 subunit suppresses the transcription of pro-inflammatory mediators such as IL-1β, IL-6 and TNFα. The anti-inflammatory effects of CLA may be due to an inhibition of NF-κB nuclear signalling.

In addition, CLA is a reported ligand of PPARα and γ (Moya-Cameron et al. 1999; Belury et al. 2002). CLA was shown to activate PPARγ by reporter assay and the regulation of iNOS by CLA was deposed by transfection of a dominant negative PPARγ into the macrophage model (Yu et al. 2002). PPARs possess anti-inflammatory activities (Chawla et al. 2001). Administration of WY14643, a PPARα ligand, impaired production of IFNγ, IL-6 and TNFα from ex vivo murine splenocytes (Cunard et al. 2002). Treatment of human endothelial cells with WY14643 or the PPARα agonist fenofibrate inhibited TNFα-induced VCAM-1 and reduced monocyte endothelial adhesion (Marx et al. 1999). PPARγ agonists, 15-deoxy-Δ-prostaglandin J2 and troglitazone, suppress monocyte elaboration of pro-inflammatory cytokines such as IL-2 and TNFα (Jiang et al. 1998). These PPARγ agonists inhibited the expression of VCAM-1 and ICAM-1 in activated endothelial cells and reduced monocyte and macrophage homing to atherosclerotic plaques in apo E-/- mice (Pasceri et al. 2000). PPARγ ligation inhibits macrophage expression of iNOS, MMP-9 and SCA. PPARγ inhibits gene expression in part by antagonising transcription factors such as AP-1, STAT and NF-κB (Ricote et al. 1998).

Treatment of THP-1 monocytes with the c9, t11-CLA and t10, c12-CLA isomers suppressed their pro-inflammatory phenotype upon activation with LPS. CLA has limited elements of the NF-κB signalling pathway. CLA may also exert its anti-inflammatory effects through PPAR activation.
CHAPTER 3: CONCLUSION

The human intervention trial, Chapter 3A, demonstrated that an isomeric blend of c9, t11-CLA and t10, c12-CLA reduced IL-2 secretion from ex vivo PBMC stimulated by Con A. In vitro investigation of the effects of c9, t11-CLA and t10, c12-CLA, Chapter 3B, demonstrated decreased IL-1β and IL-6 secretion and/or transcription induced by both isomers in LPS-activated human monocytes. The inflammatory responses elicited by Con A-activation of T lymphocytes and LPS-activation of monocytes in these studies offer a broad view of the anti-inflammatory potential of CLA in atherosclerosis. T lymphocytes, particularly CD4+ helper cells, constitute 5-20% of the inflammatory cells of the atherosclerotic plaque (De Palma et al. 2003). The CD4+ Th1 inflammatory response, producing and potentiated by IL-2 (Zhang & Nabel, 1994; Upadhya et al. 2004), is regarded as more aggressive than the alternative CD4+ Th2 inflammatory response. Furthermore, IL-2 activates monocytes (Espinoza-Delgado et al. 1995; Bosco et al. 1995). Down-regulation of IL-2 from PBMC by CLA indicates potential for inducing a less inflammatory phenotype in circulating and lesional lymphocytes. The monocyte and monocyte-derived macrophage is the predominant inflammatory cell of the atherosclerotic plaque and acts in an antigen presenting capacity to T lymphocytes. The classically activated monocyte/macrophage also has an aggressive inflammatory phenotype. TNFα and IL-1β produced by activation elicit further cytokine secretion and immune cell activation. IL-6 acts as a pro-inflammatory growth factor for other cells, increasing expression of adhesion molecule from endothelium and acute phase reactants from hepatocytes (Yudkin et al. 2000). In the present study, CLA reduced the aggressive inflammatory phenotype of activated monocytes by reducing IL-1β and IL-6 production.

The present studies indicate that CLA and its two main bioactive isomers decrease the expression and/or secretion of pro-inflammatory cytokines from immune cells that transmigrate from peripheral blood and become activated in the lesional microenvironment during atherosclerosis.
Chapter 4

The experimental work presented in this chapter contributes to work-package 1.3 of Lipgene, an EU 6th framework project.
4. EFFECTS OF FATTY ACIDS, REPRESENTING SATURATED AND UNSATURATED SERUM NEFA, ON MACROPHAGE INFLAMMATORY MEDIATORS ASSOCIATED WITH ATHEROSCLEROSIS

4.1. INTRODUCTION

Fatty acids are principally transported in the circulation esterified in lipoprotein vehicles. A proportion, however, remains non-esterified and is referred to as NEFA. In the fed state NEFA are produced by the hydrolysis of TRL at the endothelium. In the fasted state NEFA originate primarily from adipose tissue, where their deposition and mobilisation appear to be selective (Connor et al. 1996; Halliwell et al. 1996; Raclot et al. 1997). Circulating NEFA are associated with adverse health effects in the majority of published research. Their elevation has been associated with abdominal obesity, insulin resistance (Pankow et al. 2004) and increased risk of CVD (Yli-Jama et al. 2002a; Tripathy, 2003). Furthermore, the composition of circulating NEFA is markedly different between healthy subjects and those with metabolic states predisposing to CVD (Boden & Chen 1999; Yli-Jama et al. 2001; Fernandez-Real et al. 2003).

Serum concentrations of NEFA in healthy subjects have been reported at 380\(\mu\)mol/L (Yli-Jama et al. 2001), 420\(\mu\)mol/L (Yli-Jama et al. 2002b) and 630\(\mu\)mol/L (Skowronski et al. 1991). Subjects with established cardiovascular disease had a mean circulating NEFA concentration of 420\(\mu\)mol/L (Yli-Jama et al. 2001). The mean concentration of NEFA in the serum of Type 1 diabetic subjects was reported at 590\(\mu\)mol/L (Desideri-Vaillant et al. 2004). Type 2 diabetic subjects had serum NEFA concentrations ranging from 700\(\mu\)mol/L (Desideri-Vaillant et al. 2004) to 842\(\mu\)mol/L (Skowronski et al. 1991). Circulating NEFA concentrations are experimentally elevated by injection with ACTH (Connor et al. 1996), nicotinic acid and IV infusion of lipid/heparin (Boden & Chen, 1999), and fall markedly with induced physiological hyperinsulinaemia (78% in Type 2 diabetics and 83% in non-diabetic controls) (Skowronski et al. 1991). The principal fatty acids of serum NEFA from healthy subjects and overweight, post-MI and Type 2 diabetic subjects, representing groups at risk of atherosclerosis, are presented in Table 4.1. Data has been presented as percentage fatty acid composition of total serum NEFA, rather than molar concentrations, as it allows direct comparison of content to be made between subject groups, between component fatty acids and between published articles. It also allows greater understanding of serum NEFA distributions in health and disease.
Table 4.1 The percentage composition of the principal fatty acids of serum NEFA from healthy, overweight, post myocardial infarction (MI) and Type 2 diabetic subjects. Table adapted from references

<table>
<thead>
<tr>
<th>Subject group</th>
<th>Palmitic acid C16:0</th>
<th>Stearic acid C18:0</th>
<th>Oleic acid C18:1n-9</th>
<th>Linoleic acid C18:2n-6</th>
<th>EPA C20:5n-3</th>
<th>DHA C22:6n-3</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>♂ Lean</td>
<td>20.1</td>
<td>7.7</td>
<td>20.7</td>
<td>31.4</td>
<td>0.67</td>
<td>2.1</td>
<td>Fernandez-Real et al. 2003</td>
</tr>
<tr>
<td>♀ Lean</td>
<td>18.9</td>
<td>7.7</td>
<td>21.0</td>
<td>31.9</td>
<td>0.6</td>
<td>2.1</td>
<td>Fernandez-Real et al. 2003</td>
</tr>
<tr>
<td>♂ Overweight</td>
<td>19.6</td>
<td>7.8</td>
<td>21.0</td>
<td>32.1</td>
<td>0.4</td>
<td>2.0</td>
<td>Fernandez-Real et al. 2003</td>
</tr>
<tr>
<td>♀ Overweight</td>
<td>18.9</td>
<td>7.5</td>
<td>22.4</td>
<td>32.1</td>
<td>0.5</td>
<td>1.8</td>
<td>Fernandez-Real et al. 2003</td>
</tr>
<tr>
<td>non-CVD controls</td>
<td>27.0</td>
<td>10.5</td>
<td>35.5</td>
<td>13.8</td>
<td>0.3</td>
<td>1.3</td>
<td>Yli-Jama et al. 2001</td>
</tr>
<tr>
<td>♂ CVD risk</td>
<td>27.4</td>
<td>10.2</td>
<td>36.0</td>
<td>14.0</td>
<td>0.3</td>
<td>1.0</td>
<td>Yli-Jama et al. 2002a</td>
</tr>
<tr>
<td>Post MI</td>
<td>26.8</td>
<td>9.2</td>
<td>37.1</td>
<td>14.6</td>
<td>0.2</td>
<td>0.8</td>
<td>Yli-Jama et al. 2001</td>
</tr>
<tr>
<td>NIDDM (basal)</td>
<td>26.6</td>
<td>18.1</td>
<td>25.9</td>
<td>11.8</td>
<td>-</td>
<td>-</td>
<td>Boden &amp; Chen, 1999</td>
</tr>
<tr>
<td>NIDDM (post lipid/heparin infusion)</td>
<td>21.0</td>
<td>15.3</td>
<td>20.1</td>
<td>25.7</td>
<td>-</td>
<td>-</td>
<td>Boden &amp; Chen, 1999</td>
</tr>
</tbody>
</table>
Elevated NEFA, and their failed suppression, are involved in the development of Type 2 diabetes (Skowronski et al. 1991; Butler et al. 2001; Jove et al. 2005). NEFA cause peripheral and hepatic insulin resistance (Boden & Chen, 1999; Boden et al. 2003; Pankow et al. 2004), and reduce insulin-stimulated glucose uptake (Steinberg & Baron, 2002; Tripathy et al. 2003). NEFA may limit insulin signalling, secretion or access to skeletal muscle by toxicity to pancreatic β cells, as reported in Zucker rats (Butler et al. 2001; Pankow et al. 2004). It has been shown that post-lipolytic serum, a model of NEFA, is cytotoxic to cultured endothelial cells and macrophages, a model of the arterial wall (Chung et al. 1998). Interestingly, PUFA-rich post-lipolytic serum was more disruptive to endothelial barrier function, or cytotoxic to the culture model, than SFA-rich post-lipolytic serum. This toxicity is due to the detergent-like properties of fatty acids, which alter integrity of cell membrane and disrupt membrane-bound enzymes, receptors and transport systems (Chung et al. 1998).

Elevated circulating catecholamines, as occurs in autonomic nervous system dysfunction, can induce excessive lipolysis of adipose tissue and may stimulate NEFA-mediated insulin resistance. Increased hepatic flux of NEFA stimulates gluconeogenesis (Pankow et al. 2004). Acute elevation of NEFA, and their hepatic esterification, induces increased VLDL synthesis (Castro Cabezas, 2003; Julius, 2003). VLDL overproduction is the metabolic basis of familial combined hyperlipidaemia and its delayed clearance leads to increased interaction with the endothelium (Castro Cabezas, 2003). Oleic acid has been shown to stimulate hepatic VLDL production. The n-3 LC-PUFA impair VLDL assembly and secretion (Julius, 2003). In Type 2 diabetes, NEFA are significantly correlated with BMI, fasting and non-fasting glucose, cholesterol and TAG concentrations, but not age, duration of disease, HbA1C or lipoprotein (a) concentrations (Desideri-Valliant et al. 2004). Serum NEFA from subjects with established CVD correlated strongly with lipid accumulation in cultured arterial smooth muscle cells whereas serum cholesterol or TAG concentrations did not (Chung et al. 1995).

Five polymorphisms identified on the CD36 gene have been collectively associated in human males with 30% higher serum NEFA, 20% higher TAG concentration and increased risk of coronary artery disease compared to non-carrier controls (Ma et al. 2004). An exon 1 G→A polymorphism on the SRB-I gene is associated with lower circulating NEFA concentrations and carriers of the A allele, after consuming a MUFA-rich diet, display increased insulin sensitivity (Perez-Martinez et
Homozygotic carriers of the -514C allele of the hepatic lipase gene display lower circulating NEFA concentrations after consumption of a Mediterranean compared to a SFA-rich diet (Gómez et al. 2005). The common G→A polymorphism at codon 54 of the fatty acid-binding protein (FABP)-2 gene is associated with insulin resistance and, after consuming a SFA-rich diet, an increase in NEFA concentrations (Marin et al. 2005).

Elevated NEFA have been associated with coronary arrhythmia, MI and sudden death. During the normal post-prandial state the vascular endothelial is exposed to lipoproteins. Their hydrolysis to NEFA at endothelial sites by LPL may be proximally atherogenic (Weintraub et al. 1988; Berstad et al. 2003). Furthermore, the vasculature of insulin resistant subjects is constantly exposed to elevated NEFA concentrations (Steinberg & Baron, 2002). NEFA increase transmigration of macromolecules across the endothelium, induce foam cell formation (Chung et al. 1995) and the chronic inflammatory response predisposing to atherosclerosis, as outlined in Chapter 1. In vitro studies have shown that enrichment of culture media with NEFA can induce foam cell formation in cultured cells including macrophages (Chung et al. 1995). The percentage of very long chain n-3 fatty acids in serum NEFA has been shown to be inversely related to the risk of first MI (Yli-Jama et al. 2002a).

Elevated NEFA, particularly oleic acid (Berstad et al. 2003), impair endothelial-dependent vasodilation (Yu et al. 2001). They reduce release of the vascular relaxant nitric oxide (NO) (Tripathy et al. 2003) and are associated with reduced post-ischaemic flow-mediated vasodilatation of the brachial artery (Tripathy, 2003). It is postulated that NEFA contribute to reactive oxygen species generation from vascular endothelial and smooth muscle cells (Tripathy, 2003).

A number of studies have investigated the effects of NEFA on serum, leukocyte, endothelial, skeletal and smooth muscle mediators of inflammation associated with insulin resistance and atherosclerosis. Increased NF-κB binding to DNA and reduced IkB expression was observed in mononuclear cells of obese subjects with elevated serum NEFA, relative to normal weight controls (Ghanim et al. 2004). Acute elevation of NEFA, induced by lipid/heparin infusion, induced a rapid increase in nuclear p65 and NF-κB binding in mononuclear cells (Tripathy, 2003; Aljada et al. 2004). Palmitic acid increased NF-κB activation in cultured skeletal muscle cells (Jove et al. 2005). Non-esterified palmitic, oleic and linoleic acids increased, in a dose-dependent manner, IKK activity in cultured bovine aortic endothelial cells (Kim et al. 2005).
IL-6, TNFα and MMP-9 mRNA expression and protein production, and plasma CRP concentration, were significantly increased in obese human subjects with elevated serum NEFA relative to normal weight controls (Ghanim et al. 2004). Ingestion of a test meal decreased plasma levels of both IL-6 and TNFα post-prandially. This effect may have been mediated by the concomitant post-prandial increase in plasma insulin and decrease in serum NEFA (Manning et al. 2004) described earlier (Roche et al. 1998). Palmitic acid has been shown to induce IL-6 mRNA expression and secretion, reduce GLUT4 mRNA expression and reduce insulin-stimulated glucose uptake in cultured skeletal muscle cells (Jove et al. 2005). Palmitic acid induced IL-6 mRNA expression in cultured endothelial and smooth muscle cells (Staiger et al. 2004). IL-6 and TNFα production appear to be induced by serum NEFA and reduced by insulin-mediated suppression of serum NEFA. These pro-inflammatory cytokines may also induce elevation of serum NEFA. TNFα stimulates hepatic de novo fatty acid synthesis and, in chow-fed rats, has increased serum NEFA concentration (Feingold et al. 1990). IL-6, produced by macrophages and skeletal muscle (Jove et al. 2005), is associated in humans with increased plasma fatty acid, fasting TAG and fasting VLDL TAG concentrations (Fernandez-Real et al. 2003). Elevated NEFA induce hepatic TAG secretion by IL-6 (Fernandez-Real et al. 2003; Pankow et al. 2004). Plasma IL-6 correlates with NEFA palmitic acid concentration (Staiger et al. 2004) and may mediate pro-diabetic effects of palmitic acid (Jove et al. 2005).

In studies of n-3 LC-PUFA supplementation, significant positive correlations have been found between increases in plasma non-esterified DHA concentration and sVCAM-1, but not with increase in EPA (Berstad et al. 2003). A significant inverse linear association between plasma non-esterified EPA, DHA and arachidonic acid and sVCAM-1 and sICAM-1 existed normally in the study group, prior to n-3 LC-PUFA supplementation (Yli-Jama et al. 2002a; Berstad et al. 2003) These results indicate a general anti-inflammatory effect of the n-3 LC-PUFA component of serum NEFA with a potential pro-inflammatory effect following DHA supplementation.

It is well established that elevated serum NEFA is characteristic and causative in inflammatory and metabolic stress predisposing to CVD. The composition of serum NEFA also shapes the atherogenic phenotype. SFA, particularly palmitic acid, induce pro-inflammatory transcription factor activity and cytokine production. MUFA, namely oleic acid, increases pro-inflammatory transcription elements, inhibits endothelial-dependent vasodilation and stimulates hepatic VLDL production. The n-3 LC-PUFA
component of NEFA reduces hepatic VLDL production and is inversely associated with CVD. The aim of the present study was to investigate the effects of NEFA on macrophage mediators of inflammation. An LPS-activated macrophage model was used to represent the principal inflammatory cell of the human atherosclerotic plaque. Three fatty acid blends were designed to reflect serum NEFA typical of metabolic states predisposing to CVD, with gradual replacement of SFA with MUFA and n-3 LC-PUFA. The toxicity of the fatty acid blends was assessed. The effects of a range of LPS concentrations on macrophage activation were determined. The effects of the fatty acid blends on transcription and secretion of IL-1β, IL-6 and TNFα and activity of MMP-9 were investigated.

4.2 METHODS

4.2.1 Experimental model

The human monocytic cell line, THP-1, was maintained as outlined in Chapter 2.3. Viable cells were enumerated by EB/AO staining and seeded at a density of $1 \times 10^6$ cells/ml in routine media. Monocytes were plated at 1ml/well in 24-well tissue culture plates (Nunc, Roskilde, Denmark) and differentiated to macrophages by the addition of 0.1μg/ml PMA (Sigma, Saint Louis, MI, USA) for 72 hours. Routine media was removed, cells were washed twice with pre-warmed HBSS (Gibco, Grand Island, New York) and maintained for 24 hours in serum-free media, supplemented with 1% Nutridoma (Roche Molecular Biochemicals, IN, USA) to remove serum-derived fatty acids from the system. Cells were then treated with fatty acids and DMSO vehicle control for 48 hours prior to activation with LPS (Sigma, Saint Louis, MI, USA).

4.2.2 NEFA treatments

The objective of this study was to mimic, as far as possible, the exposure of macrophages to serum NEFA. Physiological serum NEFA composition and concentration were addressed and fatty acid treatments designed on their basis.

4.2.2a Treatment composition

Serum NEFA compositions were studied from the literature reviewed in the introduction (Table 4.1). Three blends of fatty acids with increasing degrees of unsaturation were designed and designated SFA, MUFA and MUFA + n-3 PUFA. They
compositionally reflected the circulating NEFA reported in metabolic states predisposing to CVD, with a gradual replacement of SFA by MUFA and n-3 PUFA.

4.2.2b Treatment concentration
A cell viability assay determined an appropriate and optimal treatment concentration for the THP-1 derived macrophage model. $1 \times 10^5$ monocytes were plated per well in 96-well microtitre plates (100μl of $1 \times 10^6$ cells/ml suspension), differentiated to macrophages and serum-starved prior to treatment with 25, 50, 100, 250 and 500μmol/L of SFA, MUFA, MUFA + n-3 PUFA or DMSO vehicle control. Control wells containing cells alone and media alone were also included. The viability of cells at 6, 12 and 24 hour time points were quantified by the MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega Corporation, WI, USA) as per manufacturer’s protocol (Chapter 2.9). Briefly, 10μl of MTS solution was added per well and incubated at 37°C for 2 hours in a humidified, 5% CO$_2$ atmosphere. Reduction of the yellow MTT tetrazolium salt to dark-blue formazan by the dehydrogenases and oxidases of metabolically active cells was measured spectrophotometrically at 570nm. The optical density of treatment wells compared to cells alone, and blanked to media, was considered a direct indication of cell viability and expressed as a percentage.

4.2.3 LPS dose-response and cytokine secretion
Secretion of pro-inflammatory mediators was used to investigate and determine the optimal dose of LPS for macrophage activation in this model. The secretion of IL-1β, IL-6 and TNFα from treated macrophages stimulated with 0, 0.001, 0.01, 0.1 and 1μg/ml of LPS for 24 hours was investigated by ELISA (Chapter 2.13).

4.2.4 Gene expression analysis
RNA was extracted from the adherent macrophages at 6 hours of LPS activation using TRIzol® reagent (GIBCOBRL, Life Technologies™) following the manufacturer’s instructions (Chapter 2.15). RNA yields were quantified spectrophotometrically and integrity assessed by visualisation of the 18s and 28s bands following denaturing agarose gel electrophoresis. RNA was DNase-treated and first strand cDNA synthesised by reverse transcription. TaqMan RT-PCR was performed for IL-1β, IL-6 and TNFα using pre-developed assay reagent kits (Assays-on-Demand; Applied Biosystems, Warrington, UK). After PCR, standard curves were constructed and the Ct readings for
each of the unknown samples were used to calculate the amount of target gene or GAPDH relative to the standard. For each sample, results were normalised by dividing the amount of target gene by the amount of GAPDH and expressed relative to DMSO vehicle control.

4.2.5 MMP gelatinase activity

The activity of secreted MMP-2 and MMP-9 was measured in the model supernatants by gelatin zymography (Chapter 2.14). Briefly, a gelatin-impregnated gel was loaded with sample in loading dye and run by SDS-PAGE. The gels were then incubated in Triton X, which removes SDS and renatures the gelatinases. Their subsequent proteolytic activity upon their substrate was revealed by Coomassie blue staining of the gels. Sites of proteolysis appeared as white bands on a dark blue background. Gels were imaged with GeneGenius and GeneSnap (Syngene, Cambridge UK). The image was then transferred to GeneTools Analysis Software (Syngene, Cambridge UK) for densitometric analysis.

4.2.6 Statistical analysis

Statistical analysis was performed with DataDesk 6.0 (Data Description Inc. NY). The distribution of data for each variable was assessed and variables transformed to normalise the distribution of data if necessary. Multiple comparisons were performed by one-way ANOVA. Individual differences were subsequently tested by Fisher's least significant difference (LSD) test after demonstration of significant inter-group differences by ANOVA. A probability of $P \leq 0.05$ was considered statistically significant.
4.3 RESULTS

4.3.1 Design of treatment composition (Table 4.2)

A number of references were compiled to investigate and compare the percentage composition of the principal fatty acids of serum NEFA from healthy, overweight, post MI and Type 2 diabetic subjects (Table 4.1). In subjects predisposed to developing CVD palmitic acid (C16:0) and stearic acid (C18:0) comprised 18.9 to 27.4% and 7.5 to 18.1% of total NEFA composition, respectively. In healthy controls C16:0 and C18:0 comprised 18.9 to 27.0% and 7.7 to 10.5% of total NEFA composition, respectively. The ratio of C16:0 to C18:0 in healthy, overweight, post MI and subjects at risk of CVD ranged from 2.5 to 2.9: 1. This ratio was reduced, 1.4 to 1.5: 1 in Type 2 diabetics. Oleic acid (C18:1n-9) ranged from 20.1 to 37.1% of total NEFA composition, being lowest in lean control and post lipid/heparin infused Type 2 diabetics. Linoleic acid (C18:2n-6) ranged from 11.8 to 32.1% of total NEFA composition. EPA (C20:5n-3) and DHA (C22:6n-3) ranged from 0.2 to 0.7 % and 0.8 to 2.1% of total NEFA composition, respectively.

Three fatty acid treatments, designated SFA, MUFA and MUFA + n-3 PUFA, were designed to reflect extremes in physiological serum NEFA composition (Table 4.2, overleaf). The percentage composition of C16:0 in the SFA, MUFA and MUFA + n-3 PUFA treatments was 30, 18 and 15 (w/w %), respectively. The percentage composition of C18:0 in the SFA, MUFA and MUFA + n-3 PUFA treatments was 20, 12 and 10 (w/w %), respectively. C16:0 and C18:0 were maintained in the fatty acid treatments at a 1.5: 1 ratio (reflecting that reported in Type 2 diabetics). The percentage composition of C18:1n-9 in the SFA, MUFA and MUFA + n-3 PUFA treatments was 20, 40 and 40 (w/w %), respectively. The percentage composition of C20:5n-3 and C22:6n-3 in the SFA, MUFA and MUFA + n-3 PUFA treatments were 0, 0 and 2.5 (w/w %), respectively.
Table 4.2 Composition of experimental NEFA treatments.

Treatments were designed, following a literature review, to represent the composition of serum NEFA of subjects predisposed to CVD. Fatty acid treatments were designated SFA, MUFA and MUFA + n-3 PUFA. The control was the fatty acid vehicle DMSO. MUFA (Oleic acid) and n-3 LC-PUFA (Eicosapentanoic and Docosahexanoic acids) are added at the expense of SFA (Stearic and Palmitic acids) Stearic and Palmitic acid are kept at the same ratio, 1:1.5, in each treatment).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>SFA</th>
<th>MUFA</th>
<th>MUFA + n-3 PUFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18:1n-9 Oleic acid</td>
<td>20</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>C18:0 Stearic acid</td>
<td>20</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>C16:0 Palmitic acid</td>
<td>30</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>C20:5n-3 Eicosapentanoic acid</td>
<td>-</td>
<td>-</td>
<td>2.5</td>
</tr>
<tr>
<td>C22:6n-3 Docosahexanoic acid</td>
<td>-</td>
<td>-</td>
<td>2.5</td>
</tr>
<tr>
<td>C18:2n-6 Linoleic acid</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

4.3.2 Dose-response effect of SFA, MUFA, MUFA + n-3 PUFA and DMSO on macrophage viability (Figure 4.1)

The viability of THP-1 derived macrophages was assessed following treatment with a range of concentrations of SFA, MUFA, MUFA + n-3 PUFA and DMSO (500, 250, 100, 50 and 25μmol/L) over a series of time points (6, 12 and 24 hours). Viability was interpreted as the expression of formazan stain, measured spectrophotometrically, of treated cells relative to untreated cells, presumed to be 100% viable. Results were consistent for 6, 12 and 24 hours and are reported for the 24 hour time point only (Figure 4.1). Cells treated with 500μmol/L SFA, MUFA and MUFA + n-3 PUFA were significantly less viable than untreated cells (P<0.001). SFA and MUFA treated cells were not significantly and/or negatively different from cells alone for the lower concentrations of fatty acids. The MUFA + n-3 PUFA treated cells were significantly less viable than cell alone at 250μmol/L (P=0.000004) and 100μmol/L (P<0.000001). Viability was not significantly different between cells alone and those treated with 50μmol/L and 25μmol/L MUFA + n-3 PUFA. DMSO treatment did not significantly and/or negatively affect cell viability at any dose or time point. The results indicate that 50μmol/L was an appropriate concentration of fatty acid and DMSO treatment for the THP-1 derived macrophage model. This was confirmed when cells treated with 50μmol/L fatty acids or DMSO for 72 hours (the maximal length of incubation in the
present experimental model) showed no significant differences in viability with untreated cells. Therefore, for all subsequent experiments of this chapter cells were treated with 50μmol/L of SFA, MUFA, MUFA + n-3 PUFA or DMSO.

**Figure 4.1** The effects of NEFA treatments on cell viability at 24 hours by MTS assay. THP-1 derived macrophages were treated with 500, 250, 100, 50 and 25 μmol/L DMSO □, SFA ■, MUFA □ and MUFA + n-3 PUFA □. Cell viability, as indicated by comparative formazan staining with untreated cells, was analysed spectrophotometrically. The values expressed are normalised to complete (100%) viability of untreated cells. Results represent the mean ± SEM of 5 independent experiments.

* indicates P≤0.001 relative to the complete viability of untreated cells at each time point.

### 4.3.3 Dose-response effects of LPS activation (0-1μg/ml) (Figure 4.2, page 160)

#### 4.3.3a IL-1β secretion (Figure 4.2a)

4.3.3a(i) Effects of SFA, MUFA and MUFA + n-3 PUFA relative to DMSO

IL-1β was detected in the supernatants of resting (non-LPS stimulated) macrophages but its concentration was not significantly different between SFA, MUFA, MUFA + n-3 PUFA and DMSO treated cells. MUFA treatment significantly increased IL-1β secretion from macrophages relative to DMSO at 0.001μg/ml LPS (P=0.03). Fatty acid treatments had no significant effects on IL-1β secretion relative to DMSO at 0.01μg/ml LPS. SFA and MUFA treatments significantly increased IL-1β secretion relative to DMSO from macrophages activated with 0.1μg/ml LPS (P=0.03 and P=0.04, respectively). All fatty acid treatments significantly increased IL-1β secretion relative to DMSO from macrophages activated with 1μg/ml LPS (P≤0.0001 for SFA, MUFA and MUFA + n-3 PUFA).
4.3.3a(ii) LPS effects within DMSO, SFA, MUFA and MUFA + n-3 PUFA groups

Secretion of IL-1β from DMSO treated cells significantly increased from resting levels when activated with 0.01 μg/ml LPS (P=0.006). There was no further significant increase in IL-1β secretion from DMSO treated cells with subsequent concentrations (0.1 μg/ml and 1 μg/ml) of LPS. Secretion of IL-1β from SFA treated cells significantly increased from resting levels when activated with 0.01 μg/ml LPS (P=0.001) and significantly increased further with 0.1 μg/ml and 1 μg/ml LPS (P=0.001 and P=0.02). Secretion of IL-1β from MUFA treated cells increased to significance with activation by 0.001 μg/ml LPS (P=0.003) and significantly increased further from preceding concentrations at 0.01 μg/ml (P=0.008), 0.1 μg/ml (P=0.003) and 1 μg/ml (P=0.004) LPS. Secretion of IL-1β from MUFA + n-3 PUFA treated cells significantly increased from resting levels at 0.01 μg/ml LPS (P=0.004) and significantly increased further at 0.1 μg/ml (P=0.004). IL-1β from MUFA + n-3 PUFA treated cells did not increase with heightened LPS activation (1 μg/ml).

Effective activation of macrophages, determined by significant increases in IL-1β secretion, was initiated and appeared to plateau at different LPS concentrations in the treatment groups. DMSO, SFA and MUFA + n-3 PUFA treated macrophages appeared to be significantly activated at 0.01 μg/ml, whereas MUFA treated macrophages were significantly activated at a more dilute concentration of LPS (0.001 μg/ml). DMSO and MUFA + n-3 PUFA treated macrophages were maximally activated at 0.01 μg/ml and 0.1 μg/ml LPS, respectively. SFA and MUFA treated macrophages continued to achieve significantly greater secretion of IL-1β up to the maximal concentration of LPS used.

4.3.3b IL-6 secretion (Figure 4.2b)

4.3.3b(i) Effects of SFA, MUFA and MUFA + n-3 PUFA relative to DMSO

IL-6 was minimally detected in the supernatants of resting (non-LPS stimulated) macrophages treated with SFA, MUFA and DMSO, but not MUFA + n-3 PUFA, and its concentrations were not significantly different between treatments (not represented on Figure 4.2b). Fatty acid treatments had no significant effect on IL-6 secretion from macrophage relative to DMSO when activated with 0.001 μg/ml and 0.01 μg/ml LPS. SFA, MUFA and MUFA + n-3 PUFA treatments significantly decreased IL-6 secretion relative to DMSO when stimulated with 0.1 μg/ml LPS (P<0.000001 for SFA, MUFA and MUFA + n-3 PUFA). SFA and MUFA + n-3 PUFA treatments significantly
decreased IL-6 secretion from macrophages relative to DMSO when stimulated with 1µg/ml LPS (P=0.01 and P=0.002, respectively) and MUFA failed to exert the significant decrease in IL-6 secretion observed at the preceding concentration of LPS.

4.3.3b(ii) LPS effects within DMSO, SFA, MUFA and MUFA + n-3 PUFA groups
Secretion of IL-6 from DMSO treated cells significantly increased from resting levels when activated with 0.01µg/ml LPS (P=0.006), and further increased from immediately preceding concentrations at 0.1µg/ml (P=0.006) and 1µg/ml (P=0.03) LPS. Secretion of IL-6 from SFA increased to significance with activation by 0.1µg/ml LPS and significantly increased further with activation by 1µg/ml (P=0.001). Secretion of IL-6 from MUFA and MUFA + n-3 PUFA treated cells significantly increased from an immediately preceding concentration of LPS only at 1µg/ml (P=0.00002 and P=0.008, respectively).

Effective activation of macrophages, determined by significant increases in IL-6 secretion, depended upon different LPS concentrations in the treatment groups. DMSO treated cells appeared to be initially significantly activated with 0.01µg/ml LPS and IL-6 secretion continued to be significantly increased in an LPS dose-dependent manner. IL-6 secretion from cells treated with SFA required a higher concentration of LPS for significant activation (0.1µg/ml) and secretion further increased significantly with the maximal concentration of LPS. IL-6 secretion increased from MUFA and MUFA + n-3 PUFA treated macrophages with sequential concentrations of LPS but only at 1µg/ml LPS was this response significant from the preceding concentration.

4.3.3c TNFα secretion (Figure 4.2c)
4.3.3c(i) Effects of SFA, MUFA and MUFA + n-3 PUFA relative to DMSO
TNFα was detected in the supernatants of resting (non-LPS stimulated) macrophages but its concentration was not significantly different between SFA, MUFA, MUFA + n-3 PUFA and DMSO treated cells. SFA, MUFA and MUFA + n-3 PUFA treatments had no effect on secretion of TNFα from macrophages activated with 0.001µg/ml LPS relative to DMSO. TNFα secretion was significantly decreased from SFA and MUFA + n-3 PUFA treated macrophages relative to DMSO at 0.01µg/ml LPS (P=0.002 and P=0.001, respectively) and remained significantly decreased from n-3 PUFA treated macrophages relative to DMSO at 0.1µg/ml (P=0.008) and 1µg/ml (P=0.002) LPS. TNFα secretion from SFA treated cells was not significantly different relative to DMSO.
above 0.01 μg/ml LPS. There was no significant difference in TNFα secretion from MUFA and DMSO treated macrophages at 0.01 μg/ml and 0.1 μg/ml LPS. At these concentrations TNFα secretion from MUFA treated cells was significantly higher than from SFA (P=0.02 and P=0.03, respectively) and MUFA + n-3 PUFA (P=0.002 and P=0.0008, respectively) treated cells. TNFα secretion from MUFA treated cells was significantly increased relative to DMSO when activated with 1 μg/ml LPS (P<0.000001) and remained significantly increased relative to SFA and MUFA + n-3 PUFA treated cells (P<0.000001).

4.3.3c(ii) LPS effects within DMSO, SFA, MUFA and MUFA + n-3 PUFA groups

Secretion of TNFα from DMSO treated macrophages increased from resting levels with activation by 0.01 μg/ml LPS (P=0.00001). There was no further significant increase in TNFα secretion with activation by 0.1 μg/ml LPS stimulation. Secretion of TNFα from DMSO treated macrophages was significantly increased from the preceding concentration of LPS at 1 μg/ml (P=0.03). Secretion of TNFα from SFA treated cells increased significantly from the preceding concentration of LPS only at 1 μg/ml (P=0.03). TNFα secretion from MUFA treated macrophages significantly increased from resting with activation by 0.01 μg/ml LPS (P=0.007). Secretion of TNFα from MUFA treated cells was not further increased with activation by 0.1 μg/ml LPS but increased significantly from this when activated with 1 μg/ml LPS (P<0.000001). Secretion of TNFα from MUFA + n-3 PUFA treated cells did not increase significantly between sequential concentrations of LPS. However, secretion of TNFα was significantly increased from that of unstimulated cells at 0.1 μg/ml LPS (P=0.01).

Effective activation of macrophages was determined by significant increases in TNFα secretion at different concentrations of LPS within the SFA, MUFA, MUFA + n-3 PUFA and DMSO groups, as before. DMSO and MUFA treated cells were significantly activated with the 0.01 μg/ml LPS. The increase in TNFα secretion observed subsequently in DMSO and MUFA treated cells was only significant at 1 μg/ml LPS. The increased secretion of TNFα from cells treated with SFA was significant only at 1 μg/ml LPS.

In summary, IL-1β secretion from treated macrophages was readily activated by lower concentrations of LPS. The response became saturated in DMSO and MUFA + n-3 PUFA treated cells, with heightened LPS activation having no effect on the secretion of
IL-1β. SFA and MUFA treated cells continued to express increased amounts of IL-1β with increased LPS activation; a true dose-response. IL-6 secretion from treated macrophages was stimulated at the penultimate concentration of LPS used and the secretory response significantly increased with the subsequent LPS doses, indicating later activation and a subsequent dose-response for DMSO, SFA, MUFA and MUFA + n-3 PUFA treatments. TNFα was secreted in significant amounts from DMSO and MUFA treated macrophage at an intermediate concentration of LPS but did not increase further until activated with the greatest dose. TNFα secretion from SFA and MUFA + n-3 PUFA increased mutedly with heightening activation by LPS.

LPS activation of macrophages is a necessary component of the present model. Resting, or unstimulated, macrophages were not affected by fatty acid treatment relative to DMSO or the compositional differences of the fatty acid blends. At 0.1 and 1μg/ml LPS SFA, MUFA and MUFA + n-3 PUFA treatments had consistent effects on IL-1β secretion relative to DMSO. At 0.1 and 1μg/ml SFA and MUFA + n-3 PUFA had consistent effects on IL-6 secretion from macrophages relative to DMSO. The suppression of IL-6 by MUFA was lost at 1μg/ml LPS. At 0.1 and 1μg/ml LPS SFA and MUFA + n-3 PUFA treatments displayed consistent effects on TNFα secretion.

Taking these results into consideration, the optimal LPS concentration for activation of the THP-1 derived macrophage treated with DMSO, SFA, MUFA and MUFA + n-3 PUFA was deemed to be 0.1 μg/ml. For all subsequent experiments 0.1μg/ml LPS was used to activate macrophages.
Figure 4.2 Secretion of IL-1β (a), IL-6 (b) and TNFα (c) from macrophages activated by a range of LPS concentrations. THP-1 derived macrophages were treated with 50μmol/L DMSO ■, SFA □, MUFA ▲ and MUFA + n-3 PUFA ○ prior to activation by 0, 0.001, 0.01, 0.1 and 1μg/ml LPS for 24 hours. Secretion of pro-inflammatory cytokines was quantified by ELISA. Results are expressed as cytokine concentration (pg/ml) in supernatant. Results represent the mean ± SEM of 6 independent experiments.

* indicates P≤0.05, ** indicates P≤0.001 relative to DMSO within LPS groups
† indicates P≤0.05, †† indicates P≤0.001 between sequential LPS dose within treatment
4.3.4 Effects of NEFA on cytokine transcription and secretion at 0.1μg/ml LPS

4.3.4a IL-1β transcription and secretion (Figure 4.3)

IL-1β transcription in macrophages at 6 hours of LPS stimulation was increased by all fatty acid treatments relative to DMSO (SFA 43.2%; MUFA 76.1%; MUFA + n-3 PUFA 149.2%) but only significantly so by MUFA + n-3 PUFA (P=0.05). There were no significant differences in IL-1β transcription between the fatty acid treatments (Figure 4.3a).

IL-1β secretion from macrophages at 24 hours of LPS stimulation was increased by all fatty acid treatments relative to DMSO (SFA 49.5%; MUFA 43.4%; MUFA + n-3 PUFA 32.5%). These increases were significant for SFA (P=0.03) and MUFA (P=0.04) but not for the MUFA + n-3 PUFA. There were no significant differences in IL-1β secretion between the fatty acid treatments (Figure 4.3b).

![Figure 4.3 IL-1β transcription in (a) and secretion from (b) THP-1 derived macrophages treated with 50μmol/L DMSO □, SFA □, MUFA □ and MUFA + n-3 PUFA □. Cells were stimulated with 0.1μg/ml LPS. Transcription was semi-quantified by RT-PCR at 6 hours and secretion quantified by ELISA at 24 hours. (a) The values expressed are normalised to GAPDH and relative to DMSO. (b) Results are expressed as cytokine concentration (pg/ml) in supernatant. Results represent the mean ± SEM of 4-6 independent experiments. * indicates P≤0.05 relative to DMSO]
4.3.4b IL-6 transcription and secretion (Figure 4.4)

IL-6 transcription in macrophages at 6 hours of LPS stimulation was significantly decreased by all fatty acid treatments relative to DMSO (SFA 45.2%, \( P=0.001 \); MUFA 26.8%, \( P=0.03 \); MUFA + n-3 PUFA 55.6%, \( P=0.0008 \)). MUFA + n-3 PUFA reduced IL-6 transcription more potently than MUFA (\( P=0.04 \)) (Figure 4.4a).

IL-6 secretion from macrophages at 24 hours of LPS stimulation was significantly decreased by all fatty acid treatments relative to DMSO (SFA 62.3%, MUFA 60.7%, MUFA + n-3 PUFA 69.8%; \( P<0.000001 \)). There were no significant differences in IL-6 secretion between the fatty acid treatments (Figure 4.4b).

![Figure 4.4](image_url)

**Figure 4.4** IL-6 transcription in (a) and secretion from (b) THP-1 derived macrophages treated with 50µmol/L DMSO ■, SFA □, MUFA △ and MUFA + n-3 PUFA ◼. Cells were stimulated with 0.1µg/ml LPS. Transcription was semi-quantified by RT-PCR at 6 hours and secretion quantified by ELISA at 24 hours. (a) The values expressed are normalised to GAPDH and relative to DMSO. (b) Results are expressed as cytokine concentration (pg/ml) in supernatant. Results represent the mean ± SEM of 4-6 independent experiments.

* indicates \( P \leq 0.05 \); ** indicates \( P \leq 0.01 \) relative to DMSO.
4.3.4c TNFα transcription and secretion (Figure 4.5)

TNFα transcription in macrophages at 6 hours of LPS stimulation was significantly increased by MUFA + n-3 PUFA relative to DMSO (286%, *P* = 0.01), SFA (P = 0.005) and MUFA (P = 0.02). SFA and MUFA had no significant effects on TNFα transcription relative to DMSO or each other (Figure 4.5a).

TNFα secretion from macrophages at 24 hours of LPS stimulation was significantly decreased by MUFA + n-3 PUFA relative to DMSO (51.8%, *P* = 0.02). SFA and MUFA had no significant effects on TNFα secretion relative to DMSO. The addition of MUFA to the predominantly saturated SFA treatment increased secretion of TNFα (36.8%, *P* = 0.06). The addition of n-3 PUFA to the MUFA treatment decreased secretion of TNFα (57.7%, *P* = 0.005). There were no significant differences in TNFα secretion between SFA and MUFA + n-3 PUFA (Figure 4.5b).

Figure 4.5 TNFα transcription in (a) and secretion from (b) THP-1 derived macrophages treated with 50μmol/L DMSO □, SFA □, MUFA □ and MUFA + n-3 PUFA □. Cells were stimulated with 0.1μg/ml LPS. Transcription was semi-quantified by RT-PCR at 6 hours and secretion quantified by ELISA at 24 hours. (a) The values expressed are normalised to GAPDH and relative to DMSO. (b) Results are expressed as cytokine concentration (pg/ml) in supernatant. Results represent the mean ± SEM of 4-6 independent experiments.

* indicates *P* ≤ 0.05; ** indicates *P* ≤ 0.01 relative to DMSO
4.3.5 Effects of NEFA on MMP-9 activity (Figure 4.6)
MMP-2 activity was inconsistently and weakly detected by zymography. Analysis of MMP-2 activity has not been included. MMP-9 activity was detected for all samples. No significant differences were observed between MMP-9 activity in the supernatants of SFA, MUFA, MUFA + n-3 PUFA and DMSO treated macrophages based on peak height of the MMP-9 proteolytic band analysed for intensity of staining. Activity was increased by SFA (8.3%) and MUFA (13.3%) relative to DMSO. Activity was decreased by MUFA + n-3 PUFA relative to DMSO (0.9%), SFA (9.3%) and MUFA (14.3%).

Figure 4.6 Gelatinolytic activity of MMP-9 secreted from THP-1 derived macrophages treated with 50μmol/L DMSO □, SFA □, MUFA □ and MUFA + n-3 PUFA □. Cells were stimulated with 0.1μg/ml LPS for 24 hours. (a) Gelatinolytic activity is presented as peak height of gelatinase bands following zymography and densitometry. Results are expressed relative to DMSO and represent the mean ± SEM of 6 independent experiments. (b) Representative bands from a zymogram.

4.4 DISCUSSION
The present model was designed to reproduce in vitro the exposure of macrophages to serum NEFA and the effect of altering serum NEFA composition on macrophage pro-inflammatory mediators involved in atherosclerosis. THP-1 monocytes were differentiated to macrophages prior to treatment with three fatty acid blends, designated SFA, MUFA and MUFA + n-3 PUFA, designed to reflect the serum NEFA composition of subjects predisposed to CVD.

Macrophages are the predominant inflammatory cell in the atherosclerotic lesion and can produced inflammatory mediators with autocrine and paracrine effects, including IL-1β, IL-6, TNFα and MMP-9. Atheroma-associated macrophages have an
unusual capacity to phagocytose large amounts of lipid and do not exhibit suppression of scavenger receptor (such as SRA and CD36) expression (Libby et al. 1996).

In the postprandial state serum NEFA are generated from the hydrolysis of TRL by endothelial-bound lipases. During fasting or after prolonged exercise serum NEFA originate principally from adipose tissue. The genesis of NEFA at the vascular endothelium may cause proximal injury, increase macromolecule transmigration across the endothelium, potentiate lesional macrophage activation and induce foam cell formation (Weintraub et al. 1988; Chung et al. 1995; Berstad et al. 2003). The typical Western diet and lifestyle elicits a prolonged post-prandial state which, in theory, increases the exposure of vascular endothelium and lesional inflammatory cells to serum NEFA.

Physiological serum concentrations of NEFA range from 380μmol/L in healthy controls (Yli-Jama et al. 2001) to 842μmol/L in Type 2 diabetics (Skowronski et al. 1991). In the present study the physiological concentrations of serum NEFA were cytotoxic to THP-1 derived macrophages. Cell viability was significantly decreased by 500μmol/L treatments of SFA, MUFA and MUFA + n-3 PUFA. SFA and MUFA treated cells were viable, relative to untreated cells, at 250μmol/L. MUFA + n-3 PUFA treated cells were viable at 50μmol/L relative to untreated cells. Therefore, 50μmol/L was deemed an appropriate, but physiologically dilute, concentration of SFA, MUFA and MUFA + n-3 PUFA to ensure maintenance of cell viability in the experimental model.

In the present study the MUFA + n-3 PUFA maintained cell viability at a 5-fold more dilute concentration than the SFA and MUFA treatments, indicating an increased cytotoxic potential of n-3 PUFA to the THP-1 derived macrophage model. Degree of fatty acid unsaturation generally increases potential for cytotoxicity. In cultured endothelial cells PUFA-rich post-lipolytic serum was demonstrated to be more disruptive to barrier function, and hence more cytotoxic, than SFA-rich post-lipolytic serum (Chung et al. 1998). LPS-activated peritoneal macrophages from mice fed a low fat diet or fish oil enriched high fat diet showed greater macrophage killing of P815 mastocytoma cells compared to mice fed high fat diets rich in coconut, olive, or safflower oils, indicating that the type, rather than content, of fat in the diet alters macrophage cytotoxicity towards target tumour cells (Wallace et al. 2000). NEFA can impair insulin secretion by inducing β-cell apoptosis (Butler et al. 2001; Pankow et al. 2004). The SFA palmitic acid was less cytotoxic than the MUFA oleic acid in apoptosis.
of insulinoma cells (Kharroubi et al. 2004). The mechanism of NEFA-induced apoptosis is unclear. It has recently been shown that, unlike cytokine-induced apoptosis, NEFA-induced apoptosis of the β cell is not mediated by NF-κB (Kharroubi et al. 2004). It has been suggested that accumulation of NEFA within the β cell may induce de novo ceramide formation and mitochondrial cytochrome C release, activate protein kinase C, inhibit protein kinase B or activate calpain-10 (Kharroubi et al. 2004). NF-κB-mediated lipotoxicity of hepatocytes has been demonstrated. Treatment of murine hepatocytes and HepG2 cells with a palmitic acid and oleic acid mix resulted in lysosomal destabilisation and release of cathepsin B into the cytoplasm. Lysosomal destabilisation induces NF-κB-dependent TNFα expression. Cathepsin B release correlates with severity of human non-alcoholic fatty liver disease (NAFDL). This indicates a potential NF-κB-mediated cytotoxic role of NEFA in, for example, the pathogenesis of NAFDL (Feldstein et al. 2004).

Activation of macrophages by LPS is an appropriate model for the classical activation of macrophages that occurs in lesional atherosclerosis. An LPS dose-response study of treated macrophages was performed to determine an appropriate concentration of LPS to use in the experimental model, the activation potential of a range of LPS concentrations and to investigate whether there may be a “saturated” response of macrophages to higher doses of LPS, masking differences in fatty acid treatment effects. THP-1 derived macrophages, treated with 50μmol/L of the three NEFA treatments and DMSO vehicle control, were activated with 0, 0.001, 0.01, 0.1 and 1 μg/ml LPS. There were no significant differences in cytokine secretion in resting (unstimulated) macrophages treated with SFA, MUFA, MUFA + n-3 PUFA and DMSO. In a non-inflammatory environment the relative concentration of SFA, MUFA and n-3 PUFA in NEFA did not affect macrophage secretory function. The results of the LPS dose-response study indicated that 0.1μg/ml was an appropriate concentration of LPS for subsequent work with the experimental model. An optimal dose was difficult to define. The results demonstrated that the initiation (or subsequent plateau) of the individual cytokines’ secretion occurred at different concentrations of LPS within the same fatty acid or DMSO group; in other words, each cytokine had a different concentration of LPS at which it would be significantly produced. Fatty acid and DMSO treatments affected cytokine secretion within the same LPS concentration groups, indicating that the individual fatty acid treatments may confer different responsiveness to LPS in THP-1 derived macrophages. It was also demonstrated that a
fully potentiated, or saturated, secretory response to LPS can occur, above which concentration there will be no significant increase in cytokine secretion despite intensified stimulation. It is important to note that ELISA quantified the accumulation of IL-1β, IL-6 and TNFα in the supernatant of stimulated cells. This represented complete cytokine secretion and indicated accumulated production. It cannot indicate a decrease in cytokine production subsequent to activated secretion if, for example, an inhibitory mechanism is induced or cell death occurs after LPS stimulation. For all experiments discussed below 50μmol/L fatty acid treatment and 0.1μg/ml LPS activation dose was used.

Transcription of IL-1β was increased by all treatments, significantly by the MUFA + n-3 PUFA, to a lesser non-significant extent by the MUFA and least by the SFA treatments. The increase in IL-1β mRNA expression was mirrored by increased secretion by all, and significantly so for SFA and MUFA, treatments. Each NEFA treatment contained 30% linoleic acid, an n-6 PUFA. Fats rich in n-6 PUFA enhance IL-1β production. TNF-induced IL-1 production from peritoneal macrophages was related in a positive curvilinear manner to linoleic acid intake (Grimble & Tappia, 1998). The reported effects of n-3 PUFA on IL-1β production are mixed. Some studies have reported that dietary fish oils enhance ex vivo rodent macrophage IL-1β production (Lokesh et al. 1990; Ertel et al. 1993) and a corn oil diet, rich in the n-3 PUFA α-linolenic acid, enhanced IL-1β production from rat peritoneal macrophages (Tappia & Grimble, 1994). Other studies report that fish oils decrease production of IL-1β (Billiar et al. 1988; Renier et al. 1993; Yaqoob & Calder, 1995) and demonstrate a reduced production of macrophage IL-1β from fish oil fed animals relative to those fed more SFAs (Tappia & Grimble 1994 and 1996; Wallace et al. 2000). It may be speculated that the over-riding increased transcription and secretion of the three NEFA treatments relative to DMSO in the present study was an effect of their high n-6 PUFA linoleic acid content. However, the increase in transcription was accentuated by n-3 PUFA. Secretion of IL-1β was significantly increased from DMSO by the SFA and MUFA treatments. The addition of n-3 PUFA did not significantly reduce IL-1β secretion relative to the other two treatments, but secretion was not significantly different from DMSO treated cells. Though the nuances of the addition of n-3 PUFA to the MUFA treatment are noteworthy, in the larger context of NEFA composition it would appear that, relative to DMSO, NEFA increase THP-1 derived macrophage IL-1β production and this may be due to their n-6 PUFA content.

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In the present study all fatty acid treatments significantly decreased IL-6 mRNA expression. The MUFA increased IL-6 transcription relative to the SFA treatment. The addition of n-3 PUFA significantly counteracted this increase, decreasing transcript levels to those observed in the SFA treated cells. All fatty acid treatments significantly reduced IL-6 secretion relative to DMSO, with no differences between the treatments. The anti-inflammatory effects appear to be general to fatty acids, although the MUFA + n-3 PUFA reduced IL-6 transcription significantly more than the MUFA treatment. Published results of the effects of fatty acids on IL-6 production conflict. IL-6 production from rat peritoneal macrophages has been correlated positively with intake of the n-6 PUFA linoleic acid (Grimble & Tappia, 1998) although in the myotubes of human skeletal muscle linoleic acid inhibited IL-6 mRNA expression and protein secretion induced by the SFA palmitic acid (Weigert et al. 2004). IL-6 production from peritoneal macrophages of rats fed fish oil and olive oil enriched high fat diets was increased compared to those of rats fed corn oil diets enriched with the n-3 PUFA α-linolenic acid (Tappia & Grimble, 1996). EPA and DHA, but not linoleic and oleic acids, increased in vitro macrophage IL-6 production (Tappia et al. 1995). In the present study an anti-inflammatory effect on IL-6 production was induced by all NEFA treatments relative to DMSO. The MUFA treatment, containing oleic acid, was least potent in suppression of IL-6 transcription and all NEFA treatments were equally capable at inhibition of IL-6 secretion. Linoleic acid inhibited the SFA-induced IL-6 production in myocytes (Weigert et al. 2004), indicating that its content in all the NEFA treatments may have induced a general anti-inflammatory effect. In macrophage models, contrary to the findings of the present study, linoleic acid (Grimble & Tappia, 1998) and fish oils (Tappia & Grimble, 1996) increased IL-6 production. These papers, however, refer to the same feeding experiment and should be compared with caution to direct in vitro results. The results of dosing cultured rat peritoneal macrophages with individual fatty acids (Tappia et al. 1995) cannot be directly compared to the present findings, where an anti-inflammatory potential of the linoleic acid base may underlie the effects of each treatment. However, the fish oils are supported by Tappia et al. (1995) as pro-inflammatory in terms of increased IL-6 production. In the macrophage and NEFA model IL-6 mRNA expression was reduced by the addition of n-3 PUFA to the MUFA treatment, indicating a suppressive effect of EPA and DHA on IL-6 production.

In the present study TNFα transcription and secretion are consistent for the SFA and MUFA, but appear contradictory for the MUFA + n-3 PUFA, treatments in the
present study. TNFα mRNA expression was significantly increased by the MUFA + n-3 PUFA treatment relative to DMSO and the other fatty acid treatments. SFA or MUFA treatments had no effect on TNFα transcription, indicating that the increase was mediated by n-3 PUFA. Secretion of TNFα was non-significantly reduced by SFA, unchanged by MUFA and significantly reduced by MUFA + n-3 PUFA treatments relative to DMSO. The increase in TNFα secretion from cells treated with MUFA compared to the SFA approached significance, and the decrease in secretion subsequent to addition of n-3 PUFA was significant. Reduced TNFα production by fatty acids, particularly those of fish oils, has been reported. TNFα secretion from the peritoneal macrophages of rats fed coconut oil, fish oil or olive oil enriched diets was decreased, though not significantly, compared to chow-fed controls, indicating a UFA effect (Tappia & Grimble, 1994). TNFα production decreased with increasing unsaturation of test feed from murine peritoneal macrophages, the effect being most potent from fish oil fed animals (Wallace et al. 2000). Other studies have also reported that fish oils decrease production of TNFα (Billiar et al. 1988; Renier et al. 1993; Boutard et al. 1994; Grimm et al. 1994; Yaqoob & Calder, 1995), concurring with the findings of reduced TNFα secretion by the n-3 PUFA component of NEFA in the present study. Increased TNFα production by fatty acids, particularly n-3 PUFA, have also been demonstrated. Murine peritoneal macrophage TNFα production was increased in animals fed a high α-linolenic acid, n-3 PUFA, diet compared to those fed a high linoleic acid, n-6 PUFA, diet (Watanabe et al. 1991). Several other studies report that dietary fish oils result in enhanced production of TNFα (Lokesh et al. 1990; Hardardottir & Kinsella, 1991 and 1992; Blok et al. 1992; Chang et al. 1992; Chaet et al. 1994; Hubbard et al. 1994; Somers & Erickson 1994) by rodent macrophages *ex vivo*. *In vitro* production of TNFα from cultured rat peritoneal macrophages was increased by treatment with eicosapentanoate and docosahexanoate but was diminished by linoleic acid treatment (Tappia et al. 1995). Furthermore, a palmitic and oleic acids mixed treatment induced a pro-apoptotic NF-κB-dependent TNFα expression in murine hepatocytes and HepG2 cells. These results may support, to some extent, the increase in TNFα transcription by the n-3 PUFA component of NEFA observed in the present study. However, the discrepancy in the present results is not clarified, with conflicting published reports lending support to both results. It may be speculated that the incongruity between transcription and secretion is due to an inappropriate alignment of transcription and secretion time points but this does not appear to be the case for IL-1β
and IL-6, where results between transcription and secretion are generally consistent. The increase in TNFα transcription at 6 hours may be transient. The TNFα gene may have an earlier response to activation than the other pro-inflammatory cytokines. Post-transcriptional modification of TNFα may occur, limiting its secretion relative to mRNA expression.

There is a preponderance of evidence linking macrophage MMP activation to atherosclerotic tissue remodelling and plaque rupture (Galis et al. 1995; Bellosta et al. 1998; Matsumoto et al. 1998; Rajavashisth et al. 1999; Morgan et al. 2004). However, the effects of fatty acids on the regulation of MMPs is not widely reported. Serum MMP-9 and active MMP-2 were reduced in pregnant rats fed a DHA-supplemented diet (Harris et al. 2001). In the present study, there was no significant effect of SFA, MUFA or MUFA + n-3 PUFA treatments on MMP-9 activity, relative to DMSO. The addition of n-3 PUFA to MUFA reduced MMP-9 activity non-significantly by 14.3%.

There is much evidence that elevated NEFA induce a pro-inflammatory phenotype. The inflammatory potential of NEFA remains difficult to define from the present study. Generalised pro- and anti-inflammatory effects are observed and there are nuances between the NEFA content in these generalised effects. Investigation of transcription indicates intercellular promotion of inflammatory mediators. In the case of the classically activated macrophage with its crucial secretory function within the atherosclerotic lesion, the secretion of pro-inflammatory mediators may be a more relevant result to consider. In this case secretion of IL-6 was generally reduced, most potently by the addition of n-3 PUFA. Secretion of TNFα was significantly reduced by the addition of n-3 PUFA. The NEFA-induced increase in IL-1β secretion relative to DMSO was non-significant for the MUFA + n-3 PUFA treatment. There were no significant effects of the SFA, MUFA and MUFA + n-3 PUFA treatments on MMP-9 activity. The anti-inflammatory effects of NEFA on IL-6 and TNFα secretion were potentiated by the addition of n-3 PUFA. Furthermore, n-3 PUFA appeared to negate a pro-inflammatory effect of SFA and MUFA treatments on IL-1β secretion.

NEFA hydrolysed from lipoproteins by endothelial-bound LPL can transmigrate into the vascular intima, are exposed to and may be phagocytosed by activated resident macrophages. The present study indicates that the n-3 PUFA composition of NEFA, represented in this case by EPA and DHA, may have anti-inflammatory potential in a macrophage model and, thus, in lesional atherosclerosis.
Chapter 5
5. EFFECTS OF THE n-3 LC-PUFA, EPA AND DHA, ON MACROPHAGE INFLAMMATORY MEDIATORS ASSOCIATED WITH ATHEROSCLEROSIS AND THE NF-κB SIGNALLING PATHWAY

5.1 INTRODUCTION

The use of n-3 LC-PUFA in treatment of human disease shows promise in terms of therapeutic cost, availability and a limited profile of side effects (Babcock et al. 2003). Epidemiological and clinical data has indicated that the n-3 LC-PUFA of fish oils are beneficial in asthma (Serhan et al. 2000; Calder, 2003), multiple sclerosis, psoriasis (Simopoulos 2002; Mori & Beilin, 2004), sepsis and burns (Lanza-Jacoby et al. 2001; Babcock et al. 2003), graft survival (Yaqoob et al. 2000), rheumatoid arthritis (Serhan et al. 2000; Arrington et al. 2001), inflammatory bowel disease (Mori & Beilin, 2004) and chronic renal disease (Li et al. 2005). Anti-tumour and anti-metastatic effects have been attributed to n-3 LC-PUFA (Narayanan et al. 2001; Babcock et al. 2003) whereas n-6 PUFA were reported to be chemopromotive (Liu et al. 2001). Fish oils may increase the length of gestation and human n-3 LC-PUFA supplementation has reduced preterm delivery rates in high-risk pregnancies from 33% to 21% (Harris et al. 2001). Fish oils have a variety of anti-atherogenic effects, most notably in the reduction of plasma TAG concentrations (Storlein et al. 1987; Harris et al. 1988; Babcock et al. 2003; Yaqoob & Calder, 2003; Mori & Beilin, 2004) by reduced endogenous VLDL production (Roche & Gibney, 1999). The n-3 LC-PUFA affect platelet and vessel wall interactions and are anti-thrombotic. They ameliorate cardiac arrhythmia and reduce sudden cardiac death (Abeywardena & Head, 2001; Thies et al. 2003; Mori & Beilin, 2004). The n-3 LC-PUFA regulate blood pressure (Mori & Beilin, 2004) and have produced moderate reductions in animal and human hypertension (Abeywardena & Head, 2001). It is proposed that DHA-induced apoptosis of vascular smooth muscle cells may regulate blood pressure by positive vascular remodelling (Diep et al. 2000). The diversity of cells and tissues upon which the n-3 LC-PUFA are reported to exert beneficial effects suggests their interaction with a ubiquitous cellular target or targets. A number of mechanisms through which n-3 LC-PUFA may work have been identified.

The most frequently proposed mechanism through which the n-3 LC-PUFA act relates to their displacement of n-6 arachidonate in phospholipid bilayers and a subsequent shunting of eicosanoid production towards a less bioactive phenotype. Dietary supplementation with n-3 LC-PUFA enhances the n-3 to n-6 fatty acid ratio in
membrane phospholipids of erythrocytes, granulocytes, platelets, endothelial cells, monocytes, brain cells and hepatocytes (Grimm et al. 2002), indicating preferential membrane incorporation of n-3 LC-PUFA. The n-3 and n-6 PUFA are substrates for production of alternative eicosanoid families (Calder, 1997). Arachidonate is the substrate for the 2-series prostaglandins and thromboxanes and the 4-series leukotrienes. EPA is the substrate for the 3-series prostaglandins and thromboxanes and the 5-series leukotrienes. The 2-series prostaglandins synthesised from n-6 PUFA are more mitogenic and pro-inflammatory than the 3-series synthesised from n-3 LC-PUFA (Bagga et al. 2003). n-3 and n-6 PUFA share the same enzymes for the synthesis of their eicosanoids (Pischon et al. 2003). Decreased availability of arachidonate in the phospholipid bilayer, competition for COX and LOX and decreased expression of COX-2 and 5-LOX leads to a decrease in n-6 PUFA derived eicosanoids (Calder, 2003). Fish oil has consistently been shown to decrease PGE\(_2\) (Lo et al. 1999a and 1999b; Lanza-Jacoby et al. 2001), LTB\(_4\) and TXA\(_2\) production (Simopoulos, 2002). Indeed, antithrombotic effects of the n-3 LC-PUFA are generally explained by the inhibition of platelet TXA\(_2\) and parallel changes in the clotting mechanism (Abeywardena & Head, 2001). It has been proposed that anti-inflammatory effects of EPA and DHA may be mediated by novel eicosanoids, including lipoxins and resolvins. Inflammatory exudates formed in murine air pouches via intrapouch injections of TNF\(\alpha\), n-3 LC-PUFA and aspirin generated several novel products including 18R-HEPE. HUVEC cells treated with aspirin and EPA generated 18R-HEPE and 15R-HEPE. These novel products were potent inhibitors of human mononuclear cell transendothelial migration and infiltration of dorsal air pouches in vivo (Serhan et al. 2000). The authors propose that generation of these novel anti-inflammatory mediators in these cell types is likely to represent significant amounts in local microenvironments, specifically in microinflammation. Another study by this group has shown that exudate obtained in the resolution phase from mice treated with aspirin and DHA produce a novel family of 17R-hydroxy-docosanoids, termed resolvins (Serhan et al. 2002). These papers indicate that n-3 LC-PUFA derivatives are not simply “less inflammatory” that those of arachidonate, but rather their derived eicosanoids and docosanoids may have novel and active anti-inflammatory functions. EPA is commonly regarded as the potent n-3 LC-PUFA in the arachidonate displacement hypothesis but a discrete role for DHA in the production of resolvins is defined here.
However, incorporation of n-3 LC-PUFA into phospholipid membranes exerts anti-inflammatory effects through mechanisms other than eicosanoid metabolism. PGE$_2$ inhibits macrophage TNF production via a negative regulatory feedback mechanism. When macrophage PGE$_2$ production is decreased, as after incubation with fish oils, TNF production would be expected to increase (Lo et al. 1999). Lo et al. (1999) demonstrated that EPA inhibits macrophage PGE$_2$ and TNF production in response to LPS stimulation in murine macrophages. Pretreatment of HSVECs with n-3 LC-PUFA inhibited the activated expression of VCAM-1, E-selectin and ICAM-1. The effect was larger for DHA than EPA which is the direct precursor of the 2-series prostaglandins and 5-series leukotrienes, the effect was unaltered by indomethacin or eicosatetranoi acid which is a common blocker of all metabolism of arachidonic acid by COX and LOX, and the effect was observed for LC-PUFA and MUFA that are not eicosanoid precursors (De Caterina et al. 2000). These data indicate that the n-3 LC-PUFA, EPA and DHA, exert anti-inflammatory effects through mechanisms independent of eicosanoid metabolism. EPA and DHA have been shown to affect a number of transcription factors and related kinases in vitro, including NF-kB (Lo et al. 1999; De Caterina et al. 2000; Novak et al. 2003; Zhou et al. 2004), PPAR (Chambrier et al. 2002; Li et al. 2005) AP-1, p44/p42 ERK, JNK/SAPK (Lo et al. 1999; Liu et al. 2001; Babcock et al. 2003) and p38 MAPK (Diep et al. 2000).

For the purposes of this thesis the effects of the n-3 LC-PUFA, EPA and DHA, on phenotypic inflammation will be discussed mainly in the context of pro-inflammatory cytokines. Published results of cell models and animal feeding overwhelmingly indicate the anti-inflammatory potential of n-3 LC-PUFA. The findings of human supplementation studies have been less consistent.

n-3 LC-PUFA emulsions have suppressed TNF$\alpha$ transcription in and secretion from in vitro murine RAW 264.7 macrophages (Babcock et al. 2002a; Novak et al. 2003). Production of IL-10, an endogenous suppressor of TNF$\alpha$, was also decreased in this model (Babcock et al. 2002b). Pre-incubation with EPA has reduced TNF$\alpha$ in LPS activated human monocytic (Zhou et al. 2004) and murine macrophage (Lo et al. 1999) cell lines. EPA and DHA suppressed tissue factor activation and elicitation of NO, TNF$\alpha$ and IL-1$\beta$ from LPS activated THP-1 cells (Chu et al. 1999). In other models, EPA and DHA decreased MCP-1 expression induced by LPS in the human kidney HK-2 cell line (Li et al. 2005) and decreased ox-LDL upregulated expression of P-selectin, ICAM-1 and monocyte binding to cultured human coronary artery endothelial cells.
DHA inhibited the expression of VCAM-1, E-Selectin and ICAM-1 in adult HSVECs after activation with virtually any stimulus able to elicit the coordinated expression of these genes (De Caterina et al. 2000). Elsewhere, an increase in TNFα production was reported by n-3 LC-PUFA treated human monocytes activated with LPS (Sato et al. 1992).

Peritoneal macrophages from atherosclerosis-susceptible mice fed an n-3 LC-PUFA enriched diet showed suppressed basal TNFα mRNA expression and a decreased ability to express TNFα and IL-1β mRNA in response to LPS (Renier et al. 1993). n-3 LC-PUFA supplemented diets decreased IL-1β (Lokesh et al. 1990) and TNFα (Lokesh et al. 1990; Wallace et al. 2000) production from activated murine peritoneal macrophages. A 10.5% fish oil diet suppressed TNFα production from porcine alveolar macrophages compared to control and mixed n-3/n-6 feeds (Turek et al. 1996). Serum MMP-9 and active MMP-2 were reduced in pregnant rats fed a DHA-supplemented diet (Harris et al. 2001). Elsewhere, however, LPS stimulated macrophages from mice fed a high fat, fish oil supplemented diet for four weeks produced higher levels of TNFα mRNA and protein than controls (Chang et al. 1995).

n-3 LC-PUFA supplementation in healthy humans has suppressed PBMC IL-1 (Meydani et al. 1991; Renier et al. 1993), IL-6 (Meydani et al. 1991; Trebble et al. 2003) and TNFα (Meydani et al. 1991; Renier et al. 1993; Trebble et al. 2003). Meydani et al. (1991) demonstrated an age-dependence to these anti-inflammatory effects, with cytokine secretion in older women more significantly decreased by n-3 LC-PUFA supplementation than in younger women. Significant inverse associations were found between dietary EPA and DHA intake and plasma levels of soluble TNF receptors 1 and 2 and CRP in healthy males (Pischon et al. 2003). PBMC secretion of IL-1β, IL-2 and TNFα were decreased in relapsing/remitting multiple sclerosis patients supplemented with n-3 LC-PUFA for 6 months (Gallai et al. 1995) and patients with chronic renal failure supplemented with n-3 LC-PUFA for 12 months (Cappelli et al. 1997). In these two study groups cytokine concentrations were in the normal range at baseline or were non-significantly different from control subjects. Although supplementation of healthy volunteers with 0.94g EPA and DHA mix or 1.9g EPA and DHA mix for 12 weeks caused a significant decrease in IL-6 production by stimulated mononuclear cells there were no effects on the production of TNFα, IL-1β, IL-2, IL-4, IL-10 or IFNγ (Wallace et al. 2003). Supplementation of healthy subjects with n-3 LC-PUFA had no effect on ex vivo IL-2, IL-1α, IL-1β, TNFα, IFNγ or IL-10 from Con A or
LPS stimulated whole blood or PBMC (Yaqoob et al. 2000). Supplementation with 3g, 6g or 9g fish oil per day had no effect on cytokine production (Blok et al. 1997). Elsewhere, there were no significant effects on monocyte expression of adhesion molecules in type 2 diabetic and non-diabetic controls supplemented with 2.1g of fish oil per day for 21 days (Sampson et al. 2001). There was no difference in ICAM-1 or VCAM-1 expression in plaques from humans undergoing carotid endoarterectomy supplemented with n-3 fish oils or control although the n-3 supplemented group displayed plaque morphologically less vulnerable to rupture (Thies et al. 2003). Genotyping of polymorphisms in TNFα and TNFβ genes has indicated why inconsistent effects of n-3 LC-PUFA have been demonstrated in human cohorts and why great variation in TNFα production has been recorded within groups. Polymorphisms in cytokine genes influence the intensity of cytokine production and inflammatory stress (Markovic et al. 2004). Circulating CRP and TAG were positively associated with the TNFα -308GG, TNFβ +252AG, IL-1β -551TT and IL-6 -174GG genotypes, and enhanced inflammation was associated with the latter three. The lipid-lowering and anti-inflammatory effects of fish oils in healthy men were influenced by BMI and possession of the TNFβ +252A allele (Markovic et al. 2004). With composite data there was no effect of fish oil supplementation for 3 months on TNF production. However, a suppressive effect of n-3 LC-PUFA was observed in subjects with inherently high pre-supplementation TNFα, irrespective of their genotype. This decrease in TNFα production was significantly greater if subjects had the TNFα -308AG rather than the TNFα -308GG genotype. In the low and middle tertiles of inherent TNFα production, sensitivity to fish oil was determined by the TNFβ +252AG genotype (Grimble et al. 2002). These data suggest that the suppressive effects of n-3 LC-PUFA is linked to the individual’s inherent level of TNFα production and to genetic variation associated with the TNFα -308 and TNFβ +252 SNPs.

The main aim of the present study is to investigate the specific effects of EPA and DHA on inflammatory mediators, IL-1β, IL-6, TNFα and MMP-9, of the classically activated macrophage, a chief cellular component of the atherosclerotic lesion. The effects of EPA and DHA on the NF-κB pathway, which signals the transcription of these inflammatory mediators, is elucidated. Additionally, the effects of EPA, DHA and DMSO vehicle control on cell viability and the dose-response of macrophages to LPS in terms of pro-inflammatory mediator secretion are investigated.
5.2 METHODS

5.2.1 Experimental model
The THP-1 monocyte derived macrophage model of Chapter 4 was repeated and elaborated on (Chapter 2.3 and 2.8). Viable monocytes were enumerated by EB/AO staining and seeded in routine media at densities outlined in the experiments below. Monocytes were differentiated to macrophages by the addition of 0.1|g/ml PMA (Sigma, Saint Louis, MI, USA) for 72 hours. Routine media was removed, cells were washed twice with HBSS (Gibco, Grand Island, New York) and maintained for 24 hours in serum-free media, supplemented with 1% Nutridoma (Roche Molecular Biochemicals, IN, USA). Cells were treated with EPA, DHA and DMSO vehicle control for 48 hours prior to classical activation with LPS (Sigma, Saint Louis, MI, USA).

5.2.2 Treatment concentration
A cell viability assay determined an appropriate and optimal EPA, DHA and DMSO concentration for treatment of the THP-1 derived macrophage model. 100μl of 1 x 10^6 monocytes/ml were plated per well in clear 96-well microplates, differentiated to macrophages and serum-starved prior to treatment with 100, 50, 25, 12.5 and 6.25μmol/L EPA, DHA or DMSO vehicle control. Control wells containing untreated cells and blank wells containing media alone were also included. The viability of cells at 6, 12 and 24 hour time points were quantified by the MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega Corporation, WI, USA) (Chapter 2.9). Briefly, the yellow MTS salt added to each well is reduced to soluble blue formazan mainly by mitochondrial dehydrogenases of metabolically active cells. Colormetric absorbance at 490nm is expressed as a percentage of the optical densities of untreated wells and represents relative viability.

5.2.3 LPS dose-response and cytokine secretion
As in Chapter 4, secretion of pro-inflammatory mediators was used to investigate and determine the optimal dose of LPS for macrophage activation in this model. 1ml of 1 x 10^6 monocytes/ml were plated per well in 24-well tissue culture plates, differentiated to macrophage and treated with EPA, DHA and DMSO as before. Secretion of IL-1β, IL-6 and TNFα from macrophages activated with 0, 0.001, 0.01, 0.1 and 1μg/ml LPS for 24 hours was investigated by ELISA (Chapter 2.13).
5.2.4 Gene expression analysis

RNA was extracted from macrophages activated with LPS for 6 hours using TRIzol® reagent (GIBCOBRL, Life Technologies™) following the manufacturer's instructions (Chapter 2.15). RNA yields were quantified spectrophotometrically, integrity assessed following denaturing agarose gel electrophoresis. RNA was DNase-treated and first strand cDNA synthesised by reverse transcription. TaqMan RT-PCR was performed for IL-1β, IL-6 and TNFα using pre-developed assay reagent kits (Assays-on-Demand; Applied Biosystems, Warrington, UK). After PCR, standard curves were constructed and the Ct readings for each of the unknown samples were used to calculate the amount of target gene or GAPDH relative to the standard. For each sample, results were normalised by dividing the amount of target gene by the amount of GAPDH and expressed relative to DMSO vehicle control.

5.2.5 MMP secretion and gelatinase activity

Secretion of MMP-9 from THP-1 derived macrophages was measured in cell supernatant by high-sensitivity ELISA (Chapter 2.13). The activity of secreted MMP-2 and MMP-9 was measured in the supernatants of macrophages activated with LPS for 24 hours by gelatin zymography (Chapter 2.14). Gelatinolytic activity in the supernatant of macrophages was renatured by Triton-X following SDS-PAGE and bands of digestion were destained against a Coomassie blue background. Gels were imaged and bands were analysed using GeneGenius, GeneSnap and GeneTools Analysis Software (Syngene, Cambridge, UK). Peak heights were normalised to those of DMSO treated cells.

5.2.6 Western immunoblotting

4mls of 1 x 10^6 monocytes/ml were plated per well in 6-well tissue culture plates, differentiated to macrophage and treated with EPA, DHA and DMSO as before. Following LPS stimulation of macrophage for 0, ½, 1, 2 and 5 hours cytoplasmic and nuclear protein extractions were performed (Chapter 2.16.1). Proteins were quantified by the Bradford method (Chapter 2.16.2). 25μg of protein for cytoplasmic extracts and 5μg protein for nuclear fractions were separated by SDS-PAGE for Western immunoblotting (Chapter 2.16.3). Antibodies (Santa Cruz Biotechnology Inc., USA) were used to detect NF-κB p65, IκBα, NF-κB p50 and IKK in nuclear and/or cytoplasmic blotted proteins of EPA, DHA and DMSO treated cells. Blots were
chemiluminescently developed (Chapter 2.16.6) and densitometry of film performed under transilluminescence (GeneGenius and GeneSnap, Syngene, Cambridge, UK). Band densities were normalised to those of DMSO treated cells (GeneTools Analysis Software, Syngene, Cambridge, UK).

5.2.7 p65 DNA consensus site binding
The activity of nuclear p65 was investigated using the ELISA-style TransAM™ NF-κB Transcription Factor Assay Kit (Active Motif Europe, Rixensart, Belgium) (Chapter 2.17). Briefly, 1.25μg of nuclear protein extracted from resting and 5 hour LPS-activated EPA, DHA and DMSO treated cells were plated per well onto a white-walled 96-well NF-κB assay plate. The plate was pre-coated with oligonucleotides containing the NF-κB consensus sequence (5'GGGACTTTCC-3') to which active NF-κB from the sample bound. Primary antibody was directed against p65 and a secondary anti-rabbit IgG antibody conjugated with HRP emitted chemiluminescence. Chemiluminescence, as measured with maximal integration time and 255 manual gains by luminometry (SPECTRAFluor Plus and XFLUOR Version 3.21, TECAN, Reading, UK), was directly proportional to p65 binding activity in the nuclear extractions.

5.2.8 NF-κB nuclear translocation by Cellomics® technology
5.2.8a Optimal cell density
An initial experiment indicated that 5 x 10⁴ THP-1 derived macrophage/ml was an appropriate cell concentration for Cellomics® KineticScan (Cellomics Inc., Pittsburgh, PA) imaging compared to the recommended 1 x 10⁵ cells/ml. Plate fixing, staining and imaging are described briefly below and in detail in Chapter 2.18.2a.

5.2.8b LPS induced NF-κB nuclear translocation – dose and time response
The effects of 0.1μg/ml and 1μg/ml LPS on nuclear translocation of NF-κB at a series of time points were investigated. 100μl of 5 x 10⁴ monocytes/ml were plated per well in 96-well black, clear-bottom microplates (Parkard ViewPlate®, Foss, Dublin). Monocytes were differentiated to macrophage as before. LPS was used to activate cells at 0 hours, 5 minutes, 15 minutes, ½ hour, 1 hour, 2 hours and 5 hours. All time points ended together and the plate was fixed and fluorescently stained for NF-κB (Cellomics Hitkit®) as per manufacturer’s instructions. The plate was imaged by the Cellomics
KineticScan Reader. NF-κB compartmentalisation was analysed using the Cellomics BioApplication (Chapter 2.18.2.b and c).

5.2.8c Effects of EPA and DHA on NF-κB nuclear translocation

100μl of 5 x 10^4 monocytes/ml were plated per well in 96-well black tissue culture plates, differentiated to macrophage and treated with EPA, DHA and DMSO as before. LPS was used to activate cells at 0 hours, 15 minutes, ½ hour, 1 hour, 2 hours and 5 hours. All time points ended together and the plate was fixed and stained for NF-κB. The plate was then imaged and NF-κB compartmental analysis performed.

5.2.9 Statistical analysis

Statistical analysis was performed with DataDesk 6.0 (Data Description Inc. NY). The distribution of data for each variable was assessed and variables transformed to normalise the distribution of data if necessary. Multiple comparisons were performed by one-way ANOVA. Individual differences were subsequently tested by Fisher’s least significant difference (LSD) test after demonstration of significant inter-group differences by ANOVA. A probability of P≤0.05 was considered statistically significant.
5.3 RESULTS

5.3.1 Dose-response effects of EPA, DHA and DMSO on macrophage viability (Figure 5.1)

The viability of THP-1 derived macrophages was assessed following treatment with a range of concentrations of EPA, DHA and DMSO (100, 50, 25, 12.5 and 6.25 µmol/L) over a series of time points (6, 12 and 24 hours). Viability was interpreted as the expression of formazan stain, measured spectrophotometrically, of treated cells relative to untreated cells, presumed to be 100% viable. At 6 and 12 hours of exposure, 100 µmol/L EPA, DHA or DMSO had no effect on macrophage viability relative to untreated cells. At 24 hours, EPA and DMSO had no significant and/or negative effect on macrophage viability relative to untreated cells within the range of concentrations studied. DHA treated macrophages were less viable than untreated cells and those treated with EPA and DMSO at 100 µmol/L (P<0.00001 for all), 50 µmol/L (P<0.00001 for all), 25 µmol/L (P=0.007, P=0.00001 and P=0.001, respectively) and 12.5 µmol/L (P=0.02, P=0.0001 and P=0.001, respectively). At 6.25 µmol/L DHA had no significant effects on cell viability relative to cells alone, EPA and DMSO (Figure 5.1). The MTS assay indicates, in a non-absolute manner, cell viability. The present results show a consistent cytotoxic effect of DHA on macrophages associated with dose and time. An optimal dose of EPA and DHA, constituting viability and efficacy, was considered to be 25 µmol/L. For all subsequent experiments of this chapter macrophages were treated with 25 µmol/L EPA, DHA or DMSO. Cell viability at 24 hours is presented in Figure 5.1.

![Graph showing cell viability at 24 hours by MTS assay](image)

Figure 5.1 Cell viability at 24 hours by MTS assay. THP-1 derived macrophages were treated with 100, 50, 25, 12.5 and 6.25 µmol/L DMSO, EPA and DHA. Cell viability, as indicated by comparative formazan staining with untreated cells, was analysed spectrophotometrically. The values are expressed normalised to complete (100%) viability of untreated cells. Results represent the mean ± SEM of 5 independent experiments.

* indicates P<0.001; ** indicates P<0.00001 relative to the viability of untreated cells.
5.3.2 Dose-response effects of LPS activation (0-1μg/ml) (Figure 5.2, page 184)

5.3.2a IL-1β secretion (Figure 5.2a)

5.3.2a(i) Effects of EPA and DHA relative to DMSO

IL-1β was detected in the supernatants of resting (non-LPS stimulated) macrophages and concentrations were not significantly different between EPA, DHA and DMSO treated cells. There was no significant difference in IL-1β secretion from EPA, DHA or DMSO treated macrophages activated with 0.001μg/ml LPS. EPA and DHA significantly decreased IL-1β secretion relative to DMSO from macrophages activated with 0.01μg/ml LPS (P=0.01 and P=0.00001, respectively), 0.1μg/ml (P=0.02 and P=0.002, respectively) and 1μg/ml LPS (P=0.0007 and P=0.000007, respectively).

5.3.2a(ii) LPS effects within DMSO, EPA and DHA groups

Secretion of IL-1β from DMSO treated cells significantly increased from resting levels when activated with 0.001μg/ml LPS (P=0.006). IL-1β secretion significantly increased from 0.001μg/ml to 0.01μg/ml LPS (P=0.000009) but did not have further effects with heightened stimulation. Secretion of IL-1β from EPA treated cells significantly increased from resting levels when activated with 0.001μg/ml LPS (P=0.01). As with DMSO treated cells, IL-1β secretion significantly increased from 0.001μg/ml to 0.01μg/ml LPS (P=0.008) but did not increase beyond this with higher concentrations of LPS. Although IL-1β secretion from DHA treated cells increased with activation, this was not significant between sequential concentrations of LPS.

Effective activation of macrophages, as determined by significant increases in IL-1β secretion, was initiated in DMSO and EPA treated cells at 0.001μg/ml LPS but became limited at the higher LPS concentrations. DHA treated cells appeared less sensitive to LPS activation and secretion of IL-1β was not significantly affected between sequential concentrations of LPS.

5.3.2b IL-6 secretion (Figure 5.2b)

5.3.2b(i) Effects of EPA and DHA relative to DMSO

IL-6 was not detected in the supernatants of resting (non-LPS stimulated) macrophages treated with EPA and DHA (not represented on Figure 5.2b). At 0.001μg/ml LPS, DHA treated cells secreted significantly less IL-6 than EPA treated cells (P=0.02) but neither fatty acid had effects on secretion relative to DMSO. With 0.01μg/ml LPS activation, DHA significantly decreased IL-6 secretion relative to DMSO and EPA (P=0.003 and 181
P=0.02, respectively). EPA and DHA significantly decreased IL-6 secretion relative to DMSO at 0.1μg/ml LPS (P=0.01 and P=0.00002, respectively). Neither fatty acid treatment affected IL-6 secretion relative to DMSO at 1μg/ml.

5.3.2b(ii) LPS effects within DMSO, EPA and DHA groups

Secretion of IL-6 from DMSO treated cells significantly increased from preceding levels of activation at 0.01μg/ml LPS (P<0.000001). Secretion significantly increased further at 0.1μg/ml LPS (P=0.02) but not with 1μg/ml LPS. As with DMSO treated cells, a significant effect of activation in EPA treated cells occurred at 0.01μg/ml LPS (P<0.000001) and at 0.1μg/ml LPS (P=0.007) but did not further increase with 1μg/ml LPS. There was no significant increase in IL-6 secretion from DHA treated cells between sequential doses of LPS until 0.1μg/ml (P=0.001) and there was no further significant increase in IL-6 secretion above this concentration of LPS.

Effective activation of macrophages by LPS, as determined by significant increases in IL-6 secretion, occurred with 0.01μg/ml LPS in DMSO and EPA treated cells. Activation was further potentiated at 0.1μg/ml but was expended by 1μg/ml LPS. DHA treated cells became sensitive to LPS at 0.1μg/ml and, as with DMSO and EPA treated macrophages, were not further activated by increased LPS concentration.

5.3.2c TNFα secretion (Figure 5.2c)

5.3.2c(i) Effects of EPA and DHA relative to DMSO

TNFα was detected in the supernatants of resting (non-LPS stimulated) macrophages but concentrations were not significantly different between EPA, DHA and DMSO treated cells. There was no significant difference in TNFα secretion from EPA, DHA or DMSO treated macrophages activated with 0.001μg/ml or 0.001 μg/ml LPS. At 0.1μg/ml LPS EPA significantly decreased TNFα secretion relative to DMSO and DHA from macrophages activated with 0.1 μg/ml LPS (P=0.01 and P=0.03, respectively) and 1μg/ml LPS (P=0.04 and P=0.0004, respectively).

5.3.2c(ii) LPS effects within DMSO, EPA and DHA groups

Secretion of TNFα from DMSO, EPA and DHA treated cells was not significantly activated by 0.001μg/ml LPS. Activation of macrophages with 0.01μg/ml LPS significantly increased TNFα secretion in DMSO, EPA and DHA treated macrophages (P=0.05, P=0.02 and P=0.03, respectively). EPA treated cells did not produce
significantly more TNFα with heightened LPS stimulation. DMSO and DHA treated cells produced significantly more TNFα at 0.1µg/ml LPS (P=0.006 and P=0.02, respectively). At maximal LPS stimulation, macrophages did not exhibit a heightened secretion of TNFα relative to that expressed at 0.1µg/ml LPS.

Effective activation of macrophages by LPS, as determined by significant increases in TNFα secretion, was not observed until cells were treated with 0.01µg/ml LPS. EPA treated cells were insensitive to heightened LPS stimulation above this concentration. DMSO and DHA treated cells were insensitive to heightened LPS stimulation above 0.1µg/ml LPS.

As in Chapter 4, macrophages demonstrated activation and "saturation" sensitivities in cytokine response to LPS stimulation, depending on fatty acid treatments. As shown previously, secretion of the individual pro-inflammatory cytokines within treatment groups is not initiated at a uniform intensity of LPS stimulation. IL-1β secretion from DMSO and EPA treated macrophages was immediately initiated by LPS and showed a positive dose response that became limited with maximal stimulation. DHA treated cells demonstrated relative insensitivity to LPS stimulation with respect to IL-1β secretion.

As with IL-1β, DMSO and EPA treated macrophages were more sensitive to LPS stimulation than DHA treated cells in eliciting IL-6. DHA treated cells responded significantly by IL-6 secretion to LPS at 0.1µg/ml. This concentration of LPS, as with secretion of IL-1β, induced maximal secretion of IL-6 from all cells. DMSO, EPA and DHA treated macrophages showed the same sensitivity to LPS with respect to TNFα secretion. As with the IL-1β and IL-6 responses, secretion of TNFα became limited at higher concentrations. The effects of EPA and DHA on IL-1β and TNFα secretion relative to DMSO remained consistent at higher doses of LPS (0.01µg/ml, 0.1µg/ml and 1µg/ml for IL-1β; 0.1µg/ml and 1µg/ml for TNFα) but the anti-inflammatory effects of DHA and/or EPA on IL-6 secretion observed at 0.01µg/ml and 0.1µg/ml LPS were lost at 1µg/ml LPS. Therefore, for all subsequent experiments 0.1µg/ml LPS was used to activate macrophages.
Figure 5.2 Secretion of IL-1β (a), IL-6 (b) and TNFα (c) from macrophages activated by a range of LPS concentrations. THP-1 derived macrophages were treated with 25μmol/L DMSO □, EPA □ and DHA □ prior to activation by 0, 0.001, 0.01, 0.1 and 1μg/ml LPS for 24 hours. Secretion of pro-inflammatory cytokines was quantified by ELISA. Results are expressed as cytokine concentration (pg/ml) in supernatant. Results represent the mean ± SEM of 5-6 independent experiments.

* indicates P≤0.05, ** indicates P≤0.001 relative to DMSO within LPS groups
† indicates P≤0.05, †† indicates P≤0.001 between sequential LPS concentration within treatment
5.3.3 Effects of EPA and DHA on cytokine transcription and secretion at 0.1μg/ml LPS

5.3.3a IL-1β transcription and secretion (Figure 5.3)

IL-1β transcription in macrophages at 6 hours of LPS activation was decreased by EPA and DHA relative to DMSO (34.2% and 60.5%, respectively) and this was significant for DHA treated cells (P=0.05). There was no significant difference between transcription from EPA and DHA treated cells.

IL-1β secretion from macrophages at 24 hours of LPS activation was decreased by EPA and DHA relative to DMSO (48.1%, P=0.02 and 66.5%, P=0.002, respectively). There was no significant difference in the potencies of EPA and DHA in this regard.

Figure 5.3 IL-1β transcription in (a) and secretion from (b) THP-1 derived macrophages treated with 25μmol/L DMSO □, EPA ■ and DHA □. Cells were stimulated with 0.1μg/ml LPS. Transcription was semi-quantified by RT-PCR at 6 hours and secretion quantified by ELISA at 24 hours. (a) The values expressed are normalised to GAPDH and relative to DMSO. (b) Results are expressed as cytokine concentration (pg/ml) in supernatant. Results represent the mean ± SEM of 4-6 independent experiments.

* indicates P≤0.05 relative to DMSO
5.3.3b IL-6 transcription and secretion (Figure 5.4)

IL-6 transcription in macrophages at 6 hours of LPS activation was significantly decreased by EPA and DHA relative to DMSO (68.8%, P=0.03 and 76.3%, P=0.01, respectively). There was no significant difference in the effects of EPA and DHA.

IL-6 secretion from macrophages at 24 hours of LPS activation was significantly decreased by EPA and DHA relative to DMSO (31.7%, P=0.01 and 69.4%, P=0.00002). DHA had a significantly greater effect than EPA (P=0.003).

![Figure 5.4](image_url)

**Figure 5.4** IL-6 transcription in (a) and secretion from (b) THP-1 derived macrophages treated with 25μmol/L DMSO ■, EPA □ and DHA □. Cells were stimulated with 0.1μg/ml LPS. Transcription was semi-quantified by RT-PCR at 6 hours and secretion quantified by ELISA at 24 hours. (a) The values expressed are normalised to GAPDH and relative to DMSO. (b) Results are expressed as cytokine concentration (pg/ml) in supernatant. Results represent the mean ± SEM of 4-6 independent experiments.

* indicates P≤0.05; ** indicates P≤0.001 relative to DMSO
5.3.3c TNFα transcription and secretion (Figure 5.5)

TNFα transcription in macrophages at 6 hours of LPS activation was decreased by EPA and DHA relative to DMSO (48.8% and 44%, respectively), significantly by EPA (P=0.04) and approaching significance for DHA (P=0.06). There was no significant difference between concentrations of TNFα secreted from EPA and DHA treated macrophages.

TNFα secretion from macrophages at 24 hours of LPS activation was significantly decreased by EPA relative to DMSO (P=0.01) and DHA (P=0.03). DHA had no significant effect on TNFα secretion relative to DMSO.

Figure 5.5 TNFα transcription in (a) and secretion from (b) THP-1 derived macrophages treated with 25μmol/L DMSO □, EPA ■ and DHA □. Cells were stimulated with 0.1μg/ml LPS. Transcription was semi-quantified by RT-PCR at 6 hours and secretion quantified by ELISA at 24 hours. (a) The values expressed are normalised to GAPDH and relative to DMSO. (b) Results are expressed as cytokine concentration (pg/ml) in supernatant. Results represent the mean ± SEM of 4-6 independent experiments.

* indicates P<0.05 relative to DMSO
5.3.4 MMP activity and secretion (Figure 5.6)
MMP-9 was detected in the supernatants of resting (non-LPS stimulated) macrophages but its concentration was not significantly difference between DMSO, EPA and DHA treated cells (mean concentrations ± SEM: DMSO 75ng/ml ± 11.1; EPA 93.9ng/ml ± 14.4; DHA 72.73ng/ml ± 8.9). MMP-9 secretion was significantly decreased in macrophages activated with 0.1μg/ml LPS treated with DHA relative to DMSO (43.4%, P=0.05) and EPA (P=0.007) treated macrophages. EPA had no effect relative to DMSO (Figure 5.6a). As in Chapter 4, MMP-2 activity was inconsistently and weakly detected by gelatin zymography and its analysis has not been included. MMP-9 activity was detected for all samples activated by 0.1μg/ml LPS. MMP-9 activity was significantly reduced in DHA relative to DMSO (P=0.03) and EPA (P=0.05) treated macrophages (Figures 5.6b and 5.6c).

![Figure 5.6](image)

Figure 5.6 MMP-9 secretion (a) and gelatinolytic activity (b) from THP-1 derived macrophages treated with 25μmol/L DMSO □, EPA ■ and DHA □. Cells were stimulated with 0.1μg/ml LPS. (a) Secretion was quantified by ELISA at 24 hours and results are expressed as ng/ml. (b) Gelatinolytic activity is presented as peak height of gelatinase bands following zymography and densitometry, normalised to DMSO. Results represent the mean ± SEM of 4-6 independent experiments. (c) Representative bands from a zymogram.

* indicates P≤0.05 relative to DMSO
5.3.5 NF-κB activation

5.3.5a LPS induced NF-κB translocation – time and dose response

The nuclear translocation of NF-κB in macrophages following 0 hours (unstimulated), 5 minutes, 15 minutes, ½ hour, 1 hour, 2 hours and 5 hours of activation with 0.1μg/ml and 1μg/ml LPS was investigated using HCS technology from Cellomics Inc.

5.3.5a(i) Cytoplasmic staining of NF-κB

The intensity of cytoplasmic staining of NF-κB is represented in pixels by the well output feature “MEANRingAvgIntenCh2” of the Cellomics compartmental analysis BioApplication. From 5 minutes to 5 hours activation with either 0.1μg/ml or 1μg/ml LPS the intensity of cytoplasmic NF-κB staining was significantly decreased from that of unstimulated cells (0 hours) (P<0.0001 for all time points). At 15 minutes of LPS activation, the intensity of cytoplasmic NF-κB staining was not significantly different from that of cells stimulated for 5 minutes. At ½ hour of LPS activation, the intensity of cytoplasmic NF-κB staining was significantly decreased from that of cells stimulated for 15 minutes with 0.1μg/ml LPS only (P=0.02). At 1 and 2 hours of LPS activation, the intensity of cytoplasmic NF-κB staining remained non-significantly different from that of ½ hour. At 5 hours of LPS activation, the intensity of cytoplasmic NF-κB staining remained non-significantly different from that of 2 hours. However, the intensity of cytoplasmic NF-κB staining significantly increased at 5 hours from that at 1 hour of activation with 0.1μg/ml and 1μg/ml LPS (P=0.03 for both). There were no significant differences in cytoplasmic NF-κB staining induced by 0.1μg/ml and 1μg/ml LPS at any of the time points investigated. The intensity of cytoplasmic NF-κB staining in cells activated with 0.1μg/ml LPS relative to unstimulated cells is presented in Figure 5.7.
Figure 5.7 Staining of cytoplasmic NF-κB at time points of LPS activation. Cells were stimulated with 0.1μg/ml LPS over a 5 hour period. Plates were fixed and stained for NF-κB with the antibody conjugated fluorophore Alexa® Fluor 488, as part of the Cellomics HitKit® protocol. The cytoplasm was distinguished from nucleus by its lack of Hoechst 34442 staining. Cells were scanned with the Cellomics KineticScan® HCS Reader and compartmental analysis of NF-κB was performed. Staining intensity is presented in pixels and results represent the mean ± SEM of 4 independent experiments.

* indicates P≤0.001 relative to unstimulated (0 hours) cells
† indicates P≤0.05 between sequential concentrations of LPS

5.3.5a(ii) Nuclear staining of NF-κB
The intensity of nuclear staining of NF-κB is represented in pixels by the well output feature “MEANCircAvgIntenCh2” of the Cellomics compartmental analysis BioApplication. At 5 minutes of LPS activation, the intensity of nuclear NF-κB staining was not significantly different from that of unstimulated cells for either 0.1μg/ml or 1μg/ml LPS. From 15 minutes to 5 hours activation with either 0.1μg/ml or 1μg/ml LPS the intensity of nuclear NF-κB staining was significantly increased from that of unstimulated cells (0 hours) (P=0.02 for 15 minutes at 0.1μg/ml LPS; P≤0.0002 for all other time points). At 15 minutes of LPS activation, the intensity of nuclear NF-κB staining was significantly increased from that of cells stimulated for 5 minutes with 0.1μg/ml and 1μg/ml LPS (P=0.007 and P=0.0005, respectively). At ½ hour of LPS activation, the intensity of nuclear NF-κB staining was significantly increased from that of cells stimulated for 15 minutes with 0.1μg/ml and 1μg/ml LPS (P=0.03 and P=0.01, respectively). At 1 hour of LPS activation, the intensity of nuclear NF-κB staining was significantly increased from that of cells stimulated for ½ hour with 0.1μg/ml LPS only (P=0.005). At 2 hours of LPS activation, the intensity of nuclear NF-κB staining was significantly decreased from that of cells stimulated for 1 hour with 1mg/ml LPS only.
At 5 hours of LPS activation, the intensity of nuclear NF-κB staining was significantly decreased from that of cells stimulated for 2 hours with 0.1μg/ml LPS only (P=0.009). At 2 hours of LPS activation, the intensity of nuclear NF-κB staining was significantly lower in cells treated with 1μg/ml LPS than those treated with 0.1μg/ml (P=0.04). At all other time points there were no significant differences in nuclear NF-κB staining induced by 0.1μg/ml and 1μg/ml LPS. The intensity of nuclear NF-κB staining in cells activated with 0.1μg/ml LPS relative to unstimulated cells is presented in Figure 5.8.

Figure 5.8 Staining of nuclear NF-κB at time points of LPS activation. Cells were stimulated with 0.1μg/ml LPS over a 5 hour period. Plates were fixed and stained for NF-κB with the antibody conjugated fluorophore Alexa® Fluor 488, as part of the Cellomics HitKit® protocol. Nuclei were identified by Hoechst 33442 staining. Cells were scanned with the Cellomics KineticScan® HCS Reader and compartmental analysis of NF-κB was performed. Staining intensity is presented in pixels and results represent the mean ± SEM of 4 independent experiments.

* indicates P≤0.05; ** indicates P≤0.001 relative to unstimulated (0 hours) cells
† indicates P≤0.05 between sequential concentrations of LPS

5.3.5a(iii) Nuclear translocation of NF-κB
Images of cellular NF-κB for unstimulated (0 minutes) and LPS stimulated (5 hours) macrophages generated by the Cellomics compartmental analysis BioApplication are presented in Figure 5.9. Staining is relatively diffuse in unstimulated cells. Staining is relatively concentrated in the nuclei of cells activated with LPS. The ratio of nuclear:cytoplasmic staining of NF-κB, represented by the well output feature “MEANCircRingAvgIntenRatioCh2” of the Cellomics compartmental analysis BioApplication, indicates nuclear translocation of NF-κB, and is presented in Figure 5.9 for macrophages activated with 0.1μg/ml LPS at a series of time points.
Figure 5.9 Cellular NF-κB staining and data integrated as ratio of nuclear:cytoplasmic NF-κB at a series of time points of LPS activation. Cellular NF-κB staining in unstimulated macrophages (a) and macrophages activated with 0.1μg/ml LPS for 5 hours (b). NF-κB is stained with the antibody conjugated fluorophore Alexa® Fluor 488 and nuclei identified by Hoechst 34442. (c) Nuclear translocation of NF-κB, represented by the ratio of nuclear:cytoplasmic staining, over time points of LPS activation. Results represent the mean ± SEM of 4 independent experiments.

* indicates P≤0.05; ** indicates P≤0.001 relative to unstimulated (0 hours) cells
† indicates P≤0.001 between sequential concentrations of LPS
5.3.5b The effects of EPA and DHA on cytoplasmic and nuclear p65 expression (Figures 5.10 and 5.11, respectively)

Cytoplasmic and nuclear p65 expression was determined by Western immunoblotting of samples stimulated with 0.1 μg/ml LPS at a series of time points. Cytoplasmic p65 expression is presented at 0, ½, 1, 2 and 5 hours of LPS activation (Figures 5.10a, b, c, d and e, respectively). Nuclear p65 expression is presented at 0, 1 and 5 hours of LPS activation (Figures 5.11a, b and c, respectively). Nuclear p65 expression was undetectable at ½ and 2 hours.

Cytoplasmic p65 expression was not affected by fatty acid treatment at 0 hours and ½ hour of LPS stimulation relative to DMSO. At 1 hour of LPS activation cytoplasmic p65 expression was significantly increased by EPA (P=0.00002) and DHA (P=0.0001). At 2 hours of LPS stimulation DHA treated cells expressed significantly more cytoplasmic p65 than DMSO treated cells (P=0.003), whereas EPA treated cells showed no significant effects. At 5 hours of LPS stimulation, cytoplasmic p65 was barely detectable in DMSO and EPA treated cells, although protein bands were observed along the length of the lanes, indicating that protein was loaded and ran through the gel from these samples. Expression of cytoplasmic p65 from DHA treated cells was significantly increased from that of DMSO treated cells (P=0.0003). Cytoplasmic p65 expression is presented in Figure 5.10.
Figure 5.10 Cytoplasmic NF-κB p65 expression in THP-1 derived macrophages treated with 25μmol/L DMSO ■, EPA ■ and DHA ■ over time points of LPS activation. Cells were stimulated with 0.1μg/ml LPS for 0 (a), ½ (b), 1 (c), 2 (d) and 5 (e) hours. Cytoplasmic proteins were extracted and separated by SDS-PAGE prior to Western immunoblotting. Blots were probed with p65 antibody and developed by chemiluminescence. Results represent the mean ± SEM of 3-4 independent experiments. Representative bands from developed immunoblots are presented.

* indicates P<0.001 relative to DMSO
Nuclear p65 expression was significantly increased by DHA treatment in unstimulated macrophages (P=0.04). At 1 hour of LPS activation DHA treated cells expressed significantly reduced nuclear p65 relative to DMSO treated macrophage (P=0.05). At 5 hours of LPS activation, nuclear p65 expression was significantly reduced in EPA and DHA treated macrophages relative to DMSO (P=0.03 for both).

Figure 5.11 Nuclear NF-κB p65 expression in THP-1 derived macrophages treated with 25μmol/L DMSO □, EPA □ and DHA □ over time points of LPS activation. Cells were stimulated with 0.1μg/ml LPS for 0 (a), 1 (b) and 5 (c) hours. Nuclear proteins were extracted and separated by SDS-PAGE prior to Western immunoblotting. Blots were probed with p65 antibody and developed by chemiluminescence. Results represent the mean ± SEM of 3-4 independent experiments. Representative bands from developed immunoblots are presented.

* indicates P≤0.05 relative to DMSO
5.3.5c The effects of EPA and DHA on cytoplasmic and nuclear IκBα expression (Figures 5.12 and 5.13, respectively)

Cytoplasmic and nuclear IκBα expression was determined by Western immunoblotting of samples stimulated with 0.1μg/ml LPS at a series of time points. Cytoplasmic IκBα expression is presented at 0, ½, 1, 2 and 5 hours of LPS activation (Figures 5.12a, b, c, d and e, respectively). Nuclear IκBα expression is presented at 0, 1 and 5 hours of LPS activation (Figures 5.13a, b and c, respectively). Nuclear IκBα expression was undetectable at ½ and 2 hours.

Cytoplasmic IκBα expression was non-significantly depressed by EPA and DHA in unstimulated cells relative to DMSO treated cells. Cytoplasmic IκBα was significantly decreased in EPA and DHA treated macrophages relative to DMSO at ½ hour of LPS activation (P=0.03 for both). At 1 hour of LPS activation, both EPA and DHA increased cytoplasmic IκBα expression relative to DMSO, significantly in the case of DHA treated macrophages (P=0.02) and approaching significance in the case of EPA (P=0.09). Cytoplasmic IκBα expression remained increased in EPA and DHA treated cells at 2 hours and 5 hours of LPS activation relative to DMSO, but this was not significant.
Figure 5.12 Cytoplasmic IkBa expression in THP-1 derived macrophages treated with 25μmol/L DMSO □, EPA □ and DHA □ over time points of LPS activation. Cells were stimulated with 0.1μg/ml LPS for 0 (a), ½ (b), 1 (c), 2 (d) and 5 (e) hours. Cytoplasmic proteins were extracted and separated by SDS-PAGE prior to Western immunoblotting. Blots were probed with IkBa antibody and developed by chemiluminescence. Results represent the mean ± SEM of 3-4 independent experiments. Representative bands from developed immunoblots are presented.

* indicates P≤0.05 relative to DMSO
Nuclear IκBα expression was not affected by fatty acid treatment in unstimulated cells relative to DMSO treated cells. Nuclear IκBα expression was significantly decreased in DHA treated macrophages relative to DMSO at 1 hour and 5 hours of LPS activation (P=0.003 for both time points). EPA significantly decreased expression at 5 hours relative to DMSO (P=0.009).

Figure 5.13 Nuclear IκBα expression in THP-1 derived macrophages treated with 25μmol/L DMSO □, EPA ■ and DHA □ over time points of LPS activation. Cells were stimulated with 0.1μg/ml LPS for 0 (a), 1 (b) and 5 (c) hours. Nuclear proteins were extracted and separated by SDS-PAGE prior to Western immunoblotting. Blots were probed with IκBα antibody and developed by chemiluminescence. Results represent the mean ± SEM of 3-4 independent experiments. Representative bands from developed immunoblots are presented. * indicates P≤0.05 relative to DMSO.

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5.3.5d The effects of EPA and DHA on cytoplasmic and nuclear p50 expression (Figures 5.14 and 5.15, respectively)

Cytoplasmic and nuclear p50 expression was determined by Western immunoblotting of samples stimulated with 0.1μg/ml LPS at a series of time points. Cytoplasmic p50 expression is presented at 0, ½, 1, 2 and 5 hours of LPS activation. Nuclear p50 expression is presented at 0, 1 and 5 hours of LPS activation. Nuclear p50 expression was undetectable at ½ and 2 hours.

Cytoplasmic p50 expression was not significantly affected by EPA or DHA treatment relative to DMSO in unstimulated cells. There was no significant difference between EPA, DHA or DMSO treated cells after ½ hour of activation with LPS. At 1 hour of LPS activation DHA treated cells expressed significantly less cytoplasmic p50 than DMSO treated cells (P=0.02). EPA and DHA increased cytoplasmic p50 relative to DMSO treated cells at 2 hours (P=0.002 and P=0.04, respectively) and 5 hours (P=0.002 and P=0.001, respectively). Results at 0, 1 and 5 hours are presented in Figures 5.14a, b and c, respectively.
Figure 5.14 Cytoplasmic NF-κB p50 expression in THP-1 derived macrophages treated with 25μmol/L DMSO □, EPA □ and DHA □ over time points of LPS activation. Cells were stimulated with 0.1μg/ml LPS for 0 (a), ½ (b), 1 (c), 2 (d) and 5 (e) hours. Cytoplasmic proteins were extracted and separated by SDS-PAGE prior to Western immunoblotting. Blots were probed with p50 antibody and developed by chemiluminescence. Results represent the mean ± SEM of 3-4 independent experiments. Representative bands from developed immunoblots are presented.

* indicates P≤0.05 relative to DMSO
Nuclear p50 expression was not significantly affected by EPA or DHA treatment at 0 hours, 1 hour and 5 hours of LPS activation relative to DMSO treated cells. Results at 0 and 5 hours are presented in Figures 5.15a and b, respectively.

Figure 5.15 Nuclear NF-κB p50 expression in THP-1 derived macrophages treated with 25μmol/L DMSO ■, EPA □ and DHA ○ over time points of LPS activation. Cells were stimulated with 0.1μg/ml LPS for 0 (a), 1 (b) and 5 (c) hours. Nuclear proteins were extracted and separated by SDS-PAGE prior to Western immunoblotting. Blots were probed with p50 antibody and developed by chemiluminescence. Results represent the mean ± SEM of 3-4 independent experiments. Representative bands from developed immunoblots are presented.

5.3.5e The effects of EPA and DHA on cytoplasmic IKK expression (Figure 5.16)
Cytoplasmic IKK expression was determined by Western immunoblotting of samples stimulated with 0.1μg/ml LPS for 0 hours, ½ hour and 2 hours (Figures 5.16a, b and c, respectively). IKK was not detected in 1 and 5 hour blots. There was no significant effect of EPA or DHA on IKK protein expression relative to DMSO in the cytoplasmic extracts of unstimulated cells. Following ½ hour of LPS activation, DHA stimulated cells expressed significantly less cytoplasmic IKK relative to EPA treated cells (P=0.03), and both remained non-significantly different to IKK expressed by DMSO treated cells. At 2 hours of LPS activation both EPA and DHA treated macrophages expressed significantly less cytoplasmic IKK than DMSO treated cells (P=0.04 and P=0.02, respectively).
Figure 5.16 Cytoplasmic IKK expression in THP-1 derived macrophages treated with 25μmol/L DMSO □, EPA □ and DHA □ over time points of LPS activation. Cells were stimulated with 0.1μg/ml LPS for 0 (a), ½ (b) and 2 (c) hours. Cytoplasmic proteins were extracted and separated by SDS-PAGE prior to Western immunoblotting. Blots were probed with IKK antibody and developed by chemiluminescence. Results represent the mean ± SEM of 3-4 independent experiments. Representative bands from developed immunoblots are presented.

* indicates P≤0.05 relative to DMSO
5.3.5f The effects of EPA and DHA on nuclear p65 binding to DNA (Figure 5.17)

Nuclear binding of the NF-κB p65 subunit was performed using the ELISA-style TransAM kit from Active Motif. Nuclear extracts from unstimulated (a) and 5 hours LPS activated (b) macrophages were analysed on the same plate. There was no significant difference in nuclear p65 binding from extracts of unstimulated macrophages treated with DMSO, EPA or DHA. At 5 hours of LPS activation the chemiluminescence generated by p65 nuclear binding was greater than that from unstimulated cells, but there was no significant effect of EPA or DHA treatment on p65 transactivation relative to DMSO.

Figure 5.17 NF-κB p65 nuclear binding in THP-1 derived macrophages treated with 25μmol/L DMSO □, EPA ■ and DHA □ over time points of LPS activation. Cells were stimulated with 0.1μg/ml LPS for 0 (a) and 5 (b) hours. Nuclear proteins were extracted and applied to an RHD-specific oligonucleotide coated plate. The plate was elaborated with primary antibody to bound p65 and conjugated secondary antibody, and developed by chemiluminescence. Results are presented as chemiluminescence relative to DMSO and represent the mean ± SEM of 4-6 independent experiments.
5.3.5g The effects of EPA and DHA on NF-κB translocation

The nuclear translocation of NF-κB in macrophages treated with DMSO, EPA and DHA was investigated using HCS technology from Cellomics. The sensitivity of this assay allowed significant NF-κB inhibitory effects of DHA to be demonstrated in unstimulated cells. Cytoplasmic NF-κB staining intensity was significantly increased by DHA treatment in unstimulated cells relative to that of cells treated with DMSO (P=0.03). Nuclear NF-κB staining intensity was significantly reduced by DHA treatment in unstimulated cells relative to that of cells treated DMSO (P=0.01) (Figure 5.18).

Figure 5.18 Staining of cytoplasmic (a) and nuclear (b) NF-κB in THP-1 derived macrophages treated with 25μmol/L DMSO □, EPA □ and DHA □. Cells were treated with fatty acids but not activated with LPS. Plates were fixed and stained for NF-κB with the antibody conjugated fluorophore Alexa® Fluor 488, as part of the Cellomics HitKit® protocol. Nuclei were identified by Hoechst 34442 staining. Cells were scanned with the Cellomics KineticScan® HCS Reader and compartmental analysis of NF-κB was performed. Staining intensity is presented in pixels and results represent the mean ± SEM of 4 independent experiments. * indicates P<0.05 relative to DMSO.
Cells treated with both fatty acids and LPS survived poorly. Cells treated with fatty acids or LPS survived well, as demonstrated by the results above and from the initial time and dose response LPS study. It seems likely that the THP-1 derived macrophages may not withstand the stress of the conditions used for Cellomics imaging. There may be a number of reasons for poor cell survival. After differentiation with PMA, THP-1 derived macrophages do not proliferate and their survival, or “shelf-life”, becomes limited. The cells thus become additionally sensitive to their culture and treatment conditions. For the Cellomics scan, macrophages were cultured in high-density black plates. Macrophages were normally cultured in lower density clear plates. The adherent macrophages may be sensitive to the quality of the plastic onto which they adhere. For the Cellomics HitKit protocol, macrophages were fixed with a 4% formaldehyde solution. This concentration may have negatively affected macrophage survival. Survival of THP-1 monocytes and derived macrophages appears, even in normal culture, to depend upon the density at which they are cultured. Macrophages seem to survive and proliferate when in close proximity to each other. Macrophages were normally cultured at $1 \times 10^6$ cells/ml. For the Cellomics assay cells were cultured at $5 \times 10^4$ cells/ml. The current protocol for macrophage culture and plate preparation with the Cellomics HitKit requires modification as it appears to negatively affect the survival of macrophages treated with EPA, DHA and DMSO prior to activation with LPS.
5.4 DISCUSSION
The anti-inflammatory effects of the n-3 LC-PUFA, EPA and DHA, have been overwhelmingly established in vitro. Animal feeding and human supplementation studies have yielded less consistent findings with respect to circulating and ex vivo secreted pro-inflammatory cytokines and cell adhesion molecules, reviewed in the introduction. In the present study, EPA and DHA suppressed the inflammatory phenotype of LPS-activated THP-1 derived macrophages. EPA and DHA significantly reduced IL-1β and IL-6 secretion. The secretion of TNFα was significantly reduced by EPA and non-significantly reduced by DHA. DHA significantly reduced the secretion and activity of the gelatinase MMP-9. Furthermore, the transcription of IL-1β was reduced non-significantly by EPA and significantly by DHA. Both EPA and DHA significantly reduced the transcription of IL-6, and EPA significantly reduced the transcription of TNFα. EPA and DHA displayed consistent anti-inflammatory effects in the THP-1 derived macrophage, in accordance with other studies that have employed monocytic cell lines (Chu et al. 1999; Lo et al. 1999; Babcock et al. 2002a and 2002b; Novak et al. 2003; Zhou et al. 2004).

The displacement of arachidonic acid from the phospholipid bilayer and consequent shunting of eicosanoid metabolism towards a less aggressive phenotype is a widely proposed mechanism through which the n-3 LC-PUFA, particularly EPA, exert their anti-inflammatory effects. The study of “fish oils” and emulsions containing both n-3 LC-PUFA has obscured the specific anti-inflammatory effects of EPA and DHA. Despite the potency of EPA over DHA in the displacement of arachidonic acid DHA, in the present and other studies (De Caterina et al. 2000; Liu et al. 2001), has demonstrated more significant anti-inflammatory effects than EPA. Furthermore, De Caterina et al. (2000) reported that the anti-inflammatory effects of EPA and DHA were not affected by indomethacin or eicosatetranooic acid, which block the activity of COX and LOX enzymes of eicosanoid metabolism. EPA and DHA are, therefore, likely to exert their anti-inflammatory effects through other mechanisms.

One of the initial experiments of the present study was an LPS dose-response study to determine the optimal concentration of LPS with which to activate the THP-1 derived macrophage. Preliminary studies employed 1μg/ml LPS. A saturating effect on cytokine secretion was observed at this concentration, whereupon the effects of EPA and DHA were either lost or unchanged from lower concentrations of LPS. The optimal concentration of LPS, eliciting the inflammatory phenotype of the macrophage while
maintaining the anti-inflammatory potential of EPA and DHA, was deemed to be 0.1 µg/ml. Interestingly, when a range of LPS concentrations was used, macrophages treated with EPA, DHA or DMSO appeared to display different sensitivity, or alertness, to the aggravating effects of classical activation. Cells treated with DMSO and EPA were activated to secrete IL-1β and IL-6 at lower concentrations of LPS than DHA treated macrophages. Cells treated with EPA became limited in their activated TNFα secretion at a lower concentration of LPS than DMSO or DHA treated macrophages. The anti-inflammatory effects of EPA and DHA have been demonstrated at single LPS concentrations, indicating their effects on activated cells. The present LPS dose-response study indicates that EPA and DHA may not just suppress the inflammatory phenotype of an activated cell, but rather mute the stimulatory effects of LPS specifically.

The stimulatory effects of LPS are mediated through the NF-κB signalling pathway (Takushima et al. 1999; Zhang & Ghosh, 2001). In the present study, the effect of LPS on cytoplasmic and nuclear compartmentalisation of NF-κB was investigated over a series of time points by Cellomics HCS technology. NF-κB began to move from the cytoplasm within 5 minutes of activation with LPS and appeared to increase in the nucleus by 15 minutes. Nuclear accumulation and cytoplasmic depletion of NF-κB continued until 1 hour of LPS activation. Compartmentalisation of NF-κB remained constant at between 1 and 2 hours of LPS activation. By 5 hours of LPS activation, however, the nuclear staining of NF-κB decreased and the cytoplasmic staining increasing, indicating that the stimulating effects of LPS activation were resolving. Thus, LPS induces NF-κB nuclear translocation very rapidly, within 15 minutes, and its effects begin to subside after 5 hours. In addition, a dose-response element to this experiment confirmed the saturated effect of stimulating THP-1 derived macrophages with 1 µg/ml LPS. There was no difference between the response to 0.1 and 1 µg/ml LPS in the timing or extent of NF-κB nuclear translocation.

The main features of the NF-κB signalling are liberation, translocation and transactivation. The effects of EPA and DHA on the liberation, translocation and transactivation of NF-κB were investigated in the time-frame of LPS activation (0 to 5 hours) indicated by the Cellomics compartmental analysis assay. NF-κB is bound in the cytoplasm to its inhibitor IκBα. The complex is dissociated by the IKK enzyme complex, which phosphorylates IκBα at serines 32 and 36 (Tak & Firestein, 2001). IκBα is subsequently polyubiquitinated by the E3 ligase complex and degraded within
the cytoplasm (Baldwin, 1996; Karin & Delhase, 2000), liberating NF-κB. NF-κB dimers translocate to the nucleus, escorted by karyopherin carrier proteins. The p50/p65 heterodimer is the most studied, with the p65 subunit found in “early” active NF-κB complexes. Overexpression of p50 represses the transcription of target genes such as TNFα and accumulates with delayed kinetics following macrophage activation with LPS (Baer et al. 1998). Nuclear IκBa represses transcription of target genes by binding and shuttling NF-κB from the nucleus. In addition, transcription of target genes may be repressed by the restrictive nature of chromatin architecture, which can present a physical barrier to NF-κB binding to DNA (Perkins, 1997; Ma et al. 2004). Transactivation, therefore, represents the binding of NF-κB, namely the active p65 subunit, to DNA. NF-κB liberation, translocation and transactivation were investigated in the present study.

The liberation of NF-κB was investigated in the present study by Western immunoblotting for expression of the inhibitory IκBa and the complex dissociating enzyme IKK. Cytoplasmic IκBa expression was significantly reduced by EPA and DHA at ½ hour of LPS activation and increased significantly by DHA at 1 hour of LPS activation relative to DMSO. Both EPA and DHA non-significantly increased cytoplasmic IκBa at 2 and 5 hours of LPS activation, relative to DMSO. The increase in cytoplasmic IκBa in EPA and DHA treated macrophages, though not consistently significant, indicates increased capacity for maintaining NF-κB in the cytoplasm. DHA significantly reduced nuclear IκBa at 1 and 5 hours of LPS activation and EPA significantly reduced nuclear IκBa at 5 hours of LPS activation, relative to DMSO. IκBa continuously shuttles in and out of the nucleus, removing nuclear NF-κB. Its nuclear reduction and cytoplasmic accumulation towards the end of the LPS activation time-frame may represent the removal of NF-κB from the nucleus and retention in the cytoplasm. Alternatively, as IκBa is itself a target gene of p65-induced transcription, its accumulation in the cytoplasm of EPA and DHA treated macrophages could indicate increased p65 activity in the nucleus. Cytoplasmic IKK expression was non-significantly lower in DHA treated macrophages at 0 and ½ hours of LPS activation and significantly lower in EPA and DHA treated macrophages at 2 hours of LPS activation, relative to DMSO. This decreased expression of IKK indicates a decreased potential for dissociation of NF-κB from IκBa. The effects of EPA and DHA on the components of NF-κB activation, namely IκBa and IKK, appear to be inhibitory.
The translocation of NF-κB was investigated in the present study principally by Western immunoblotting. The subunits of the p50/p65 heterodimer were investigated separately. The cytoplasmic expression of the NF-κB p65 subunit was significantly increased by EPA and DHA at 1 hour of LPS activation and by DHA at 2 and 5 hours of LPS activation, relative to DMSO. This directly indicates retention of the transcriptionally active NF-κB subunit in the cytoplasm by the n-3 LC-PUFA, particularly DHA. Nuclear expression of the NF-κB p65 subunit was significantly decreased by DHA at 1 hour of LPS activation and by EPA and DHA at 5 hours of LPS activation. These results indicate suppressive effects of EPA and DHA on nuclear translocation of p65. HCS technology from Cellomics was also used to investigate the effects of EPA and DHA on nuclear translocation of NF-κB. In unstimulated cells, the technique was sensitive enough to detect a significant increase in cytoplasmic and decrease in nuclear accumulation of NF-κB in DHA, relative to DMSO, treated macrophages. The LPS activated THP-1 derived macrophages did not withstand the cumulative stresses of sample preparation for HCS scanning and the protocol for sample preparation requires further modification. The cytoplasmic expression of the NF-κB p50 subunit was reduced in EPA and DHA treated cells, significantly by DHA, at 1 hour of LPS activation relative to DMSO. At 2 and 5 hours of LPS activation, however, both EPA and DHA significantly increased cytoplasmic expression of p50. Nuclear p50 was non-significantly reduced at 5 hours of LPS activation by EPA and DHA, relative to DMSO. This subunit is translocated into the nucleus with p65. Mentioned earlier, it accumulates with delayed kinetics in LPS activated macrophages and its overaccumulation, particularly as a homodimer, represses the transcriptional activity of the active subunit. The present results do not indicate an inhibitory effect of p50. Its cytoplasmic accumulation has been shown but no effect of accumulation in the nuclei of cells treated with EPA or DHA is evident, relative to DMSO.

The nuclear binding of the NF-κB p65 subunit was investigated in the present study by an ELISA style assay. The alternative, and more usual, technique is electromobility shift assay (EMSA). Despite reduced nuclear translocation of NF-κB in macrophages treated with EPA and DHA at 0 and 5 hours of LPS activation, there was no effect on binding of the p65 subunit, relative to DMSO.

The results of the present study indicate that EPA and DHA suppress the liberation and translocation of NF-κB. n-3 LC-PUFA have been shown, in a number of other studies, to suppress the pro-inflammatory transcriptional regulation of NF-κB.
LPS activated *in vitro* murine macrophages displayed decreased IκBα phosphorylation at serine 32, NF-κB activation and binding when pre-treated with an n-3 LC-PUFA emulsion (Novak *et al.* 2003). Pre-incubation of human monocytes with EPA partially prevented LPS-induced phosphorylation and degradation of IκBα, NF-κB activation and nuclear translocation of p65 (Zhou *et al.* 2004). Macrophages incubated in EPA rich media displayed suppression of p65/p50 at baseline and after LPS exposure (Lo *et al.* 1999). DHA has also been shown to inhibit the activation of the NF-κB system of transcription factors (De Caterina *et al.* 2000).

In the present study EPA and DHA significantly inhibited the transcription of NF-κB regulated genes such as IL-1β, IL-6 and TNFα. However, EPA and DHA did not inhibit the binding of NF-κB to nuclear oligonucleotide sequences representing a genomic p65 consensus binding site.

Nuclear translocation of NF-κB does not indicate binding to DNA. As outlined above, the binding of p65 to DNA is tightly regulated by nuclear IκBα, p50 and by the repressive architecture of chromatin. Modifications, such as acetylation, ubiquitination, methylation or SUMOylation, of the DNA package are required for p65 access to transcription factor-promoter complexes (Vermeulen *et al.* 2002). In the present study, however, it is incongruous that the transcription of NF-κB regulated genes was suppressed by EPA and DHA but p65 binding was not.

Nuclear binding of NF-κB does not indicate transactivation. NF-κB is capable of binding to DNA without being transcriptionally active. The p38 MAPK inhibitor SC203580, bcl2 and bcl-xL have been shown to inhibit transactivation without affecting the nuclear localisation of NF-κB (Perkins 1997). Vitamin C has been shown to inhibit IKK activation, IκBα phosphorylation and induction of IL-8, an NF-κB dependent gene without affecting the DNA binding of NF-κB (Bowie & O’Neill, 2000). The authors of this paper demonstrate that inhibition of upstream NF-κB activation by Vitamin C was via activation of p38 MAPK. Furthermore, a κB element is not always required for the transactivation of NF-κB (Perkins, 1997). The RHD of NF-κB associates with a large number of b-Zip containing transcription factors. p65 stimulates AP-1 DNA binding and activation through AP-1 sites in the absence of a κB element (Liu, Le & Nian, 2001). EPA and DHA may potentially suppress the transactivation of NF-κB associated with a non-κB promoter element. Therefore, the suppression of NF-κB signalling by n-3 LC-PUFA must be considered in terms of other transcription factors and related kinases.
n-3 LC-PUFA have been shown to modulate activation of members of the MAPK family. Unstimulated murine macrophage RAW 264.7 cells exhibited decreased p44/p42 activation, decreased JNK/SAPK phosphorylation and increased p38 phosphorylation when pre-treated with an n-3 LC-PUFA emulsion. n-3 LC-PUFA pretreated LPS-stimulated macrophages demonstrated decreased p44/p42 and AP-1 activation and decreased phosphorylation of JNK/SAPK (Babcock et al. 2003). EPA rich media inhibited LPS-stimulated p44/p42 phosphorylation and AP-1 activation in RAW 264.7 macrophages (Lo et al. 1999b). DHA inhibited both TPA- and EGF-induced AP-1 activation in a murine epidermal cell line (Liu et al. 2001) and activated p38 in cultured vascular smooth muscle cells (Diep et al. 2000). Diep et al. (2000) report that DHA induced apoptosis through the activation of PPARα in a p38 dependent manner. PPAR activation inhibits the production of pro-inflammatory cytokines and NF-κB activation. EPA and DHA increased PPARγ mRNA and protein activity and decreased NF-κB activation in human renal HK-2 cells and these effects were abolished by the addition of the PPARγ inhibitor BADGE (Li et al. 2005). Serum n-3 LC-PUFA concentrations correlated positively with PPARγ mRNA levels in adipose tissue, and EPA, but not DHA, induced a significant increase in PPARγ1, but not PPARγ2, mRNA expression in cultured human adipocytes (Chambrier et al. 2002). It has been demonstrated that carriers of the Pro12Ala polymorphism of the PPARα2 gene presented a greater decrease in serum TAG in response to n-3 LC-PUFA supplementation than carriers of the Pro12Pro genotype (Lindi et al. 2003).

In the present study fatty acid treatments appeared to affect the sensitivity of macrophages to the concentration of LPS at which they become phenotypically activated in terms of cytokine secretion. [14C]DHA has been demonstrated to become significantly incorporated into the phosphotidylethanolamine pool found in the inner plasma membrane, a strategic position from which to alter intracellular signal transduction pathways (De Caterina et al. 2000). Jordan & Stein (2001) proposed that n-3 LC-PUFA alter the properties of the plasma membrane and receptor-ligand binding. Pre-treatment of THP-1 monocytes with EPA or DHA reduced fluorescein-isothiocyanate (FITC)-conjugated LPS binding by approximately 70% and significantly reduced CD14 upregulation (Chu et al. 1999). Unsaturated fatty acids have been demonstrated to inhibit TLR4, the LPS receptor, activation (Lee et al. 2001). Moreover, DHA inhibited the activation of all TLRs tested (Lee et al. 2003). n-3 LC-PUFA may block LPS transmembrane signalling by altering ligand binding to toll-like receptor 4.
In the present study, anti-inflammatory effects of the n-3 LC-PUFA on IL-1β, IL-6 and TNFα secretion and transcription in LPS activated THP-1 macrophages were demonstrated. In addition, MMP-9 secretion and activation were reduced by DHA. These proteins and their transcripts are under the regulation of the NF-κB signalling pathway. EPA and/or DHA were demonstrated to inhibit IKK expression, to increase cytoplasmic expression of IκBα and to retain the active NF-κB p65 subunit in the cytoplasm. Neither fatty acid affects p65 binding to a consensus sequence representing genomic DNA. It is likely that EPA and DHA affect related transcription factors and kinases, including AP-1 and p38 MAPK. The n-3 LC-PUFA may also affect the activation of TLR4 by LPS. The suppression of this fundamental and evolutionarily conserved membrane signalling complex would represent a major anti-inflammatory paradigm. The gross anti-inflammatory effects of EPA and DHA are relatively well established in vitro, but the cellular signals that mediate these effects are not yet resolved.
Chapter 6

Discussion
6. GENERAL DISCUSSION

The Central Statistics Office attributed 39% of deaths in Ireland in 2003 to CVD. Atherosclerosis, the principal cause of CVD, has a strong inflammatory component.

The presence of inflammation in the arterial wall was identified in the 19th century by European pathologists. The “response to injury” theory propounded by Ross (1993) and others in the 1990s defined the role of inflammation in the aetiology of atherosclerosis. The cells that contribute to this inflammatory process include endothelial cells, smooth muscle cells, dendritic cells, T lymphocytes, monocytes, macrophages and foam cells. T lymphocytes and monocytes accumulate at prime sites of lesion development in infancy and are initiative, constitutive and active in the history of atherosclerosis, from the “fatty streak” to the complicated lesion. Fatty acids have been shown to modulate immune responses. The main objective of this thesis was to investigate the effects of a number of fatty acids on mediators of inflammation associated with atherosclerosis. Specifically, the effects of PUFA on pro-inflammatory elements of activated T lymphocytes, monocytes and macrophages were studied.

A human intervention trial demonstrated that CLA supplementation may modulate elements of the Th1 inflammatory response in a cohort of healthy middle-aged men. The secretion of IL-2 from ex vivo Con-A stimulated PBMC was significantly decreased following 8 weeks of supplementation with a 50:50 blend of c9, t11-CLA and t10, c12-CLA. When the study group was divided by circulating CRP concentrations, CLA supplementation appeared to significantly reduce TNFα secretion in the “high” group (CRP>0.8mg/L) and to significantly increase TNFα secretion in the “low” (CRP<0.8mg/L) group. CLA supplementation had no effect on the secretion of IL-10 from PBMC, on systemic IL-6, CRP and fibrinogen concentrations or on plasma total cholesterol, HDL, LDL, TAG and fasting glucose levels.

The isomer specific effects of c9, t11-CLA and t10, c12-CLA, relative to the vehicle control DMSO were investigated in vitro using the THP-1 monocytic cell line. Both isomers significantly reduced IL-1β secretion from LPS activated monocytes. Transcription of IL-1β was reduced by both isomers, significantly by t10, c12-CLA. Secretion of IL-6 was significantly reduced by c9, t11-CLA. Transcription of IL-6 was significantly reduced by both isomers. Secretion of TNFα was not affected by CLA treatment of monocytes. The anti-inflammatory effects of the CLA isomers were mirrored, to some extent, by linoleic acid. Palmitic acid generally displayed pro-
inflammatory effects in the context of this experiment. These studies demonstrate anti-inflammatory potential of CLA on activated T lymphocytes and monocytes, inflammatory cells of atherosclerosis. However, the crux of the matter, that CLA is anti-inflammatory *in vivo* and *relevant* as an anti-inflammatory agent, has yet to be resolved.

Cytokine production varies between individuals. Secretion and transcription of cytokines as study outputs are, therefore, inherently prone to variation and demanding on statistical power. *In vitro* and animal studies, reviewed in Chapter 3, have demonstrated anti-inflammatory effects of CLA. Human studies have shown this to a much lesser extent. The sensitivity of the individual to the effects of PUFA has been shown to depend on genotype (Markovic *et al.* 2004). In the human intervention trial, CLA significantly modulated the secretion of TNFα on the basis of circulating CRP levels, which have a strong genetic association and are relatively invariant. Studies of human supplementation with PUFA may benefit from and yield more consistent results with genotype information of the study participants. It is likely that anti-inflammatory effects of PUFA, such as CLA, would be more convincingly demonstrated in humans with this approach. Indeed, a homogenous cell system, such as the THP-1 monocytic line, displays sensitivity to PUFA in cytokine production that *ex vivo* cells do not.

The present study showed that both CLA isomers suppress cytokine production relative to DMSO, *in vitro*. Additionally, linoleic acid was shown to suppress cytokine production. Linoleic acid is often used as a control for CLA, and it seems likely that the effects of CLA may be lost or obscured by this practice. The placebo used in the present human supplementation trial contained a blend of fatty acids typically found in the Western diet and, by virtue of this, demonstrated no effects on the inflammatory mediators investigated. The anti-inflammatory effects demonstrated by linoleic acid *in vitro* pose the question of whether the effects of CLA are of its own accord or due to its derivation from linoleic acid. However, the CLA isomers and linoleic acid demonstrated different potencies in their effects on pro-inflammatory cytokine production in the THP-1 model. These fatty acids were shown previously by our group to down-regulate NF-kB:DNA binding in the nuclei of PBMC with different potencies.

The present investigation demonstrates anti-inflammatory potential of CLA in activated *ex vivo* PBMC and *in vitro* monocytes. These effects may be further elucidated by CLA and isomer-specific supplementation of genotyped cohorts.

Elevated serum NEFA concentrations are atherogenic. Additionally, the composition of serum NEFA appears to influence their atherogenicity. The main
The objective of Chapter 4 was to investigate the effects of fatty acids, representative of serum NEFA, on macrophage inflammatory mediators associated with atherosclerosis. Three blends of fatty acids were designed. The SFA blend reflected the composition of NEFA reported in subjects with metabolic syndromes and those at risk of CVD. In the MUFA blend, a proportion of SFAs were replaced with oleic acid, maintaining reported physiological concentrations throughout. In the MUFA + n-3 PUFA blend, SFA were further replaced by the n-3 LC-PUFA, EPA and DHA. The concentrations of NEFA typically found in the circulation were toxic to the THP-1 macrophage model. However, the extent of macrophage exposure to NEFA in the atherosclerotic microenvironment is difficult to define. Therefore, the composition of serum NEFA was the factor by which the inflammatory response of activated macrophage was investigated.

IL-1β transcription was increased by all NEFA treatments relative to DMSO, significantly by MUFA + n-3 PUFA. Secretion of IL-1β was increased by all NEFA treatments relative to DMSO, significantly by SFA and MUFA. Transcription of IL-6 was significantly decreased by all NEFA treatments. Secretion of IL-6 was significantly decreased by all NEFA treatments relative to DMSO. The addition of MUFA increased IL-6 secretion relative to SFA and the addition of n-3 PUFA decreased IL-6 secretion relative to MUFA. Transcription of TNFα was significantly increased by the MUFA + n-3 PUFA treatment. Secretion of TNFα was significantly decreased by the MUFA + n-3 PUFA treatment relative to DMSO. None of the NEFA treatments affected MMP-9 activity, although activity was non-significantly reduced by the addition of n-3 PUFA relative to the MUFA treatment. The effects on cytokine secretion were consistent over a range of LPS concentrations. The increase in IL-1β appeared to be a general fatty acid effect, perhaps due to the n-6 content of the NEFA blends. Each treatment contained a constant concentration of linoleic acid. The over-riding effects of altering NEFA composition were, in this experiment, limited. However, the addition of n-3 PUFA to the MUFA treatment appeared to exert some anti-inflammatory potential.

There are two sources of serum NEFA; post-prandial TRL and, during fasting or prolonged exercise, adipose tissue. The composition of circulating NEFA resembles the fatty acid composition of the test meal. However, there is a stronger correlation between adipose tissue TAG content and serum NEFA (Yli-Jama et al. 2001). To what extent would altering the composition of dietary fatty acids affect NEFA composition? An extended post-prandial state is typical of the Western lifestyle. It is likely, then, that TRL are a prime source of circulating NEFA. Thus, altering the composition of dietary
fatty acids should, in theory, be reflected in the composition of circulating NEFA. However, the correlation between adipose tissue and circulating NEFA is reported to be stronger than that between diet and circulating NEFA. Selective deposition and mobilisation of fatty acids from adipose tissue indicates restrictive expression of fatty acids in circulating NEFA. In the present study, some anti-inflammatory potential was demonstrated by increasing the content of n-3 PUFA in the NEFA blend. Fatty acids are selectively mobilised from adipose tissue in order of chain length and degree of saturation with PUFA, such as EPA, release >MUFA>SFA. In the fasting state or following prolonged exercise it is, therefore, likely that a less inflammatory composition of serum NEFA circulates by virtue of adipose “gate-keeping”. Since post-prandial NEFA resembles to some extent fatty acids of the diet, it is likely that increasing n-3 PUFA intake may also elicit a less inflammatory contingent of circulating NEFA.

*In vitro* experiments generally do not employ blends of fatty acids. The effects of isolated fatty acids on inflammatory mediators are commonly investigated. In the present study, the blends of fatty acids represented those found circulating physiologically in NEFA. The anti-inflammatory potential of individual components was masked, in some instances, by generalised fatty acid effects. This may be more reflective of the *in vivo* situation than studies that use single fatty acids. However, *in vitro* experiments are inherently artificial. In the present study, the THP-1 monocytic cell line was used. For preliminary studies cell lines are ideal. As mentioned earlier, their homogenous nature generally produces consistent and powerful results. The hypothesis should be, thereafter, applied to a more physiological model; e.g. a mixed culture of leukocytes, such as *ex vivo* PBMC. PBMCs are directly exposed to circulating NEFA *in vivo*. PBMC exposed to NEFA, elevated by infusion with lipid emulsion and injection with ACTH or heparin, altered by ingestion of a test meal, prolonged fasting or exercise, could be cultured *ex vivo* and activated to secrete pro-inflammatory mediators. As mentioned earlier, it would be important to understand the genotype of participants in such a study to minimise variation in cytokine secretion between individuals. Additionally, it may be interesting to establish an *in vitro* model to investigate the selective mobilisation of fatty acids from adipocytes. The NEFA composition of adipocytes treated with blends of fatty acids could be investigated before and after activated lipolysis. Furthermore, the media into which adipocytes release their NEFA could be analysed.
EPA and DHA were demonstrated to possess potent anti-inflammatory effects on activated macrophages in the experimental work of Chapter 5. Transcription of IL-1β was reduced by both n-3 LC-PUFA relative to DMSO, significantly by DHA. Secretion of IL-1β was significantly reduced by EPA and DHA. Transcription and secretion of IL-6 was significantly reduced by EPA and DHA. Transcription of TNFα was reduced by both n-3 LC-PUFA, significantly by EPA relative to DMSO. Secretion of TNFα was significantly reduced by EPA. Secretion and activity of MMP-9 was significantly reduced by DHA. Within the NEFA treatment of Chapter 4, EPA and DHA exerted mild anti-inflammatory potential; in their isolated capacity anti-inflammatory effects were potent. Western immunoblot analyses indicated that EPA and DHA modulate elements of the activated NF-κB signalling pathway, including downregulated translocation of the p65 subunit to the nucleus, its retention in the cytoplasm and downregulation of cytoplasmic IKK expression. In unstimulated cells investigated by HCS technology from Cellomics Inc., DHA significantly reduced nuclear compartmentalisation of NF-κB. However, by an ELISA-style binding assay, EPA and DHA had no effect on p65 binding to an oligonucleotide consensus sequence, indicating no effect on DNA binding to κB promoter elements. EPA and DHA may affect the binding of p65 to other non-κB nuclear elements or the interaction of p65 with other transcription factors, such as AP-1, and related kinases, such as p38 MAPK. EPA and DHA may work upstream of IKK. EPA and DHA could directly interfere with the activation of the cell by blocking effective interaction of LPS with TLR4. Given the function of fatty acids in membrane structures, including the phospholipid bilayer and lipid rafts, and the unique structure of the n-3 LC-PUFA, it seems likely that EPA and DHA may function at the membrane. The mechanism of the anti-inflammatory effects of the n-3 LC-PUFA requires further investigation.

Minimising artificiality in a cellular model is desirable. Likewise, minimising artificiality in the investigation of cellular biomolecules is important. Techniques such as RT-PCR and Western immunoblotting that investigate intracellular events generally involve the destruction of the cell and extraction of material from cellular compartments. Cellomics, the knowledge of cellular phenotype and function, employs the entire cell and, thus, naturalises the investigation. In the present study LPS activated THP-1 derived macrophages, pre-treated with EPA, DHA and DMSO, did not withstand plate fixing, staining and scanning for NF-κB with the Cellomics KineticScan Reader. The protocol needs to be modified, as outlined in Chapter 5. Cellomics Inc.
currently supply HitKits for ATF-2, c-Jun, ERK MAPK, NF-κB, NFAT-1, p38 MAPK, PKCa, STAT1, STAT2 and STAT3 activation. It will be of interest to investigate the effects of EPA and DHA on a number of these signalling molecules, particularly ERK and p38 MAPK in macrophages.

T lymphocytes, monocytes and macrophages have been studied, more or less, in isolation in the present thesis. Their pro-inflammatory activation is shown to be inhibited by PUFA. Further work in this area is essential. The effects of PUFA on DC function must be investigated. The effects of PUFA on APC activation of lymphocytes needs to be studied; perhaps by culturing lymphocytes in the media of PUFA-treated activated macrophages or DCs. Co-culture of PUFA treated immune cells with smooth muscle cells or endothelial cells would establish a more complex *in vitro* model of atherosclerosis. The mechanisms of the anti-inflammatory effects of PUFA must be elucidated by simple and more physiological *in vitro* models before application in human intervention trials, where genotyping of participants will deepen our understanding of how PUFA affect mediators of inflammation association with atherosclerosis *in vivo*.
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