The Pleiotropic Effect of Statins on Immune Cell Function

Dianne Zoë Hillyard
BSc (Hons)

Thesis submitted for the degree of PhD

University of Glasgow
Faculty of Medicine
Division of Cardiovascular and Medical Sciences

October 2006

Acknowledgements

I would first and foremost like to thank Dr Angus Cameron for persuading me to do this PhD and for his enormous help and encouragement.

I would also like to thank my supervisor, Dr Alan G Jardine for allowing me to do this PhD, and everyone else in the lab, especially Kenny and Jackie for their support. Also, Alan Macintyre (and everyone else in the Department of Surgery) for their regular supply of blood.

Finally, I wish to acknowledge the funding support received from The British Heart Foundation and Darlinda's Charity for Renal Research without which this work would not have been possible.

Author's declaration

I declare that this thesis has been composed by me and is a record of work performed by me (unless otherwise stated). It has not previously been submitted for a higher degree.

Dianne Z Hillyard

October 2006

Summary

Statins are 3-hydroxy, 3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, which block the cholesterol biosynthetic pathway to lower serum total and LDL-cholesterol. However, the cholesterol pathway also provides a supply of isoprenoids (farnesyl and geranylgeranyl) for the prenylation of signalling molecules, which include the Ras and Rho families of GTPases. Prenyl groups provide a membrane anchor that is essential for the correct membrane localisation and function of these proteins. An alternative explanation for the disruption of signalling pathways is that cholesterol depletion disrupts cholesterol and sphingolipid rich micro-domains - termed membrane rafts. Rafts play a central role in signal transduction in immune effector cells by providing a platform that physically concentrates receptors, downstream kinases and adaptor proteins involved in signalling pathways. Clustering of raft components upon receptor ligation and cross-linking increases the local concentration of signalling molecules, facilitating or triggering the generation of signals.

In this thesis, the pleiotropic effects of statins on the disruption of prenylation and membrane rafts were investigated in immune effector cells. T cells and NK cells were extracted from various groups of patients treated with statins and proliferation and cytotoxicity respectively, measured *ex vivo*. In a study of patients with cardiovascular disease, a 23% reduction in T cell proliferation and 43% reduction in NK cytotoxicity were observed. In a normal volunteer study where healthy subjects received simvastatin (40mg per day) for 4 weeks, a comparable reduction in T cell proliferation was not apparent, however a 30% reduction in NK cytotoxicity was observed. *In vitro* statin treatment further reduced proliferation and cytotoxicity whereas addition of farnesyl and geranylgeranyl transferase inhibitors had little or no effect. Addition of farnesol and geranylgeraniol groups failed to rescue statin inhibited T cell proliferation indicating that inhibition of prenylation is likely to have less of an impact than membrane raft disruption. A final study of *ex vivo* NK cytotoxicity was performed in a diverse group of patients. It revealed a trend towards higher levels of cytotoxicity at higher cholesterol levels and although statin therapy reduced NK cell killing in individuals, statin-treated patients had levels of NK cell killing appropriate for their total cholesterol.

The effect of statins on prenylation of small GTPases was studied in the U937 monocyte cell line and primary human T cells. U937 cells treated with statins were separated into cytosolic and membrane components and visualised by western blot. Reductions of GTPases were observed in membrane compartments with corresponding increases in cytosolic compartments. Unprenylated Ras was also observed as a band shift. Ras and Rac activity was reduced in primary human T cells treated with statin. Corresponding downstream signalling molecules ERK and p38 were also reduced by statin treatment and this was confirmed in the U937 cell line.

Cholesterol reduction in membrane rafts by statins were then analysed using the U937 cell line and the NK92MI cell line. Both fluvastatin and simvastatin reduced membrane raft associated proteins LAT and Lyn in a dose dependent manner in U937 cells and NK92MI cells. FTI and GGTIs had no effect on rafts and mevalonate rescued the statin inhibition, indicating that prenylation did not affect rafts and cholesterol depletion within the rafts could be recovered by restoration of cholesterol biosynthesis. Similar results obtained from the cholesterol chelator MβCD, confirmed that statins reduced cholesterol within the membrane to alter the density of rafts.

The functional effect of statins on Fc receptor signalling in monocytes was investigated using the U937 cell line. Tyrosine phoshorylation of cross-linked FcγR was time and dose dependently inhibited by statins, as was the corresponding downstream signalling molecules ERK and p38. FcγR immune complex internalisation and trafficking and release of MMP-1, IL-6, IL-8 and IL-10 inflammatory cytokines was reduced dose dependently by fluvastatin and simvastatin and unaffected by FTI and GGTI. ERK and p38 inhibitors demonstrated that IL-6 release involves both the ERK and p38 signalling pathways whereas MMP-1 release involves the ERK pathway, but not the p38 pathway.

The functional effects of statins on lymphocytes were finally investigated in human T cells and the NK92MI cell line. Primary human T cell proliferation was measured *in vitro*. Fluvastatin reduced proliferation while mevalonate rescued the inhibition and FTI and GGTI had no effect. Statins minimally reduced NK cell proliferation and dose dependently reduced NK cytotoxicity as measured in the

NK92MI cell line. Mevalonate rescued the inhibition and FTI and GGTI had no effect. Low concentrations of MβCD in the presence of cholesterol increased NK cytotoxicity, but at higher concentrations acted as a chelator to remove cholesterol from membranes and reduced cytotoxicity. I concluded that the pleiotropic effect of statins was due to raft disruption rather than inhibition of prenylation.

In conclusion, this thesis has demonstrated that statins have a multitude of applications and effects. The pleiotropism of statins due to reduced prenylation was observed *in vitro* by western blot in multiple signalling pathways and confirmed with the use of FT and GGT inhibitors in these pathways. However, the lack of functional effect of FT and GGTIs indicated that prenylation has a lesser impact on the functions of immune effector cells than cholesterol depletion of rafts. The comparable results obtained with MβCD indicated that the reduction of cholesterol in the membrane by statins was disrupting rafts and therefore disrupting signalling pathways to a greater extent than prenylation inhibition. It is however likely to be a combination of both processes that contributes to the pleiotropic effect of statins.

Contents

Tables

Figures

Publications and abstracts

Publications

1. Hillyard DZ, Cameron AJ, McIntyre AH, Hadden MH, Marshall HE, Johnston N, Jardine AG. **Inhibition of proliferation and signalling mechanisms in human lymphocytes by fluvastatin**. *Clin Exp Pharmacol Physiol.* 2002;29:673-8.

2. Hillyard DZ, Jardine AG, McDonald KJ, Cameron AJ. **Fluvastatin inhibits raft dependent Fcgamma receptor signalling in human monocytes.** *Atherosclerosis.* 2004;172:219-28.

3. Hillyard DZ, Cameron AJ, McDonald KJ, Thomson J, MacIntyre A, Shiels PG, Panarelli M, Jardine AG. **Simvastatin inhibits lymphocyte function in normal subjects and patients with cardiovascular disease**. *Atherosclerosis.* 2004;175:305-13.

4. Mohamed AS, Thomson J, McDonald KJ, Hillyard DZ, Mark PB, Elliott HL, Jardine AG. **Circulating endothelial cells in renal transplant recipients.** *Transplant Proc.* 2005;37:2387-90.

5. Hillyard DZ, Nutt CD, Thomson J, McDonald KJ, Wan RK, Cameron AJ, Mark PB, Jardine AG. **Statins inhibit NK cell cytotoxicity by membrane raft depletion rather than inhibition of isoprenylation**. *Atherosclerosis.* 2006 (e published ahead of print).

Meeting abstracts

1 Hillyard DZ, Cameron AJM, MacIntyre A, Jardine AG. **Mechanism of the immunosuppressive effects of fluvastatin in human lymphocytes**, *MRS, London*, November 2001. (Poster presentation. Award for best poster.)

2 Hillyard DZ, Cameron AJM, MacIntyre A, Jardine AG. **Mechanism of the immunosuppressive effects of fluvastatin in human lymphocytes**. *ATC, Washington DC*, April 2002. (Poster presentation and oral presentation for top 10 posters.)

3 Hillyard DZ, McDonald KJ, Cameron AJM, Jardine AG. **Statins (HMG-CoA reductase inhibitors) inhibit immune complex internalisation and trafficking in the U937 monocyte cell line.** *The Renal Association, London*, October 2002. (Poster presentation.)

4 Hillyard DZ, Cameron AJM, McDonald KJ, Thomson J, MacIntyre A, P Shiels, Jardine AG. **Simvastatin inhibits natural killer cell cytotoxicity in normal subjects**. *BTS, Birmingham*, April 2004. (Poster presentation.)

5 Hillyard DZ, Nutt CD, McDonald KJ, Thomson J, Mark PB, Jardine AG. **Statins inhibit Natural Killer cell cytotoxicity through raft depletion rather than inhibition of isoprenylation.** *ASN, Philadelphia,* October 2005. (Poster presentation.)

Abbreviations

Ab	Antibody
ACS	Acute coronary syndrome
ADCC	Antibody dependent cell mediated cytotoxicity
APC	Antigen presenting cell
ATP	Adenosine 5'-triphosphate
BCR	B cell receptor
BLNK	B-cell LiNKer protein
BSA	Bovine serum albumin
CAD	Coronary artery disease
CAM	Cell adhesion molecule
cDNA	Complementary DNA
CRP	C-reactive protein
CTLA	Cytotoxic T-lymphocyte antigen
CyA	Cyclosporin
CYP	Cytochrome P 450
DAG	Diacylglycerol
DAP10/12	DNAX activating protein of 10/12kDa
dATP	Deoxy Adenosine 5'-triphosphate
dbcAMP	Dibutyryl cyclic adenosine 5'-triphosphate
ddH_2O	Double distilled water
DGAT	Diacylglycerol acyltransferase
dGTP	Deoxy Guanosine 5'-triphosphate
DH	Dbl homology domain
DMEM	Dulbecco's modified eagles medium
DNA	Deoxy-ribonucleic acid
DRM	Detergent resistant membrane
dsRNA	Double stranded ribonucleic acid
DTT	Dithiothreitol
dTTP	Deoxy thymidine 5' triphosphate
EC	Endothelial cell
ECL	Enhanced chemiluminesence
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase

F(ab)	Antigen binding fragment of an immunoglobulin
FasL	Fas Ligand
Fc	Constant region of an immunoglobulin
FcαR	Receptor for the constant region of IgA
FcεR	Receptor for the constant region of IgE
FcγR	Receptor for the constant region of IgG
FcμR	Receptor for the constant region of IgM
FcRn	Neonatal Fc receptor
FCS	Foetal calf serum
FITC	Fluoroscein isothiocyanate
FTase	Farnesyl transferase
GAP	GTPase activating protein
GDF	GDI displacement factors
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanine 5'-diphosphate
GEF	Guanine nucleotide exchange factor
GM1	Ganglioside1
GMCSF	Granulocyte–macrophage colony stimulating factor
GPI	Glycosyl phosphatidyl inositol
Grb-2	Growth factor receptor-bound protein-2
GGTase	Geranylgeranyl transferase
GTPase	Small guanine nucleotide binding proteins or small g proteins
HA	Haemagglutinin
HBSS	Hanks' balanced salt solution
HDL	High density lipoprotein
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMG-CoA	3-hydroxy, 3-methylglutaryl coenzyme A
HRP	Horse radish peroxidase
hsCRP	High sensitivity C-reactive protein
Hsp	Heat-shock protein
IC	Immune complex
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-gamma
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M

IgSF	Immunoglobulin super family
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
$InsP_3$	Inositol trisphosphate
$InsP_4$	Inositol tetrakisphosphate
IPTG	Isopropylthiogalactoside
IRI	Ischemiareperfusion injury
ITAM	Immunoreceptor tyrosine based activation motif
ITIM	Immunoreceptor tyrosine based inhibitory motif
ITSM	Immunoreceptor tyrosine based switch motif
JNK/SAPK	c-Jun N terminal kinase
KIR	Killer immunoglobulin-like receptor
LAT	Linker for activation of T-cells
LDL	Low density lipoprotein
LFA-1	Leukocyte function antigen-1
LIR	Leukocyte inhibitory receptor
mAb	Monoclonal Antibody
MAPK	Mitogen activated protein kinase
MβCD	Methyl-β-cyclodextrin
MCP-1	Monocyte chemotactic protein-1
M-CSF	Monocyte-colony stimulating factor
MEK	MAPK/ERK kinase
MEKK	MAPK/ERK kinase kinase
MHC	Major histocompatability complex
MI	Myocardial Infarction
MICA/B	MHC class I chain related A or B
MIP	Macrophage inflammatory protein
MKK	MAP kinase kinase
MKKK	MAP kinase kinase kinase
MLB	Magnesium lysis buffer
MMP-1	Matrix metalloproteinase-1
Mϕ	macrophage
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MTOC	Microtubule organising centre
NADPH	Nicotinamide adenine dinucleotide phosphate
NCR	Natural cytotoxicity receptor

NFAT	Nuclear factor of activated T-cells
NFκB	NFκB
NK cell	Natural Killer cell
NPC1L1	Niemann-Pick C1-like 1
NTB-A	NK, T and B cell antigen
oxLDL	Oxidised LDL
PA	Phosphatidic acid
PAI	Plasminogen activator inhibitor-1
PAMPS	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PDGF	Platelet–derived growth factor
PDK	PIP_3 dependent kinase
PGI2	Prostaglandin 12
PH domain	Pleckstrin homology domain
PI 3-kinase	Phosphatidyl inositol 3 kinase
PI	Phosphatidyl inositol
PIP	Phosphatidyl inositol phosphate
PIP_2	Phosphatidylinositol bisphosphate
PIP_3	Phosphatidyl inositol trisphosphate
PKB	Protein kinase B
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
Poly I:C	polyinosinic-polycytidylic acid`
PPAR-γ	Peroxisome proliferator activated receptors
PTB domain	Phosphotyrosine binding domain
PTK	Protein tyrosine kinase
PVR	Poliovirus receptor
RANTES	Regulated upon activation normal T cell expressed and secreted
RBD	Ras binding domain
RCT	Reverse cholesterol transport
RGS	Regulators of G protein signalling
Rho	Ras-homologue
RNA	Ribonucleic acid

RT	Room temperature
RT-PCR	Reverse transcriptase-polymerase chain reaction
RXR	Retinoid X receptor
SAP	Signalling lymphocyte activation molecule-associated protein
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylmide gel electrophoresis
SE	Standard error of the mean
SH2 domain	Src homology 2 domain
SH3 domain	Src homology 3 domain
SHIP	SH2 containing inositol phosphatase
SHP	SH2 containing protein tyrosine phosphatase
SLP-76	SH2 domain containing linker protein - 76 kDa
SMC	Smooth muscle cell
TBS	Tris buffered saline
TBST	Tris buffered saline containing Tween-20
TCA	Trichloracetic acid
TCR	T cell receptor
TEMED	N,N,N',N',-Tetramethylethylenediamine
TGF-β	Transforming growth factor-beta
TIMP-1	Tissue inhibitors of metalloptoteinase
TIR	Toll/IL-1 receptor homology domain
TLR	Toll-like receptor
TMB	Tetra-methylbenzidine
TNF	Tumour necrosis factor
t-PA	Tissue plasminogen activator
TRAIL	TNF related apoptosis inducing ligand
tRNA	Transfer ribonucleic acid
TxA2	Thromboxane A2
VCAM-1	Vascular cell-adhesion molecule-1
VLA-4	Very late antigen

Chapter 1: Introduction

1.1 Cholesterol pathway

1.1.1 Products of mevalonate

Statins are 3-hydroxy, 3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors that block the cholesterol biosynthetic pathway[1] (Figure 1.1). Cholesterol is required for the synthesis of steroid hormones, vitamin D, bile acids and lipoproteins and it is therefore important to maintain intracellular levels. Reduction of intracellular cholesterol stores activate sterol-responsive-element-binding proteins, leading to increased transcription of the LDL receptor gene and expression of cell surface receptor and aids the clearance of LDL from plasma, therefore restoring intracellular homeostasis. Other products of HMG-CoA include, dolichol, haem, ubiquinone and isoprenylated proteins. Cells can also regulate mevalonate disposition. Non-sterol pathway enzymes have higher affinities for mevalonate-derived substrates than sterol pathway enzymes. The first committed enzyme of sterol synthesis, squalene synthetase, is suppressed by the presence of sterols, thereby limiting incorporation of mevalonate into further sterols and favouring the high affinity non-sterol pathways.

1.1.2 Biochemistry

1.1.2.1 HMG-CoA biochemistry

Conversion of HMG-CoA to mevalonate, catalysed by HMG-CoA reductase, is the rate limiting step of the cholesterol pathway and proceeds in three stages, the first and third of which are reductive[2] (Figure 1.2).

1.1.2.2 Statin biochemistry

Statins share a structural component that is similar to, but more bulky and

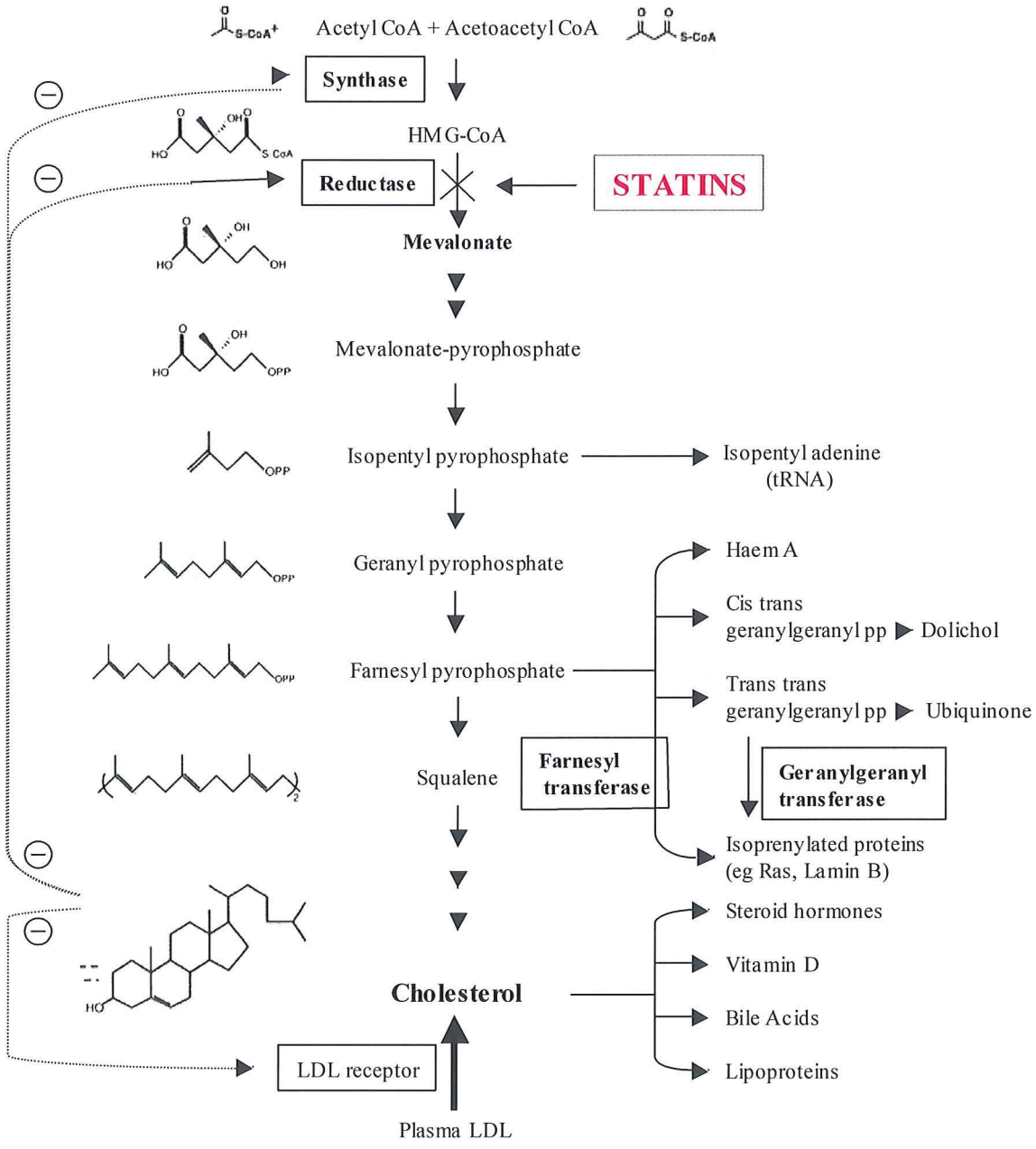


Figure 1.1 The Mevalonate pathway.

Cholesterol can be produced from two different sources: 1) endogenously by synthesis from mevalonate metabolism and 2) exogenously from receptor-mediated uptake of plasma LDL. The balance of sources is achieved through feedback regulation. In the absence of LDL high activities of HMG-CoA synthase and reductase are maintained, and decline in the presence of LDL. When cholesterol accumulates, LDL receptors are repressed to prevent further uptake.

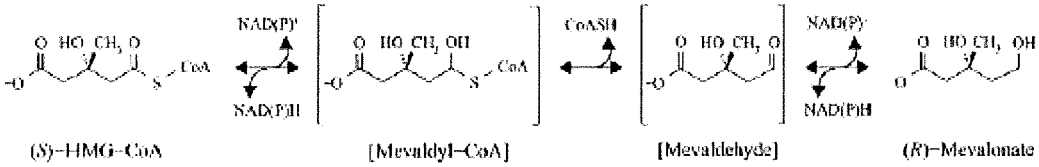


Figure 1.2 The reaction catalyzed by HMG-CoA reductase.

The reversible four-electron reductive deacylation of HMG-CoA to mevalonate involves mevaldyl-CoA and mevaldeyde as enzyme-bound intermediates.

hydrophobic, than the HMG portion of HMG-CoA. This interacts with the active site (CoA recognition site) of HMG-CoA reductase and competes with HMG-CoA for binding. A conformational change in the reductase allows movement of flexible C terminal alpha helices to expose a shallow hydrophobic binding site for the bulky hydrophobic groups of the statin molecule. The statin side chains mimic mevalonate and therefore sterically hinder substrate binding by blocking access of the substrate to the enzyme's active site[3]. The affinity of statins for the active site is several orders of magnitude higher than HMG-CoA, which leads to effective displacement of the natural substrate and inhibition of endogenous cholesterol synthesis[4].

Mevastatin was first identified in the mould *Penicillium citrinium*[5] *and Aspergillus terreus* naturally produces lovastatin. Lovastatin, simvastatin and pravastatin have a hydronapthalene ring that interacts with the coenzyme A recognition site and a hydroxy acid side chain that mimics mevalonate[6]. Simvastatin and pravastatin (Type 1 statins) are produced from lovastatin by chemical modification (Figure 1.3). Fluvastatin (Type 2 statin) was the first totally synthetic HMG-CoA reductase inhibitor and is a mevalonolactone derivative of a fluorophenyl-substituted indole ring. The fluorophenyl indole portion of fluvastatin mimics CoA in interacting with HMG-CoA reductase and the side chain mimics mevalonate (Figure 1.3).

The mean inhibitory concentrations for some statins are shown in table 1.1.

1.1.3 Statin Pharmacology

Atorvastatin, cerivastatin, lovastatin and simvastatin utilise the P450 (CYP) 3A4 pathway for metabolism or biotransformation[7] where as fluvastatin metabolism occurs via CYP2C9[8]. Pravastatin and rosuvastatin does not use the CYP pathway significantly. The concomitant use of 2 drugs that are both metabolised by the CYP3A4 pathway compete for that pathway which results in increased serum concentrations of both drugs[9]. Other drugs that may cause concern when used in conjunction with atorvastatin, cerivastatin, lovastatin and simvastatin are CYP3A4 inhibitors cyclosporin, erythromycin, the antidepressants fluoxetine,

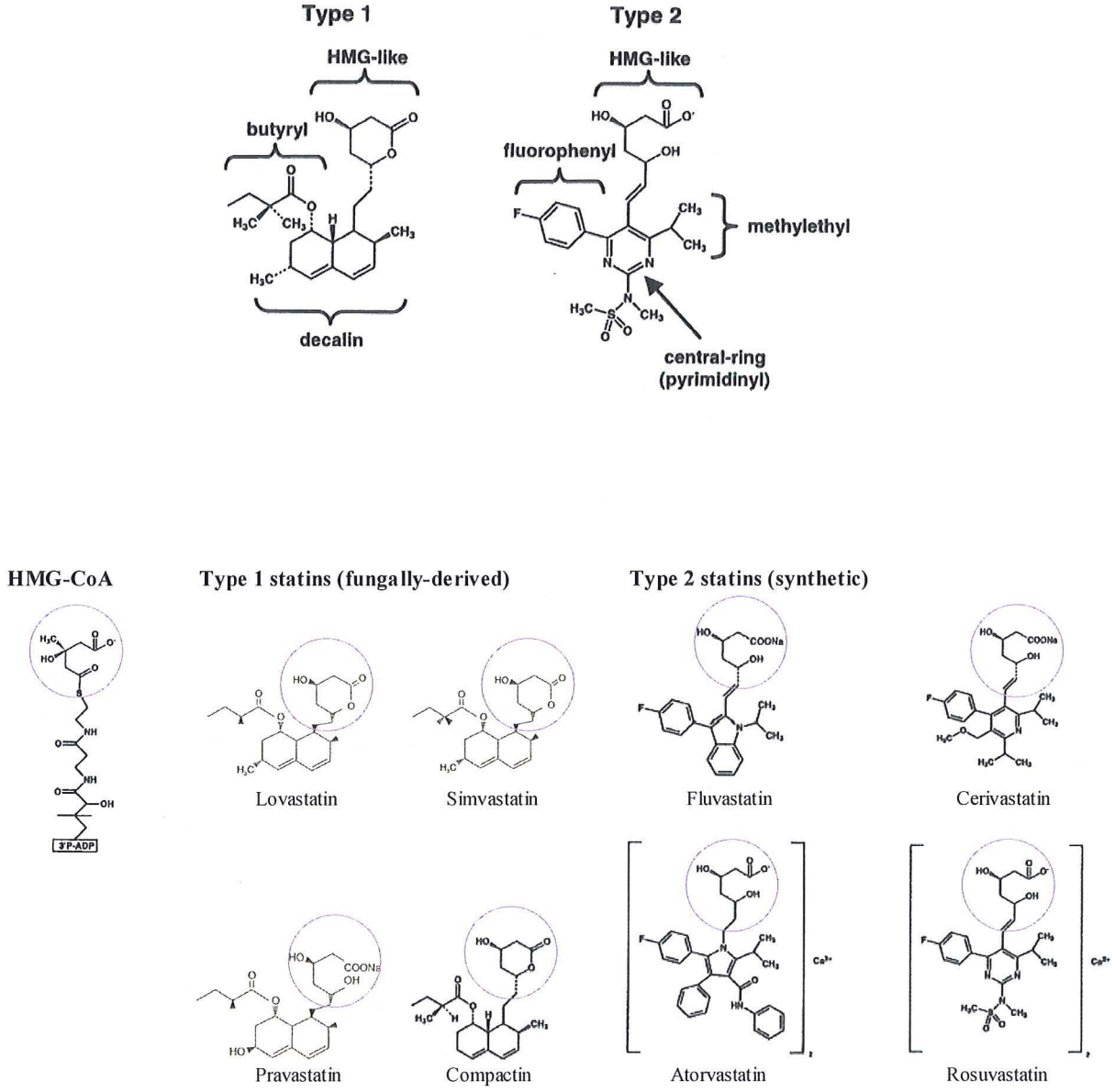


Figure 1.3 Structures of statins

Type 1 and type 2 statins contain an HMG-like moiety, covalently linked to a rigid hydrophobic group. In addition, type 1 statins contain a decalin ring whereas type 2 contains a fluorophenyl group and a methylethyl group.

Statin	IC_{50}	Statin	IC_{50}
		Cerivastatin	10
Simvastatin	9	Atorvastatin	10
Pravastatin	7	Rosuvastatin	2
Compactin	23	Fluvastatin	18

Table 1.1 Median inhibitory concentrations of statins

Median inhibitory concentrations (IC_{50}) of statins (μM) for inhibition of HMG-CoA reductase.

fluvaxamine, nefazdone and seraline, the protease inhibitors indinavir, nelfinavir, ritonavir and saquinavir, calcium channel blocker mibefradil, conjugated oestrogen diltiazem, grapefruit juice. Care should also be taken when combining fluvastatin with alprenolol, diclofenac, diazepam and warfarin.

Pravastatin is more hydrophilic when compared to the other statins. It has a short half life and is not affected by food intake[6]. Hydrophilic statins are distributed more selectively in hepatic cells than hydrophobic ones. Hydrophilic statins (i.e. pravastatin) cannot penetrate the membrane of extrahepatic cells because they consist of lipid bilayers and therefore cannot reach the intracellular enzyme. However hepatic cell membranes contain organic anion transporters, which take hydrophilic material into the cell. Conversely, hydrophobic statins are lipophils and can enter extrahepatic and hepatic cells and not only inhibit cholesterol synthesis but also other essential substances of the mevalonate pathway in many extrahepatic tissues[10].

1.2 Isoprenylated proteins

1.2.1 Prenylation

The inhibition of mevalonate synthesis also prevents the synthesis of isoprenoid intermediates. The 15-carbon farnesyl pyrophosphate and the 20-carbon geranylgeranyl pyrophosphate are lipid attachment molecules for the post-translational prenylation of various important cell-signalling molecules[11]. Protein prenylation allows the covalent attachment, subcellular localization and intracellular trafficking of membrane-associated proteins. Heterotrimeric G protein subunits and nuclear lamins are prenylated but the largest group is the small GTPase family of molecular switch proteins. The Ras superfamily is structurally classified into five major subfamilies: Ras, Rho/Rac, Rab, Sar1/ARF and Ran[12]. The functions can be broadly classified as follows: Ras mainly regulate gene expression; Rho/Rac/Cdc42 regulate cytoskeletal reorganization and gene expression; Rab and Dar1/ARF regulate intracellular vesicle trafficking and Ran

regulate nucleocytoplasmic transport during the G1, S and G2 phases of the cell cycle[12].

1.2.2 Transferases

Farnesyl transferase (FTase) catalyses farnesylation, while geranyltranferases (GGTases I and II) catalyse geranylgeranylation[13]. FTase and GGTase I are heterodimers of Zn^{2+} metalloenzymes that share an α subunit and have homologous but distinct β subunits[11]. GGTase II is also a $\alpha\beta$ hetrodimer but is distinct from GGTase I and doubly geranylgeranylates proteins of the Rab small G protein family that end in CC or CaC. The prenyl groups farnesyl diphosphate and geranylgeranyl diphosphate, are added to the thiol group of the cysteine residue in the CaaX motif, where 'a' is any aliphatic amino acid and X determines which prenyl group is added: if serine, methionine, glutamine or alanine the protein is farnesylated and if leucine or phenylalanine it is geranylgeranylated[14-16].

1.2.3 Cleavage of aaX and methylation

Following prenylation, two further post-translational modifications occur. The aaX is cleaved from the s-prenyl protein by the endoprotease Rce-1[17], resulting in a C-terminal prenylated cysteine that is carboxymethylated. The removal of the last three amino acids may relieve steric hindrance and allow insertion of the farnesyl group into the lipid bilayer[18]. The final modification is methylation of the new carboxy terminus by a carboxymethylase, using s-adenosylmethionine as the methyl donor[19] (Figure 1.4). Methylation adds hydrophobicity to farnesylated proteins by neutralising the negative charge on the ionised carboxyl group, which could cause repulsion from the negatively charged head groups of membrane phospholipids[18]. Geranylgeranyl is already more hydrophobic than farnesyl, thereby facilitating lipid-lipid interactions[16].

1.2.4 Exceptions to the rule

The substrate specificity rules for FTase and GGTase noted above are not absolutely stringent, with many examples of cross-prenylation[14]. For example,

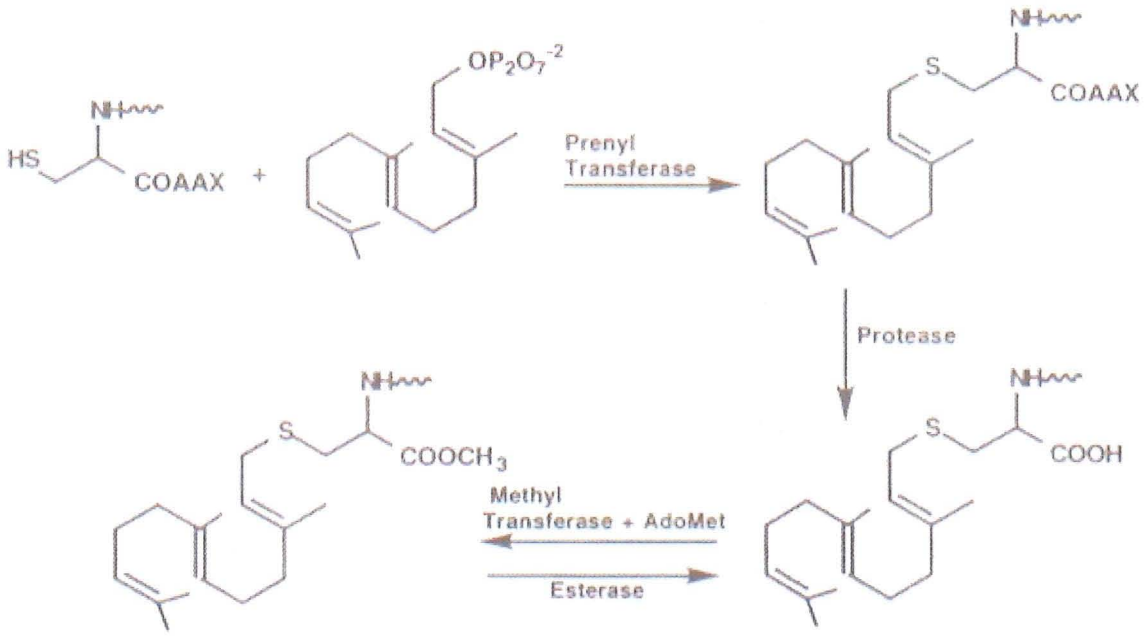


Figure 1.4 Isoprenylation/methylation pathway for CAAX proteins

(Adapted from Rando [20])

when K-RasB which has an FTase CaaX box is inhibited by FTase inhibitors, it becomes geranylgeranylated by GGTase I[21]. The reaction is made possible by an upstream polybasic sequence that alters GGTase I substrate specificity[22]. Furthermore, RhoB contains a GGTase I CaaX box but is found in both farnesylated and geranylgeranylated forms in cells, due to the ability of GGTase I to both geranylgeranylate and farnesylate this substrate[23].

1.2.5 Palmitoylation, myristolyation and acylation

A second signal is required for stable membrane association of prenylated proteins. Palmitoylation and myristolyation are known to localise proteins to membranes[24, 25]. Palmitoylation of Ras proteins has been shown to stabilize the association of prenylated proteins with the plasma membrane[16] and may play an important role in membrane localization and protein function. However not all Ras proteins are palmitoylated (e.g. K-Ras), but instead have a polybasic run of six positively charged lysine residues, which may increase the avidity of membrane binding through ionic interactions with negatively charged membrane head groups[26] (Figure 1.5).

Protein S-acylation has been proposed to be involved in cooperation between prenylation and carboxymethylation, regulating cycling between intracellular membrane compartments to control signalling activity[27]. Thus, post-translational modifications that follow prenylation may be potential candidates as factors that confer membrane-binding or functional specificity to the prenylated proteins.

By inhibiting HMG-CoA reductase, statins reduce the availability of these intermediate metabolites and therefore the activity of key cell-signalling molecules. Given the central role these small GTP-binding proteins have in determining cell function, it is not surprising that the immune response will be modified independently of their plasma lipid-lowering effect.

The role of some prenylated proteins in mediating immune function are listed in Table 1.2

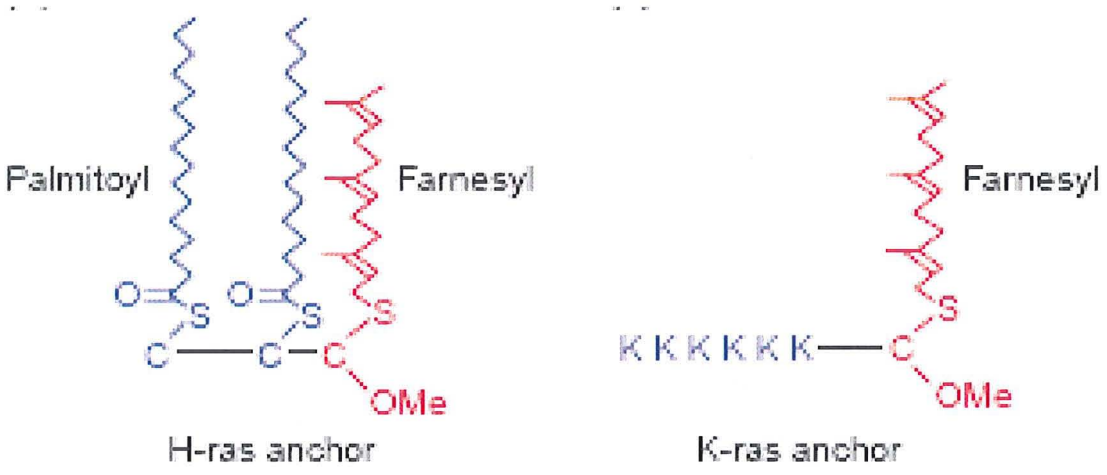


Figure 1.5 Palmitoyl and polybasic domains of Ras

H-Ras is doubly palmitoylated and K-Ras has a polybasic run of six positively charged lysine residues, which localise them to the plasma membrane. (Adapted from Parton et al[28])

Prenylated protein	Function
Leukocyte motility	
CDC42, RAC1	Forward cell movement through formation of membrane protrusions (lamellipodia and filopodia)
CDC42, RAC1, RHOA	Formation of focal complexes and podosomes
CDC42	Directional leukocyte migration: macrophage and lymphocyte chemotaxis
RHOA	Leukocyte tail retraction during transmigration
RAP1	Increased leukocyte substrate motility and migration
RAC1	LFA1-induced T cell motility
Antigen uptake, processing and presentation	
CDC42, RAC	Cytoskeleton remodelling for antigen endocytosis and immunological synapse formation
RAB proteins	Antigen processing and presentation
CDC42, RHO	Antigen presentation
RAC1, RAC2	Formation of mature DC dendrites; polarized short-range migration to T cells for T cell priming
RAP1	Regulation of LFA1 avidity for ICAM1
Leukocyte activation, proliferation and function	
RAP1	TCR clustering and increased LFA1 avidity for ICAM1 after activation by TCR ligation; increased integrin-mediated adhesion after chemokines activation
RAS	Regulation of transcription factors that control cytokine transcription after ligation of TCRs, and some cytokine receptors
RAC1	T cell fate in the thymus; regulation of actin cytoskeletal dynamics for clustering of TCRs, adhesion molecules and signalling receptors, and immunological synapse formation; IL-2 secretion and T cell proliferation after TCR ligation
RAC2	CD4+ T cell differentiation to Th1 cells through activation of IFNγ gene expression
RHOA	Regulation of BCR signalling and B-cell proliferation
CDC42, RAC1, RAS, RHO	Cell cycle progression and proliferation
RAC, RHO	PKCθ-dependent AP1 and NF-κB activation during proliferation; JNK activation in Th cell differentiation; cytoskeletal reorganisation, receptor clustering and IL-2 secretion after CD3 stimulation
CDC42, RAC, RHO	Inhibition of CTL- and CD95-induced apoptosis
RAB27A	Exocytosis of lytic granules in CTLs

Continued on next page....

....*Continued from previous page.*

Phagocytosis	
CDC42, RAC1	Formation of the phagocytic cup
RAC	Induction of NADPH oxidase production of superoxide-mediated phagosome function
RHO	Superoxide formation during phagocytosis
RAP1, RHO	Complement-mediated phagocytosis
Leukocyte transvascular migration	
RAP1	Chemokine-receptor-mediated integrin activation and lymphocyte transvascular migration through ICAM1 and VCAM1; PECAM1-mediated leukocyte signalling to increase integrin adhesion
RAS, RHO	Expression of monocyte MMP9 for leukocyte migration
RHOA	Induction of high-affinity state of LFA1
RAP1, RAP2	CXC-chemokine-ligand-12-induced B cell migration
RAP2	LFA1 and VLA4-mediated B cell adhesion
CDC42,RAC1	Chemokine-induced lymphocyte polarization and directional migration
RAC2	Neutrophil migration
Endothelial-cell immune function	
RHO	Formation and maintenance of the endothelial cell docking structure; ICAM1-mediated endothelial cell signalling for lymphocyte migration; endothelial cell actin cytoskeletal reorganization for monocyte migration; endothelial cell junction opening; inhibition of eNOS and NO production; adhesion-receptor clustering on endothelial cell s for monocyte adhesion and spreading; LPS-induced ICAM1 expression and TNF-induced E-selectin expression by endothelial cells
RHO, RAC1	Induction of ICAM1, VCAM1 and E-selectin expression through activation of NF-κB family members
RAC1	VCAM1-mediated endothelial cell signalling for NADPH oxidase activation, ROS production, junction opening and monocyte transvascular migration
RAP1	Increased endothelial-cell junction assembly by reducing leukocyte transmigration

Table 1.2 Role of prenylated proteins in mediating immune function

(Adapted from Greenwood et al[29])

1.3 Activation of small G proteins

1.3.1 GDIs

Monomeric small guanine nucleotide binding proteins (also referred to as GTPase) cycle between an inactive GDP-bound state and an active GTP-bound state[20]. In the resting state, activation is prevented by guanine nucleotide dissociation inhibitors (GDIs). GDIs maintain Rho GTPases as soluble cytosolic proteins by forming high-affinity complexes in which the isoprenoid moiety of the GTPase is shielded from the solvent by its insertion into the hydrophobic pocket formed by the immunoglobulin-like β sandwich of the GDI. GDIs inhibit the dissociation of GDP from Rho proteins, preventing GTPase activation by guanine nucleotide exchange factor (GEFs)[30]. GDIs are also able to prevent interactions with effector targets by interacting with the GTP-bound form of the GTPase[31]. Through these multiple actions, GDIs act as a major regulator of Rho GTPase activity and function, but are themselves also regulated by other proteins termed GDI displacement factors, or GDFs. (Figure 1.6)

1.3.2 GEFs

Following dissociation of GDIs, the small G proteins are activated by guanine nucleotide exchange factors (GEFs) which contain a Dbl homology (DH) domain that catalyses the exchange of GDP for GTP[32]. They also have an adjacent C-terminal pleckstrin homology domain (PH), which binds to lipid products of PI3K. PH domains localize Dbl proteins to plasma membranes, and cooperation between DH and PH domains facilitate exchange activity[33]. The GDP-GTP exchange reaction is the rate-limiting step in the GTP-binding/GTP hydrolytic cycle of GTPases and the GEFs have therefore been proposed as key regulators of the GTPases.

1.3.3 GAPs

Inactivation occurs via hydrolysis of GTP, a slow process that is catalysed by GTPase-activating proteins (GAPs). GAPs are classified according to their GTPase

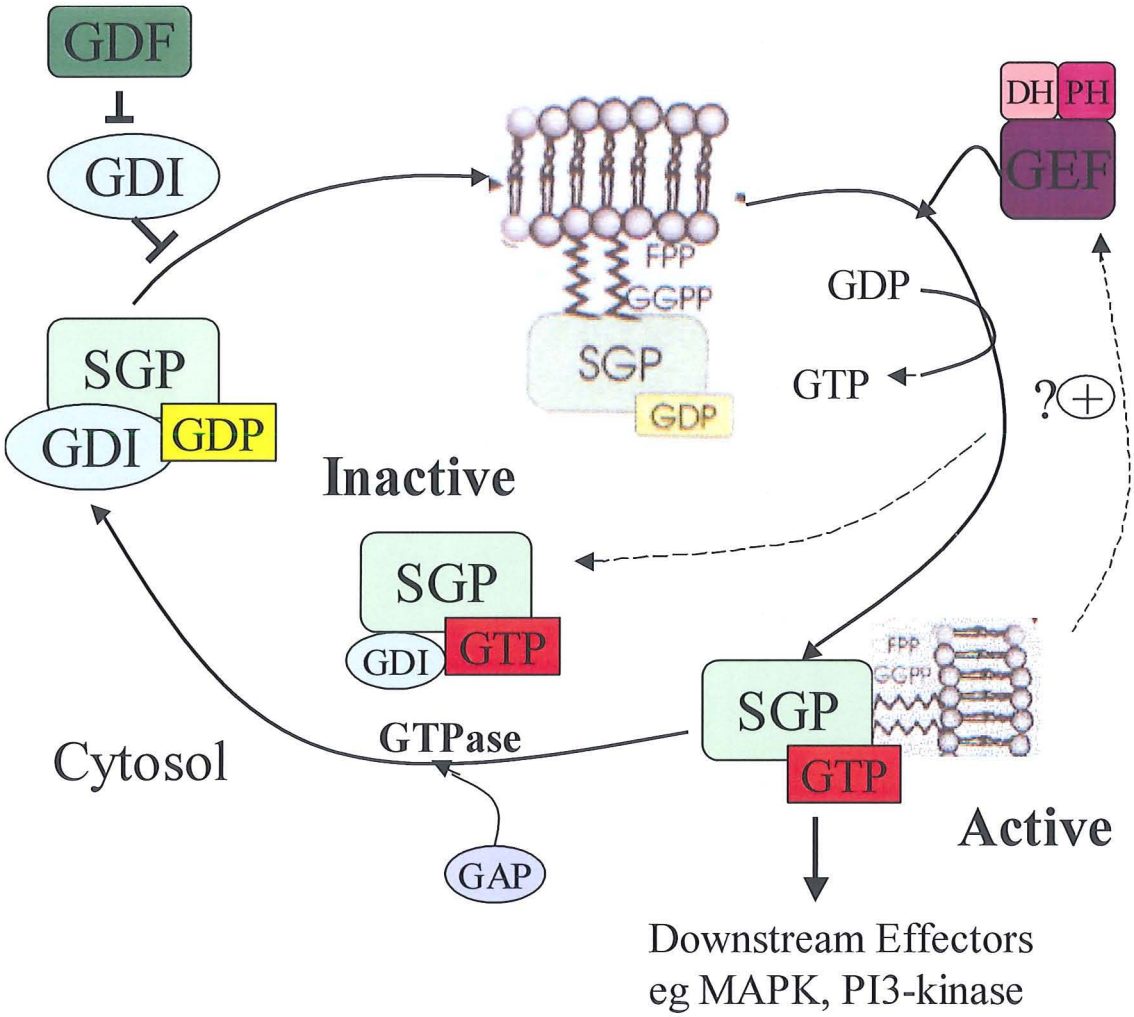


Figure 1.6 GTPase cycle

Small G proteins are found in the cytosol bound to GDIs, which are negatively regulated by GDFs. Cellular signals stimulate release of GDI and dissociation of GDP by GTP binding which is catalysed by GEFs. Farnesylated and geranylgeranylated residues bind the active form of GTPase to the cell membrane. Intrinsic GTPase activity stimulated by GAPs leads to inactivation. (Adapted from McTaggart[34])

subfamily (Ras-GAP, Rap-GAP, etc) with sequence homology within subfamilies but not between families[35]. GAPs contain a phosphoinositide-binding PH domain but also commonly contain a Src homology domain (SH3) that binds proline-containing peptides[36].

Regulators of G protein signalling (RGS) act functionally as GAPs and interact with heterotrimeric G protein α subunits to accelerate the GTP hydrolysis rate of the Gα subunit[37].

1.4 Rafts

Lipid rafts are compartmentalized membrane microdomains into which membrane lipids and proteins sort, allowing specific interactions that modulate signal transduction, membrane trafficking, cytoskeletal organization and motility, polarization and pathogen entry[38]. Lipid rafts are highly dynamic, laterally mobile, assemblies that float freely within the liquid disordered membrane bilayer. Rafts are liquid-ordered domains enriched in sterols (including cholesterol), glycosphingolipids and sphyngomyelin that are more tightly packed than the surrounding non-raft phase of the bilayer (Figure 1.7). The tighter packing is due to the saturated hydrocarbon chains in raft sphingolipids (such as gangliosides) and phospholipids compared with the unsaturated fatty acids of phospholipids in the non-raft phase[39].

Lipid rafts were initially identified as detergent resistant membrane (DRM) fractions. Upon membrane solubilization by some mild non-ionic detergents (such as Triton X-100 or Brij) at low temperatures, rafts remain insoluble and because of their high lipid content, float to a low density following sucrose gradient centrifugation[40]. Rafts can be visualized by cholera toxin B subunit (CT-B), which binds to and cross-links with glycosphingolipid GM1.

Lipid rafts have been subdivided into caveolar and non-caveolar rafts that co-exist in distinct membrane areas. Caveolae are flask-shaped membrane invaginations enriched in cholesterol and caveolin. Caveolin is thought to stabilize the

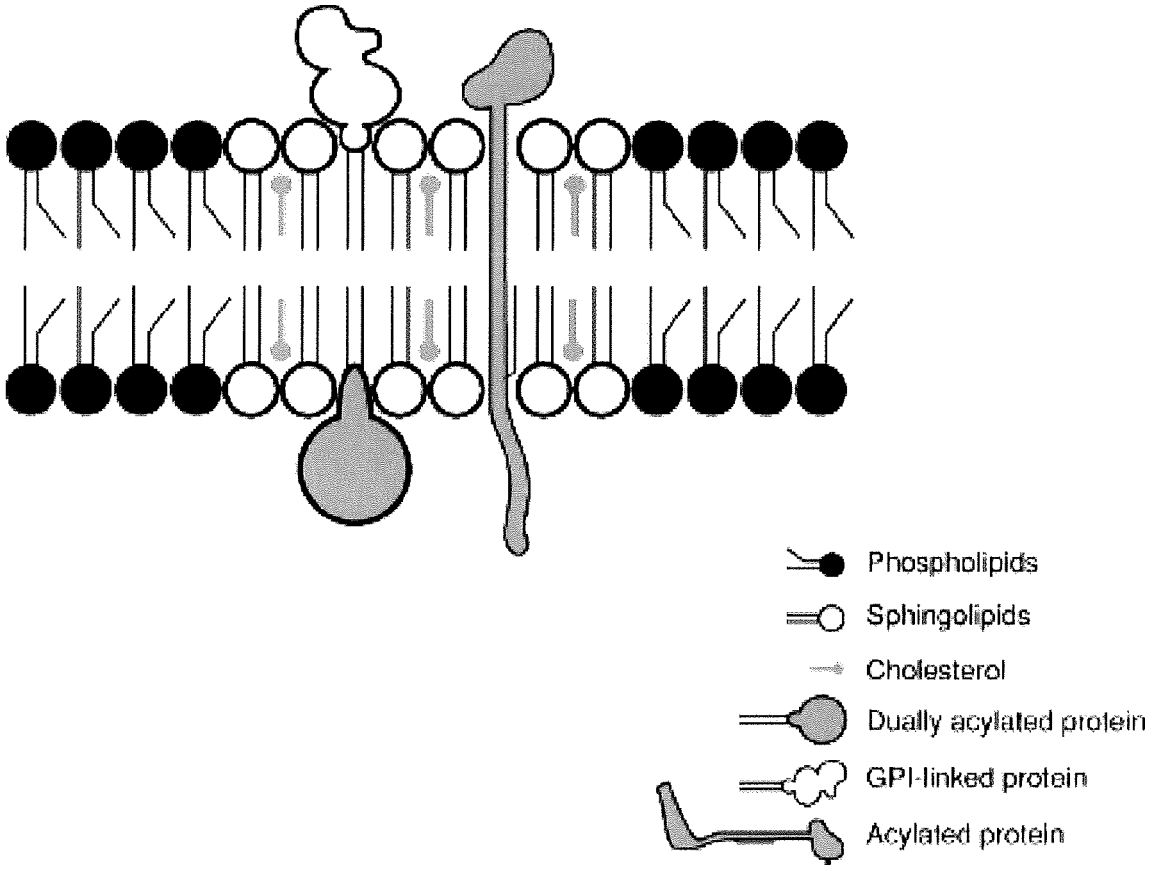


Figure 1.7 Lipid raft model

Lipid rafts are membrane microdomains that are highly enriched in tightly packed sphingolipids and cholesterol. Raft associated proteins include GPI-anchored proteins that contain saturated acyl chains (e.g. Lyn and Lck). Transmembrane receptors are usually excluded from rafts. (Adapted from Dykstra et al [41])

invaginated structure by its hairpin-like palmitoylated structure. Unlike caveolar rafts, non-caveolar rafts are flat and heterogeneous in protein composition[42].

Many cell surface proteins localize to the liquid disordered regions, however some proteins selectively partition into the rafts domains. These include glycosylphosphatidylinositol (GPI) –anchored proteins because their phosphoinositide anchors typically have saturated acyl chains[43]. Palmitoylated and myristoylated proteins (e.g. flotillin), doubly acetylated proteins (e.g. Src-family kinases)[44], phospholipid bound proteins (e.g. annexins), and cholesterol bound transmembrane proteins (e.g. caveolins) also associate with ordered domains. Prenylated membrane anchors (for example, on Ras superfamily GTPases) have a preference for disordered domains because of the unsaturation of the isoprenyl groups[45, 46]. However, these preferences on the basis of hydrophobic membrane anchors may be outweighed by interactions with other proteins or by headgroup interactions.

To engage in membrane function, rafts usually have to cluster together and can be accomplished from both sides of the plasma membrane[47]. Antibodies, antigens or raft-lipid-binding proteins such as cholera toxin B, cluster rafts on the extracellular side of the membrane whereas raft-clustering proteins such annexins, flotillins or other scaffolding proteins could serve as clustering agents for the rafts in the cytoplasmic leaflet. Clustered rafts can sequester specific sets of signalling and other proteins and could serve as platforms to execute functions in membrane trafficking, signalling and polarisation[48].

Evidence for a functional role of lipid rafts came from studies of haematopoietic cells. The high-affinity IgE receptor (FcεRI), the T-cell receptor (TCR) and the B cell receptor (BCR) are multichain immune receptors, which translocate to lipid rafts upon crosslinking[49]. In resting mature T cells, the TCR is excluded from lipid rafts. However, upon engagement, the TCR associates with rafts where the ζ chain of the CD3 and Zap-70 become phosphorylated[50]. Adaptor molecules such as LAT and SLP-76 are then recruited. Lck, LAT and certain other proteins constitutively associate with lipid rafts at least in part because of their fatty acid modifications. When T cells recognise the antigen presented on the surface of antigen presenting cells (APCs), polarisation of lipid rafts and raft-associated proteins occurs through

their association with the immunological synapse, where the T cell contacts the APC[51].

MHC class II molecules colocalize with GM1 and partition into insoluble membranes following cross-linking with specific antibodies[52]. Other studies demonstrated that, when disrupted by cholesterol depletion, a constitutive association of class II molecules with lipid rafts on APC, diminished antigen presentation to T cells[53].

NK cells express an array of inhibitory and activating receptors that together allow NK cells to distinguish normal healthy cells from target cells for lysis[54]. A study has shown that conjugate formation of NK cells with sensitive tumour cells results in the actin-dependent redistribution of the raft marker GM1 to the contact site in a Src- and Syk-dependent fashion[55]. Significantly, both the redistribution of rafts and cytotoxicity were blocked by the engagement of an inhibitory receptor in a SHP-1-dependent fashion[55]. In another study, photobleaching experiments showed that CD94/NKG2A receptors move freely within the plasma membrane and accumulate at the site of ligand contact where mobility becomes restricted. Lipid rafts are excluded from the site of receptor contact with the ligand, which suggests that immobilization of the CD94/NKG2A receptors at ligation sites not only promote sustenance of the inhibitory signal, but by lipid rafts exclusion prevent formation of activation signalling complexes[56]. As the integration of signals from multiple receptors is essential for NK cell activation, the role of lipid rafts in NK cell signalling may be pivotal.

The major lipid of the membrane, cholesterol, is a critical component that controls lipid phase separation and stabilizes lipid rafts. Drugs that modify membrane cholesterol concentration and disrupt membrane domain organization include: drugs that sequester cholesterol (filipin, nystatin, and amphotericin), form membrane pores (saponin, digitonin, and streptolysin O), deplete cholesterol (methyl-β-cyclodextrin (MβCD)), impair cholesterol trafficking (progesterone), inhibit cholesterol biosynthesis (statin), or perturb raft stability (exogenous addition of cholesterol, gangliosides, or polyunsaturated fatty acids).

In general, cholesterol depletion impairs receptor activation and downstream signalling, consistent with an important role for lipid rafts in the organization of signalling components.

1.5 Immunology and signalling

1.5.1 Monocytes and macrophages

Macrophages participate in the production, activation and regulation of all immune effector cells. These cells belong to the mononuclear phagocyte lineage, being derived from blood monocytes that terminally differentiate to give rise to tissue macrophages. Probably the most significant role of these cells is to engulf and destroy foreign and aberrant material, including infectious agents, immune complexes, virus infected cells and cancer cells.

Monocytes and macrophages have central roles in innate and adaptive immunity[57]. Receptors on the surface of these cells directly bind to pathogens and result in internalisation and disposal. These cells also express receptors for the complement system and C-reactive protein (CRP), which mediate internalisation of complement and CRP opsonised pathogens. These innate functions of the immune system serve as a first line of defence against pathogens. In the adaptive response, production of antigen specific antibodies by B-cells results in either the formation of antibody:antigen complexes, known as immune complexes, or the opsonisation of pathogens through antibody binding to surface antigens. The antibodies are then recognised by Fc receptors and the resultant aggregation of these receptors by immune complex or opsonised particle leads to their internalisation through the process of endocytosis for immune complexes or phagocytosis for opsonised particles.

1.5.1.1 Monocytes and lymphocyte regulation

Alongside their central roles in immune complex clearance and inflammation, monocytes and macrophages, also interact and coordinate lymphocyte responses. Monocytes and macrophages are also a major source of the immune regulatory cytokines IL-10 and IL-12 which regulate the type of lymphocyte response. In addition, macrophages are able to present internalised antigen to T-cells via MHC class II. This receptor is the most effective method of stimulating T-cell activation[58].

1.5.1.2 Monocytes and Macrophages are highly heterogeneous

Mononuclear cells such as monocytes and macrophages are highly heterogeneous, attributable to differences in their maturation status and also tissue specific variation[59]. This heterogeneity makes *in vitro* study of these primary cells very difficult. Primary human mononuclear cells provide more difficulty due to genetic variation and disease status differences between donors. To overcome this difficulty, a number of human monocyte-like cell lines have been established, including U937, HL60 and THP-1[60] and are widely used as models for studying human mononuclear cells. U937 cells are widely used as a model for elucidating immune response signalling pathways and data from U937 cell-based experiments have been applied to cardiovascular models of inflammation[61-63].

1.5.1.3 Fc Receptors

Three functionally and structurally distinct classes of Fcγ receptors are expressed by human monocytes and macrophages[64]. FcγRI is a high affinity IgG receptor that binds monomeric IgG at low, physiological concentrations, whereas FcγRII and FcγRIII bind with low affinity and thus interact only with IgG aggregates or immune complexes. FcγR are activated when clustered at the cell surface by immune complexes, and signal through an immunoreceptor tyrosine based activation motif (ITAM)[65]. For FcγRI and FcγRIII the ITAM is situated within the FcγR associated γ-chain subunit, whereas FcγRIIa contains an ITAM in the cytoplasmic tail. Though the exact mechanism remains unclear, aggregated FcR

are recruited to cholesterol and sphingolipid rich plasma membrane rafts as a platform for signal transduction[66, 67], resulting in phosphorylation of key tyrosine residues within associated ITAMs and creating a docking site for recruitment of cytoplasmic signalling molecules such as Syk tyrosine kinase, the p85-p110 PI3-kinase complex, PLC-γ and LAT (Linker for Activation of T-cells). LAT is a membrane associated 36-38 kDa adapter protein targeted to rafts by palmitoylation initially shown to couple the T cell receptor to calcium flux, ERK activation and IL-2 production[68]. In myeloid cells, LAT is phosphorylated in response to FcγR clustering and thus recruits key SH2 domain proteins - Grb-2, p85 and PLC-γ - to the FcγR signalling complex[69].

1.5.2 Lymphocytes

Lymphocytes are the principle cells of adaptive immunity with their ability to recognize antigens. They arise from bone marrow lymphoid stem cells to defend against invading pathogens and to prevent or reduce reinfection[70]. Lymphocytes mature in lymphoid organs: B cells are bursa of Fabricius (or bone marrow) derived and T cells migrate from bone marrow to mature in the Thymus.

1.5.2.1 T cells

Unchallenged T cells recirculate between blood and lymph nodes, awaiting antigen presentation by antigen presenting cells (APCs)[70]. T cells are stimulated by contact of the T cell receptor with an APC. The T cell rapidly proliferates and differentiates into effector cells or mature into memory T cells, which lodge in the spleen and site of first infection, ready to respond to re-infection. T helper cells (CD4+) interact in antigen specific contacts with B cells and provide cell surface and cytokine signals that drive the B cells toward antibody production. Th1 helper cells activate macrophages toward phagocytosis of intracellular microbes by secretion of cytokines including IFN-γ. Th2 secretes cytokines including IL-4 and IL-5 to stimulate eosinophil/mast cell-mediated immune reactions and to down regulate Th1 responses. Cytotoxic T-lymphocytes (CD8+) recognize virally or microbially infected cells (or cancerous cells) and directly induce cellular death by release of cytoplasmic granules that contain membrane pore-forming proteins and enzymes.

1.5.2.1.1 Adhesion molecules

Lymphocyte function-associated antigen (LFA-1) is a heterodimeric glycoprotein belonging to the β_2 integrin family and consists of a 180-kDa α-chain (CD11a) and a 95-kDa β-chain (CD18)[71]. LFA-1 is involved in lymphocyte recirculation and leukocyte extravasation to sites of inflammation[72]. It is also important for effective T-cell activation by APCs by providing a potent costimulatory signal for TCR-activated T cells[73]. T-cells appear to require two signals for activation[74], the first trigger being from recognition of antigen in the form of antigenic peptide bound to cell-surface proteins encoded by the major histocompatibility complex displayed on the surface of antigen presenting cells. The second co stimulatory signal, for example, ICAM-1 (CD54) on APC binds to LFA-1 on the T-cell. Similarly, VLA-4 (a β1 integrin) binds to VCAM-1 and controls the progression of leukocyte migration. Resting leukocytes constitutively express LFA-1, which needs to be converted from a low-affinity state to a high-affinity state by activation from either a response to chemokines or engagement of the T-cell receptor[75].

Lovastatin, mevastatin and simvastatin but not pravastatin were found to bind directly to LFA-1 via the "lovastatin site" (L-site), which is located on the inserted (I)-domain of the LFA-1 α chain, distant from the ICAM binding site. A conformational change via an allosteric mechanism independent of HMG-CoA reductase activity, stabilizes the low-affinity form of LFA-1 and does not allow the transition to the high-affinity, ligand binding conformation[76, 77]. An increase in ligand binding can also be brought about by lateral association of integrins into clusters on the plasma membrane called lipid rafts, which increases ligand binding avidity by providing multiple contact sites[78]. Inactive LFA-1 is excluded from membrane rafts by cytoskeletal tethers and upon activation are untethered and relocated into rafts[79, 80]. However LFA-1 is not palmitoylated, a characteristic of many raft transmembrane proteins, so is thought to be localised to rafts by complex formation with other membrane proteins[79]. The prenylated GTPase - Rho, regulates receptor clustering into membrane rafts[81]. Rac and CDC42 mediate the assembly of smaller adhesive complexes associated with lamellipodia and filopodia, therefore inhibition of prenylation by statins has the potential to disrupt regulation and movement of integrins.

Therefore blockade of LFA-1 and VLA-4 interactions with their ligands is a potential target for immunosuppression and for controlling inflammation and autoimmune diseases. Whether this is achieved by statins binding directly, disrupting rafts, reducing prenylation of regulatory signalling molecules or by monoclonal antibodies[82, 83] is equally obtainable.

1.5.2.2 NK cells

Natural killer cells constitute approximately 10% of peripheral blood mononuclear cells and are bone marrow derived, CD56+ CD16+ CD3-, large granular lymphocytes, that function in innate immune responses, a critical role in early host defence against infections and cancers as well as rejection of grafts[84]. They exert their effector function, without prior sensitisation, by direct lytic mechanisms and production of immunoregulatory cytokines and chemokines[85]. A balance between activating and inhibitory receptors regulates NK activation.

1.5.2.2.1 Cytotoxicity

NK cells store perforin (which creates pores in target cell membranes) and granzymes (enters perforin pores and induce apoptosis) in preformed cytoplasmic granules[86]. A tight conjugate between the NK cell and its target is required for direct delivery of granule components and cytotoxicity[87] and leads to the formation of an immunological synapse[88]. This is a rapid process and can be complete within 20 minutes[89].

1.5.2.2.1.1 Adhesion molecules

Adhesion molecules are also necessary for cytotoxicity. NK cells express LFA-1 (CD11a), LFA-2 (CD2), CD44, CCD49e, LFA-3 (CD58), ICAM-1 (CD54) and L-selectin (CD62L), which interacts with the endothelium, for trafficking to secondary lymph organs[90]. LFA-1 binding of NK cells to target ICAM-1 adhesion molecules activates the signal transduction pathway of Vav-Rac1-Erk to rearrange the cytoskeleton for cytotoxicity[87] and gathers the receptors in lipid rafts[91]. Adhesion molecules therefore not only participate in conjugate formation but also trigger the earliest activatory signal transduction events.

1.5.2.2.1.2 Activating receptors

Activating receptors in NK cells lack intrinsic signalling domains, therefore require coupling to adaptor proteins that contain ITAM motifs for signal transduction[92]. ITAMs contain the consensus sequence YxxL6-8xYxxL/I (where x is any amino acis), with 6-8 amino acids between the two YxxL/I elements. Adaptor protein family members containing ITAMs include CD3γ, CD3δ, CD3ε, ζ, Igα, Igβ, FcεRIγ and DAP12 of which NK cells express ζ, FcεRIγ and DAP12[93]. FcεRIγ and ζ form disulfide-bonded homo or heterodimers and DAP12, homodimers only[94]. When receptor engages its ligand, tyrosines in the ITAM are phosphorylated, usually by Src family kinases, which results in recruitment through SH2-domain binding and activation of Syk or ZAP-70 cytoplasmic tyrosine kinases.

1.5.2.2.1.2.1 Antibody dependent cellular cytotoxicity (ADCC) signalling

FcεRIγ and ζ associate with CD16 (FcγRIIIA) when crosslinked with IgG-coated target cells in antibody-dependent cellular cytotoxicity. This results in the association of the receptor complex with Src family PTKs (including Lck, Fyn, Yes and Lyn), which phosphorylates FcεRIγ and ζ. Syk family PTKs (including ZAP-70 and Syk) then become activated, which induces phospholipase Cγ1 tyrosine phosphorylation and the activation of phosphatidylinositol-3 kinase (PI3K). PI3K leads to the formation of critical membrane bound second messengers that are docking sites for proteins containing pleckstrin homology (PH) domains, such as PLCγ and the family of three VAV guanine exchange factors. Ras, Rho and Rac are substrates of VAV, which then activate the Rac substrate (PAK) p21-activated kinase and mitogen-activated protein kinase (MAPK), extracellular regulated kinase (ERK). Shc also becomes phosphorylated and binds Grb2, a Src homology 2 (SH2) and SH3 domain containing protein. The SH2 domain binds LAT (Figure 1.8)[86]

1.5.2.2.1.2.2 ITAM bearing receptors – non-MHC class I binding

Natural cytotoxicity receptors (NCR) include NKp30 and NKp46 which interact with hetero or homodimers of FcεRIγ and ζ, are expressed in all resting and

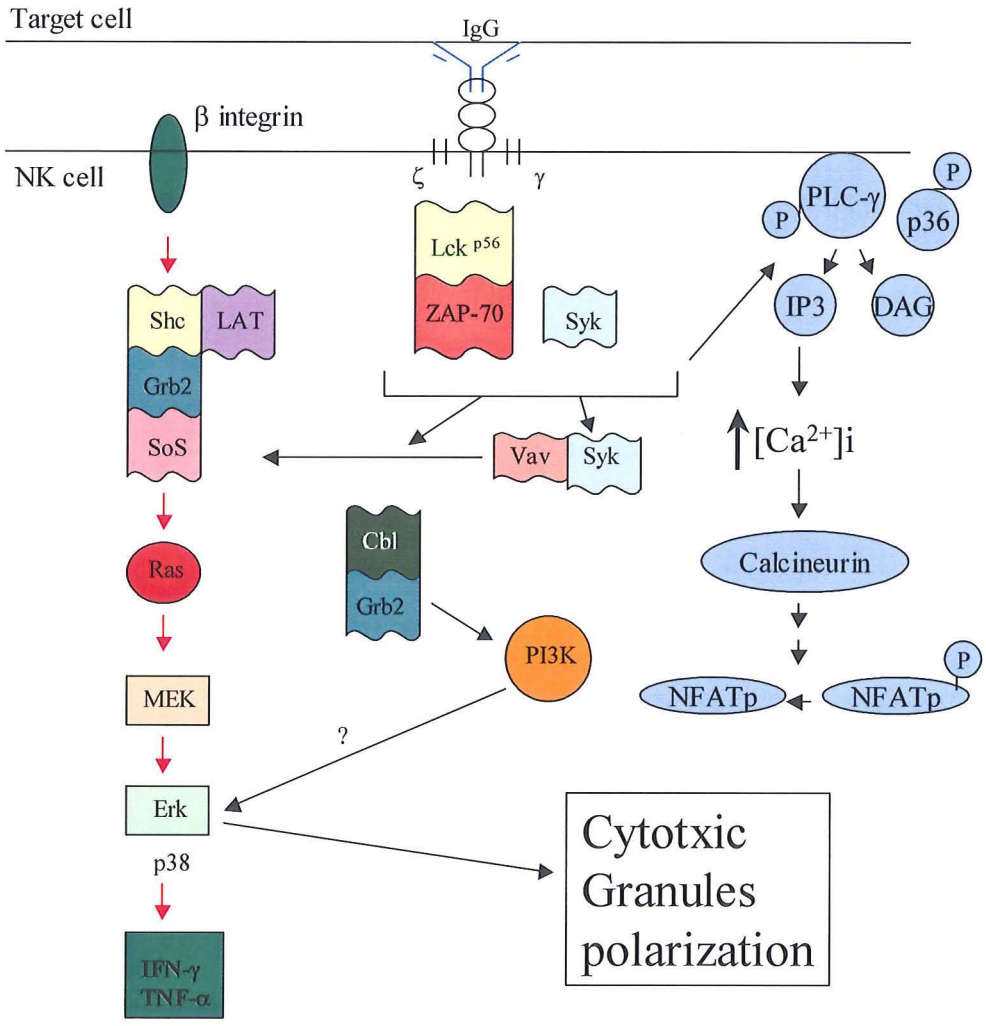


Figure 1.8 Signalling events in NK cell mediated cytotoxicity.

Signalling during ADCC, initiated upon engagement of FcgRIIIA by IgG-target cell recognition. (Adapted from Perussia [86] and Leibson [95])

activated NK cells and provide the most accurate surface markers for identifying human NK cells. NKp44 which couples with DAP12 is also NK specific, but is expressed only upon long term IL-2 induced activation[96]. NKp44 and NKp46 recognize virus specific haemagglutinins and therefore facilitate NK lysis of virally infected cells.

1.5.2.2.1.2.3 ITAM bearing receptors – MHC class I binding

Activating killer immunoglobulin-like receptors (KIRs) contain two or three extracellular immunoglobulin-like domains and lack an inhibitory signalling motif, but are highly homologous to the inhibitory members of the KIR family. They also bind the same HLA ligands (Table 1.3) with lower affinity than the inhibitory counterparts. KIR2DS, KIR3DS belong to this group of receptors and couple with DAP12.

The stimulatory isoforms of the NKG2 receptor family are the NKG2C and E receptors. They form heterodimers with CD94 and recognize HLA-E.

1.5.2.2.1.2.4 ITAM independent receptors

NKG2D is a C-type lectin expressed on all NK cells[97] and differs from other NKG2 proteins because it only shares 20% amino acid identity, is a homodimeric receptor and does not bind HLA-E (Figure 1.9). NKG2D is a receptor for the MHC-like molecules MICA/B stress inducible ligands, primarily expressed by epithelial cells[97] (Figure 1.10). MICA and B differ from MHC class I molecules in that they lack the ability to bind proteins or associate with β2-microglobulin and are not induced by IFN-γ [98]. MICA and B have been considered as cell-stress response genes as they are regulated by heat shock promoter elements[97]. MICA can be upregulated during certain infections[99], resulting in enhanced recognition by CD8+αβ or γδ T cells. NKG2D has a charged amino acid residue in the cytoplasmic domain and requires DAP10 for function. DAP10 contains a different tyrosine-based motif - YxxM[100, 101]. Engagement of NKG2D results in tyrosine phosphorylation of DAP10, recruitment and activation of PI3K and AKT[102] (Figure 1.11). NK cells activated with IL-2 in culture associates with both DAP12 and DAP10. DAP12 activates SYK or ZAP70 PTKs whereas DAP10 activates PI3K.

Receptor	Ligand
Immunoglobulin-like receptors	
KIR2DS1	HLA-C, C2
KIR2DS2	HLA-C, C1
KIR3DS1	HLA-B Bw4?
Lectin-like receptors	
CD94/NKG2C or E	HLA-E
Cytotoxicity receptors	
NKG2D	MICA, MICB, ULBP
NKp30	Unknown
NKp44	Unknown cellular ligand, viral HA
NKp46	Unknown cellular ligand, viral HA
CD16	IgG
DNAM-1	PVR (CD155), nectin-2 (CD112)
NKp80	* Unknown
CD59	* Unknown
NTB-A	* NTB-A
2B4	* CD48
CD2	* LFA-2
LFA-1	ICAM
TLR	PAMPS, e.g. dsRNA

* Co receptors as well as activatory receptors.

Table 1.3 Natural killer cell activatory receptors and ligands

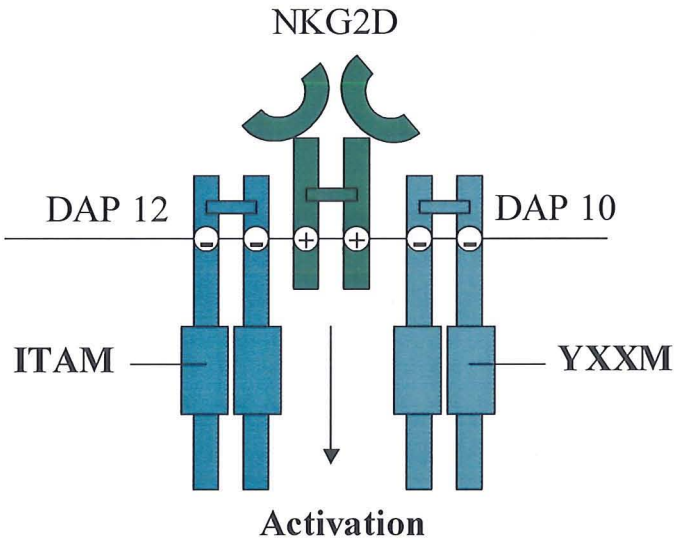
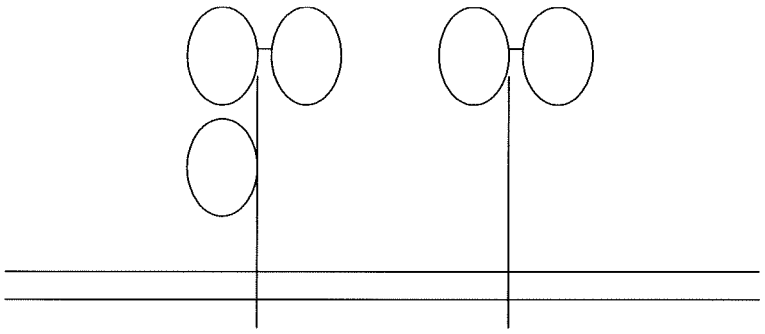


Figure 1.9 NKG2D receptor

NKG2D receptor is a type 2 transmembrane homodimer that contains charged residues in the transmembrane segments and associates with DAP10 and DAP12 to provide activating signals. (Adapted from Raulet[102])



MICA/MICB ULBPs/RAET1s

Figure 1.10 Domain structures of NKG2D ligands

MHC class I chain-related protein A and B contain an α3-like domain, but fail to bind β2-microglobulin – the light chain of MHC class I molecules. (Adapted from Raulet[102])

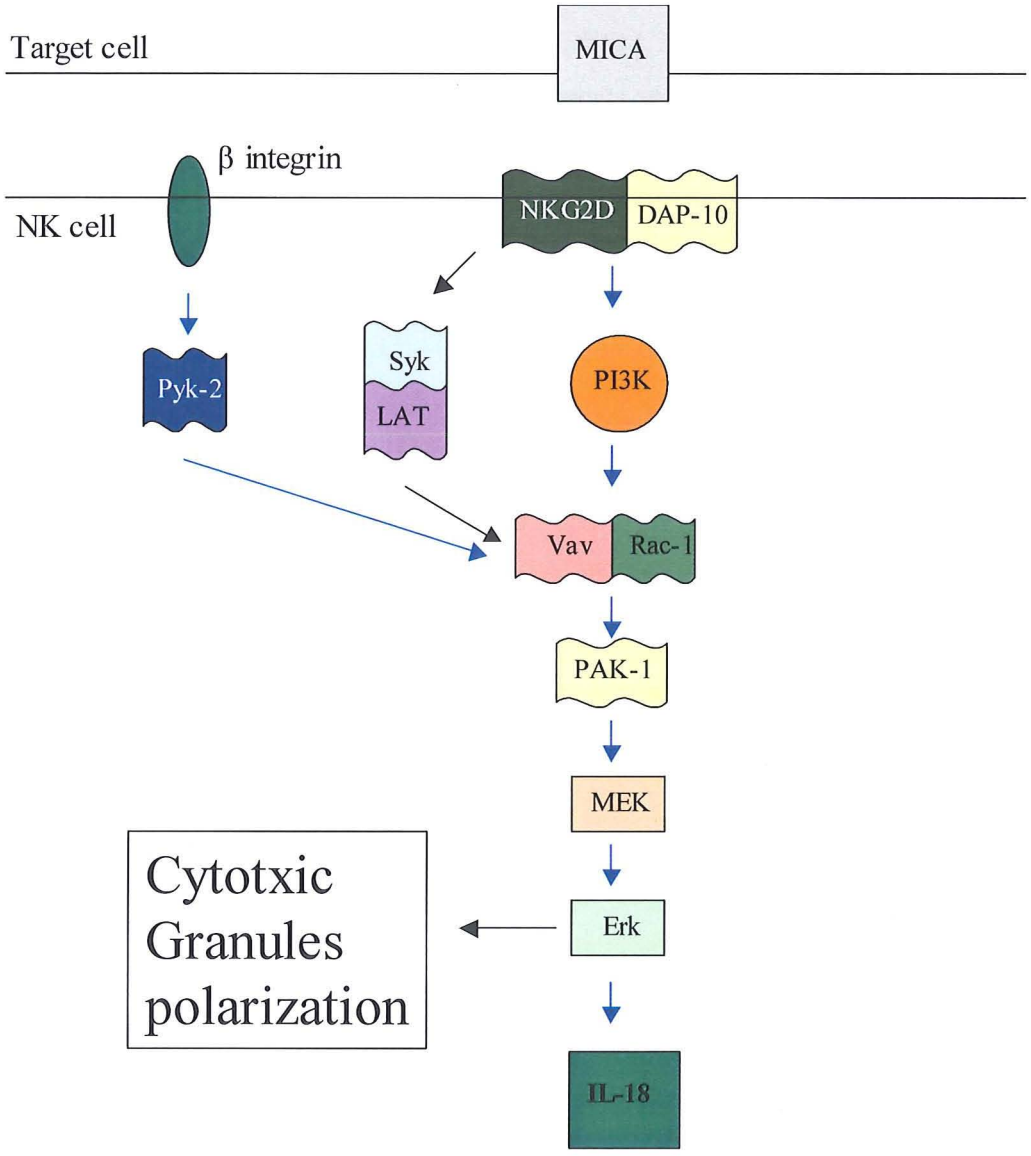


Figure 1.11 Signalling events in NKG2D activating receptor

Signalling pathway during spontaneous cytotoxicity, initiated upon engagement of NKG2D receptor with MICA ligand is independent of Ras. (Adapted from Perussia[86])

Activation of both leads to killing and cytokine release.

Toll-like receptors (TLR) are a family of innate immune receptors which recognize conserved pathogen-associated molecular patterns (PAMPs), eg LPS[103]. Ten mammalian TLRs have been identified to date, classified as TLR1 to TLR10. While individual TLRs bind specifically to one or two ligands, the TLR family as a whole will bind a wide range of microbe-derived ligands. These include LPS and LTA, bound by TLR4, PGN and lipoproteins, bound by TLR2, and unmethylated bacterial DNA, bound by TLR9. TLR3 has been shown to bind polyinosinic-polycytidylic acid (polyI:C) which is a synthetic analogue of double stranded RNA (dsRNA)[104].

The TLR family all share a similar structure. Embedded in the plasma membrane the typical TLR possesses a large extracellular portion made up of between 12 and 23 leucine-rich repeat domains with a cysteine-rich domain flanking the transmembrane region. The intracellular portion consists of a Toll/IL-1 receptor homology domain (TIR).

TLR activation leads to an immune response mainly through gene regulation via the NF-κB transcription factor system. This TLR-mediated immune response is available to a wide range of cell types that form the first line of defence against an immune challenge. Macrophages, neutrophils and dermal, gut and lung epithelial cells all express various TLRs on their surfaces. Upon exposure to microbes they are then recruited from the plasma membrane to phagosomes where they help facilitate the destruction of invading cells.

1.5.2.2.1.3 Costimulatory receptors

Co receptor's ability to signal depends on the simultaneous co-engagement of one or another triggering receptor and may help amplify the signal. 2B4 and NTB-A are members of the CD2 Ig superfamily and contain an immunoreceptor tyrosine based switch motif (ITSM) – TxYxxV/I. ITSM recruits the signalling lymphocyte activation molecule-associated protein (SAP) upon activation[105]. They recruit SHP-1 (or 2[106]), SAP, LAT, PLCγ and Grb2[107]. The ligand for 2B4 is CD48, a GPI-anchored surface molecule expressed by mainly B cells.

A similar co receptor function has been demonstrated for NKp80[108]

1.5.2.2.1.4 Polarisation of granules

Resting NK cells contain cytotoxic granules scattered around the cytoplasm. Activation leads to the reorganisation of the actin cytoskeleton and the polarisation of the microtubule organising centre (MTOC) toward the immune synapse[88]. Granule polarisation is triggered by the Src – Syk - PI3K – VAV-1 – Rac-1 – PAK – MEK – ERK-2 pathway[109].

1.5.2.2.1.5 Granule exocytosis

Activated PLCγ produces inositol-1,4,5-trisphospate (IP3) which induces Ca^{2+} release from the intracellular stocks and diacylglycerol (DAG), leads to activation of protein kinase C (PKC) (Figure 1.8). The elevated intracellular free Ca^{2+} $[Ca^{2+}]i$ is required for granule release[95].

1.5.2.2.2 Inhibitory receptors

NK cells are programmed to kill and therefore require inhibitory signals to override the activating signals and prevent death of normal autologous cells. Few receptor types are expressed by every NK cell, which leads to functional heterogeneity based upon the combination of cell surface receptors expressed. This is likely to translate into some degree of antigen or pathogen specificity.

1.5.2.2.2.1 MHC inhibitory receptors

HLA class I expression normally maintains NK cell tolerance. However, during viral infections or cancer, inhibition is removed by reduced HLA class I expression (i.e. the 'missing self' hypothesis[110]). Killer immunoglobulin-like receptor family is comprised of 15 receptors of both inhibitory and activatory members. Inhibitory receptors are characterized by immunotyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails, where as activatory receptors recruit adaptor molecules (Table 1.4). ITIMs contain the consensus sequence IVLSxYxxL/V. Healthy cell

Receptor	Ligand
Immunoglobulin-like receptors	
KIR2DL1	HLA-C, C2
KIR2DL2/3	HLA-C, C1
KIR3DL1	HLA-B Bw4
KIR3DL2	HLA-A A3, A11
Lectin-like receptors	
CD94/NKG2A	HLA-E

Table 1.4 Natural killer cell inhibitory receptors and ligands.

HLA ligands bound to inhibitory KIRs results in the inhibition of NK cell activation and protection from lysis.

Three immunoglobulin-domain KIRS, KIR3DL1 recognizes a subgroup of HLA-B molecules expressing the serological Bw4 epitope[111]. It is a 70-kDa protein containing three extracellular immunoglobulin domains (3D) and a long (L) intracellular domain that contains two ITIMs. Ligation results in phosphorylation of ITIMs and recruitment of phosphatases, SHP-1 and SHP-2, that inhibit activation pathways[112, 113] by dephosphorylation of downstream signalling proteins such as Vav1. KIR3DL1 is sensitive to the peptide presented by the HLA molecule. Unlike T-cell receptors, which display very fine peptide specificity, KIR3DL1 shows a broader specificity, with several unrelated peptides capable of preventing KIR3DL1 recognition and thus leading to KIR3DL1$^+$ NK cell activation. This allows NK cell detection of viral infection or transformation as a result of perturbation in the inhibitory signal to KIR upon presentation of viral or tumour derived peptide. Figure 1.12 depicts other KIR.

CD94/NKG2A heterodimer belongs to the lectin-like family of receptors[114], which recognize the non-classical class I HLA-E molecule[56]. HLA-E binds and presents the leader peptide of most HLA-A, -B and –C molecules and non-classical HLA-G[115]. While KIRs are sensitive to individual HLA changes, they are complemented functionally by CD94/NKG2A, which responds to changes in global HLA expression. This allows NK cells to respond to both subtle and major alterations in HLA-expression caused by different pathological situations.

LILR family encode inhibitory receptors that bind MHC class I. LILRB1 binds with low affinity to a conserved region in the $\alpha 3$ domain of essentially all HLA class I glycoproteins, including HLA-A, B, C, E, F and G.

A model for activation of NK cell cytotoxicity was suggested as ligand binding to stimulatory receptors leads to tyrosine phosphorylation of Vav1 and subsequent Vav1-dependent actin cytoskeleton rearrangements forming a stable NK cell immunologic synapse. SHP-1 located at the periphery cannot prevent the stimulatory activities i.e. Vav1 dephosphorylation cannot occur and leads to activation. The activating signals responsible for initiating cytotoxicity may

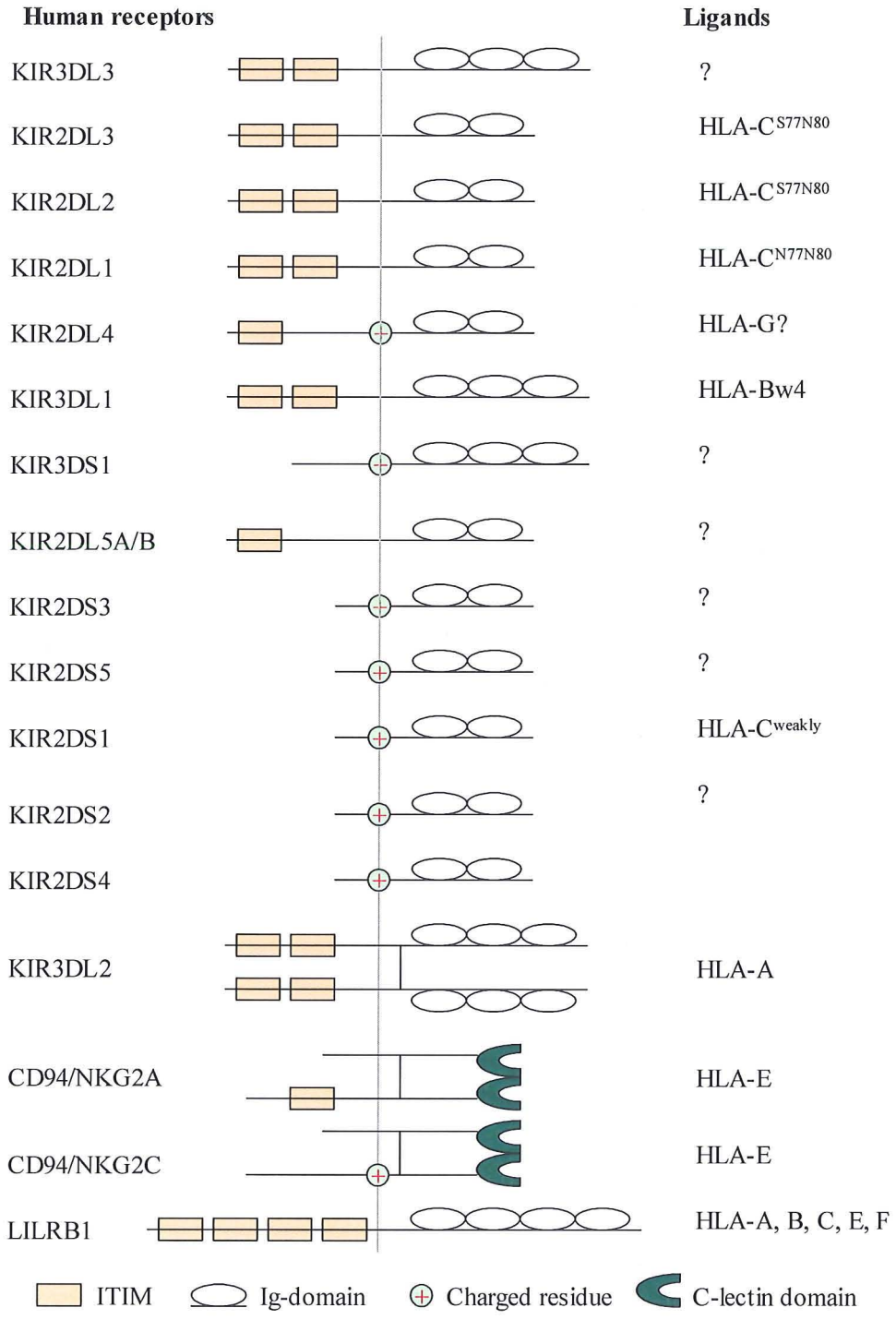


Figure 1.12 Human NK cell receptors for MHC class I

Inhibitory KIR LILRB1 and CD94/NKG2A contain ITIMs in their cytoplasmic domains. CD94/NKg2C and the KIR lacking ITIMs and containing a charged residue in their transmembrane domains are likely to pair with DAP12 signalling adapter. KIR2DL4 is an exception; it possesses an ITIM in its cytoplasmic domain and is associated with FcεRIγ signalling adapter. (Adapted from Lanier[116])

converge on downstream signalling molecules such as Vav1[117].

1.5.2.2.3 NK cell inactivation and apoptosis

After an NK cell delivers its lethal hit to a susceptible target, it may either recycle to kill again, become inactivated or undergo activation induced cell death. MHC class I inhibition is transient where as inactivation induced by NK-sensitive targets is more sustained and decreases the capacity to mediate subsequent killing of additional targets. Exposure to IL-2 increases NK responsiveness and recovery of cytotoxic function, however IL-2 or IL-12-activated NK cells are also more susceptible to activation-induced cell death if they subsequently bind to susceptible targets or FcR-specific ligands[95]. FcR-initiated cell death is dependent on tyrosine kinase activation and calcium signalling and is due to the induction of FasL.

1.5.2.2.4 Cytokine release

NK cells express the Tumour necrosis factor (TNF) family ligands; TNFα, Fas ligand (FasL) and TNF related apoptosis inducing ligand (TRAIL). These molecules bind to death domain containing receptors on target cells leading to perforin-independent apoptosis[118]. NK cells constitutively express receptors for IL-2 and IL-15, which markedly enhance the ability of these cells to kill ab-coated targets, or cells that lack MHC molecules.

NK cells are a source of cytokines and chemokines including IFNγ which can control tumour cell proliferation[119] or kill infected cells[120]. IFNγ can elicit innate and adaptive immune responses by increasing the expression of MHC class I and II molecules and by activating macrophages. Various inflammatory cytokines (IL-12, IL-18 and IFNα/β) or cross-linking of activating receptors induces IFNγ production by NK cells. NK cells also secrete IL-5, IL-10, IL-13, GM-CSF, MIP-1a/b and RANTES[121], and chemokines which recruit other effector cells during the immune response[122].

Overall, the combination of various activating receptors and different signalling pathways gives multiple choices to NK cells. There are at least two levels of redundancy, one at receptor level, with the existence of numerous receptors that can trigger cytotoxicity, and the other at the intracellular level with signalling molecules that share partially overlapping functions. Moreover, the strength of the activating signal, the nature of the receptor-ligand systems and the different environmental situations are integrated inputs that participate to decide whether to switch on signalling pathways specific for cytotoxicity or IFNγ production, or both, thus modulating effector functions.

1.6 Inflammation and Atherosclerosis

1.6.1 The inflammatory response

Inflammation is the physiological process which occurs in response to damage induced by multiple agents, including pathogens, foreign bodies, complement opsonised particles and immune complexes, as well as physical injury[123]. In the inflammatory response, soluble mediators and cellular components act in concert to eliminate the cause of damage. While this response is key to host defence, it is also clear that inflammation can also result in tissue destruction. In inflammation, soluble inflammatory mediators are released which recruit and activate the cellular components of inflammation, including neutrophils, monocytes and lymphocytes. Many of these proinflammatory mediators are synthesised and released by macrophages and monocytes. These include the cytokines TNF-α, IL-1 (α and β), IL-6 and IL-8 together with other inflammatory mediators such as prostaglandins and eicosanoids. Further, in the resolution of inflammation, macrophages are responsible for clearing recruited leukocytes, such as neutrophils and for the release of anti-inflammatory mediators such as transforming growth factor β (TGF-β) and IL-10. Thus, monocytes and macrophages play a key role in the initiation, coordination and resolution of the inflammatory response.

Other cells also play key roles in the inflammatory response[123]. Release of IFN-γ by NK cells and T-cells is important in the priming and activation of monocytes and macrophages. Neutrophils are phagocytic cells, which are rapidly recruited in response to inflammatory stimuli. These cells play a key role in the clearance of foreign materials and cellular debris. They, therefore, express high levels of phagocytic receptors, including Fc receptors and complement receptors. Eosinophils and mast cells play a central role in inflammation associated with allergy. Central to this response are IgE and its receptors (e.g. FcεRI and CD23) expressed on these cells.

1.6.2 Atherosclerosis and the immune response

Atherosclerosis is a major cause of cardiovascular disease and stroke. Plaques (focal lesions) in the arterial tree are composed of cell debris, lipid (mainly cholesterol), inflammatory cells, such as macrophage foam cells and a fibrous cap of smooth muscle cells and collagen[124]. Rupturing of plaques promotes thrombus formation and leads to ischaemia in end organs such as the brain or heart[125].

1.6.2.1 Local responses

Atherosclerosis is a chronic inflammatory disease in which both cell-mediated and humoral immune responses participate. Hypercholesterolemia and hypertension initiate the inflammatory activation of the vascular endothelium. Immune cells are recruited across the endothelial barrier into the arterial intima. Platelet glycoproteins activate endothelium via engagement of surface molecules[126]. Excess LDL penetrates the artery wall, facilitated by proteoglycans of the extracellular matrix that bind LDL, where it is prone to oxidation[127]. Phospholipids released from LDL activate endothelial cells, which then express vascular cell-adhesion molecule-1 (VCAM-1)[128]. Haemodynamic strain of oscillating flow changes, common at arterial branches, also promotes VCAM-1 expression[129]. Monocytes and T cells display the VCAM-1 receptor, VLA-4 and therefore adhere to the endothelium. Chemokines such as monocyte chemoattractant protein-1 (MCP-1), RANTES, fractalkine and others, also recruit monocytes and T cells to the lesion.

Monocyte-colony stimulating factor (M-CSF) induces monocytes entering the plaque to differentiate into macrophages that express pattern recognition receptors, such as scavenger receptors and TLRs[130]. PAMPs such as endotoxins, endogenous molecules such as heat-shock proteins (HSPs), apoptotic cell fragments and oxidized LDL (oxLDL) are taken up and destroyed via the scavenger receptor pathway. TLRs also bind molecules with pathogen-like patterns, but in contrast initiate an activation signal pathway[131]. Cholesterol contained in the LDL accumulates in the macrophage cytoplasm and transform the macrophage into a foam cell. TNF produced by neighbouring immune cells and IL-1 by immune and vascular cells, trigger a signal transduction pathway similar to TLRs[132] to produce inflammatory cytokines, chemokines, proteases, vasoactive agents, cytotoxic oxygen and nitrogen radical molecules.

Endothelial nitric oxide synthase (eNOS) and thrombomodulin confer anti-adhesive and anti thrombotic properties to the vessel wall and thereby maintain bloodflow, however these favourable factors are reduced in response to inflammatory cytokines and oxLipids.

Antigens such as oxLDL, Hsp-60 and microbial antigens activate T cells in the atherosclerotic artery. CD4+ Th1 predominate and secrete INFγ, which leads to activation of macrophages and endothelial cells. INFγ improves the efficiency of antigen presentation and augments synthesis of TNF and IL-1 synergistically instigating the production of inflammatory and cytotoxic molecules in macrophages and vascular walls. However, regulatory T cells act to inhibit atherosclerosis by producing IL-10 and TGFβ[133, 134].

1.6.2.2 Systemic responses

Systemic reactions include humoral immunity and acute phase reactants[135]. Antibodies reactive to oxLDL are abundant in atherosclerosis. IgM reactive to phospholipid epitopes, cross-react with oxLDL, apoptotic bodies and the capsule of *Streptococus pneumoniae*, all of which contain phosphocholine[136]. These antibodies may contribute to the elimination of oxLDL and dead cells as well as

defence against pneumococcal infections. Atheroprotective immunity can be induced (in animals) by immunization with oxLDL[137] or Hsp-65[138].

Acute phase reactants include CRP, serum amyloid A, fibrinogen, pentraxin-3 and others. CRP is an independent risk factor for coronary artery disease[139], and likely reflect inflammation in the coronary artery rather than the ischemic myocardium[140]. CRP acts together with complement to eliminate microbes and C3, C4 and terminal C5b-C9 complement complexes occur in atherosclerotic lesions[141].

1.6.2.3 Plaque rupture

Activated macrophages, T cells and mast cells at sites of plaque rupture[142, 143] produce several types of molecule – inflammatory cytokines, proteases, coagulation factors, radicals and vasoactive molecules – that destabilise lesions. Inflammation interferes with the integrity of the interstitial collagen matrix by blocking the formation of new collagen fibres and therefore formation of stable fibrous caps, and by stimulating the destruction of existing collagen, initiating thrombus formation[144]. TGFβ, Platelet–derived growth factor (PDGF) and IL-1 stimulate smooth muscle cells to produce collagen. However INFγ, produced by T cells in the plaque, inhibits both basal collagen production and the stimulatory effects of TGFβ, PDGF and IL-1[145].

Matrix metalloproteinases (MMPs)[146] and cysteine proteases[147] have been implicated in plaque activation. CD40 ligand and IL-1 produced by T cells promote the production of collagen-degrading enzymes by macrophages including MMP-1, MMP-8 and MMP-13[148]. MMP are synthesized and secreted as inactive proenzymes, activated by TNFα, tryptase and chymase[146]. CD40 ligand also stimulates the production of tissue factor, when exposed to factor VII, initiates the coagulation cascade[144].

1.7 Anti inflammatory effects of statins

Statins interfere with the pro inflammatory pathway of adhesion and migration at the protein expression and function levels. For example, Lovastatin and cerivastatin have been shown to reduce expression of the ICAM-1 ligand - CD11b on monocytes and inhibit adhesion of leukocytes to endothelial cells[149, 150]. Table 1.5 summarizes the inflammatory pathways and mediators affected by HMG-CoA reductase inhibitors. Reduction in adhesion molecule synthesis also occurs in other cell types e.g., E-selectin and VCAM-1 on EC[149, 150].

Statins diminish the expression and function of proatherogenic cytokines such as IL-6, IFN-g and TNFα in macrophages[151]. Stains may also repress the activation of T lymphocytes by inhibiting MHC class II antigen expression[152].

Statins regulate expression of chemokines MCP-1, IL-8 and RANTES in cultured monocytes, ECs and SMCs[153, 154]. Inhibition of sterol synthesis via squalestatin was not comparable in a study, suggesting that the in vivo regulation of cytokine/chemokines production by statins depends on the biosynthesis of nonsterol compounds arising from mevalonate[155]. MCP-1 induction (as well as IL-1β and TNFα) reduced by statins is related to a decrease in NFκB activation[156].

Statins may mediate apoptosis. Concentrations as low as 10 nM induce the expression of proapoptotic enzymes caspase-3 and –9 and limit the expression of Bcl-2, an inhibitor of apoptosis[157, 158]. Reduced prenylation of p21-RhoB, a central regulator of apoptosis, by statins in SMCs, promotes apoptosis[159]. In atherogenesis, apoptosis promotes the formation if the lipid core by the death of foam cells and influences the SMC content in the fibrous cap[160].

Statins lower the expression and function of a broad range of MMPs including interstitial collagenases, MMP-1 and MMP-13, gelatinases MMP-2 and MMP-9, and stromelysin MMP-3 in most cell types involved in atherogenesis including macrophages[161, 162]. Statins also augment the expression of TIMP-1 in SMCs and macrophages, which limits extracellular matrix breakdown, and thus render lesions less prone to rupture[163]. Mechanisms regulating MMP/TIMP expression

Process/pathway	Mediator	Cell type
Adhesion ⇩	↓ CD11b, LFA-1 (also via direct binding)	Mϕ and T cells to endothelium; peripheral blood
	↓ ICAM-1 (and sICAM-1), VCAM-1 (and sVCAM-1)	
	↓ E-selectin, L-selectin	
Migration ⇩	↓ MCP-1, IL-8, RANTES	EC, SMC, Mϕ, T cells
Proliferation ⇩	↓ SMC, EC (via p21[Waf1/Cip1])	
Endothelial function ⇧	↑ eNOS	EC
	↓ LDL oxidation, Endothelin-1	
Matrix degredation ⇩	↓ Interstitial collagenases MMP-1/-13	
	↓ Gelatinases MMP-2/-9, Stromelysin MMP-3	
	↑ TIMP-1	
Apoptosis ⇧	↑ Caspase-3, Caspase-9	EC, SMC,Mϕ
	↓ Prenylation of p21RhoB, Bcl-2	
Thrombosis ⇩	↓ Tissue factor, Factor VIIa, t-PA, Tx2, TxB2	EC, Mϕ, platelets; peripheral blood
	↓ Platelet aggregation	
	↑ Fibrinogen, PAI-1,PGI2	
Inflammatory mediators	↓ CD40/CD40L, sCD40L	
⇩	↓ IL-1β, IL-6, TNF-α, CRP, cyclooxygenase 2, Serum amyloid A	
	↓ MHC II, Th1 (IFN-γ, IL-12)	
	↑ PPAR-g, Th2 (IL-4, IL-10, TGF-β)	

Table 1.5 Inflammatory pathways and mediators affected by HMG-CoA reductase inhibitors.

Arrows indicate enhanced or diminished activation/expression of pathway/mediator after statin administration. (Adapted from Schonbeck et al[164])

may involve NF-κB activity and prenylation of Rho proteins[165].

Statins modulate lesional procoagulant activity and platelet activity. They reduce tissue factor expression[166], the primary initiator of the extrinsic pathway in macrophages and ECs. They also promote fibrinolytic activity by diminishing plasminogen activator inhibitor-1 expression and enhancing tissue-plasminogen activator in EC and SMCs[167]. Statins inhibit fibrinogen expression and thrombin formation, and reduce platelet aggregation and deposition in diseased vessels[168]. Reduced expression of COX-2, TxA2 or TxB2 and enhanced synthesis of prostacyclin by statins contribute to diminished platelet activation[169, 170].

Statins reduce the susceptibility of LDL to oxidation by lowering the amount of substrate available for oxidation[171] and reducing macrophage superoxide formation thereby decreasing oxygen production[172]. Fluvastatin and lovastatin bind to phospholipid on the surface of LDL and thus prevent diffusion into the lipoprotein core, of free radicals, generated under oxidative stress[173]. Atorvastatin has also been shown to have direct antioxidant effects by protecting LDL, VLDL and HDL from oxidation[174].

Statins can directly upregulate eNOS expression and prevent its downregulation by oxidized LDL[175]. Endothelium dependent vasodilation is increased by simvastatin after four weeks treatment[176].

1.8 Clinical trials and the effects of statins

Several clinical trials have suggested that benefit of statin therapy is greater than that expected on the basis of LDL reduction alone.

1.8.1 Scandinavian Simvastatin Survival Study (4S)

The secondary prevention trial, Scandinavian Simvastatin Survival Study (4S)[177] established the importance of treating the hypercholesterolaemic patient with cardiovascular disease. A total of 4444 patients with serum cholesterol 212–

309mg/dl (5.5–8.0mmol/l) and angina or prior myocardial infarction (i.e. stable coronary disease) were given either simvastatin (dose range 10–40mg) or placebo and followed up for a median of 5.4 years. LDL cholesterol was reduced by 35% and total cholesterol by 25%, in the simvastatin group. Treatment with simvastatin significantly reduced the total mortality primary end-point (RR 0.70, 95%CI 0.58–0.85), as well as major coronary events (defined as coronary death, myocardial infarction, or resuscitated cardiac arrest, RR 0.66, 95%CI 0.59–0.75), and coronary mortality (RR 0.58, 95%CI 0.46–0.73). Statin therapy also reduced the need for coronary revascularization with bypass surgery or angioplasty (RR 0.63, 95%CI 0.54–0.74). There was no difference between the treated and placebo groups in noncardiovascular deaths. This study established that hypolipidaemic therapy was safe and reduced morbidity and mortality in hypercholesterolaemic patients with IHD. It concluded that all patients with elevated cholesterol levels and known previous cardiovascular heart disease should receive statins to limit future adverse events.

Post hoc analysis of the 4S study suggested that the benefit provided by simvastatin was indeed related to the change in LDL cholesterol[178].

1.8.2 West of Scotland Coronary Prevention study (WOSCOPS)

The primary prevention trial, The West of Scotland Coronary Prevention study (WOSCOPS)[179] investigated whether hypolipidaemic therapy was beneficial in hypercholesterolaemic men without a prior history of myocardial infarction. 6595 men aged 45–64 years, at high risk of coronary events were enrolled i.e. middle-aged men with high lipid levels - mean total cholesterol 27222mg/dl (7.03 ±0.57mmol/l) and elevated BMI (26 ±3.1kg/m_2), and over a third were current smokers. They were treated with either 40mg/day of pravastatin or placebo, and followed up over an average of 4.9 years. LDL cholesterol was reduced by 26% and total cholesterol by 20% with pravastatin. Pravastatin therapy reduced the primary end-point of non-fatal myocardial infarction or coronary deaths by 31% (95%CI 17–43%), revascularization procedures by 37% (95%CI 11–56%) and cardiovascular mortality by 32% (95%CI 3–53%). The reduction in total mortality

of 22% was of barely significant (p=0.051) and there was no difference in non-cardiovascular mortality. This suggests that in subjects with no previous heart disease, treatment of elevated lipid levels does not achieve as great a benefit as it would in a group with known cardiovascular disease, (such as in the 4S population). The reduction of coronary events in WOSCOPS was profound, and high-risk hypercholesterolaemic patients not previously known to have IHD would still benefit from treatment of hyperlipidaemia.

Post hoc analysis of the WOSCOPS study[180] applied the Framingham coronary heart disease model and accurately predicted the risk in the placebo group but underestimated the risk reduction in the pravastatin group by 31%. The benefits of LDL reduction with statins also appear to occur earlier than observed with other cholesterol lowering therapies such as cholestyramine and ileal bypass surgery[181, 182], although they did provide long term risk reduction. It was concluded that LDL reduction alone might not account for the observed benefits of statin therapy.

1.8.3 The Cholesterol and Recurrent Events (CARE) study

Data from The Cholesterol and Recurrent Events (CARE) study suggests that pravastatin, in a process independent of LDL lowering, directly attenuates the adverse effects of inflammation[183]. Subjects examined had average cholesterol levels of 209 ±17mg/dl (5.4±0.4mmol/l) and a history of myocardial infarction. 4159 patients had follow-up for a median five years. LDL cholesterol was reduced by 28% and total cholesterol by 20%. Pravastatin treatment compared to placebo reduced the primary end-point (defined as coronary death or non fatal myocardial infarction) by 24% (95%CI 9–36%). There was no significant difference in cardiovascular, non-cardiovascular or total mortality. However, the significant reduction in the primary end-point could be accounted for by the marked reduction in myocardial infarction. Pravastatin therapy also lowered the need for revascularization procedures and the incidence of strokes were significantly reduced. Patients with a prior myocardial infarction (secondary prevention), hypolipidaemic therapy is important even if cholesterol levels are not highly elevated. However, the absence of coronary mortality reduction, and the lower percentage reduction of major coronary events in the CARE study compared to 4S,

suggests that it is the patient who is both hyperlipidaemic and high-risk who will benefit most from therapy.

In a stratified analysis, patients with persistent inflammation, measured by elevation of hs-CRP and serum amyloid A, were at increased risk of recurrent cardiovascular events[184]. This was shown in the placebo study group who's risk of recurrent events were: relative risk=2.81, p=0.007 compared to in the pravastatin group: relative risk=1.29, p=0.5. Although the two groups had identical LDL baseline levels, the proportion of recurrent cardiac events prevented by pravastatin was 54% among those with high CRP levels and 25% without inflammation. Pravastatin therapy also reduced median hs-CRP over five years, compared with placebo[185]. Taken together the data suggests pravastatin has clinically important anti-inflammatory properties in addition to LDL cholesterol lowering.

1.8.4 Long Term Intervention with Pravastatin in Ischemic Disease (LIPID) study

The placebo-controlled, Long Term Intervention with Pravastatin in Ischemic Disease (LIPID) study enforced the importance of hypolipidaemic therapy in secondary prevention[186]. Subjects enrolled had a broad range of initial total cholesterol levels, from 155 to 271mg/dl (4.0–7.0mmol/l) and a history of myocardial infarction or unstable angina with. After a mean follow-up of 6.1 years 9014 patients were enrolled. Pravastatin reduced LDL cholesterol by 25% and total cholesterol by 18%. The primary end-point was coronary mortality and pravastatin treatment reduced this by 24% (95%CI 12–35%). Total mortality was reduced by 22% (95%CI 13–31%). There was also a 24% reduction of major coronary events (coronary death and non fatal infarction), 20% reduction in coronary revascularization and 19% reduction in strokes. Subgroup analysis of LIPID showed the benefit of hypolipidaemic therapy over all ranges of total cholesterol levels, however benefit was seen most in those with the highest LDL cholesterol.

1.8.5 Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TEXCAPS)

The placebo controlled, randomised trial, Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TEXCAPS)[187] investigated the effects of lovastatin on a healthy population with average risk and normal cholesterol levels of 221 ±21mg/dl (5.71 ±0.54mmol/l) but having low HDL cholesterol: ‹45 mg/dl for men (1.16mmol/l) and ‹47mg/dl (1.22mmol/l) for women. Treatment with lovastatin significantly reduced LDL cholesterol by 25%, total cholesterol by 18%, and increased HDL cholesterol by 6%, after a year. 5608 men and 997 women received a mean follow-up of 5.2 years, the primary end-point of first major coronary event, defined as myocardial infarction, unstable angina or sudden death, was highly significantly reduced (RR 0.63, 95%CI 0.50–0.79). A similar marked improvement was seen in risks of myocardial infarction, unstable angina and coronary revascularization. There was no difference in total mortality between the two groups[188] with 80 deaths in the lovastatin group, and 77 in the placebo group (RR 1.04, 95%CI 0.76–1.42). Over two-thirds (115) were from non-cardiovascular causes, which highlight the fact that in a group of people not at high risk of coronary deaths, therapy to lower cholesterol cannot do much to lower mortality. Non-statin hypocholesterolaemic drugs have revealed similar findings of a reduction in cardiac end-points without total mortality reduction in primary prevention trials. AFCAPS/TEXCAPS concluded that patients at high risk of adverse cardiovascular events should be treated with statin even though their cholesterol levels are not elevated.

1.8.6 Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering (MIRACL) study

The Myocardial Ischaemia Reduction with Aggressive Cholesterol Lowering (MIRACL) study[189] looked at the acute coronary syndromes (ACS) and the effect of early use of atorvastatin. 3086 patients with unstable angina or non-Q-wave myocardial infarction were randomly assigned to atorvastatin 80 mg/day or placebo at 24–96 h of admission. Atorvastatin treatment reduced LDL cholesterol

by 52% and total cholesterol by 34%. After a follow-up of 16 weeks, atorvastatin therapy produced a significant reduction in the primary end-point, defined as death, non-fatal myocardial infarction, cardiac arrest or recurrent symptomatic ischaemia (RR 0.84, 95%CI 0.70–1.00). Unfortunately, there was no significant change in death, non-fatal myocardial infarction or cardiac arrest, and the benefit was seen in the reduction of recurrent ischaemia. This study concluded that more benefit from other therapeutic interventions would arise when dealing with unstable angina and myocardial infarction[190], i.e. quick, increased, coronary flow which is not obtainable with hypolipidaemic therapy.

1.8.7 Atorvastatin Versus Revascularization Treatment (AVERT) study

The Atorvastatin Versus Revascularization Treatment (AVERT) study investigated the value of aggressive cholesterol reduction in comparison with coronary angioplasty[191]. 341 patients with stable angina recommended for percutaneous intervention were studied. 177 were advised to have angioplasty and 164 were given 80 mg/day atorvastatin instead of angioplasty. Atorvastatin treatment reduced LDL cholesterol by 46% and total cholesterol by 31%. After a follow-up of 18 months, 13.4% of patients assigned to the aggressive lipid-lowering therapy had had an ischaemic event, compared to 20.9% in the angioplasty group (risk reduction 36%, p=0.048, not significant after adjustment for interim analysis). It took significantly longer to reach an ischaemic event in the atorvastatin group (RR 0.64, 0.33–0.95, p=0.03). The study confirmed the safety of aggressive lipid reduction therapy, and suggested that it may be as effective as angioplasty in managing patients with stable angina pectoris. It has been shown that angioplasty does not reduce the incidence of MI or coronary mortality[192], as MIs are not due to the progression of previously narrowed plaques, but the sudden disruption of mildly stenotic lesions[125].

1.8.8 Heart Protection (HPS) study

The Heart Protection (HPS) study established the benefit of statin therapy in patients at high risk of atheromatous disease, regardless of initial lipid levels, in

preventing adverse events[193]. 20 536 patients at high risk of coronary mortality due to prior coronary disease (secondary prevention), presence of non-coronary atheromatous disease or diabetes were recruited and followed-up for a mean of five years. Patients were randomised to receive simvastatin 40 mg/day or placebo. Simvastatin reduced LDL cholesterol by 1.0mmol/l (29.4%) and total cholesterol by a mean of 1.2mmol/l (20.3%). Primary end-point was total mortality. Simvastatin produced a highly significant reduction in total mortality (RR 0.87, 95%CI 0.81–0.94, p=0.0003), in major coronary events (RR 0.73, 95%CI 0.67–0.79, p<0.0001), in strokes (RR 0.75, 95%CI 0.66–0.85, p<0.0001) and in revascularization procedures (RR 0.76, 95%CI 0.70–0.83, p<0.0001). There was no effect on noncardiac mortality and no increase in non-haemorrhagic stroke. Women, the elderly, and patients with prior non-cardiac atheromatous disease saw a benefit. The value of statin therapy in diabetic patients was seen in the reduction of cardiovascular disease in the 5963 diabetics recruited[194]. The proportional reduction in adverse events was the same in all categories of lipid levels, even in those with initially low LDL and total cholesterol.

1.8.9 The Lescol Intervention Prevention Study (LIPS)

The secondary prevention Lescol Intervention Prevention Study (LIPS)[195] assessed the efficacy of fluvastatin treatment and supported the view that the protective effect of statins is a class effect.

1.8.10 The Prospective Study of Pravastatin in the Elderly at Risk (PROSPER) trial

The mixed secondary and primary prevention study, The Prospective Study of Pravastatin in the Elderly at Risk (PROSPER) trial assessed hypocholesterolaemic therapy in older patients. 5804 patients aged 70–82 years were randomised to either pravastatin 40 mg/day or placebo. Patients recruited either had a history of existing vascular disease (coronary, cerebral or peripheral) or were at risk because of smoking, hypertension or diabetes. Pravastatin therapy reduced LDL cholesterol by 34%, and increased HDL cholesterol by 5% after three months. The primary end-point was composed of coronary death, non-fatal myocardial

infarction, and fatal or non-fatal stroke. After mean follow-up of 3.2 years, the primary end-point was significantly reduced (RR 0.85, 95%CI 0.74–0.97, p=0.014), due to the lowered risk of coronary death and non-fatal myocardial infarction (RR 0.81, 95%CI 0.69–0.94, p=0.006). There was no significant change in stroke incidence (RR 1.03, 95%CI 0.81–1.31, p=0.8). However, there were more frequent new cancer diagnoses in the pravastatin group (RR 1.25, 95%CI 1.05–1.51, p=0.02), supporting the previous concern about the potential carcinogenic dangers of statin therapy[196]. Meta analysis was performed on previous statin trials, and showed no overall increase in cancer occurrence whether patients were on pravastatin or other statin drugs.

1.8.11 The Anti-hypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial—Lipid Lowering Trial component (ALLHAT-LLT)

The mixed primary and secondary prevention trial, Anti-hypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial—Lipid Lowering Trial component (ALLHAT-LLT) randomised patients aged >55 years with hypertension and one other risk factor to either pravastatin 20–40 mg/day (n=5170) or usual care (n=5185)[197]. With pravastatin, LDL cholesterol was reduced by 17% and total cholesterol by 10%. There was no difference in the primary end-point of all-cause mortality between the pravastatin and usual care groups (RR 0.99, 95%CI 0.89–1.11, p=0.88) after a mean follow-up of 4.8 years. With 86% of patients having no prior coronary disease it was not surprising that there was no difference in coronary heart disease events (RR 0.91, 95%CI 0.79–1.04, p=0.16). Generally healthy patients are at risk of dying from non-cardiovascular causes, and therefore any benefit from statin therapy in reducing coronary mortality is obscured. The unexpected failure of ALLHAT-LLT to show a reduction in coronary heart disease events was attributed to the use of hypolipidaemic therapy in the 'usual care' patients. 26.1% of the 'usual care' patients were on statins, 2.4% were on other hypolipidaemic drugs and 16.2% of the pravastatin group ended up receiving no lipid-lowering therapy at all, after six years[198].

1.8.12 Higher dose statin trials (PROVE-IT, REVERSAL and ALLIANCE)

Patients in The Pravastatin or Atorvastatin Evaluation and Infection Therapy—Thrombolysis in Myocardial Infarction (PROVE-IT)[199] were randomised to standard therapy with 40 mg/day pravastatin, or to aggressive therapy with atorvastatin 80 mg/day as were phase Z of the A to Z trial[200]. In showing a trend favouring early and intensive statin therapy that was more pronounced over a longer follow-up period, the A to Z trial supports the findings of MIRACL and PROVE-IT which similarly studied ACS patients. Although ACS patients will benefit clinically from high-dose statin therapy, liver and muscle enzymes should be carefully monitored given the increased incidence of adverse effects. The PROVE-IT trial also showed that statin therapy decreases CRP levels in association with better clinical outcomes, regardless of cholesterol levels[201].

Reversal of Atherosclerosis with Aggressive Lipid Lowering (REVERSAL) trial[202] addressed whether intensive statin therapy would be beneficial in the stable non-acute coronary patient and successful halt to atheroma progression was demonstrated.

The Aggressive Lipid-Lowering Initiation Abates New Cardiac Events (ALLIANCE) study suggested that aggressive statin treatment was safe and significantly reduced cardiovascular events in patients with known coronary disease, without elevation of liver enzymes, rhabdomyolysis or increased cancer

The treating to New Targets (TNT) recruited patients with stable IHD already receiving 10mg/day of atorvastatin and either continued with that level or increased to 80mg/day atorvastatin. High dose statin reduced cardiovascular outcomes but also increased adverse events. It is therefore important to treat the correct patient with high doses of statin to maximize the risk-benefit ratio.

1.8.13 The Anglo-Scandinavian Cardiac Outcomes Trial - Lipid Lowering Arm (ASCOT-LLA)

The primary prevention study, The Anglo-Scandinavian Cardiac Outcomes Trial - Lipid Lowering Arm (ASCOT-LLA)[203] recruited 19,342 hypertensive patients with at least three other cardiovascular risk factors, thus at higher risk of cardiovascular event than those in ALLHAT-LLT. Patients were randomised to either 10 mg/day atorvastatin or placebo, with a primary end-point of nonfatal myocardial infarction and fatal coronary heart disease. In the atorvastatin group, 100 events occurred and in the placebo group, 154 events (HR 0.64, 95%CI 0.50–0.83, p=0.0005) after a median follow up of 3.3 years. Incidence of chronic stable angina, strokes, and total coronary and cardiovascular events and procedures, were all significantly reduced. Statistical significance was not reached with reduction in total mortality (HR 0.87, 95%CI 0.71–1.06, p=0.16). ALLHAT-LLT and ASCOT-LLA suggest that the high-risk hypertensive patient will benefit from statin therapy, while the hypertensive with no previous cardiovascular disease and at low risk will not.

1.8.14 A Study to Evaluate the Effect of Rosuvastatin on Intravascular Ultrasound-Derived Coronary Atheroma Burden (ASTEROID)

A Study to Evaluate the Effect of Rosuvastatin on Intravascular Ultrasound-Derived Coronary Atheroma Burden (ASTEROID)[204] assessed whether very intensive statin therapy (40mg/day rosuvastatin) could regress atherosclerosis. The percent atheroma volume was measured by Intravascular ultrasound. Average LDL cholesterol achieved was 60.8mg/dL (53.2% reduction) and HDL cholesterol increased by 14.7%. Treatment to LDL cholesterol levels below currently accepted guidelines accompanied by significant HDL cholesterol increases, regressed atherosclerosis in coronary disease patients.

1.9 Non statin lipid lowering trials

Low levels of HDL-C have long been associated with an increased cardiovascular events risk[205, 206]. Elevated HDL-C levels are protective as they drive reverse cholesterol transport (RCT) which results in the net delivery of systemic cholesterol back to the liver for disposal as bile salts or to steroidogenic organs for conversion to steroids (e.g. adrenalin)[207]. Atherosclerotic disease is prevented as long as RCT outpaces accumulation of cholesterol in vessel walls. HDL reduces oxidation of LDL via activity of paraoxonase[208] and platelet activating factor acetyl hydrolase[209], rendering it less atherogenic. HDL stimulates eNOS activity and increases NO and arterial vasodilation[210]. HDL decreases VCAM-1 expression [211] and decreases platelet aggregability[212].

1.9.1 Fibrates

The peroxisome proliferator-activated receptors (PPARs) (i.e., PPAR-a, -β and -γ) form the steroid nuclear receptor superfamily[213]. All PPARs are activated by fatty acids and derivatives. When PPAR-α binds a hypolipidemic fibrate, the activated complex interacts with the retinoid X receptor (RXR). The PPAR-RXR heterodimer regulates the transcription of a variety of genes involved in inflammation, as well as lipoprotein and fatty acid metabolism by binding to the peroxisome proliferator response elements within the promoters of these genes. Fibrates stimulate lipoprotein lipase and apolipoprotein (apo)A-V and inhibits apoC-III expression (which is a lipoprotein lipase inhibitor) and drives metabolism of triglycerides in VLDL and chylomicra[214]. Hepatic uptake and esterification of free fatty acids is increased, and mitochondrial free fatty acid uptake and the resulting free fatty acid oxidation is increased. Plasma HDL-C increases are dependent partly on hepatic overexpression of apoA-I and apoA-II and upregulation of HDL-C proteins[215, 216]. PPARs are also expressed in atherosclerotic lesions and fibrates reduce serum CRP levels[217].

PPAR-alpha activators; bezafibrate, fenofibrate and gemfibrozil, reduce triglycerides and increase HDL-cholesterol, without significantly affecting LDL-

cholesterol, to slow down coronary artery luminal narrowing and decrease coronary artery disease mortality in secondary prevention trials.

1.9.1.1 Helsinki Heart Study

The Helsinki Heart Study found a 34% reduction in the combined CVD endpoint in the gemfibrozil treatment group that was independently related to an HDL-C increase of 10%[218].

1.9.1.2 Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial (VA-HIT)

Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial (VA-HIT) study[219] found an 11% decrease in CVD events for every 5mg/dL increment in HDL-C when treated with gemfibrozil.

Fibrate use did not confer an independent risk reduction in CVD in patients with low baseline HDL-C and High TG levels. Reductions of 30% in triglycerides were not independently predictive of a reduction in CVD risk in both studies.

1.9.2 Niacin

Niacin (Nicotinic acid, Vitamin B3) is a precursor for nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphatase (NADP). The reduced coenzyme forms participate in red-ox reactions of many metabolic processes[220]. Chronic deficiency causes pellagra.

Niacin (at doses of 1-3g/day) is also effective at increasing HDL levels[221]. Mobilization of fatty acids and triglyceride levels are decreased by inhibition of lipolysis in adipose tissue. Niacin inhibits triglyceride production at two synthetic sites: fatty acid synthesis from acetate and esterification of fatty acids to form triglyceride[222]. Niacin inhibits directly and non competitively microsomal diacylglycerol acyltransferase (DGAT), a rate limiting enzyme in triglyceride synthesis, which limits apoB lipidation, resulting in delayed translocation of apo B

across the ER membrane. The result is increased intracellular apoB degradation and subsequent decreased VLDL/LDL and inhibition of the formation of atherogenic small dense LDL particles. Niacin does not stimulate hepatic biosynthesis of HDL. Instead, the HDL subfraction containing ApoA-I without ApoA-II (LP-AI) which acts as a cholesterol acceptor, is increased in the serum[223].

1.9.2.1 The Cholesterol-lowering Atherosclerosis study (CLAS)

The Cholesterol-lowering Atherosclerosis study (CLAS) randomised patients to niacin (3-12g/day) combined with bile acid sequesterant colestipol (30g/day) or to diet/placebo[224]. LDL decreased by 39%, triglycerides by 16% and HDL rose by 35%. After two years follow up Quantitative coronary angiography showed regression of lesions in 16% of patients compared to 2% on diet/placebo.

1.9.2.2 sAfety and tolerability stUdy of a modified-release nicoTinic acId formuLation in sUbjects with dySlipidaemia and low HDL-cholesterol (NAUTILUS)

The multiceNtre, open, uncontrolled sAfety and tolerability stUdy of a modified-release nicoTinic acId formuLation in sUbjects with dySlipidaemia and low HDL-cholesterol (NAUTILUS) recruited 566 patients with dyslipidaemia where lipids were inadequately controlled by four weeks of diet treatment and had low HDL-cholesterol (< 1.03mmol/L [< 40mg/dL] in men and < 1.29mmol/L [< 50mg/dL] in women), and had triglycerides < 9.03mmol/L (< 800mg/dL)[225]. Prolonged-release nicotinic acid was well tolerated, and the study supports its use in the management of patients with low HDL-cholesterol at elevated cardiovascular risk.

1.9.2.3 ARBITER 2 study

The ARBITER 2 study recruited patients on stable treatment with statins, average LDL cholesterol 2.3mmol/L, mean duration of statin treatment five years[226]. They all had CHD and low HDL cholesterol and were randomised to placebo or 1g/day PR nicotinic acid (Niaspan) in combination with statin treatment for one year. Niacin increased HDL cholesterol by 21%. Carotid intima-media thickness

progressed in the placebo/statin group but remained unchanged in the nicotinic acid/statin group, showing a beneficial effect of Niacin.

1.9.3 Infusion of HDL

Intravenous infusion of HDL or bioengineered HDL induces the regression of atheromatous plaques[227].

1.9.4 Ezetimibe

Ezetimibe binds to Niemann-Pick C1-like 1 (NPC1L1) receptors to dose dependently inhibit cholesterol absorption. Ezetimibe localizes at the brush border of the small intestine[228] and does not affect the absorption of triglycerides, fatty acids bile acids or fat soluble vitamins[229]. Ezetimibe is glucuronidated in the intestine and undergoes enterohepatic recirculation, repeatedly returning to its site of action and minimizing systemic exposure, explaining its long half life[230].

1.9.4.1 Ezetimibe Monotherapy trials

Ezetimibe reduced LDL cholesterol in patients with hypercholesterolaemia in two randomised, placebo-controlled phase 3 trials. 892 patients treated with 10mg/day ezetimibe decreased LDL-C by17.7% compared with placebo ($p<0.01$) and increased HDL-C by 1.3% compared with 1.6% decrease with placebo ($p<0.01$)[231]. In another trial 827 patients treated with 10mg/day ezetimibe decreased LDL-C by 18.2% and increased HDL-C by 1% compared with 1.6% decrease with placebo ($p<0.01$)[232].

1.9.4.2 Ezetimibe plus statin trials

Ezetimibe was combined with four different statins in randomised double blind placebo controlled studies. Patients were randomised to Ezetimibe at 10mg/day alone or with statin at various concentrations or placebo (Table 1.6). Ezetimibe with statin lowered LDL-C more effectively than statin alone in each study.

	Change in concentration (%)						
	No of patients	TC	LDL-C	HDL-C	TG	ApoB	Ref
Atorvastatin	248	-32	-44	4	-25	-36	
+ ezetimibe	255	-41	-56	7	-33	-45	[233]
Simvastatin	263	-26	-36	7	-17	-30	
+ ezetimibe	274	-37	-51	9	-24	-41	[234]
Pravastatin	205	-17	-25	7	-8	-20	
+ ezetimibe	204	-27	-39	8	-18	-30	[235]
Lovastatin	220	-18	-25	4	-11	-21	
+ ezetimibe	192	-29	-40	9	-22	-33	[236]

Table 1.6 Percent changes in serum lipids:Statin monotherapy versus Ezetimibe plus statin

CRP was also significantly reduced with combination of statin plus ezetimibe compared with statin plus placebo (9.7% v 0%, $p<0.05$) in another study[237].

1.9.5 Program on the Surgical Control of the Hyperlipidemias (POSCH) trial

The Program on the Surgical Control of the Hyperlipidemias (POSCH) trial refuted a special benefit of statins beyond lipid changes[238]. Ileal bypass surgery lowered LDL by 38% and reduced major CHD events by 35%, which was almost identical to statins.

1.10 Other uses of Statins

The anti-inflammatory and immunomudulatory nature of the pleiotropic effects of HMG CoA reductase inhibitors[239] on prenylation allow a wide variety of uses.

1.10.1 Immunomodulation

1.10.1.1 Transplant Rejection

Hyperacute rejection begins within minutes, with intravascular thrombosis initiated by binding of pre existing antibodies to HLA and complement to the endothelium. Secretion of von Willebrand factor mediates platelet adhesion and aggregation. Acute rejection, tissue injury mediated by T cells, macrophages and antibodies, follow within the first week. T cells respond to MHC molecules and antigens. Activated CD8+ T cells cause direct lysis of graft cells and CD4+ T cells produce cytokines that recruit and activate inflammatory cells to cause necrosis. Antibodies made during the humoral response bind to vessel walls and activate complement. Chronic rejection is characterised by fibrosis and graft arteriosclerosis, occurring over a prolonged period. Remodelling of the graft extracellular matrix produces scarring which leads to progressive graft dysfunction and loss[240].

1.10.1.2 Current Immunosuppression

1.10.1.2.1 Corticosteroids

Glucocorticoids were the first immunosuppressants used in transplantation. They affect gene expression of many cytokines on T and B cells, macrophages, granulocytes and monocytes[240].

1.10.1.2.2 Antimetabolites

Azathioprine and Mycophenolate mofetil inhibit DNA and RNA synthesis by blocking *de novo* synthesis of purine and therefore clonal expansion of T and B cells[241].

1.10.1.2.3 Calcineurin inhibitors

Cyclosporin is the most commonly used immunosuppressive agent. As it is T cell selective, the incidence of infection is lower. Cyclosporin binds to cyclophillin and inhibits the calcium/calmodulin-activated protein phosphatase calcineurin. Calcineurin activates the cytoplasmic component of the transcription factor, nuclear factor of activated T cells (NFAT). Cyclosporin blocks NFAT and therefore the transcription of cytokines such as IL-2 and the growth and differentiation of T cells[240]. Cyclosporin also induces the synthesis of the immunosuppressive cytokine TGF-β[57].

Tacrolimus binds FK binding protein 12 which also inhibits calcineurin.

1.10.1.2.4 Target of rapamycin inhibitors

Rapamycin (sirolimus) also binds FK-binding proteins, but does not inhibit calcineurin. It binds the kinase, target of rapamycin, preventing the translation of cell cycle regulation mRNA and T cell proliferation[242].

1.10.1.2.5 Monoclonal antibodies

The monoclonal antibody OKT3, against CD3 causes non specific T cell depletion. CD3 facilitates T cell receptor signalling which is critical in CD4+ activation by antigen and in CD8+ cells to bind and lyse target cells[243].

Daclizumab is a humanised mAb and basiliximab a human/mouse chimeric mAb against CD25, a key unit of the IL-2 receptor and therefore inhibits T cell proliferation[244].

1.10.1.2.6 Statins

Statins reduce isoprenylation of proteins, such as Ras and Ras-related proteins, which are important in T-cell activation and effector function and are pivotal in the development of allograft rejection during organ transplantation[245].

Statins have been reported to effectively repress the induction of MHC-II expression by interferon-γ in a dose-dependent manner. Messenger RNA experiments established that the specific mechanism by which MHC-II induction inhibited by statins was a selective, repressive effect on the induction of expression of the promoter IV region of the MHC-II transactivator (*CIITA*) gene. Inhibition of inducible expression of MHC-II molecules, in the considerable variety of cells that normally become MHC-II-positive by the effect of interferon-γ, has multiple functional consequences. Such conditions include repression of the activation of endogenous CD4+ T lymphocytes and recognition of MHC-II molecules by CD4+ T lymphocytes in an allogeneic context after organ transplantation[152].

In response to several stimuli, including the T-cell receptor cross-linking with MHC-II complex, LFA-1 binds to intercellular adhesion molecule-1 (ICAM-1) and provides a potent costimulatory signal for activated T cells. Statins bind directly to a novel allosteric site within LFA-1 (see section 1.5.2.1.1) thus inducing immunosuppression. CD40 is also a costimulatory molecule and its signalling, via an activation of vascular cells, has been shown to induce inflammatory responses with expression of adhesion molecules and secretion of proinflammatory

cytokines, chemokines, matrix metalloproteinases, and tissue factor[246] and been implicated in allograft rejection after organ transplantation[247].

Pravastatin has been reported to decrease coronary artery intimal lesions while depressing IgG alloantibody levels, suggesting a role for the humoral immune response in the development of cardiac allograft vasculopathy[248].

Given these beneficial effects, early statin therapy (regardless of cholesterol level) should be an integral part of the immunosuppressive regimen in transplantation.

1.10.1.3 Statin transplant trials

A number of studies confirmed the benefit of statins in cardiac transplantation[249, 250] and showed not only a decrease in the development of cardiac allograft vasculopathy and improved survival, but also a surprising reduction in the number of rejection episodes. The "effect of pravastatin on outcomes after cardiac transplantation" trial involved 97 cardiac-transplant patients treated with or without pravastatin. Cholesterol levels, survival, rejection with haemodynamic compromise, the development of cardiac allograft vasculopathy, and decreased natural-killer-cell cytotoxic effects were significant first-year benefits seen in the statin-treated patients and suggested an immunomodulatory effect[250]. The pravastatin-treated group had greater 10-year survival than the control group (68% versus 48%; $P = 0.026$) and greater 10- year freedom from the combined endpoint of angiographic graft vasculopathy, death, or both (43% versus 20%; $P = 0.009$)[251]. A similar effect was found in a study with simvastatin[249].

A randomised open label renal transplant study of 48 patients randomised to treatment with or without pravastatin together with cyclosporin, azathioprine and corticosteroids, showed after four months a significantly lower incidence of rejection[252]. However, a multicentre randomised trial of 346 patients double blind randomised to fluvastatin with cyclosporin, corticosteroids and azathioprine showed no effect on rejection rate, severity of rejection or graft function during the first three months after renal transplantation. Nevertheless, the lipid profiles were altered beneficially with a trend toward a smaller number of mild rejection biopsies in the fluvastatin group.

The Assessment of LEscol in Renal Transplantation trial (ALERT) was a large multicentre, randomised, double blind, placebo-controlled trial in 2102 renal transplant patients with total cholesterol 4.0-9.0 mmol/L (154.7-348 mg/dl)[253]. The reduction in the primary endpoint (total major adverse cardiac events) of 17 % was not significant despite a favourable result for the fluvastatin group [risk ratio 0.83 (95% CI 0.64-1.06), p=0.139]. The rates of cerebrovascular events, noncardiovascular deaths, all-cause mortality, and the renal composite endpoint of graft loss or doubling of serum creatinine were similar in both groups. This could have been due to the stability of the renal patients at entry, comprising of long term survivors with good graft function. An alternative primary endpoint such as in the WOSCOPS study of cardiac death or definite nonfatal MI would have been significant. The absence of an effect on graft loss or doubling of serum creatinine was unexpected because chronic allograft nephropathy shares pathophysiological mechanisms with atherosclerosis.

The Study of Lescol (fluvastatin) in Acute Rejection (SOLAR) trial was another multicentre, randomised, double blind, placebo-controlled trial in 364 patients receiving 40 mg fluvastatin or placebo in combination with cyclosporin[254, 255]. There was no difference in the primary endpoint - treated first acute rejection rate [86 (47.3%) fluvastatin vs. 87 (47.8%) placebo] and no significant difference in the secondary endpoints - severity of rejection, steroid resistant rejection or mean serum creatinine at three months.

The beneficial effect of statins is more prominent in heart transplant recipients where there is established evidence of arterial injury to the donor heart. Although there is a lack of immunosuppressive effect in kidney transplant recipients, the patients still derive a cardiovascular benefit from statins, similar to the general population[256].

1.10.2 Stroke

The relationship between dyslipidaemia and stroke is less clearly documented than CAD. However, the LIPID and CARE trials demonstrated a relative risk reduction of stroke of 19% and 31%, respectively in subgroup analyses[183, 186]. In the primary prevention setting of WOSCOPS, the relative risk reduction was 11%. The

beneficial effect in these studies resulted from a reduction in non-haemorrhagic strokes[257]. No data exist on statin benefits in the secondary prevention of stroke in patients without known CAD. PROSPER focused on a high-risk elderly population but showed no reduction on stroke rate perhaps due to the low stroke incidence in the study population and short follow-up of 3.2 years[258]. The relative reduction in ischaemic strokes (28%) in the HPS paralleled that of major coronary events[193]. CARDS even reported a 48% risk reduction for strokes in diabetics, higher than that for acute coronary events[259]. Taken together, there is clear support of a beneficial effect of statins on ischaemic stroke risk, which is comparable to that on coronary events.

1.10.3 Angiogenesis

HMG-CoA reductase inhibition has a biphasic, dose-dependent effect on angiogenesis that is lipid independent and related to geranylated proteins. The effect of prenylation inhibition alters endothelial apoptosis and vascular endothelial growth factor signalling. Statins have proangiogenic effects at low therapeutic concentrations but angiostatic effects at high concentrations that are reversed by geranylgeranyl pyrophosphate[260].

1.10.4 Rheumatoid arthritis

Rheumatoid arthritis, a chronic inflammatory disease that destroys synovial joints, is associated with systemic as well as local inflammation and with an increased risk of cardiovascular disease not fully explained by traditional cardiac risk factors. Statins influence multiple steps in the inflammatory process, including leukocyte migration and adhesion, T-cell stimulation, nitric oxide bioavailability, generation of free radicals, and angiogenesis. Recent studies show that statins may provide mild anti-inflammatory benefit in rheumatoid arthritis[261, 262].

1.10.5 Osteoporosis

Lovastatin inhibits osteoclasts in vitro by interference with isoprenylation[263]. Statins may therefore have a therapeutic potential in osteoporosis prevention and/or treatment[264].

1.10.6 Dementia

Studies have suggested reductions in risk of dementia and Alzheimer's disease with statin treatment[265, 266]. There is evidence to suggest a relation between lipids and vascular changes involving the brain in dementia. These associations include: the ε4 apolipoprotein allele (APOEε4) is a risk factor for Alzheimer's disease; the effect in cell culture of cholesterol on degradation of the amyloid precursor protein; the abnormal appearance of microvascular endothelial cells in affected brain areas in Alzheimer's disease; and a possible role of the LDL receptor-related protein in Alzheimer's disease. The precise mechanisms by which any of these factors might be associated with dementia are poorly understood.

1.10.7 Psoriasis

Psoriasis is thought to involve dendritic cells and T cells becoming activated through the formation of an immunological synapse which results in NF-κB activation, and release of cytokines (e.g. TNF-α), chemokines, proteases and other inflammatory mediators, which induce a wound-healing-like phenotype in the epidermis that is characterized by keratinocyte proliferation[267]. T cells migrate into the epidermis, which involves interactions between LFA1 on T cells and ICAM1 on endothelial cells and also between VLA1 on T cells and type 4 collagen basement membrane constituents. In a chronic psoriatic plaque, it is thought that there is a continuous cycle of T-cell and dendritic-cell activation. The immunomodulatory activities of statins may therefore be useful in the treatment of psoriasis[76]. It has been shown that pharmacological doses of orally administered lovastatin do not worsen the course of psoriasis[268] and though gemfibrozil, has been reported to exacerbate psoriasis, statins do not[269]. The condition of some psoriatic subjects improves when a statin is administered[193]. Disruption of the

skin barrier as the result of decreased production of cholesterol by keratinocytes can be reversed by application of cholesterol[270]. It is most likely that the beneficial effects of statins on immunocytes outweigh their untoward effect on surface lipids. Topical or systemic use of statins, either alone or in combination with topical cholesterol, may thus prove beneficial against psoriasis.

1.10.8 Multiple sclerosis

MHC class II genes are associated with susceptibility to MS[271]. Induction of MHC class II in the CNS during the pathogenesis of MS is at the centre of a destructive cascade of inflammatory events targeting the white matter and the underlying axon in this disease. Statins inhibited MHC class II upregulation on antigen presenting cells, reducing the activation of pro-inflammatory CD4 T cells[152]. Statins also downregulated ICAM-1 and bind directly to LFA-1, preventing the movement of lymphocytes into the brain. INOS and TNFα play a role in MS and were suppressed by statins. MMP-9 is upregulated in MS and reduced by statins[272]. MMP-9 is involved in migration of T cells across the blood brain barrier, disruption of the blood brain barrier and degradation of extracellular matrix and myelin in MS[273].

1.10.9 Adverse effects

Statins are generally well tolerated, however measurement of transaminase levels are necessary to determine liver and muscle toxicity. Myopathy (muscle pain or weakness) is associated with raised levels of creatine kinase, usually greater than 10 times the upper limit of normal. Rhabdomyolysis and acute renal failure may result if myopathy is not recognised, but is reversible if the drug is stopped promptly. Symptoms can also include fever and nausea and occurs in about 1 in 1000 patients, but is dose related[274].

Cerivastatin is 16-80 times more likely to cause fatal rhabdomyolysis than any other statin[275]. Rhabdomyolysis, which lead to kidney failure, was responsible for 52 deaths worldwide and 385 non-fatal cases reported among 700,000 cerivastatin users required hospitalisation. Drug-drug interactions were implicated as 12 out of

31 fatalities were using gemfibrozil concomitantly. This lead to the withdrawal of cerivastatin from the world market in 2001 and highlights the importance of pharmacological aspects in statin toxicity[276].

1.11 Aims and hypothesis

Statins have revolutionised the treatment of cardiovascular disease. Several studies have shown that long-term statin therapy reduces mortality of patients with hyperlipidaemia and ischaemic heart disease. A prospective meta-analysis of 14 randomised trials found that there was a 12% proportional reduction in all-cause mortality per mmol/L reduction in LDL cholesterol[277]. This reflected a 19% reduction in coronary mortality and 21% in combining myocardial infarction or coronary death, in the need for coronary revascularisation, in fatal or non-fatal stroke and in any such major vascular event. Statin therapy can safely reduce the 5-year incidence of major coronary events, coronary revascularisation, and stroke by about one fifth per mmol/L reduction in LDL cholesterol, largely irrespective of the initial lipid profile or other presenting characteristics.

Although primarily developed to reduce cholesterol there is increasing evidence that statins have cholesterol independent actions. These pleiotropic effects are believed to be mediated by reduction of isoprenoid intermediates, farnesyl and geranylgeranyl, in the cholesterol biosynthetic pathway[239, 278]. Thus, statins may inhibit prenylation of signalling molecules including the Ras and Rho family of GTPases and the γ-subunit of G-proteins that is essential for the correct membrane localisation and function of these proteins. An alternative explanation is that statins reduce the density of cholesterol-rich membrane micro-domains or rafts[66, 279], thus limiting the function of receptors that are localised to membrane rafts. Both of these mechanisms are involved in lymphocyte activation and may explain the reported immunosuppressive actions of statins[152, 280].

The aims of this thesis were to explore the potential mechanisms of the anti-inflammatory and immunosuppressive effects of statins specifically using *in vivo* and *in vitro* methods to measure the effect of statins on prenylation and rafts. I examined the effects of statins on lymphocyte functions of T cell proliferation and

NK cytotoxicity *in vivo* and hypothesized that the pleiotropic effects of statins would reduce these functions by the disruption of signalling pathways either by reduced prenylation of small G proteins or disruption of membrane rafts. I demonstrated *in vitro* that statin reduced the prenylation of small GTPases such as Ras and Rac and therefore activation of downstream signalling molecules such as ERK and p38 in monocytes and T cells. An alternative mechanism for inhibition of signalling pathways was also demonstrated by disruption of cholesterol lipid rafts by statins. Functional effects of statins on; monocyte trafficking and endocytosis, cytokine release and MMP-1 production; T cell proliferation; and NK cytotoxicity was shown to be reduced by statin treatment and concluded, by the lack of effect of farnesyl and geranylgeranyl transferase inhibitors, that this was most likely due to disruption of lipid rafts, rather than inhibition of prenylation.

Chapter 2: Materials and methods

2.1 Materials

2.1.1 Biochemicals

Unless otherwise stated, all chemicals were purchased from Sigma (Poole, UK). Tissue culture products including foetal calf serum (FCS), L-glutamine, RPMI 1640 penicillin and streptomycin were purchased from Gibco, (Paisley, UK).

2.1.2 Immunochemicals

The commercial antibodies used in this study are listed below in table 2.1.

2.2 Cell biology techniques

2.2.1 Cell culture

2.2.1.1 U937 cell line

The human haematopoietic cell line U937 was derived from a patient with generalized histiocytic lymphoma[281]. U937 cells were routinely cultured in RPMI 1640 medium supplemented with FCS (10% v/v), glutamine (2 mM), penicillin (10 U/ml) and streptomycin (10 μg/ml). They were maintained at 37°C in a water saturated atmosphere containing 5% carbon dioxide at a density of between 5 x 10^5 and 2 x 10^6 cells/ml, as measured using a Neubauer haemocytometer. They were routinely sub-passaged twice weekly by centrifugation for 5 min at 400 x g and resuspended in fresh complete medium. Typically, cells were maintained in 75 cm^2 tissue culture flasks in a volume of 10-20 mls.

1 **Specificity** (clone)	**Species**	2 **Usage**	3 **Source**
Anti-human CD19 IgG-1 (SJ25-C1)	Mouse	IP	Caltag
Anti-mouse IgG:magnetic beads	Goat	IP	Biomag
Anti-mouse Ig	Rabbit	T cell prolif	Dako
Anti-human CD3 IgG	Mouse	T cell prolif	Serotec
Anti-human LFA-1 (CD11a)	Mouse	FACS	R&D Systems
Anti-human VLA-4 (CD49)	Mouse	FACS	Merck
Anti-human LCA (CD45)	Mouse	FACS	Dako
Anti-mouse: FITC	rabbit	FACS 2ndry Abs	Sigma
pTyr IgG_{2b} (4G10)	Mouse	W	Upstate
pERK p44/42 MAP Kinase (Thr202/ Tyr204)	Rabbit	W	NEB
ERK	Rabbit	W	NEB
pp38 MAP Kinase (Thr180/Tyr182)	Rabbit	W	NEB
p38	Rabbit	W	NEB
Ras (RAS10)	Mouse monoclonal	W	Upstate
Human IgG	Human	IP, FACS, X-link	Serotec
^{125}I-labelled anti-human IgG	Sheep	T/E	Amersham
LAT	Rabbit	W	Upstate
Lyn	Rabbit	W	Santa Cruz
Fyn	Rabbit	W	Santa Cruz
FcγRI IgG1 (10.1)	Mouse	IP, FACS	Calbiochem
FcγRI IgG1 (22)	Mouse	IP, FACS, X-link	Medarex
FcγRII IgG1 (KB61)	Mouse	IP, FACS, X-link	DAKO
FcγRII IgG_{2a} (IV3)	Mouse	IP, FACS, X-link	Medarex
FcγRIII IgG1 (3G8)	Mouse	FACS	Medarex
CD89 IgG1 (A3)	Mouse	IP, FACS, X-link	Serotec
γ-chain	rabbit	W	NEB
SHIP	goat	W, IP	Santa Cruz
Syk	rabbit	W	Santa Cruz
anti-mouse:HRP	Sheep	W 2ndry Ab	Amersham
anti-rabbit:HRP	Donkey	W 2ndry Ab	Amersham
anti-goat:HRP	Rabbit	W 2ndry Ab	Sigma

Continued on next page.....

.....*Continued from previous page*

Table 2.1 Antibodies

1 Clone number and/or species in which antibody was raised.

2 FACS; fluorescent activated cell scanning. IP; immunoprecipitation. T/E; trafficking and endocytosis. W; western blotting. X-link; crosslinking studies.

3 manufacturer: -

Amersham, Amersham, UK
Biomag; Polyscience, Warington PA, USA
Calbiochem, Beeston, UK
Caltag laboratories, Towcester,UK
DAKO Ltd, Cambridgeshire, UK
Medarex, NJ, USA
Merck, (VWR International Ltd), Lutterworth, UK
NEB, New England Biolabs, MA, USA
R&D Systems, Abingdon, UK
Santa Cruz, Santa Cruz Biotechnology, Santa Cruz, CA, USA
Serotec, Oxford, UK
Sigma, Poole, UK
Upstate, Upstate Biotechnology, Lake Placid, USA

2.2.1.2 K562 cell line

The human erythroleukemic cell line K562 was established from the pleural effusion of a 53-year-old female with chronic myelogenous leukaemia in terminal blast crises[282]. The cell population has been characterised as highly undifferentiated and of the granulocytic series[283]. K562 cells were cultured in the same way as the U937 cell line and maintained in 25 cm^2 tissue culture flasks in a volume of 10 mls.

2.2.1.3 NK-92MI cell line

NK-92MI is an interleukin-2 (IL-2) independent Natural Killer Cell line derived from the NK-92 cell line by stable transfection with human IL-2 cDNA in a retroviral vector by particle-mediated gene transfer. The parental NK-92 cell line was an IL-2 dependent cell line derived from peripheral blood mononuclear cells from a 50 year old Caucasian male with rapidly progressive non-Hodgkin's lymphoma[284]. NK-92MI cells were routinely cultured in Alpha minimum essential medium without ribonucleosides and deoxyribonucleosides and containing 2 mM L glutamine, 1.5g/L sodium bicarbonate, 0.2mM inositol, 0.1mM 2-mercaptoethanol, 0.02mM folic acid, 12.5% horse serum, 12.5% foetal bovine serum, penicillin (10 U/ml) and streptomycin (10 μg/ml). They were maintained at 37°C in a water-saturated atmosphere containing 5% carbon dioxide.

2.2.1.4 Human peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated typically from 40mls of freshly drawn human blood into lithium heparin tubes then centrifuged on a Ficoll-Hypaque gradient (1.077g/ml) (Histopaque–1077, Sigma). The cells at the interface were removed and washed with HBSS supplemented with 2% FCS, 25mM HEPES, 100U/ml penicillin and 100μg/ml of streptomycin (HBSS+). The cellular pellet was resuspended in 20 ml of HBSS+ and incubated on a 15cm plate at 37°C for 45 min, to allow monocytes to adhere. Non adherent cells were collected from the plate and incubated independently with Mouse IgG-1 monoclonal antibody to human CD19 then Goat anti-mouse IgG magnetic beads to

remove B cells and leave a cell population of T cells and NK cells. The enriched T/NK cells were counted and resuspended at a concentration of 2 x10^6/ml in complete RPMI 1640 medium. The plate of monocytes was washed thoroughly (up to 10 times) with HBSS+ to remove non-adherent cells.

2.2.2 Proliferation assays

2.2.2.1 T cell proliferation assay

Flat-bottomed 96 well immulon 4 plates (Dynatech, Billingshurst, UK) were coated with Rabbit anti-mouse Ig in 0.1M sodium hydrogen carbonate (pH 8.2) for 2 hours at 37 °C and washed with PBS (0.07 M Sodium Chloride, 75 mM disodium hydrogen orthophosphate, 5 mM sodium dihydrogen orthophosphate). Mouse anti-human CD3 Ig was cross-linked, to stimulate T-cell proliferation. Replicates of six wells were seeded at a density of 2 x 10^5 cells/well in 0.2ml of medium with either complete medium, simvastatin (Merck (VWR International Ltd), Lutterworth, UK), geranylgeranyl transferase inhibitor (GGTI-286), farnesyl transferase inhibitor I (FTI I) (Calbiochem-Novabiochem, Beeston, UK), or a combination of inhibitors with concentrations as indicated in individual experiments. Cells were incubated at 37°C for 72 hours and pulsed with 1 μCi [^{3}H]thymidine (Amersham International plc, Amersham, UK) for a further 18 hours. Cells were harvested onto glass fibre filtermats (Wallac EG & G Instruments Ltd, Milton Keynes, UK) and the [^{3}H]Thymidine incorporated into replicating DNA counted by Betaplate (1205) flatbed liquid scintillation counter (Wallac, Milton Keynes, UK).

2.2.2.2 Alamar blue assay

The AlamarBlue assay incorporates a fluorometric/colourimetric growth indicator based on metabolic activity. A 96 well plate with 1-2 x 10^4 cells per well were treated with various preparations in replicates of 6 for 24 hours. 22 μl of AlamarBlue was added for a further 24 hours and fluorescence determined in a PerkinElmer LS55B fluorimeter (λ_{exc} = 530nm, λ_{em} = 590nm).

2.2.3 Natural Killer cell cytotoxicity assay

Enriched T/NK cells or NK-92MI cells were seeded at a density of 1 x 10^6 cells/ml in 2.0ml of medium with either 1 μm simvastatin, 10 μm GGTI-286, 10 μm FTI I, or a combination of inhibitors, for 48-72 hours. The human Caucasian chronic myelogenous leukaemia cell line, K562, was used as a target for NK cells as they lack MHC class I and II antigens[285]. Briefly, 2 x 10^6 cells/ml were incubated with 5MBq of ^{51}chromium at 37°C for one hour and washed thoroughly. Effector cell concentration was established and equalised in all samples. Target cell concentration was then adjusted to 12.5:1 and 25:1 E/T ratio. One hundred μl of effector cells and 100 μl of target cells were mixed in V-bottomed plates in replicates of six. A negative control of 100 μl of target cells plus 100 μl of media and a positive control of 100 μl of target cells plus 100 μl of 10% Triton X-100 lysis buffer were also prepared. After a four hour incubation at 37°C, plates were centrifuged at 400g (1000 rpm) for five minutes and 100 μl of supernatant collected and radioactivity counted by gamma counter (Packard Cobra gamma counter). Percentage killing was calculated: (sample – negative control)/(positive control – negative control) x 100[286].

2.2.4 Flow cytometry.

2.2.4.1 T/NK cells

Enriched T/NK cells at a density of 1 x 10^6 were incubated with either 0.2% BSA in PBS, or 1 μg of primary antibody: anti-human LFA-1 (CD11a), anti-human VLA-4 (CD49), anti-human LCA (CD45), or the isotype control mouse IgG1 negative control – human, to control for non specific labelling, for 45 mins on ice. After 2 washes with PBS, fluoroscein isothiocyanate (FITC) labelled anti-mouse secondary ab was incubated for 45 mins on ice and in darkness. After a further two washes in PBS, at least 10,000 cells were analysed by FACScan using System II software version 3.0 (Coulter, High Wycombe, UK).

2.2.4.2 U937 cells

Typically 1x10^6 cells were washed and resuspended in 100µl ice cold PBS with 1µg of relevant monoclonal antibody (mAb). Where appropriate, 3 µM monomeric human IgG was added to block non-specific interactions of monoclonal antibodies with Fc receptors. Following 45 mins incubation on ice cells were washed twice and incubated for a further 45 mins with a fluoroscein isothiocyanate (FITC) conjugated goat anti-mouse IgG secondary antibody (1:100 dilution). Irrelevant isotype control antibodies were used to control for non-specific labelling.

2.2.5 Determination of apoptosis in U937 cells

To exclude a significant pro-apoptotic effect of statins, U937 cells were treated with 10 µg/ml puromycin or 1mM H_2O_2[287] as positive controls and various concentrations of simvastatin and fluvastatin.

2.2.5.1 Caspase flow cytometric apoptosis kit

The caspase activity assay (Oncogene, Beeston, UK) detected activation of caspases by flow cytometry in living cells. The assay was based on the cleavage of $(aspartyl)_2$ – Rhodamine 110 (D_2R), a reported substrate for members of the caspase family proteases. The caspase substrate D_2R is non fluorescent, however upon cleavage by cellular caspases, the released Rhodamine 110 gives rise to fluorescence that can be measured at excitation of 488nm and emission of 530nm.

A pellet of 1x10^5 cells was resuspended in 0.3 ml of D_2R incubation buffer with 10mM DTT and 1 µl D_2R reagent. After 10-20 mins incubation at 37 °C in the dark, cells were analysed by flow cytometry using FL-1 channel (Ex/Em=488/530 nm).

2.2.5.2 Morphology of apoptotic cells

Cells were cytospun onto gelatin/chrom alum coated slides at 50 x g (500rpm) and their morphology scored by counting nuclear condensation (performed by Lorna Murray, Centre for Inflammation Research, University of Edinburgh).

2.3 Serum measurements

2.3.1 Lipid Profiles

A 7 ml tube of blood was allowed to clot then centrifuged at 2000 x g (3000 rpm) to collect serum. One ml of serum was used in a rapid semi- micro method for the separation of lipoprotein fractions that uses a bench top ultracentrifuge[288].

2.3.2 C-Reactive Protein Measurement

CRP concentrations were determined using a high sensitivity latex-enhanced immunoturbidimetric assay, CRP-UL (Wako Chemicals GmbH, Neuss, Germany) on a Cobas Fara centrifugal analyser (Roche Diagnostics, Lewes, East Sussex, UK). Six µl of serum was mixed with 270 µl of the kit buffer solution and incubated for 5 mins at 37 °C. Sixty µl of latex reagent was mixed and measured at a wavelength of 700 nm after a further 5 min incubation.

2.4 Biochemical Assays

2.4.1 Cell lysate preparation

For general lysate preparations, cells in suspension were lysed by the addition of ice-cold lysis buffer (1% (v/v) Triton X-100, 50 mM Tris-HCl, pH 7.5, 0.25% (w/v) sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM vanadate and 1 mM NaF) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 µg/ml each chymostatin, leupeptin, antipain and pepstatin). Following 30 min tumbling at 4°C lysates were clarified at 15,000 x g (13,000 rpm) for 15 mins to remove insoluble cell debris. Lysates were either used immediately or stored at -20 °C prior to electrophoresis.

2.4.2 Determination of protein concentration

Protein concentrations were routinely determined for all cell lysates and membrane preparations. Protein concentrations were estimated using the Bio-Rad protein assay system (Bio-Rad Laboratories, Hemel Hempstead, UK), which is based on the Bradford Coomassie brilliant blue dye binding system. A range of bovine serum albumin (BSA) concentrations were used to construct a standard curve and samples were suitably diluted to allow their protein level to fall within the linear range of this curve. Protein concentrations of all samples and standards were routinely measured in duplicate on a Beckman DU640B spectrophotometer at a wavelength of 595 nm.

2.4.3 Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using the NuPAGE Pre-Cast Gel System (Invitrogen, Paisley, UK). The NuPAGE system works with bis-tris buffered (pH 6.4) polyacrylamide gels, available at 3 different acrylamide concentrations (10% (w/v), 12% and 4-12% gradient gels) allowing different separation ranges to be selected. By varying the running buffer used, the separation range can be altered further. Samples were denatured in NuPAGE LDS (Lithium Dodecyl Sulphate) sample buffer by heating to 70°C for 10 min. For reduced proteins, 50 mM dithiothreitol was added prior to sample denaturing. For a detailed protocol, including gel separation ranges, please refer to the manufacturers instructions.

2.4.4 Western blot analysis

Proteins were transferred to nitrocellulose (Schleicher and Schuel GmbH) using the Novex XCell II blotting apparatus. Transfer efficiency was ascertained by staining with Ponceau S solution for five minutes followed by washing with dH_2O. Ponceau S was removed by washing with TBS (20 mM Tris.HCl, pH 7.6, 136 mM NaCl) containing 0.1% (v/v) Tween 20 (TBST). Nitrocellulose membranes were blocked in TBST with 4% (w/v) non-fat powdered milk (e.g. Marvel) for at least 1 hour at RT. Typically, blots were incubated with primary antibodies at a final

concentration of 0.1-1 μg/ml in 4% milk-TBST overnight at 4°C. Blots were then washed vigorously three times for five minutes with TBST and incubated with a HRP conjugated secondary ab diluted 1 in 2000 for 2 hours at RT. Blots were washed extensively with large volumes of TBST (typically 5 x 5 min washes) followed by a final wash in H_2O prior to developing. Blots were developed using the enhanced chemiluminescence (ECL) detection system (Amersham, Amersham, UK). To strip blots for reprobing, blots were washed in acid strip buffer (100 mM glycine, 150 mM NaCl, pH 2.6) for 20-45 minutes.

2.4.5 Detection of phosphotyrosine containing proteins by Western blot

Proteins phosphorylated on tyrosine residues were detected by Western blot using the monoclonal antibody, 4G10 (Upstate Biotechnology). This antibody gave a very poor signal and high background using the standard blotting protocol outlined above (section 2.4.4) so the following modified protocol was developed. Following membrane blocking with TBST 4% milk, blots were incubated with a 1:1000 dilution of 4G10 (Upstate Biotechnology) overnight at 4°C in TBS with 0.05 % (v/v) Tween 20 and 0.5 % (w/v) milk. Membranes were washed 3 x 5 minutes with TBST and incubated for two hours with donkey anti-mouse IgG: HRP (1:4000, Amersham) in TBST with 4% (w/v) milk. Membranes were then rinsed four times with large quantities of dH_2O, washed 5 x 5 min with TBST with vigorous agitation followed by four further rinses with ddH_2O. Blots were developed as described above (section 2.4.4).

2.4.6 Detection of activated MAPK by Western blot (ERK and p38)

To detect activated forms of the MAPKs, lysates were prepared as described in section 2.4.1. Typically, 10 μg of total cell lysate were electrophoresed and blotted. Activated forms of ERK, p38 and JNK were detected by Western blot using rabbit polyclonal antibodies raised against peptides corresponding to the dual phosphorylated active sites of the different MAP kinases. All blots for activated

MAPKs were stripped and reprobed with polyclonal antibodies, which recognise all forms of the MAPK being assayed to ensure comparable loading of cell lysates between samples. For antibody specificities see table 2.1.

2.4.7 ERK and p38 activation in human T cells.

Human T cells prepared from PBMC (section 2.2.1.4) were stimulated on anti CD3 coated 96 well plates for 24, 48 and 72 hours. A shorter time course of 10, 30 and 120 minutes was examined by first incubating the cells with fluvastatin and mevalonate before being stimulated. All samples were then lysed with ice cold Lysis Buffer (section 2.4.1). Samples were western blotted and probed for ERK or p38 with phospho specific p44/42 MAP Kinase antibody and phospho specific p38 MAP Kinase antibody.

2.4.8 Fc receptor aggregation

2.4.8.1 FcγRI aggregation with human IgG

U937 cells were harvested by centrifugation (5 min, 400 x g), washed and resuspended in ice cold RPMI, and incubated with 1μM monomeric IgG for 45 mins at 4°C to occupy surface FcγRI. Cells were then washed twice with RPMI to remove unbound ligand. Following this cells were resuspended in RPMI, 25 mM HEPES, 1% FCS and a 1:50 dilution of goat anti-human IgG was added to crosslink FcγRI bound IgG. Cells were then warmed to 37°C for the times specified.

2.4.8.2 Fc receptor aggregation with heat aggregated IgG

2.4.8.2.1 Preparation of heat aggregated IgG

IgG was heat aggregated by heating human IgG at a concentration of 50mg/ml to 65°C for 20 mins. The aggregated IgG was pelleted by micro centrifugation at 15,000 x g (13,000 rpm) for 30 mins, then washed 3 or 4 times with PBS. The protein concentration was measured by Bradford's assay as in section 2.4.2 with the ratio – 22.4 mg/ml of protein measured is equal to 50mg/ml of IgG.

2.4.8.2.2 Fc receptor aggregation with heat aggregated IgG

Heat aggregated IgG was added to cells at concentration of 100 μg/ml and heated for the times indicated.

2.4.9 Membrane preparation

Cells were washed twice in cold nuclear preparation buffer (10 mM Tris-HCl, pH 7.4, 2 mM magnesium chloride, 140 mM sodium chloride). Cells were then lysed by three liquid nitrogen freeze-thaw cycles in nuclear preparation buffer containing 2% (v/v) Tween 40, protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1μg/ml each chymostatin, leupeptin, antipain and pepstatin) and phosphatase inhibitors (1mM vanadate and 1mM NaF). Cell debris and nuclei were removed by centrifugation at 15 000 x g (13,000 rpm) for 5 min and membranes were precipitated by ultra centrifugation (Beckman Instruments, High Wycombe, UK) of the supernatant at 100 000 x g (55,000 rpm), 4°C for 1 hour[289, 290]. Following ultra centrifugation, the supernatant represents the cytosolic fraction. Membrane pellets were resuspended in cold nuclear preparation buffer.

2.4.10 Endocytosis and trafficking

FcγR mediated internalisation and degradation of IgG immune complexes was as described by Harrison[291]. Briefly, cells were loaded with IgG as above (2.4.8) and after removal of unbound IgG, FcγR bound IgG was aggregated with ^{125}I-labelled sheep anti-human IgG (Amersham, Buckinghamshire, UK) for 20 minutes on ice. Unbound crosslinking antibody was removed by washing and cells warmed to 37°C for specified times to allow internalisation and trafficking of immune complexes. At time 0, duplicate aliquots of cells were counted to provide a measure of total cell activity. After incubation at 37°C, immune complex (IC) internalisation was then determined by washing the cells in ice cold acidified PBS (pH 2) to remove surface bound IC and non-acid releasable radioactivity used as a measure of internalisation. Duplicate cell aliquots were removed and acid washed at each time point and internalisation expressed as a fraction of total cell activity. After warming to 37°C internalised cell radioactivity falls, accompanied by the

appearance of trichloroacetic acid (TCA) soluble radioactivity in the supernatant, representing IC degradation. To determine the rate of degradation, TCA (20% w/v) soluble radioactivity in the supernatant was determined in duplicate at each time point and expressed relative to total cell activity.

2.4.11 Lipid raft isolation

Cells were lysed with MNE (25mM MES, pH6.5, 150mM NaCl, 2mM EDTA) containing 1% Triton X-100 (v/v) plus protease and phosphatase inhibitors and subjected to sucrose gradient centrifugation[40] at 287,500 x g (45000 rpm) for 18 hours in a SW60 ti rotor (Beckman Instruments, High Wycombe, UK). The samples were mixed (1 in 2) with 80% sucrose MNE (1ml), with 30% (2ml) then 5% sucrose MNE (1.3ml) layered on top.

2.4.11.1 Lipid raft fractions

Ten 450µl samples were collected from the top and analysed by western blot.

2.4.11.2 Lipid raft samples

The top 1ml was removed and discarded; while the next 1.5ml (corresponding to fractions 3-5) was collected, with the sucrose content diluted at least 1 in 2 by MNE. Raft pellets were collected by microcentrifuging at 15,000 x g (13,000 rpm) for 15 mins at 4°C and resuspended in lysis buffer prior to western blot analysis. LAT, Lyn or Fyn abs were used as markers for rafts.

2.4.11.3 Lipid raft densitometry

PBMC were extracted (as in section 2.2.1.4) from 35mls of blood collected in lithium/heparin tubes from normal volunteers before and after taking 40mg of simvastatin per day for 28 days. Lipid rafts were collected (as in section 2.4.11 and 2.4.11.2) with the exception that the PBMCs were lysed in 1.5 mls of MNE 1% Triton X-100 and split into 3 separate samples before being centrifuged. The triplicate raft samples were resolved beside duplicate total samples (diluted 1 in 5)

and probed for LAT, Lyn and Fyn. Protein levels were measured by densitometry (Quantity One 4.2.1, Bio-Rad, Hemel Hempstead, UK). A ratio was calculated between total protein levels and raft protein levels, to assess whether statin treatment could reduce raft levels *in vivo*.

2.5 Molecular biology

2.5.1 RNA preparation

Total cellular RNA was isolated by TRIzol (Invitrogen, Paisley, UK) extraction. Cells were lysed by resuspending cell pellets in 1ml TRIzol per 10^7 cells for 5 minutes at RT. Tubes were shaken vigorously with 0.2ml of chloroform then left at RT for 3minutes. Samples were centrifuged at 12000 x g (13,000 RPM) in the cold room. The aqueous layer, which contains the RNA, was then removed to a fresh tube and the RNA precipitated with 0.5ml of isopropanol followed by centrifugation (12 000 x g, 10 min, 4°C). RNA pellets were washed with 75% ethanol, resuspended in an appropriate volume of diethylpyrocarbonate (DEPC) treated water and stored at -70°C. RNA concentration was determined by measuring absorbance at 260 nm and purity was estimated by checking the 260 nm:280 nm absorbance ratio (RNA has a ratio of roughly 2:1).

2.5.2 RT-PCR

For reverse transcriptase PCR (RT-PCR) cDNA was prepared from total RNA using an Invitrogen cDNA cycle kit with oligo dT primers. Briefly, reactions were carried out in 20 µl containing 1 µg total RNA, RNase inhibitor, 5 mM dNTPs, 4 mM sodium pyrophosphate, 4 µl Invitrogen 5 x RT reaction buffer and 0.5 µl AMV reverse transcriptase. Reactions were carried out at 42°C for 1 hour followed by phenol chloroform extraction and ethanol precipitation. cDNA was aliquoted and frozen at -70°C until used in PCR reactions. Aliquots of cDNA were amplified by PCR as follows. To amplify FcγRIIa and FcγRIIb mRNA, a single crossreactive forward primer was used (FcγRII-FOR) with reverse primers specific for either

FcγRIIa (RIIa-REV - Table 2.2) or FcγRIIb (RIIb-REV - Table 2.2). For FcγRIIb these primers flank the alternative FcγRIIb2 splice site to allow identification of different splice variants by PCR product size. PCR reactions were carried out in 50μl containing 20pmole of each primer, 2.5mM $MgCl_2$, 5μl Promega Taq 10x reaction buffer and 2 units Promega Taq polymerase. PCR reactions were continued for 20 to 40 cycles with each cycle comprising 1 minute at 93°C (denaturing), 1 minute at 50°C (primer annealing) and 1 minute at 72°C (product extension).

2.5.3 Agarose gel electrophoresis of DNA

PCR DNA products were typically electrophoresed at 100 V through 1-2% (w/v) agarose in TAE buffer (40mM Tris-acetate, 1mM EDTA) containing 0.5 μg/ml ethidium bromide. Bands were visualised on an ultraviolet transilluminator and photographed with a Polaroid instant camera.

2.6 Manufactured kits

2.6.1 Ras Assay.

The Ras small GTPase was assayed using an Upstate biotech Ras activation assay kit (TCS Biologicals, Claydon, Buckingham, UK). The kit comprised of glutathione agarose bound GST fusion proteins of the Ras binding domain (RBD) of Raf-1, which binds Ras:GTP. Briefly, cells were stimulated and then lysed with Mg^{2+} lysis buffer (MLB – 25mM HEPES, pH 7.5, 150mM NaCl 1% Igepal CA-630, 10mM $MgCl_2$, 1mM EDTA and 2% glycerol). Typically, 500μg cell lysate at a concentration of 1 mg/ml was used for each assay sample. Active Ras was precipitated with 5 μl Raf-1 RBD agarose for 30 minutes. Precipitates were washed three times with 500 μl MLB followed by SDS-PAGE (section 2.4.3) and Western blotting (section 2.4.4). Ras was detected with specific mAb.

Target	Name	Sequence (5' → 3')	Product size
β actin	β actin-for	GGG GTA TGC CCT CCC CCA TGC CAT CCT GCG	482bp
	β actin-rev	TTG GCG TAC AGG TCT TTG CGG ATG TCC ACG	
FcγRI	I-for	CAT GTG GTT CTT GAC AAC TCT GCT CC	876bp
	I-rev	TGA AAC CAG ACA GGA GTT GGT AAC TGG	
FcγRIIa	II-for	GCA CAG GAA ACA TAG GCT ACA CG	717 + 594bp
	IIa-rev	GGT ATC TTC TTA GAA AGT CCC	
FcγRIIb	II-for	GCA CAG GAA ACA TAG GCT ACA CG	287bp
	IIb-rev	GGT GAT TGT GTT CTC AGC CCC	
FcγRIII	III-for	ATG TGG CAG CTG CTC CTC CCA ACT G	567bp
	III-rev	GGT GAT GTT CAC AGT CTC TGA AGA CAC	
FcαR	CD89-F2	CGC TTA AGA TGG ACC CCA AAC AGA CCA C	880bp
	CD89-R2	GCT CTA GAT TAC TTG CAG ACA CTT GGT G	
Fcα/μR	amr-for1	GAC AAC TAC CAA GGC TGA TAG G	702bp
	amr-rev	TCT GTC CCT CAG GGT CCT GGA T	
PIGR	PIGR-for	GCC CGA GCT GGT TTA TGA AG	694bp
	PIGR-rev	AGC CGT GAC ATT CCC TGG TA	
FcRn	FcRn-for	CAA AGC TTT GGG GGG AAA AG	359bp
	FcRn-rev	TGC AGG TAA GCA CGG AAA AG	
Gamma chain	GAM3-for	GCC CAA GAT GAT TCC AGC AG	458bp
	GAM3-rev	CCG TAA ACA GCA TCT GAG C	

Table 2.2 Oligonucleotide sequences

2.6.2 MMP-1 ELISA

MMP-1 levels from the supernatant of U937 cells treated with statin or prenyl inhibitors for 48 hours, crosslinked as in section 2.4.8.1 or with heat aggregated IgG (section 2.4.8.2) overnight were measured by an Oncogene ELISA kit (Calbiochem, Beeston, UK). The MMP-1 ELISA assays was a "sandwich" enzyme immunoassay employing two monoclonal antibodies. A monoclonal antibody, specific for human MMP-1 protein, was immobilised onto the surface of the plastic wells provided in the kit. The samples and standards were pipetted into the wells and the capture antibody bound any MMP-1 protein present. Unbound material was washed away and monoclonal horseradish peroxidase (HRP)-conjugated anti–MMP-1 antibody added. Following incubation and washing, the chromogenic substrate tetra-methylbenzidine (TMB) was catalysed from a colourless solution to blue by the HRP, then yellow by 2.5N sulphuric acid. The intensity of colour was proportional to the amount of MMP-1 protein present. Absorbance was measured at 450 nm within 30 mins and concentrations determined from the standards.

2.6.3 CB array

IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12p70 levels from the supernatant of U937 cells treated with statin or prenyl inhibitors for 48 hours, crosslinked as in section 2.4.8.1 or with heat aggregated IgG (section 2.4.8.2) overnight or LPS overnight were measured by cytometric bead array kit (BD Biosciences, Oxford, UK). Six bead populations with distinct fluorescence intensities, coated in antibodies specific for the proteins above were mixed with PE detection reagent and 50 μl of sample supernatant (or standard). After 3 hours incubation and a wash with 1 ml wash buffer, the beads were resuspended by vortexing with wash buffer. The FACS machine with CellQuest software installed was prepared with the cytometer setup beads provided in the kit. Samples were then analysed by the FACS and calculated from standard curves by the software.

Chapter 3: Pleiotropic effect of statins *in vivo*

3.1 Introduction

Inhibition of HMG-CoA reductase by statins lowers serum total and LDL-cholesterol and is of proven benefit in the treatment of patients with a spectrum of cardiovascular diseases[177, 179, 292]. However, actions independent of serum lipid lowering may play an important role in the beneficial effects of statins in atherosclerosis[128] and *in vivo* effects on lymphocytes may provide a readily accessible model for the *in vivo* actions of statins on other cell types.

I therefore carried out three separate *in vivo* studies of patients receiving statin therapy. The first (Study 1) was a sampling exercise of patients with cardiovascular disease in which the lymphocyte functions of T cell proliferation, NK cytotoxicity and cell adhesion molecules (CAMs) were measured to investigate the potential lipid independent, immunomodulatory effects of statins which play an important role in atherosclerosis. The second study (Study 2) was an interventional study, which investigated the effects of 40mg per day of simvastatin, on leukocyte functions of T cell proliferation and NK cytotoxicity in normal human subjects. The aim of this study was to confirm the known benefit of statins in lowering lipids in patients with elevated cholesterol, to translate this effect to normal subjects without elevated cholesterol and to assess the effect of statins on immune cell functions without the interference form other medications. The final study (Study 3) was another sampling study of a diverse variety of patients and concentrated on natural killer cell activity as this cell type showed the most *in vivo* evidence of a statin effect.

I hypothesised that the pleiotropic effects of statins would reduce lymphocyte functions of T cell proliferation and NK cytotoxicity, *in vivo* by the disruption of signalling pathways either by reduced prenylation of small G proteins or reduced raft cholesterol. Statins would also reduce the amounts of CAMs reaching the membrane by reducing the pro inflammatory cytokines which stimulate production of CAMs[293] as well as by exclusion from lipid rafts which retains LFA-1

in an inactive form[79]. Simvastatin also binds directly to LFA-1 causing an allosteric change which blocks the binding of ICAM-1 to LFA-1[76].

3.2 Results

3.2.1 Study 1: Study of patients with cardiovascular disease

Blood samples were collected from hospital in-patients and out patients, targeting patients admitted for coronary angiography and with angina pectoris or myocardial infarction. Renal transplant recipients receiving cyclosporin immunosuppressive therapy in conjunction with or without statin treatment were also targeted. Recruitment was not restricted to the dose of simvastatin taken which ranged from 10 to 80 mg of simvastatin per day. Samples were obtained from 16 patients who were not receiving statin therapy (13 men; median age 54 years (range 35 to 83 years)); 17 patients receiving statin therapy (16 men; median age 62 years (range 40 to 76)); 18 patients receiving cyclosporin (15 men; median age 42 years (range 28 to 64)) and 8 patients receiving both cyclosporin and simvastatin (4 men; median age 46 years (range 38 to 71)).

3.2.1.1 T cell proliferation (Figure 3.1)

Lymphocytes were extracted and T cell proliferation measured by tritiated thymidine incorporation. A 23% reduction in *ex-vivo* T cell proliferation was observed between the statin treated and untreated group (Figure 3.1). The cyclosporin group was reduced by 12% compared to untreated (but was statistically insignificant) and cyclosporin plus statin reduced by 51%. *In vitro*, addition of 1μm simvastatin resulted in significant reductions in all four groups: no treatment 34 ±17% (mean ±SD), statin 23 ±18%, cyclosporin 53 ±35%, and cyclosporin plus statin 37 ±41%. In vitro addition of FTI and GGTI had no significant effect in any group.

3.2.1.2 Natural Killer cell cytotoxicity (Figure 3.2)

From the same pool of lymphocytes extracted for T cell proliferation, NK

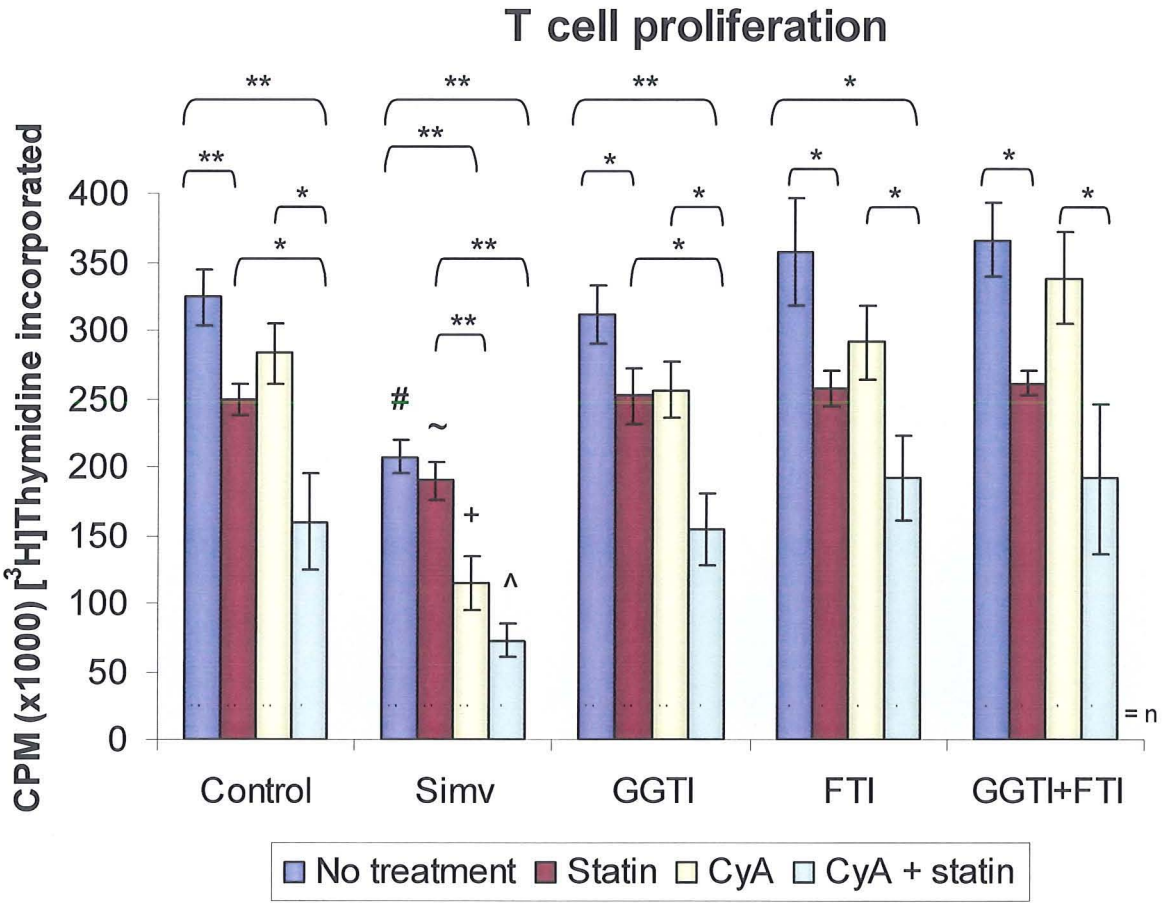


Figure 3.1 Ex vivo and in vitro comparisons of T cell proliferation from groups of cardiovascular patients before receiving statin therapy, after statin therapy, receiving cyclosporin therapy or cyclosporin and statin therapy.

Blood samples were obtained from the 4 groups of patients in study 1 and T cell proliferation stimulated by anti-CD3 ab was measured by thymidine incorporation. Control cells compared *ex vivo,* showed a reduction in proliferation of 23% between the untreated group and the statin group. Addition of 1 μM simvastatin *in vitro* resulted in significant reductions of proliferation in all groups: 34 ±17% no treatment, 23 ±18% statin, 53 ±35% and 37 ±41% plus statin. GGTI and FTI had no effect on T cell proliferation. [*($p < 0.05$, **($p<0.005$. # $p<0.001$ vs no treatment, control. ~ $p<0.005$ vs statin, control. + $p<0.001$ vs CyA, control. ^ $p<0.05$ vs CyA + statin, control. Students two sample t test.]

cytotoxicity was measured by ^{51}Cr release. Statin treated patient's NK cytotoxicity was 43% lower than untreated patients and cyclosporin patient's was 54% lower (Figure 3.2). *In vitro* addition of 1 μM simvastatin resulted in significant reductions in all groups: no treatment 76 ±15%; statin 87 ±10%; cyclosporin 80 ±38%. As with T cell proliferation, FTI and GGTI had no significant effect on NK cytotoxicity *in vitro*.

3.2.1.3 Cell Adhesion Molecules (Figure 3.3)

Again from the same pool of lymphocytes, FACS analysis was performed to measure changes in levels of LFA-1 and VLA-4 cell adhesion molecules. VLA-4 levels remained unaffected by *in vivo* statin or cyclosporin (Figure 3.3). LFA-1 levels appear to have been reduced by *in vivo* statins and increased by cyclosporin +/- statin. However, these results were statistically insignificant.

3.2.2 Study 2: Normal Volunteer Study

Thirteen healthy subjects were recruited to the study, 8 men and 5 women, median age 36 years (range 26 years to 46 years). Treatment with simvastatin, 40mg per day, resulted in a predictable effect on plasma lipid sub-fractions in normal subjects without hyperlipidaemia (Table 3.1). Total cholesterol was reduced by 23 ±12% (mean ±SD) during treatment and LDL cholesterol by 36 ±16%. HDL cholesterol was increased by 13 ±20%. Triglyceride levels were not reduced significantly. I also measured CRP, as an inflammatory marker that could potentially follow changes in immune cell function. Although there was a trend towards reduced levels during active treatment, the measured levels were low in keeping with the absence of illness in the study population and the reduction did not achieve statistical significance.

3.2.2.1 T cell proliferation (Figure 3.4)

Lymphocytes extracted from volunteers before and after simvastatin treatment, were assayed for proliferation by CD3 stimulation (Figure 3.4). There were no significant differences in proliferation of T cells isolated from the pre- and post-

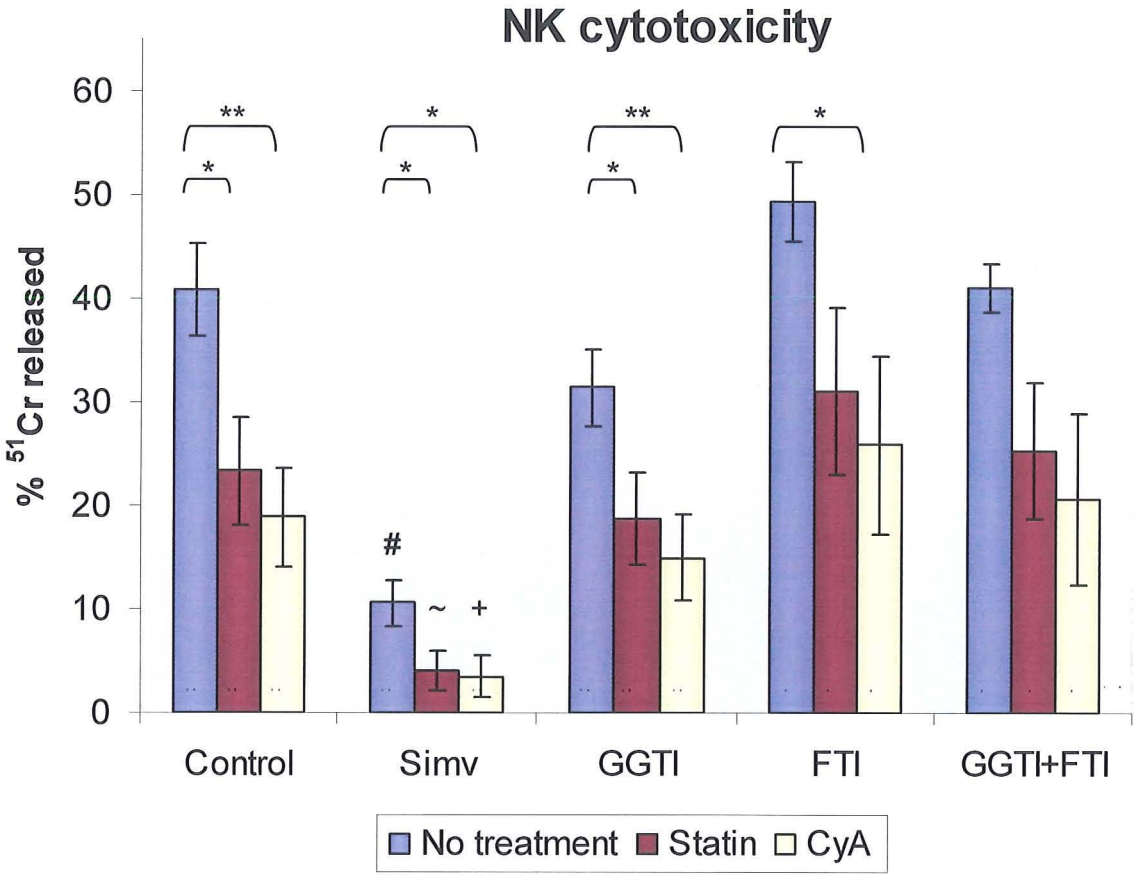


Figure 3.2 Ex vivo and in vitro comparisons of natural killer cell cytotoxicity from groups of cardiovascular patients before receiving statin therapy, after statin therapy, receiving cyclosporin therapy or cyclosporin and statin therapy.

NK cytotoxicity was measured by ^{51}Cr release. Control cells compared *ex vivo*, showed a reduction in cytotoxicity of 43% between the untreated group and the statin group. Addition of 1μM simvastatin *in vitro* resulted in significant reductions of cytotoxicity in all groups: 76 ±16% no treatment, 87 ±10% statin, and 80 ±38% cyclosporin. GGTI and FTI had no effect on NK cytotoxicity. [*($p<0.05$, **($p<0.005$. # $p<0.001$ vs control, ~ $p<0.003$ vs control, + $p<0.01$ vs control. Students two sample t test.]

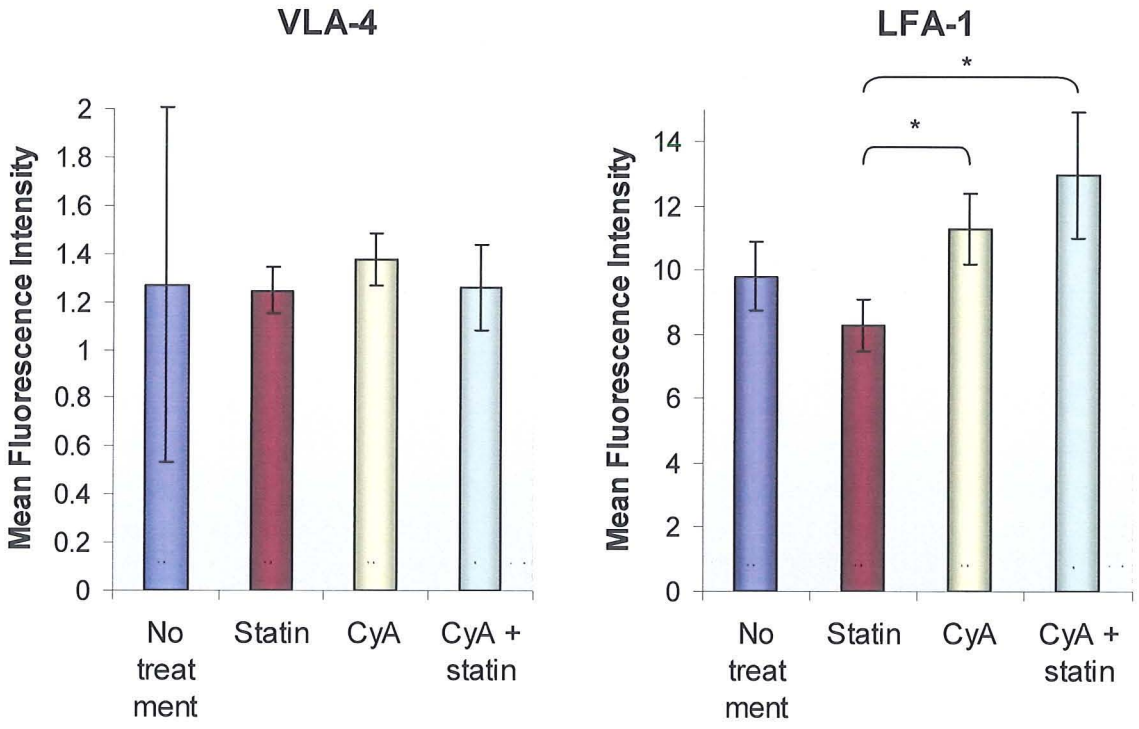


Figure 3.3 Ex vivo flow cytometry analysis of adhesion markers on T lymphocytes from cardiovascular patients.

VLA-4 and LFA-1 adhesion molecules were unaffected by the patients' treatments. LFA-1 changes were statistically insignificant compared to the normal group. [*$p<0.05$. Students two sample t test.]

		Control 1	Simvastatin	Control 2	p-value *Control 1 v Statin*	*Statin v Control 2*
Triglycerides	Mean (±SE)	1.11 (±0.09)	1.22 (±0.17)	1.08 (0.14)	0.45	0.67
mmol/L	Median	1.10	1.10	0.95		
Total Cholesterol	Mean (±SE)	**5.02** (±0.15)	**3.85** (±0.16)	**5.03** (±0.21)	**<0.001**	**0.002**
mmol/L	Median	4.94	3.93	4.97		
VLDL Cholesterol	Mean (±SE)	0.32 (±0.05)	0.25 (±0.03)	0.32 (±.08)	0.27	0.35
mmol/L	Median	0.30	0.24	0.25		
LDL Cholesterol	Mean (±SE)	**3.45** (±0.18)	**2.12** (±0.12)	**3.35** (±0.21)	**<0.001**	**0.001**
mmol/L	Median	3.41	2.16	3.15		
HDL Cholesterol	Mean (±SE)	**1.26** (±0.09)	**1.43** (±0.11)	**1.35** (±.07)	**0.022**	**0.006**
mmol/L	Median	1.29	1.44	1.34		
hs-CRP	Mean (±SE)	1.33 (±0.58)	1.27 (±0.54)	1.17 (±0.70)	0.79	0.73
mg/L	Median	0.45	0.28	0.35		

Table 3.1 Simvastatin treatment reduced cholesterol and LDL levels in normal volunteers.

Lipid profiles were measured before and after taking simvastatin. Total cholesterol and LDL cholesterol were significantly reduced by simvastatin (24% and 39% respectively). HDL cholesterol levels increased by 12%. The changes in other lipids and CRP levels were not significant.

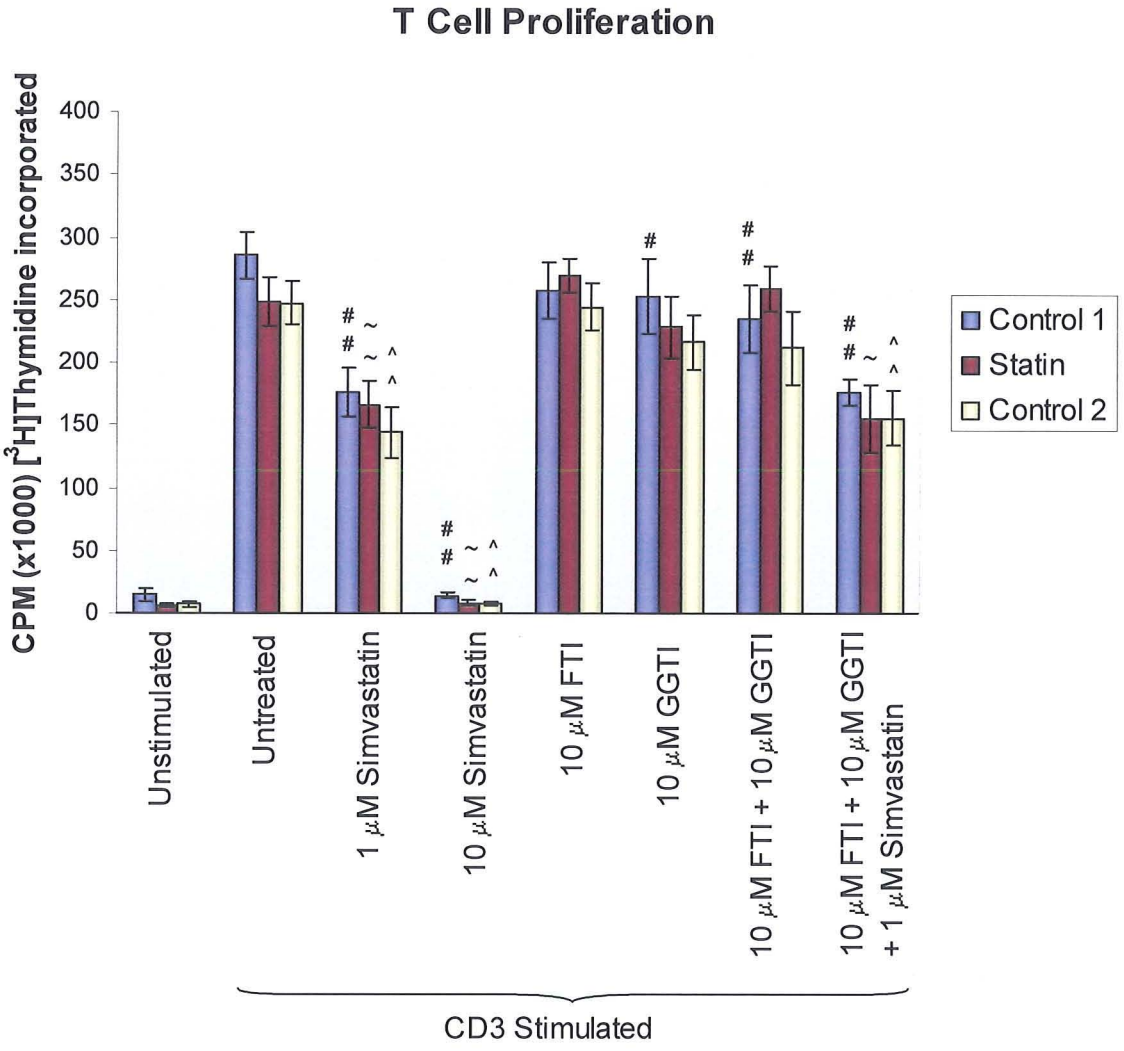


Figure 3.4 Simvastatin reduced T cell proliferation in vitro.

T cell proliferation was measured by [3H]Thymidine incorporation into lymphocytes extracted from normal volunteers before and after taking simvastatin (Study 2). Kruskal-Wallis analysis showed no significant differences between the three groups. Addition of 1μM simvastatin *in vitro* resulted in significant reductions of proliferation in all three groups (control 1 – 39 ±18%, statin – 33 ±24%, control 2 – 43 ±20%) and 10μM simvastatin (control 1 – 95 ±3%, statin – 97 ±2%, control 2 – 97 ±3%). GGTI produced a statistically significant reduction in proliferation only in the control 1 group - 14 ±15%. [# $p < 0.005$ vs control 1, untreated, stimulated. ~ $p < 0.005$ vs statin, untreated, stimulated. ^ $p < 0.005$ vs control 2 untreated, stimulated.] (Data sets are the mean ±SE, n=13.)

treatment control phases, or the simvastatin treatment phase of the study. I then added simvastatin, *in vitro*, to examine the hypothesis that cells from statin-treated subjects would be more susceptible to the *in vitro* effects of statins even in the absence of an obvious *in vivo* effect. Addition of 1μM simvastatin caused a 39 ±18% reduction in T-cell proliferation and 10μM simvastatin, a 95 ±3% reduction, confirming the well-established *in vitro* effects of lymphocyte proliferation[294]. Oral simvastatin therapy did not influence the response of T cells to *in vitro* statin therapy. Addition of a geranylgeranyl transferase inhibitor *in vitro* also reduced lymphocyte proliferation, by 14 ±15%, whereas addition of a farnesyltransferase inhibitor produced a non-significant reduction of 10 ±19%. Addition of both prenyl inhibitors together reduced proliferation by 20 ±22.7% ($P < 0.001$). Oral simvastatin therapy did not influence the *in vitro* response to prenyl transferase inhibitors, either alone or in combination.

3.2.2.2 Natural Killer cell cytotoxicity (Figures 3.5 and 3.6)

NK cell cytotoxicity was measured in lymphocytes extracted from volunteers before and after taking simvastatin. Simvastatin reduced NK cytotoxicity *ex vivo* by 30 ±33% in untreated cells, the levels returning to normal following cessation of therapy (Figure 3.5). Addition of simvastatin or prenyl transferase inhibitors *in vitro* resulted in a reduction in NK cytotoxicity. Thus, addition of 1μM simvastatin to control (period 1) cells reduced NK cytotoxicity by 56 ±24%. Farnesyl and geranylgeranyl transferase inhibitors had a lesser effect, producing reductions of 9 ±10% and 21 ±18% respectively. Combinations of inhibitors did not show increased or additive effects. Similar reductions, although less significant, were observed in the statin treatment and washout phase (Control 2) in response to *ex vivo* treatment with simvastatin and prenyl transferase inhibitors. Overall, there was a close relationship between reduction in LDL and inhibition of NK cell killing (Figure 3.6).

3.2.2.3 Simvastatin and membrane rafts (Figures 3.7 and 3.8)

An alternative mechanism for the pleiotropic actions of statins is through the disruption of membrane cholesterol-rich rafts. I performed western blot analysis

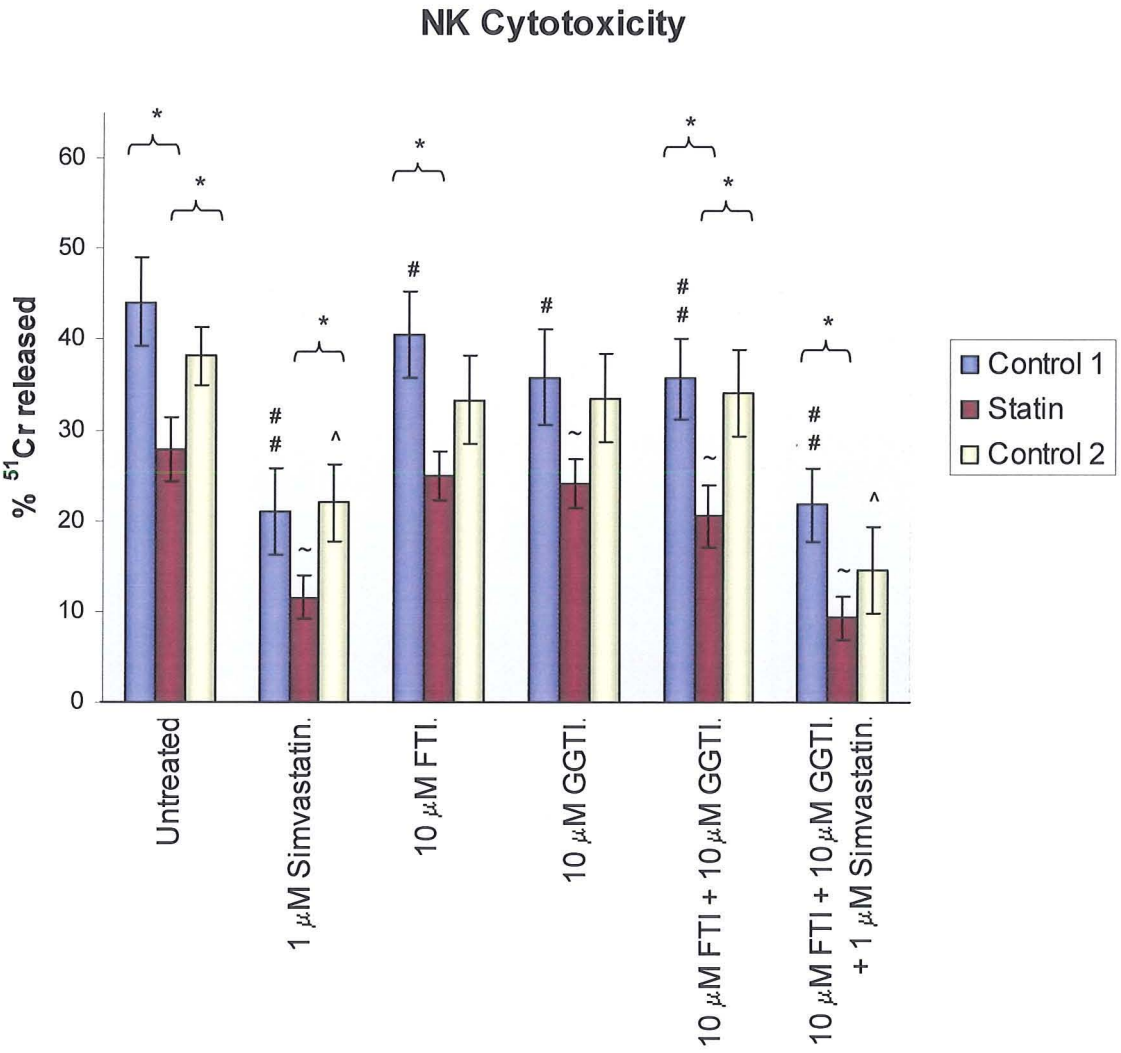


Figure 3.5 Simvastatin reduced NK cytotoxicity ex vivo and in vitro.

Natural killer cell cytotoxicity was measured by their ability to lyse K562 target cells and release [51]chromium. NK cytotoxicity in untreated cells was reduced by 30 ±33% after taking simvastatin. Adding simvastatin or prenyl transferase inhibitors *in vitro* also resulted in statistically significant reductions. One μM simvastatin added to control group 1 was enough to reduce NK cytotoxicity by 56 ±29%. Farnesyl and geranylgeranyl transferase inhibitors were less effective, producing reductions of 9 ±10% and 21 ±18%) respectively. Combinations of inhibitors did not have any additive effects. Similar reductions *in vitro* were observed in the statin groups and control group 2. (*{ $p < 0.05$. # $p < 0.005$ vs control 1, untreated. ~ $p < 0.005$, vs statin, untreated. ^ $p < 0.008$ vs control 2 untreated.) (Data sets are the mean ±SE, n=13.)

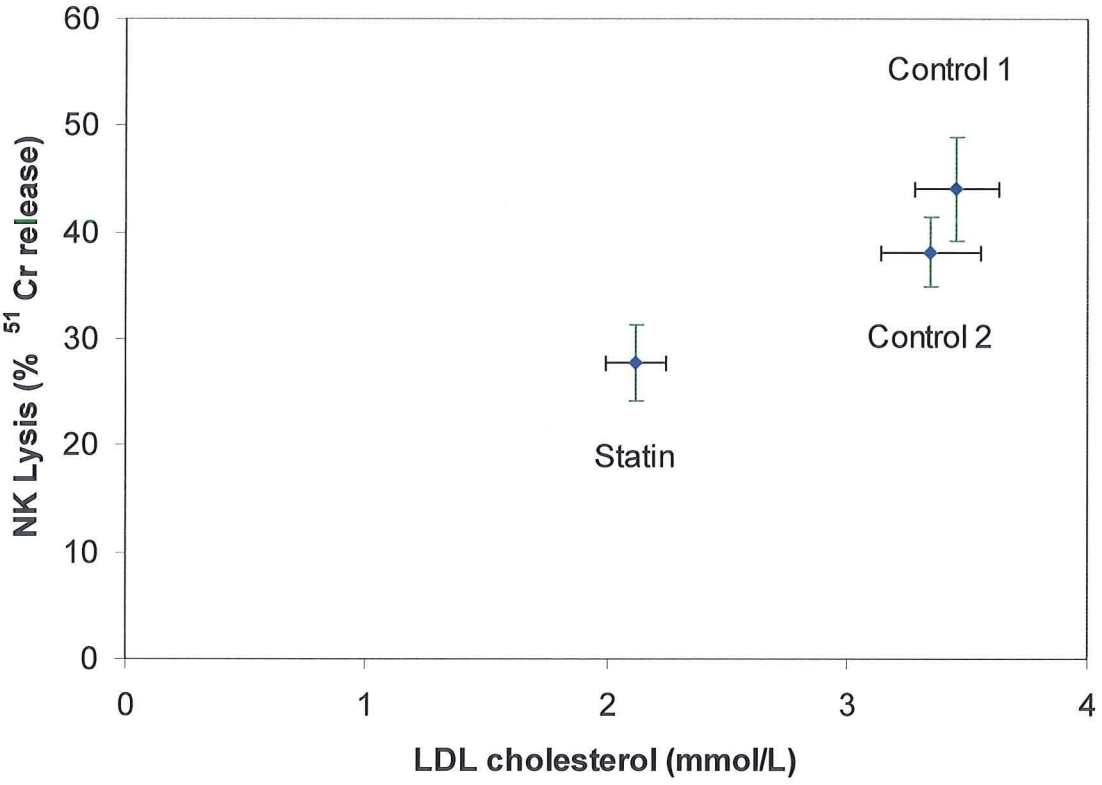


Figure 3.6 Simvastatin treatment reduced NK cytotoxicity in line with LDL levels.

NK cytotoxicity was plotted against LDL cholesterol levels to show the reduction by statin was in line with the reduction in LDL cholesterol. (Data sets are the mean ±SE, n=13)

of human leukocytes obtained from normal volunteers and treated them *in vitro* with 5μM simvastatin. I then probed with antibodies against the known raft associated proteins, LAT and Lyn. Figure 3.7 shows the results of this experiment, with reduction of the raft fraction of treated cells. Having established this technique, I examined cells from subjects before and after simvastatin treatment to identify changes in raft fractions of the cell membrane.

Leukocyte preparations were made from peripheral blood of normal volunteers before and after taking simvastatin 40 mg/day. The samples were subjected to sucrose gradient centrifugation and the presence of raft-associated proteins measured in the raft fraction by western blot. The intensity of Lyn and Fyn bands was determined by densitometry and a ratio calculated between whole cell levels of protein and raft levels. The ratios were plotted on a bar graph and the general trend was for a reduction in rafts (Figure 3.8). Although there was a clear trend towards reduction of the raft fraction during treatment this failed to achieve statistical significance.

3.2.2.4 Farnesol and geranylgeraniol fail to rescue simvastatin inhibition (Figure 3.9)

To further investigate the effect of simvastatin on prenylation and its role in immunomodulation, I attempted to rescue simvastatin inhibition of T cell proliferation by addition of excess prenyl groups, farnesol and geranylgeraniol (Figure 3.9). Farnesol and geranylgeraniol individually or in combination did not significantly rescue simvastatin inhibition where as mevalonate, which is further upstream of the cholesterol pathway, did.

3.2.3 Study 3: Human NK study (Figure 3.10)

A total of 146 patients and normal subjects had samples taken for measurement of NK cytotoxicity using standard methodology. The population sampled was deliberately diverse. The average age was 56.3 ± 16.7 years (mean ±SD), 58.9% were male, 20.9% were cigarette smokers, 12% had diabetes, 31% had treated hypertension, 16.5% had coronary heart disease and 33.5% were on

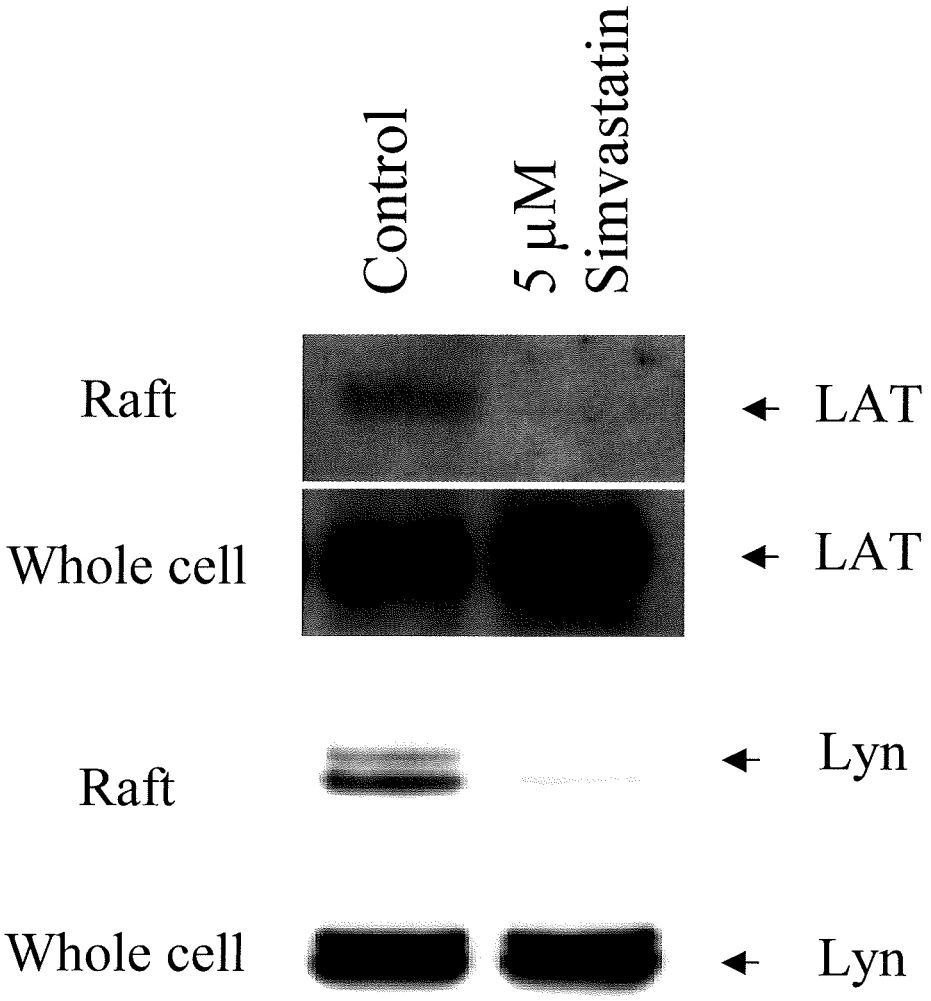


Figure 3.7 Simvastatin treatment reduced raft levels in leukocytes.

Western blot of leukocytes treated *in vivo* with 5µM simvastatin showed a reduction in LAT and Lyn levels and therefore rafts.

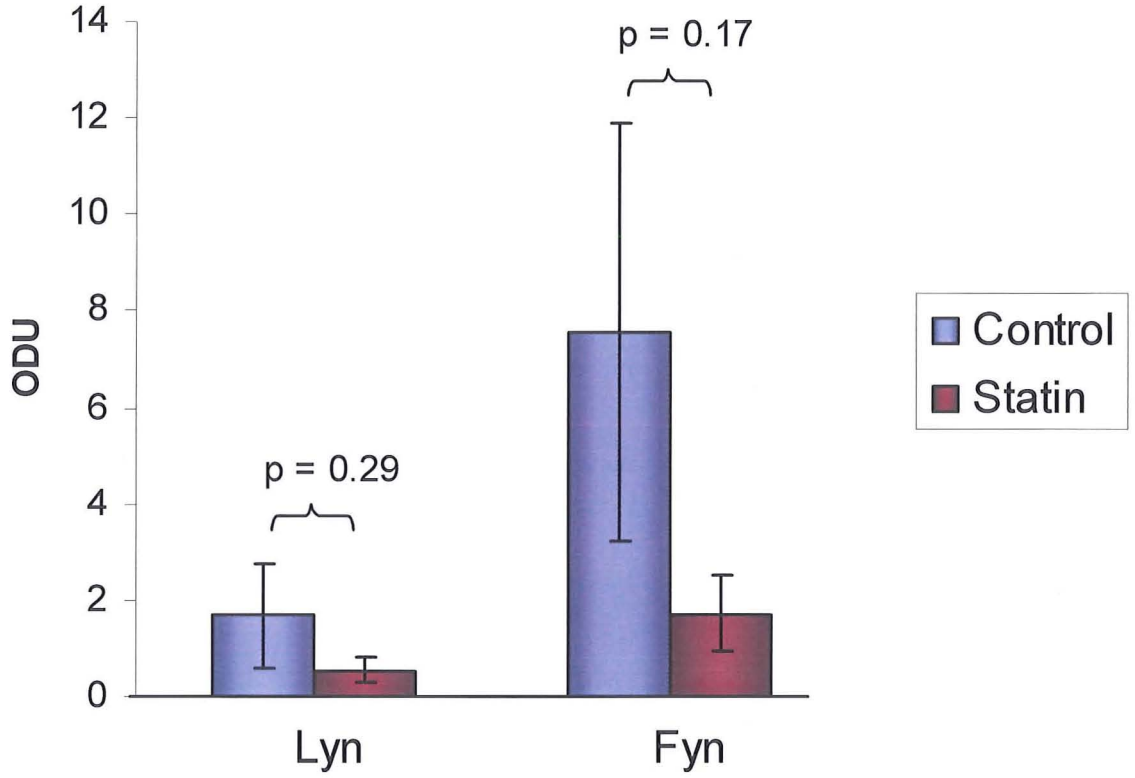


Figure 3.8 Simvastatin treatment reduced the western blot band intensities of raft levels in leukocytes

Band intensities of Lyn and Fyn on western blots were measured by densitometry. A ratio between whole cell levels and raft content was calculated and plotted on a bar graph. The general trend was a reduction in raft levels, however the small number of samples used made the figures statistically insignificant. (Data sets are the mean ±SE, n=7)

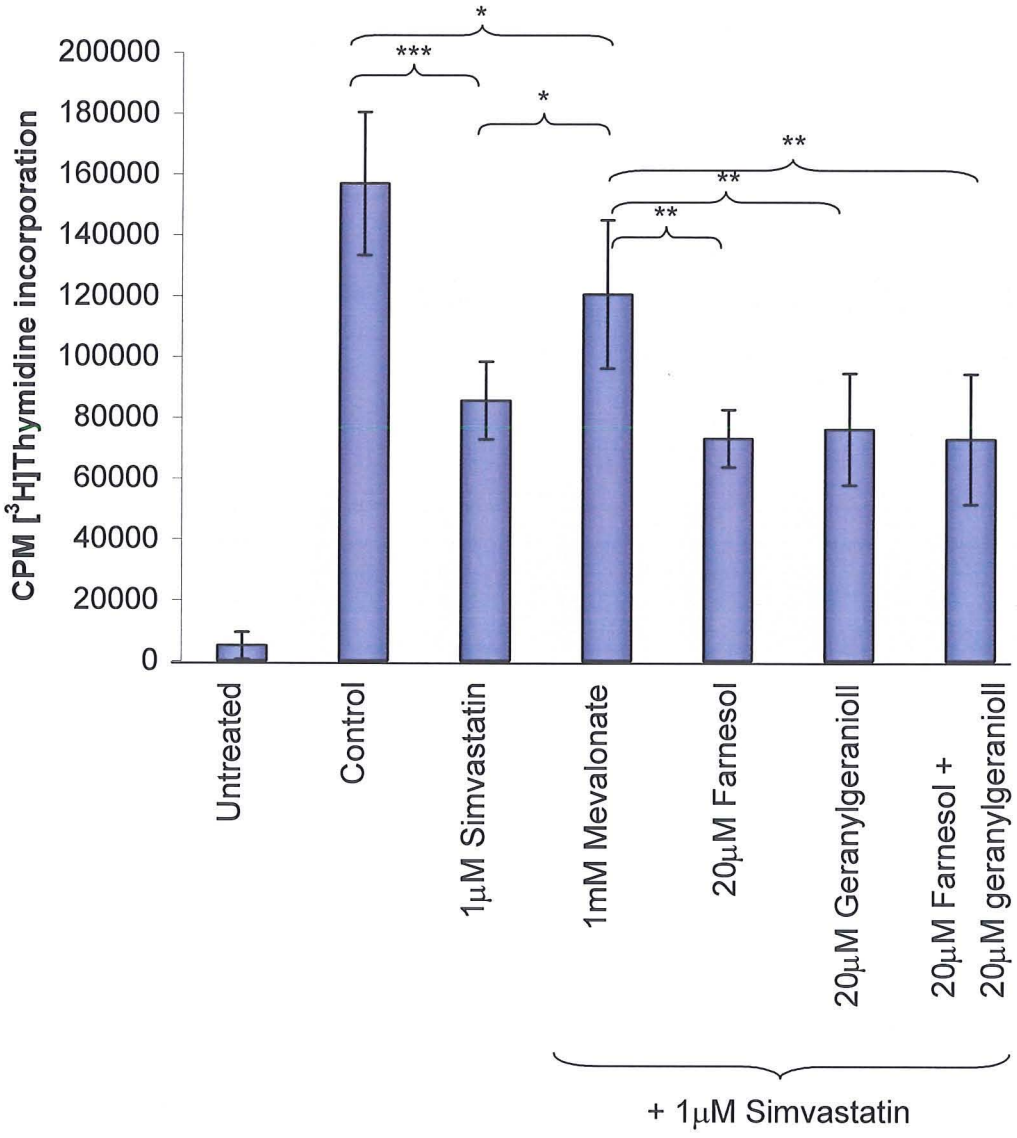


Figure 3.9 Farnesol and geranylgeraniol failed to rescue simvastatin inhibited T cell proliferation.

Mevalonate, farnesol and geranylgeraniol were added 24 hours after initial stimulation. Mevalonate partially rescued proliferation where as farnesol and geranylgeraniol did not significantly. (Data sets are the mean ±SD, n=6. *{ p=0.06, **{ p<0.05, ***{ p<0.005)

immunosuppressive therapy. 30.4% of subjects were on statin therapy; the type and dose were not recorded. In this population the average non-fasting total cholesterol was 4.76 ±1.08 mmol/L, weight was 77.38 ±15.93 kg, and blood pressure was 134.6 ±20.9/74.9 ±11.7 mmHg. In the normal subjects the average non-fasting total cholesterol was 4.77 ±1.06 mmol/L, weight was 77.4 ±15.9 kg, and blood pressure was 134.8 ±20.3/75.0 ±11.3 mmHg. Overall, in all subjects and patients, the proportion of target K562 cells lysed, when incubated in a 1:25 ratio with a crude plasma lymphocyte preparation, was 52.9 ±19.4% (range 10-89%).

The relationship between total cholesterol and NK cell killing is shown in Figure 3.10. There was a trend towards higher levels of cytotoxicity at higher cholesterol levels and although statin therapy reduces NK cell killing in individuals, statin-treated patients have levels of NK cell killing appropriate for their total cholesterol. The possible determinants of NK cell function were investigated by regression analysis. These data are shown in Table 3.2. The best-fit model identified total cholesterol and weight as the main factors associated with cytotoxicity; whereas statins, immunosuppressive therapy, gender, age or blood pressure were not independently associated.

3.3 Discussion

The involvement of inflammatory mechanisms in atherosclerosis is now well established where both T lymphocytes and NK cells have been implicated in experimental and human atherosclerosis[295, 296]. Statins are known to inhibit proliferation and to modulate function of lymphocytes and NK cells *in vitro*. However, *in vivo*, statins appear to inhibit NK cell function in transplant recipients receiving concomitant immunosuppressive therapy but there is little evidence of impaired T cell or NK cell function in other groups (Study 1). Since the prevailing view is that these, potentially immunosuppressive, effects are *in vitro* effects with limited *in vivo* or clinical relevance, I sought to determine the effects of simvastatin in normal subjects.

Study 2 was not randomised. All patients were studied before and after treatment

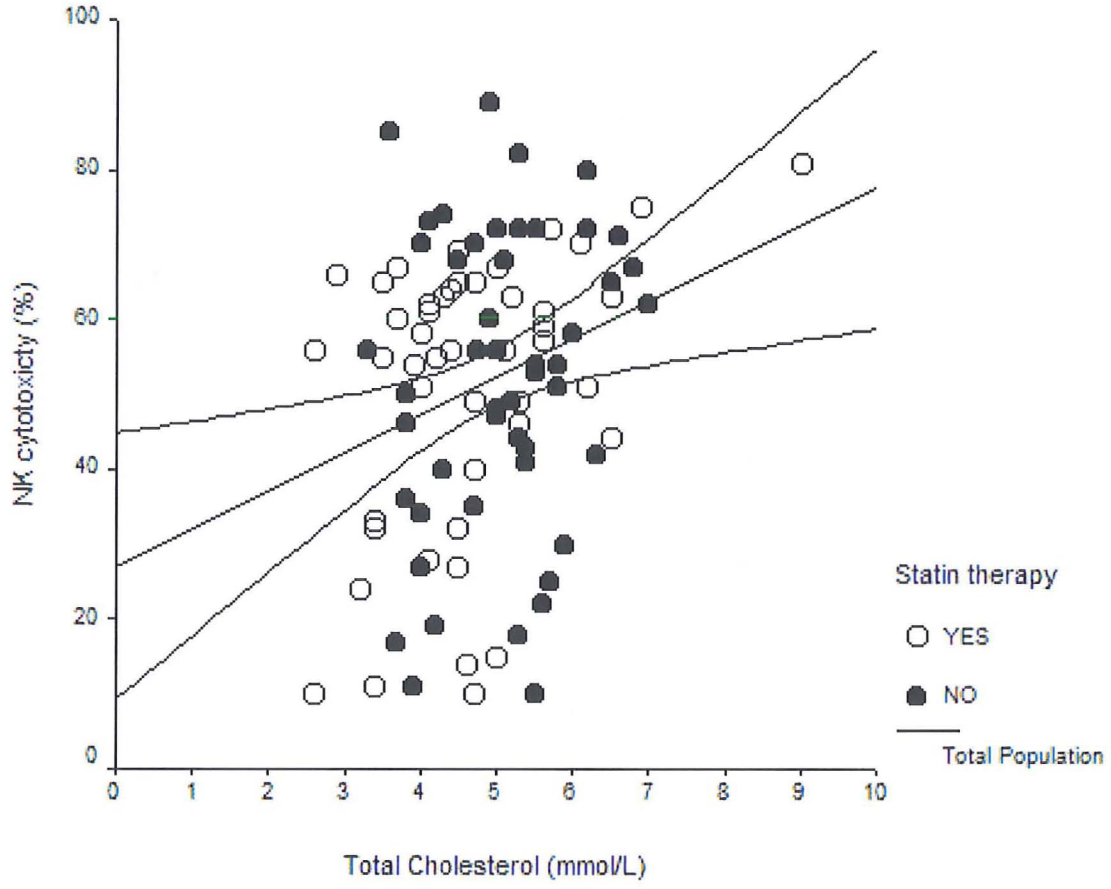


Figure 3.10 Relationship between total cholesterol and NK cell cytotoxicity.

Crude lymphocyte preparations were extracted from peripheral blood samples from a mixed group of patients and normal subjects. NK cytotoxicity was measured by lysis of ^{51}Cr labelled K562 target cells. NK cytotoxicity (% killing) is shown on Y axis vs., random total cholesterol concentration (mmol/L) on the X axis. The corresponding multivariate analysis is shown in Table 3.2.

Variable	Beta (SD)	Significance
Constant	8.09 (17.69)	
Age (years)	-0.03 (0.11)	0.90
Sex (F vs., M)	-2.24 (3.09)	0.44
Smoker (Y vs., N)	2.23 (4.33)	0.61
Statin (Y vs., N)	-2.33 (3.85)	0.55
TC (mmol/L)	4.82 (1.66)	<0.01
Weight (Kg)	0.22 (0.12)	0.07
SBP (mmHg)	0.05 (0.10)	0.59
ImRx (Y vs., N)	-2.26 (3.68)	0.54

(ii) Stepwise Model ($r=0.29$, $r^2 = 0.09$, $F=7.23$, $P=0.001$)

Variable	Beta (SD)	Significance
Constant	10.72 (11.41)	
TC (mmol/L)	4.80 (1.54)	<0.01
Weight (Kg)	0.25 (0.11)	0.02

Table 3.2 Relationships with NK cytotoxicity - linear regression analysis.

The best-fit model identified total cholesterol and weight as significant independent predictors of cytotoxicity; statins, immunosuppressive therapy, gender, age or blood pressure were not independently associated.

with simvastatin for four weeks, and once more at least four weeks after the cessation of therapy. Reassuringly, a significant reduction in LDL cholesterol of 36 ±16% and a similar reduction in total cholesterol were observed. HDL-cholesterol was increased by 13 ±20.2% and, despite the fact that the lipid levels in this study were normal and much lower than the majority of patients enrolled in clinical trials I observed a quantitatively similar, beneficial effect on plasma lipid levels[177, 179, 193, 203] (Table 3.1).

Study 2 sought to determine whether or not, T cell proliferation was affected by oral statin therapy. A standard lymphocyte proliferation assay, using CD3 cross-linking for specificity was utilised. There was no significant difference in the *in vitro* proliferation of T lymphocytes isolated from subjects during statin therapy. Moreover, although *in vitro* treatment with simvastatin (at concentrations in the micromolar range that are consistent with the peak levels achieved during simvastatin treatment) inhibited T cell proliferation, cells isolated during simvastatin treatment were no more susceptible to subsequent *in vitro* exposure. The absence of an *in vivo* effect of statins on T cell proliferation is consistent with the absence of an effect of statins on infection and malignancy rates in large scale trials[177, 179, 193, 203]. Alternatively this may reflect either the insensitivity of the assay system or the fact that T cell proliferation is such an important biological response that it may bypass inhibition of a single signalling mechanism.

Natural Killer cell function is more sensitive to *in vivo* statin use[250, 285]. A clear reduction in NK cell cytotoxicity during statin treatment was observed and was reversed following withdrawal of simvastatin. The changes in NK cell function paralleled those changes in LDL-cholesterol, reflecting the influence of statin therapy (Figure 3.6). As with lymphocyte proliferation, there may be differences in sensitivity of the assay so that it is easier to detect a reduction in NK cell killing than in lymphocyte proliferation. Alternatively, NK cell activation and killing may be more susceptible to blockade of statin dependent signalling pathways, whether they be dependent on isoprenoids or membrane rafts[56].

Having shown that simvastatin inhibits NK cell cytotoxicity in normal subjects during short-term treatment, I sought to investigate the influence of statins in patients receiving statin therapy for conventional indications. In this preliminary

study (Study 3) I did not restrict sampling to patients without concomitant disease or to those on statin therapy alone. However, it is unusual in the clinical practice for patients to receive statin monotherapy, or to have an indication for statin treatment in the absence of co-morbid disease. Thus, the patients sampled are typical of the patients receiving statin therapy in the practice. Although statin treated patients were older, there was no significant relationship between age and NK cell cytotoxicity (p=0.418). The fact that statin recipients had reduced NK cell cytotoxicity, is consistent with the findings in normal subjects and also previous reports that NK cell cytotoxicity is reduced in transplant recipients on statin therapy[250]. Although T cell proliferation was reduced in statin recipients, this does not match the findings in normal subjects and should be viewed with caution. Overall, the pattern of *in vitro* effects of statins, and prenyl transferase inhibitors in these patients (whether or not they received statin therapy) is similar to the findings in normal subjects.

I examined the potential importance of isoprenoids by the use of isoprenyl transferase inhibitors in the first two studies. *In vitro*, these agents inhibit the proliferation of lymphocytes and NK cell cytotoxicity to a more limited extent than statins. Both agents together are more effective than either agent alone, suggesting that geranylgeranylation and farnesylation may offer alternative pathways, either for individual proteins or for physiological processes. FTI and GGTI plus statin therapy had no greater effect than statin alone *in vitro*, consistent with the ability of statins to block the common pathway to isoprenoid generation. There was no additional effect of these agents (alone or in combination) on cells isolated from subjects during oral statin therapy. Although these observations are interesting and confirm the efficacy of both farnesyl and geranylgeranyl transferase inhibitors *in vitro*, the effects of these inhibitors are limited in comparison to statins. Whilst the specificity and potency of these prenyl transferase inhibitors may differ, and are likely to be less than that of commercially available statins, these observations are also consistent with the notion that mechanisms other than inhibition of prenylation are involved.

To investigate the effect of the prenylation process on the immunomodulatory effect of simvastatin I have shown that addition of excess prenyl groups failed to rescue T cell proliferation. However, addition of mevalonate (which is further

upstream in the cholesterol pathway and could therefore restore cholesterol synthesis) did recover T cell proliferation. Providing prenyl groups to restart the prenylation of signalling molecules could not rescue proliferation, demonstrating the importance of cholesterol and raft structure over prenylation in the mechanism of proliferation.

This alternative explanation for the actions of statins on lymphocytes is the disruption of membrane rafts. These cholesterol rich membrane microdomains act as cell membrane platforms for receptors and signal molecules. The raft fractions in the cell membrane were separated by sucrose gradient centrifugation and probed for the presence of known raft associated proteins, specifically Lyn and Fyn in lymphocytes. Lyn is of particular interest as its translocation to the cell membrane is independent of prenylation. The data from study 2 show a reduction in raft-associated proteins in the samples from seven subjects, during statin therapy. Although the failure to achieve statistical significance reflects the fidelity of methodology that is seldom subjected to statistical analysis, it is consistent with an effect of oral simvastatin to deplete membrane rafts in circulating cells.

Blockade of LFA-1 and VLA-4 interactions with their ligands is a potential target for immunosuppression and for controlling inflammation and autoimmune diseases. Statins can prevent these interactions by disrupting membrane rafts. Inactive LFA-1 is tethered to the cytoskeleton, and if unable to move into lipid rafts where it would normally become activated, it would therefore remain inactive. Rho, Rac and Cdc42 are prenylated GTPase signalling molecules which regulate receptor clustering[81] and treatment with statins would prevent them controlling and moving the integrins. Some statins have been found to bind directly to LFA-1[76] preventing the conformational change to a high affinity state. I measured the adhesion markers LFA-1 and VLA-4 on T cells from the cardiovascular group of patients (Study 1) by FACS. Statin treatment however had no statistically significant effect on the amount of either of these markers perhaps due to the highly variable levels on normals. The anti-LFA-1 and anti-VLA-4 antibody used was not specific enough to distinguish between activated and inactive forms, therefore could not distinguish between the forms within or outwith lipid rafts. Treatment with statins would only alter the position on the membrane not the overall amount. The type of statin used could also have affected the results as

some statins bind directly to LFA-1. This could have sterically prevented the FACS antibody from binding and as the patients were not all on the same statin, makes the results difficult to interpret.

In conclusion, these preliminary studies demonstrate that a short oral course of simvastatin has a detectable effect on NK cell cytotoxicity. Accumulating evidence indicates a multifactorial mode of action for statins in the reduction of morbidity and mortality from cardiovascular disease. Thus, in addition to lowering LDL-cholesterol levels, statins block leukocyte recruitment to atherosclerotic lesions, inhibit monocyte antigen processing and production of inflammatory mediators, and reduce NK functions. NK cells have been implicated in atherosclerosis and are known to target cells that do not express HLA class I on the cell surface, including cells that have been infected by viruses[285, 295]. Whether this effect on NK cells contributes to the overall beneficial effects of statin therapy in cardiovascular disease is not clear and will require further investigation. However, the mechanism by which statins inhibit NK cell killing is likely to provide an insight into the signalling mechanisms disrupted by statin therapy and that contribute to their pleiotropic effects in other cell types[86, 239].

The possibility that reduced NK cytotoxicity may reflect an underlying relationship between cholesterol synthesis, membrane cholesterol and cell function has not been considered[297]. In the human NK study (Study 3), I found that statin treated patients did not have lower NK cell cytotoxicity than non-statin treated patients but that the overall relationship was with total cholesterol (Figure 3.10; Table 3.2). The patient selection for this study was not focused on one particular group but was deliberately wide-ranging to provide a spectrum of age, underlying disease and drug therapy to allow comparison of potential influences. The analysis is preliminary and will require further investigation in larger, more homogeneous groups. However, the findings concur with the previous study in normal subjects where there was a close relationship between reduction in NK cell cytotoxicity and lipid levels in normal subjects receiving statins. Thus, rather than being directly due to statin treatment this change may be a consequence of changes in cholesterol levels, both in the circulation and in the cell membrane.

Although this was a small-scale, pilot study that recruited normal subjects and patients over a wide age range, a range of drug therapy and underlying pathology, it poses questions about the relationship between circulating lipids and membrane lipids, and the wider effects on cell membrane structure and signalling. NK cells may simply provide an accessible cell type and the relationship between lipids and immune cell function[297-299] may be applicable to all cells.

Chapter 4: Effect of fluvastatin on prenylation and signalling

4.1 Introduction

Chapter 3 confirmed the effectiveness of statins in reducing LDL cholesterol and reducing lymphocyte functions *in vivo* and *in vitro*[179, 300]. These pleiotropic effects, independent of cholesterol reduction, are believed to be mediated by reduction of isoprenoid intermediates, farnesyl and geranylgeranyl[239, 278]. Addition of these isoprenoids to a variety of signal molecules[301] by specific transferases, promotes the membrane association of otherwise hydrophilic proteins. Hence, inhibition of prenylation (by statins or specific prenyl transferase inhibitors) may lead to functional inactivation of signalling proteins, such as those of the Ras superfamily, involved in lymphocyte function. The Ras superfamily of small GTPases can be subdivided into five major families (Ras, Rho/Rac, Rab, Arf and Ran)[302, 303]; the Ras family, are farnesylated; whereas Rho, Rab and Rac are geranylgeranylated[21].

Ras and Rho GTPases link extracellular stimuli to downstream signalling molecules such as mitogen-activated protein kinases (MAPK) including extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinase (JNK) and the p38 MAPK[302]. In general ERK is activated by mitogenic factors, while JNK and p38 are activated by stress inducing agents or pro-inflammatory cytokines. The dependence of these pathways on isoprenylated signal proteins offer a potential mechanism of action for statins in lymphoid cells and may explain the potential immunosuppressive actions of these compounds.

The aim of this chapter was to explore the potential mechanisms of the immunosuppressive effects of statins by examining the effect of HMG-CoA reductase inhibition by fluvastatin on Ras-dependent ERK activation and p38 activation in human lymphocytes *in vitro*.

I hypothesised that fluvastatin would reduce the prenylation of small GTPases such as Ras and Rac and therefore affect activation of downstream signalling molecules such as ERK and p38.

4.2 Results

4.2.1 U937 Membrane preparations (Figure 4.1)

To assess the effect of fluvastatin on prenylation and membrane association of small G-protein signalling molecules, U937 cells were treated overnight with 20μM fluvastatin or 20μM fluvastatin plus 1mM mevalonate. They were then separated into their cytosolic and membrane components by ultracentrifugation and Western blots performed.

Figure 4.1 showed the levels of Ras in the membrane compartment were reduced by treatment with fluvastatin (panel 1 lane 2) and rescued by mevalonate (lane 3). The reduction in prenylation of Ras by HMG-CoA reductase inhibitor removed the membrane anchor and caused the unprenylated Ras to remain in the cytosol (lane 5). A similar result was obtained with Rac (panel 3), but did not show the rescue by mevalonate or movement to the cytosol as well. The samples were re run on a higher resolution gel (12% polyacrylamide with MOPS buffer), to separate the prenylated form of Ras from the unprenylated form and to observe the band shift effect (panel 2). The negative control (lane 1) showed more Ras in the smaller form (prenylated form) and less in the larger form (unprenylated). However, fluvastatin treatment (lane 2) showed less of the smaller prenylated form and more of the unprenylated larger form, which had moved into the cytosolic fraction (lane 4). Lyn is a member of the src family kinase, which has a palmitoylated membrane anchor rather than a prenylated anchor, so was used as an equal loading control for the membrane fraction. Shp-1 has a cytosolic location in unstimulated cells and was therefore used as the loading control for the cytosolic fraction.

4.2.2 Measurement of FcγRI levels on U937 cells by FACS (Figure 4.2)

U937 cells were treated overnight with 10μM fluvastatin or 10μM fluvastatin plus 1mM mevalonate. FcγRI was measured by FACS using an anti-human FcγRI antibody and FITC conjugated anti-mouse Ig to determine whether fluvastatin

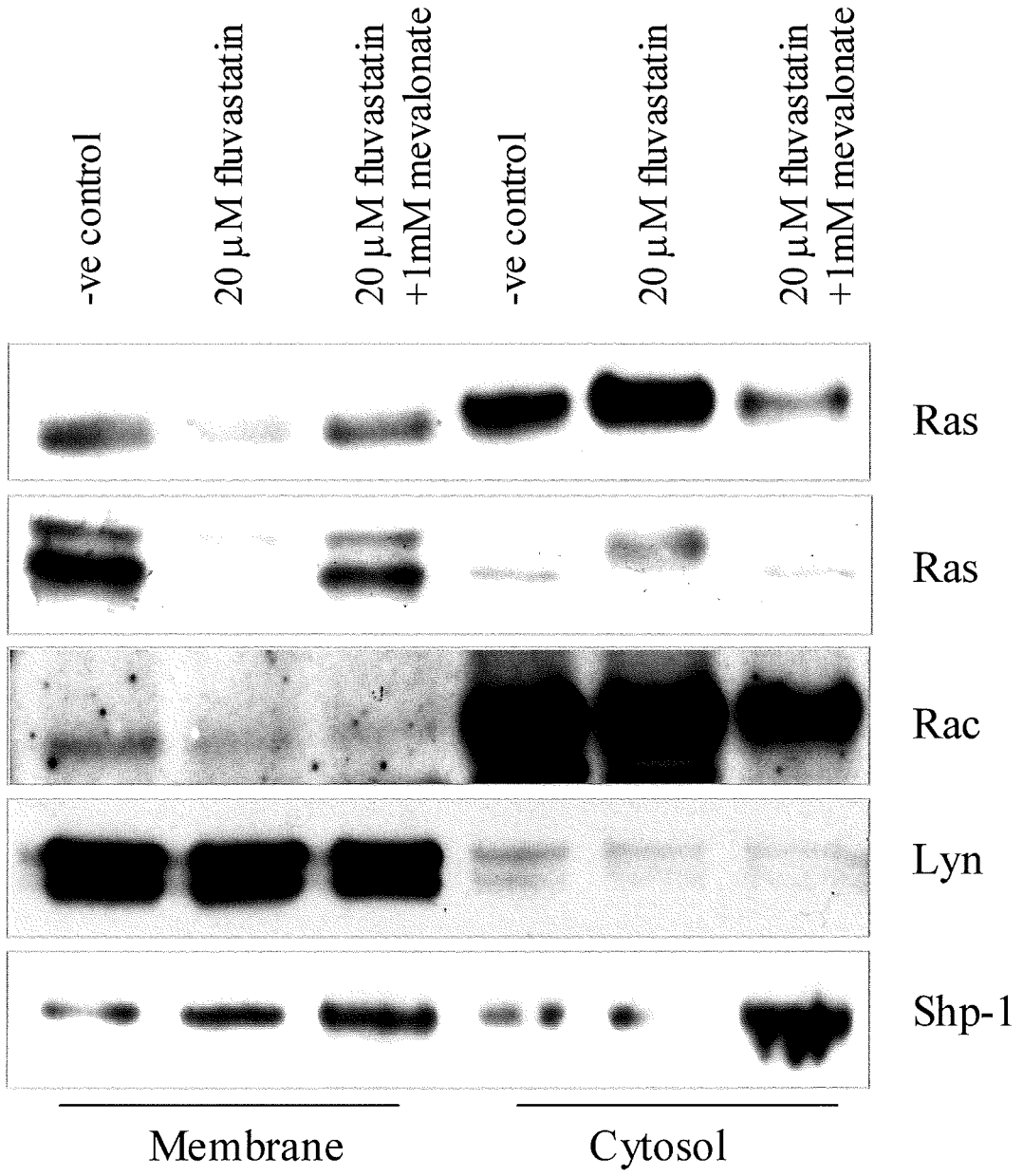


Figure 4.1 Fluvastatin reduced Ras and Rac levels in the membrane of U937 cells.

U937 cells were treated overnight with 20µM fluvastatin with or without 1mM mevalonate and separated into their cytosolic and membrane components by ultracentrifugation. Fluvastatin treatment reduced the levels of Ras and Rac in the membrane and consequently increased them in the cytosol (panel 1 and 3). A band shift in Ras was demonstrated in panel 2 with fluvastatin treated cells containing higher levels of unprenylated Ras in both membrane and cytosolic fractions. Lyn and Shp-1 were used as loading controls. This was representative of 3 experiments.

affected Fc receptor numbers. Autofluorescence was controlled for by addition of secondary antibody only. Mean fluorescence intensity was measured and FcγRI levels were not affected by either treatment (Figure 4.2).

4.2.3 Ras activity

4.2.3.1 Ras activity assay in U937 cells (Figure 4.3)

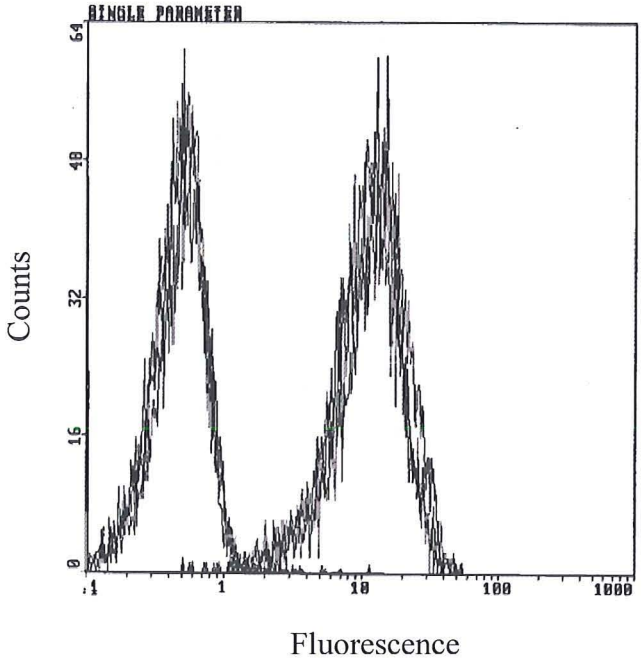
Ras is a prenylated G protein signalling molecule which cycles between an inactive GDP-bound state and an active GTP-bound state and couples CD3 to the ERK proliferative pathway in T cells [20]. U937 cells were treated overnight with 20μM fluvastatin or 20μM fluvastatin plus 1mM mevalonate to assess the effect of fluvastatin on reducing prenylation of Ras and therefore affecting its activity. Cells were stimulated by cross-linked IgG. Ras activity was measured by kit from Upstate Biotech and phosphotyrosine levels were measured by western blot using the supernatant from the Ras assay. Figure 4.3 showed a reduction in Ras activity when treated with fluvastatin (top panel lane 4 reduced compared to lane 2) and full recovery when rescued with mevalonate (lane 6). The same also applied to tyrosine phosphorylation (bottom panel) of proteins in the supernatant, with a partial rescue by mevalonate.

4.2.3.2 Ras activity assay in human primary T cells (Figure 4.4)

To confirm the effect of fluvastatin on the prenylation of Ras was not confined to a cell line, I repeated the Ras activity experiments in T cells extracted from normal volunteers. I also further investigated the effect of reduced Ras prenylation by addition of farnesyl and geranylgeranyl transferase inhibitors.

T cells were treated for 72 hours with 20 μM fluvastatin, with or without mevalonate (in the final 18 hours), 10 μM FTI and/or 10 μM GGTI inhibitor and stimulated by crosslinked anti-human CD3 antibody. Ras activity was measured by kit and phospho ERK levels were measured by western blot using the supernatant from the ras assay. Figure 4.4 showed reduced Ras activation by addition of fluvastatin and FTI and GGTI together, but not FTI or GGTI

A



B

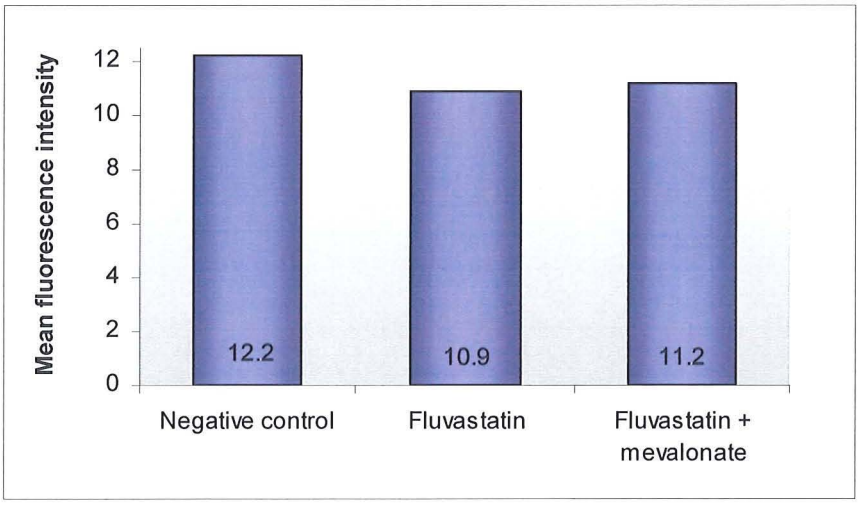


Figure 4.2 Fluvastatin treatment did not affect FcγRI levels on U937 cells.

U937 cells were treated overnight with 10μM fluvastatin or 10μM fluvastatin plus 1mM mevalonate. FcgRI levels were measured by FACS. (A) FACS overlay graph and (B) mean fluorescence intensity values showed FcγRI levels were not affected by either treatment.

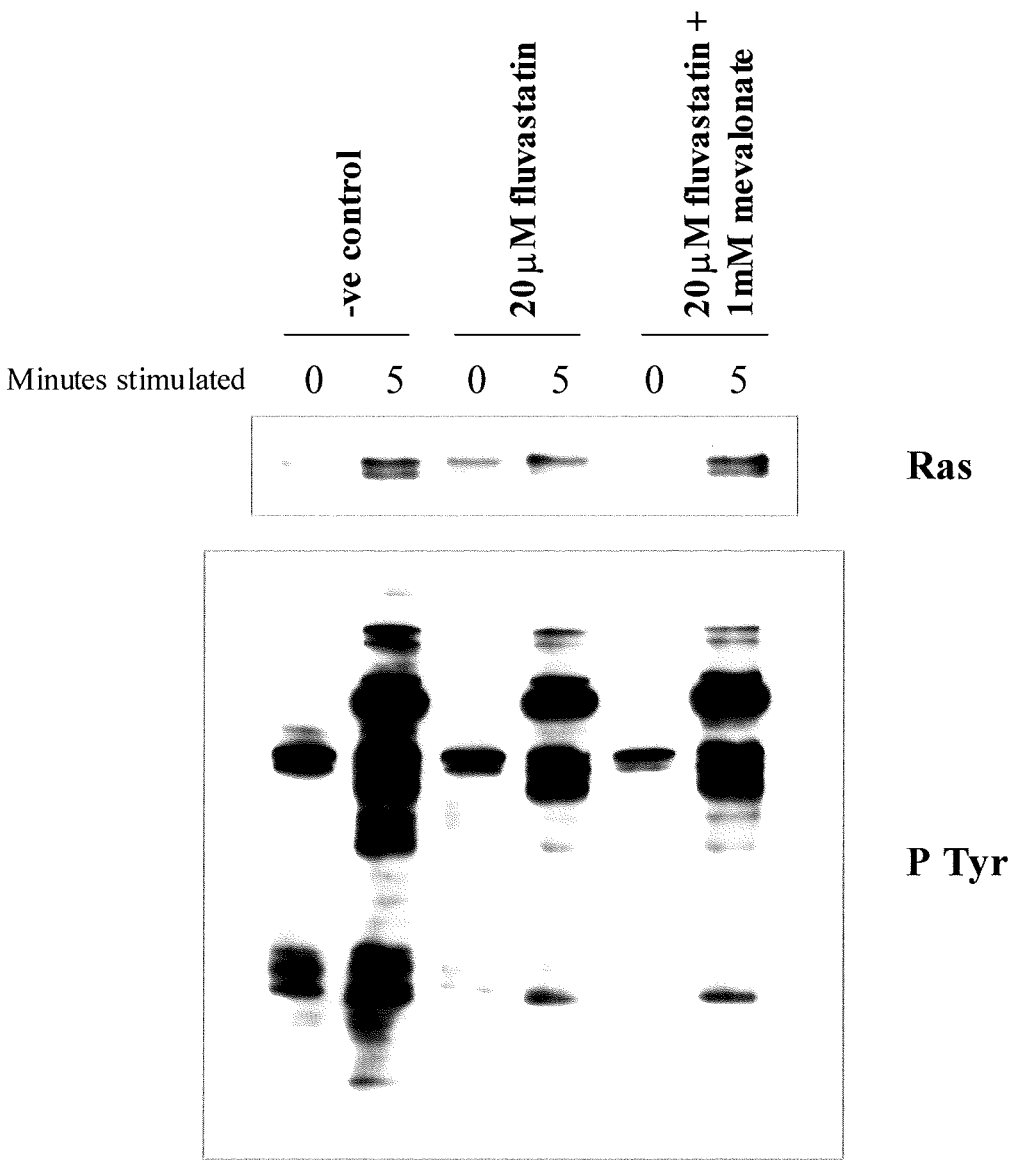


Figure 4.3 Ras activity and tyrosine phosphorylation was reduced by fluvastatin and rescued by mevalonate in the U937 cell line.

U937 cells were treated overnight with 20µM fluvastatin or 20µM fluvastatin plus 1mM mevalonate and cross linked with IgG. Ras activity was measured (top panel) and was reduce by fluvastatin treatment and rescued by mevalonate. The supernatant from the ras assay was western blotted and probed with anti-phosphotyrosine antibody. Similarly, tyrosine phosphorylation was reduced by fluvastatin and some protein's tyrosine phosphorylation was rescued by mevalonate. This was representative of 2 experiments.

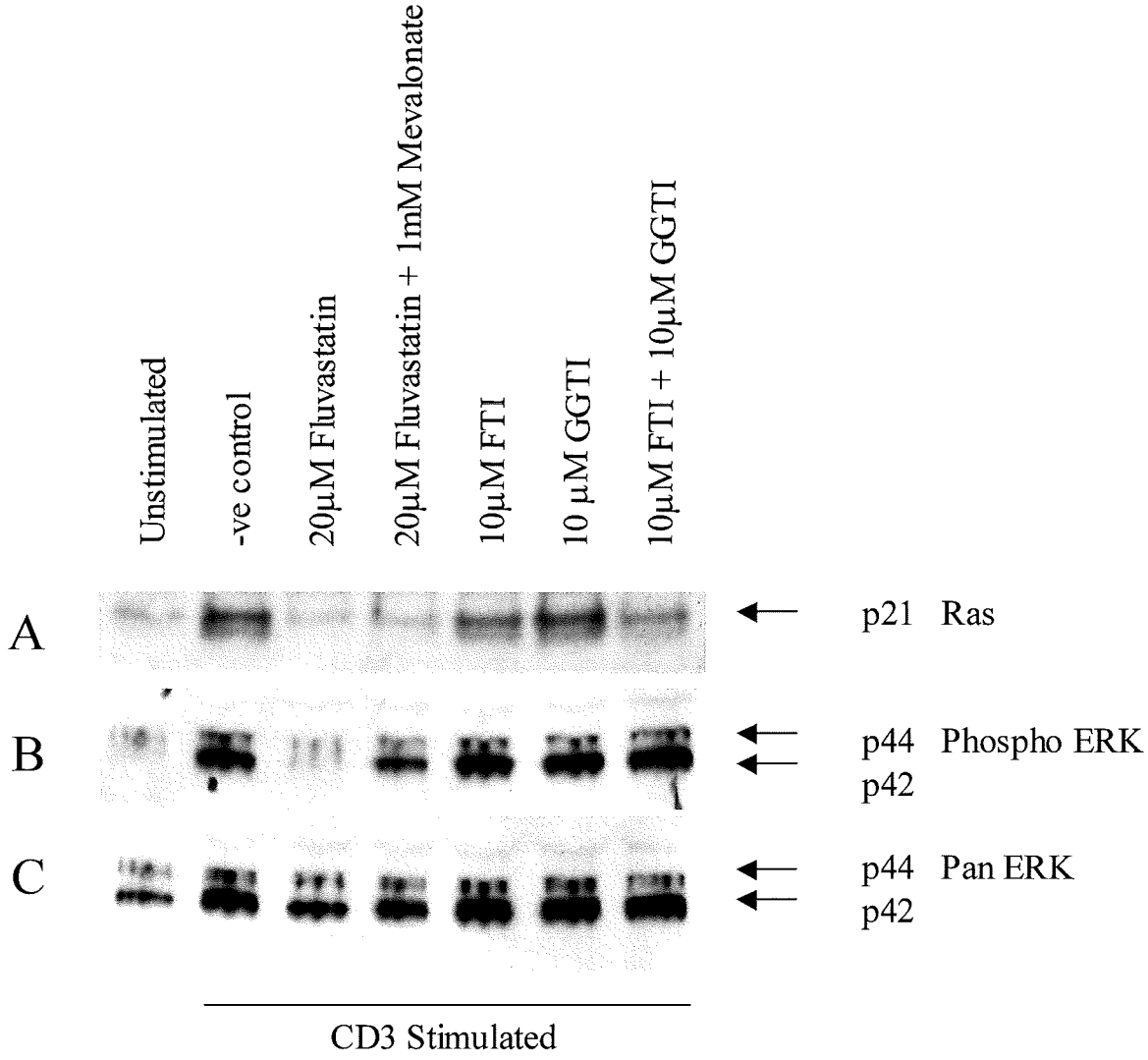


Figure 4.4 Ras activation and ERK phosphorylation was reduced in human primary T cells by fluvastatin

Human T cells were extracted from a normal volunteer and stimulated via CD3 and treated for 72 hours. (A) Ras activation was reduced by addition of fluvastatin (lane 3) and a combination of FTI I and GGTI-286, (lane 7) but not by each transferase inhibitor alone (lane 5 and 6). (B) ERK phosphorylation was also reduced by addition of fluvastatin (lane 3) but not by transferase inhibitors (lane 5-7). ERK phosphorylation could also be rescued by the addition of mevalonate (lane 4). (C) Pan ERK confirmed comparable gel loading. This blot was representative of two experiments.

individually. Ras is normally farnesylated, therefore should be unaffected by GGT inhibitors but is alternatively geranylgeranylated when treated with FTI inhibitors[21]. Consistent with this, addition of both inhibitors resulted in some reduction of Ras activation. ERK phosphorylation was correspondingly reduced by addition of fluvastatin but was unaffected by the addition of FTI, GGTI or both inhibitors together. ERK could also be rescued by mevalonate unlike Ras suggesting an alternative pathway might be involved[304, 305].

4.2.4 Rac activity assay with human primary T cells (Figure 4.5)

Rac is the isoprenylated protein that couples CD3 to the p38 stress pathway. I therefore investigated the effect of fluvastatin and prenyl inhibitors on Rac and p38. The same samples from section 4.2.3.2 were assayed for Rac activity by a Rac specific kit and Phospho p38 levels were measured by western blot using the supernatant from the ras assay. Figure 4.5 showed reduced Rac activation by addition of fluvastatin and GGTI but not FTI. Rac is normally geranylgeranylated[306], therefore would be unaffected by FT inhibitors. Consistent with this, addition of both inhibitors resulted in some reduction of Rac activation. p38 phosphorylation was correspondingly reduced by addition of fluvastatin but was unaffected by the addition of FTI, GGTI or both inhibitors together. p38 could also be rescued by mevalonate unlike Rac suggesting an alternative pathway might be involved[307].

4.2.5 Erk Phosphorylation

4.2.5.1 ERK phosphorylation in primary human T cells (Figure 4.6)

I investigated the Ras-ERK pathway in primary human T cells further by examining a time course of CD3 stimulation. CD3 ligation resulted in the sustained activation of both ERK1 and ERK2 at all time points examined (Figure 4.6). The inclusion of 20μM fluvastatin to the assay significantly reduced ERK activation in response to CD3 ligation at 24 hours, while at 48 hours and 72 hours ERK activation was almost entirely abolished. Addition of Mevalonate 18 hours

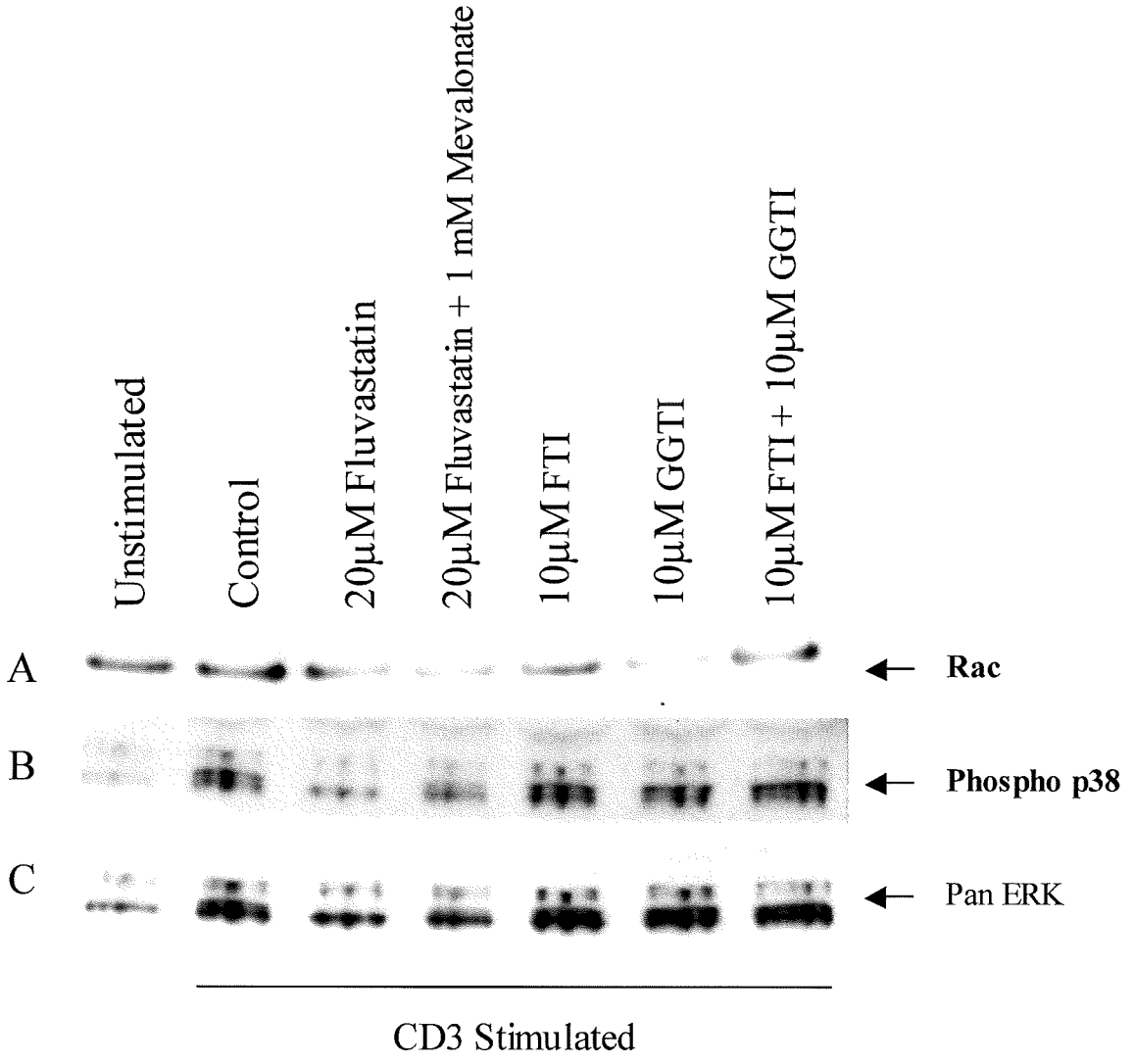


Figure 4.5 Rac activation and p38 phosphorylation was reduced by fluvastatin.

The same samples from figure 4.2.3.2 were assayed for Rac activity in the same way as Ras. (A) Rac activation was reduced by addition of fluvastatin (lane 3) and by GGTI (lane 7), but not by FTI (lane 5). (B) p38 phosphorylation was also reduced by addition of fluvastatin (lane 3) but not by transferase inhibitors (lane 5-7). p38 phosphorylation could also be rescued by the addition of mevalonate (lane 4). (C) Pan ERK confirmed comparable gel loading.

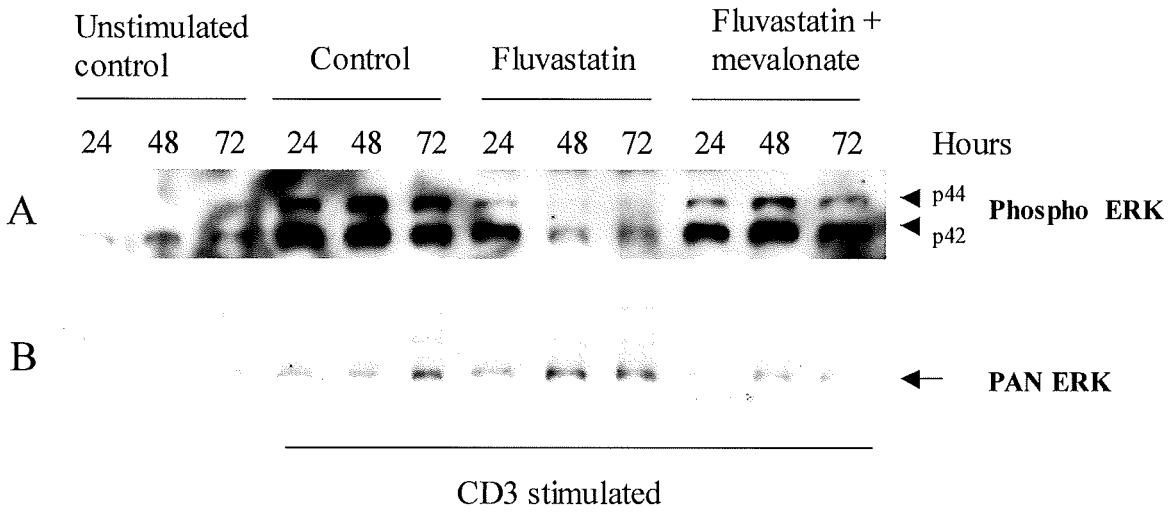


Figure 4.6 ERK phosphorylation stimulated via CD3 was reduced by fluvastatin in primary human T cells over a time course of 72 hours.

Human T cells were extracted from a normal volunteer and stimulated via CD3 over a time course of 72 hours. (A) A small reduction in ERK phosphorylation was apparent in fluvastatin treatment at 24 hours (lane 7), however a greater reduction was observed at 48 and 72 hours (lane 8 and 9). Mevalonate rescued ERK phosphorylation at all time points (lane 10-12). (B) Pan ERK confirmed equal gel loading. This blot was representative of two experiments.

prior to cell lysis completely reversed the effects of fluvastatin at all time points examined. This was consistent with the inhibitory effects of fluvastatin being mediated by reduced isoprenoid synthesis.

4.2.5.2 ERK phosphorylation in U937 monocyte cell line (Figure 4.7)

I further investigated the Ras-ERK pathway in the human monocyte cell line U937. FcγR were aggregated with IgG at 37°C for 3, 10 and 30 minutes and control U937 cells were incubated with cross-linking anti-human IgG alone. FcγR aggregation caused dual phosphorylation of both p44 and p42 ERK (Figure 4.7). No increase in phosphorylation of either kinase was seen in controls. Pre-treatment with 10μM fluvastatin for 48 hours reduced FcγR mediated activation of ERK and at all time points.

4.2.6 p38 phosphorylation

4.2.6.1 p38 phosphorylation in primary human T cells (Figure 4.8)

The Rac/Rho-p38 pathway plays a role in lymphocyte activation and cytoskeletal regulation[302]. Therefore p38 activation was assessed using a phospho-specific p38 MAP Kinase (Thr180/Tyr182) antibody. CD3 ligation resulted in a transient rise in p38 phosphorylation with highest levels observed at 10 and 30 minutes (Figure 4.8), returning to baseline by 120 minutes. This activation pattern contrasts to the sustained activation observed for ERK (Figure 4.6). As a result of this short time course of p38 activation it was necessary to culture cells with fluvastatin prior to CD3 ligation. Thus, cells were incubated with 10μM fluvastatin for 48 hours and then plated onto anti-CD3 coated plates for 0, 10, 30 and 120 minutes. Figure 4.8 demonstrated that pre-treatment of cells with fluvastatin entirely uncoupled CD3 from the p38 pathway at all time points examined. Addition of mevalonate to the cells for 18 hours prior to CD3 ligation partially restored CD3 coupling to p38 activation. Analysis of ERK activation in the same cell lysates (Fig 4.8 (C)) indicated that the ERK pathway was also uncoupled by fluvastatin over this short timecourse of stimulation though the addition of mevalonate was able to restore

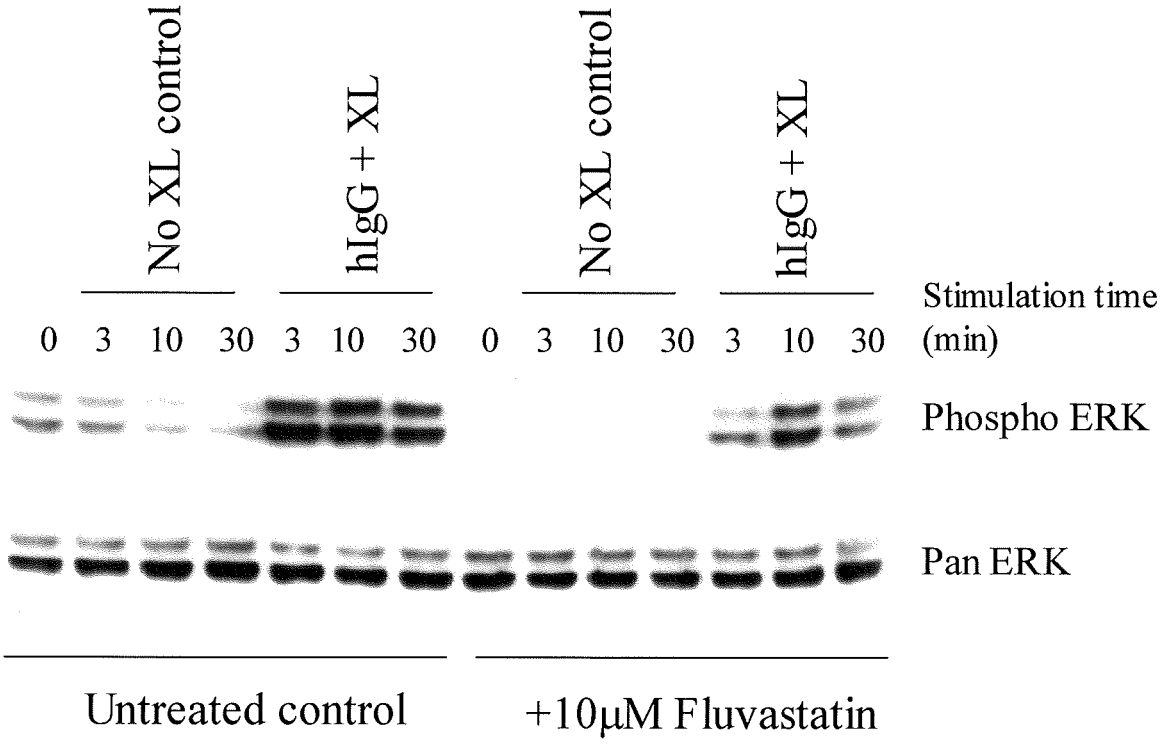


Figure 4.7 Fluvastatin blocks Fcγ receptor activation of ERK

U937 cells were cultured for 48 h in control medium or in 10 μM fluvastatin. FcγR were aggregated with human IgG followed by crosslinking goat anti-human IgG at 37°C for the times indicated (FcγR XL). Control cells were incubated with goat anti-human IgG alone (no XL control). Fluvastatin treatment reduced phospho ERK levels. This blot was reperesentative of 3 individual experiments

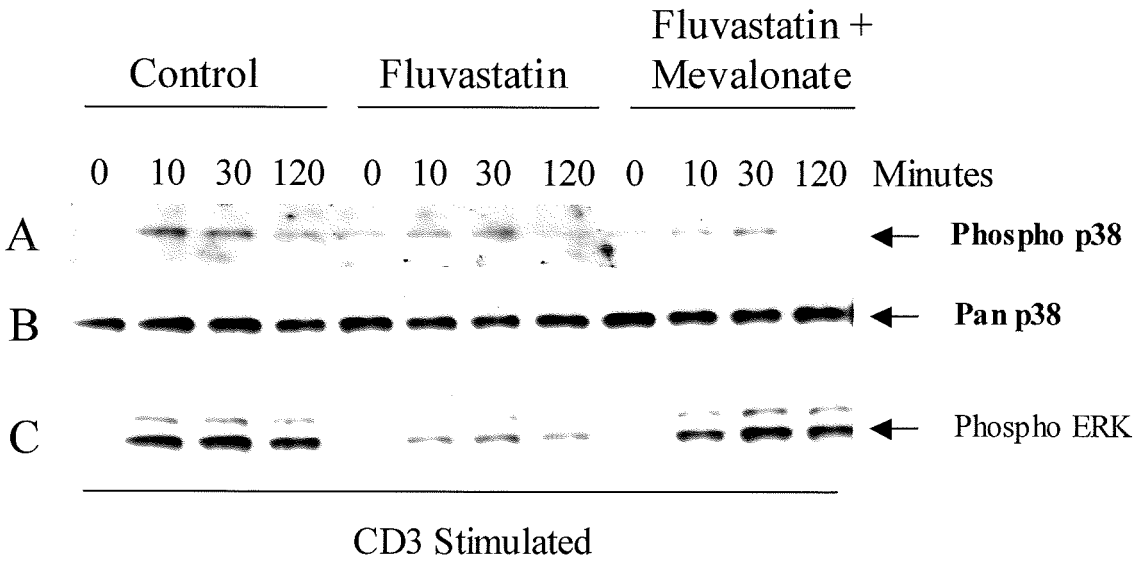


Figure 4.8 p38 phosphorylation in T cells stimulated via CD3 was reduced by addition of fluvastatin and rescued by mevalonate.

Human T cells were extracted from a normal volunteer and stimulated via CD3 over a time course of 2 hours. (A) p38 phosphorylation was apparent at 10 and 30 min but had diminished by 120 min (lane 2-4). Fluvastatin treatment reduced this phosphorylation (lane 6-8) and mevalonate rescued it (lane 10-12). (B) Pan p38 confirmed equal gel loading. (C) ERK phosphorylation was reduced at these smaller time points by being first incubated with fluvastatin before being stimulated. This blot is representative of two experiments.

ERK activation entirely. This differing sensitivity to mevalonate rescue may reflect differences in the rate of turnover of different small G-proteins. Unfortunately, my attempts to assess activation of Rac/Rho family members, which lie upstream of p38, in response to CD3 ligation proved unsuccessful due to a lack of assay sensitivity.

4.2.6.2 P38 phosphorylation in U937 monocyte cell line (Figure 4.9)

I further investigated the Rac-p38 pathway in the human monocyte cell line U937. FcγR were aggregated with IgG at 37°C for 3, 10 and 30 minutes and control U937 cells were incubated with cross-linking anti-human IgG alone. FcγR aggregation caused phosphorylation of p38 (Figure 4.9). No increase in phosphorylation was seen in controls. Pre-treatment with 10μM fluvastatin for 48 hours reduced FcγR mediated activation of p38 and at all time points.

4.3 Discussion

Several studies have suggested that statins have immunosuppressive actions[152, 308] including reduction in the severity and frequency of acute allograft rejection episodes. Fluvastatin has potential benefits in transplant patients receiving calcineurin inhibitors (CNI) since the metabolism of fluvastatin is not inhibited by the CNI, CyA or tacrolimus[255, 309, 310]. Thus, fluvastatin was investigated both for its impact on rejection and cardiovascular events in renal transplant recipients.

Statins have been shown to have a number of pleiotropic effects, specifically inhibition of proliferation of vascular smooth muscle cells and macrophage function. These effects are independent of cholesterol and are reversible by mevalonate, geranylgeranyl or farnesyl[311] implicating a role for isoprenylated proteins in this process. Many small GTPases, involved in cell proliferation pathways, require isoprenylation to enable membrane localisation. I have used specific inhibitors of geranylgeranyl and farnesyl transferase to attempt to dissect out the pathways involved in cell proliferation. I targeted ERK and p38, two MAPK pathways known to play a central role in the proliferation and activation of

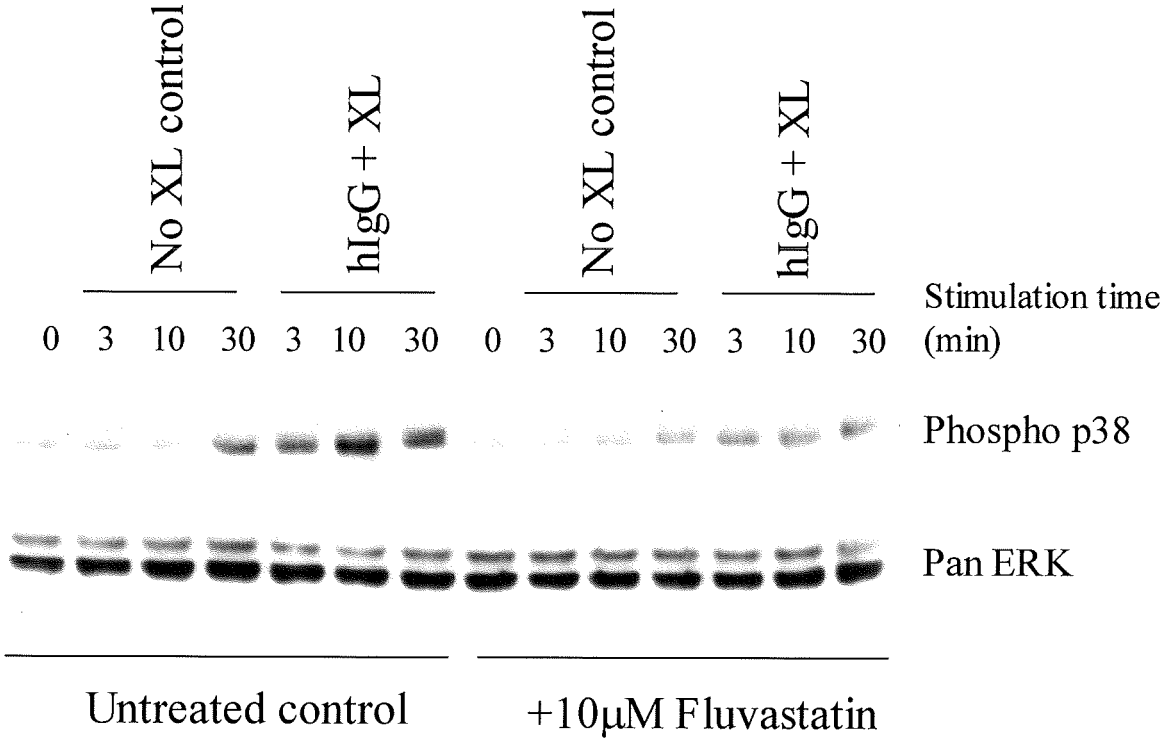


Figure 4.9 Fluvastatin blocks Fcγ receptor activation of p38

U937 cells were cultured for 48 h in control medium or in 10 μM fluvastatin. FcγR were aggregated with human IgG followed by crosslinking goat anti-human IgG at 37°C for the times indicated (FcγR XL). Control cells were incubated with goat anti-human IgG alone (no XL control). Fluvastatin treatment reduced phospho p38 levels. This blot was reperesentative of 2 individual experiments.

lymphocytes[312] both of which are reliant on small isoprenylated GTPases for activation; ERK on Ras and p38 on rho/rac.

ERK activation by anti-CD3 cross-linking is evident within minutes and persists for up to 72 hours. This effect is abrogated at 24 hours in fluvastatin treated cells (figure 4.6) and abolished at 48 and 72 hours. Activation is completely restored by inclusion of both statin and mevalonate ("mevalonate rescue") consistent with the effects being mediated by isoprenoids. Figure 4.6 also shows two molecular weight subfractions of phospho ERK (p44 ERK1 and p42 ERK2), the larger of which is less abundant. In keeping with the known dependence of ERK on upstream Ras activation, CD3 stimulation results in increased p21 Ras expression that is significantly reduced by fluvastatin pre-treatment (Figure 4.4). In these experiments, 20 μM fluvastatin was used because this provided clearer results, although a similar pattern was seen with 5 μM fluvastatin.

Another pathway involved in lymphocyte function is the p38 MAP kinase pathway, downstream of the rho/rac family of GTPases. I found that fluvastatin significantly reduced phospho p38 production in response to CD3 stimulation. Mevalonate rescue was less clearly demonstrated for p38 MAP kinase. This is likely to reflect differences in the susceptibility of different pathways to mevalonate rescue, reflecting the different G protein turnover rates between Ras and Rac/Rho. The time course of changes in the phosphorylation of p38 was much shorter than ERK, lasting a few hours rather than several days. The observation that the inhibitory effects of statins take many hours to develop is consistent with intracellular depletion of substrate; the time course of mevalonate rescue consistent with re-synthesis of substrate. Thus, the limited recovery may reflect the short time-course of activation of p38 in T cells.

Overall, the data demonstrated that exposure to fluvastatin *in vitro* is associated with reduction in ERK and p38 activity, consistent with inhibition of isoprenylation of G proteins involved in signal transduction. The time scale of the observed effects suggested that depletion of substrate is important and may explain the limited effects observed *in vivo* (chapter 3), at least in stable patients. It may be that under circumstances where there was more active disease, and faster cell turnover, such as during acute allograft rejection [308] that substrate

supply is limited. It remains to be seen whether changes in signal transduction can be seen in vivo in lymphocytes and other immune-mediator cells, and in other cell types (e.g. vascular smooth muscle cells) involved in atherosclerosis. The availability of specific inhibitors of geranylgeranyl and farnesyl transferase, some of the latter are in clinical trials, offers a novel approach. The combination of these agents with statins may allow the combination of depletion of isoprenoids and inhibition of enzymatic isoprenylation, with numerous potential applications including immunosuppression.

Chapter 5: Effects of statins on lipid rafts.

5.1 Introduction

Chapter 4 explored the effect of reduced prenylation of small GTPase molecules on signalling pathways. However, an alternative explanation for the pleiotropic effects of statins is due to disruption, or depletion, of cholesterol rich membrane micro-domains (membrane rafts)[66, 279]. The reduced density of rafts limits the function of receptors localised to these domains.

NKG2D inhibitory receptors are excluded from lipid rafts and prevent formation of activation signalling complexes. Therefore reduction of lipid rafts by statins maintain NK cells in a non cytotoxic state[56].

FcγR are activated when clustered at the cell surface by IC, and signal through an immunoreceptor tyrosine based activation motif (ITAM)[65]. Aggregated FcR are recruited to cholesterol and sphingolipid rich plasma membrane rafts as a platform for signal transduction[66, 67, 69], resulting in phosphorylation of tyrosine residues within associated ITAMs and recruitment of cytoplasmic signalling molecules including Syk tyrosine kinase, PI3-kinase complex, PLC-γ and LAT (Linker for Activation of T-cells). Importantly, LAT is targeted to rafts by palmitoylation rather than isoprenylation. Disruption of lipid rafts by statins would therefore interfere with FCγR signalling pathways.

Since tyrosine kinase activation is independent of prenylated proteins, I hypothesised that statins may limit FcγR and NK inhibitory receptor signalling and function by disrupting membrane rafts. The aim of this chapter was to examine the disruption of formation of lipid rafts by cholesterol depletion whether by statin or MβCD. I examined the effect of fluvastatin in a dose dependent manner on rafts in the U937 human monocyte cell line, primary human lymphocytes, and NK92MI cells. I also examined the effect of FTI and GGTI to assess the effect of prenylation on raft disruption as well as the effect of apoptosis. Raft disruption may provide a further mechanism by which statins may exert their

immunomodulatory effects. This mechanism is likely to apply to other immune receptors that signal through membrane rafts, including the T cell and B cell receptors.

5.2 Results

5.2.1 Raft fractions

5.2.1.1 Protein measurement of raft fractions (Figure 5.1)

Resting U937 cells and U937 cells treated with 10μM fluvastatin were subjected to sucrose density centrifugation to isolate rafts. Ten 400μl fractions were collected and total protein content measured by Bradford's assay (Figure 5.1). Fluvastatin reduced the total protein levels in the raft fractions (3-5).

5.2.1.2 Western blot of raft fractions (Figure 5.2)

As a marker of cellular membrane raft integrity I assessed the distribution of LAT, a 36kD phosphoprotein that is palmitoylated and localised to rafts independent of receptor engagement[69]. I also examined the distribution of the src tyrosine kinase, Lyn which is myristoylated[313] and associates with rafts. Proteins were resolved by SDS-PAGE and western blotting of sucrose gradient fractions. Fractions 8-10 represent Triton X-100 soluble proteins, while fractions 3-5 correspond to low-density lipid rafts. In resting U937 cells, both LAT and Lyn were present within the Triton X-100 soluble fractions and the raft fractions (Figure 5.2, panel 1 and 3) but pre-treatment with 10μM fluvastatin for 48 hours caused a substantial reduction in raft associated LAT and Lyn (Figure 5.2, panel 2 and 4). The transferrin receptor is excluded from lipid rafts and was unaffected by fluvastatin treatment (Figure 5.2 panel 5.and 6).

5.2.2 FACS of cell surface receptors on U937 cells (Figure 5.3)

To determine whether changes in receptor expression could account for the inhibitory effects of statins, flow cytometry was used to examine U937 cell

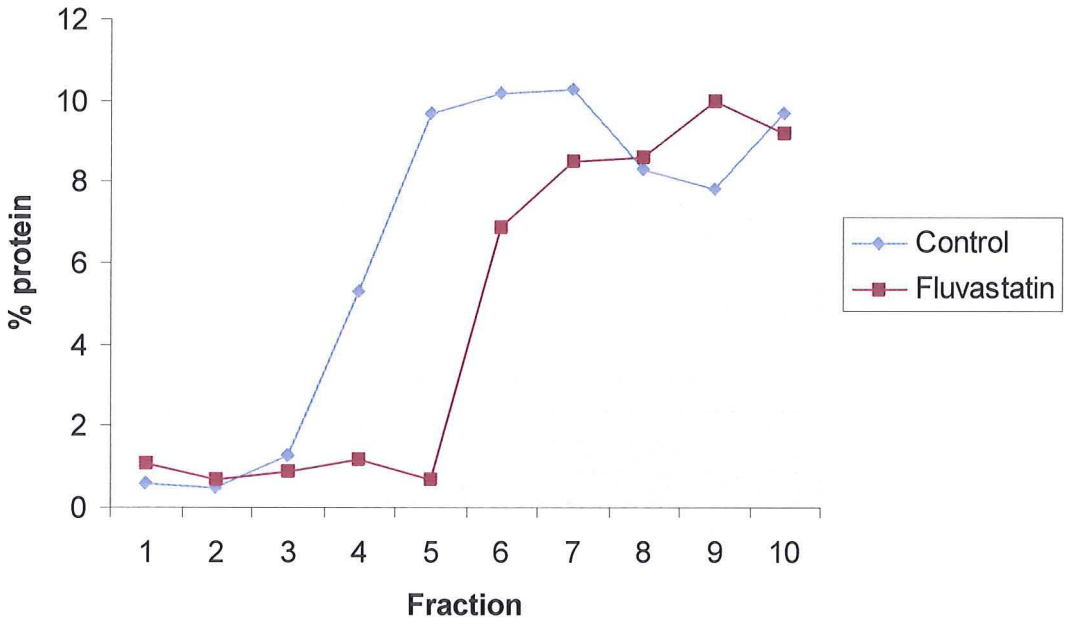


Figure 5.1 Measurement of proteins within sucrose density gradient of U937 cells.

U937 cells treated with and without 10μM fluvastatin were subjected to sucrose density centrifugation and the protein content from 400μl fractions measured by Bradford's assay. Protein levels were expressed as percentage of total protein. Rafts were expected to be around fraction 4 and fluvastatin reduced the total protein content within this fraction.

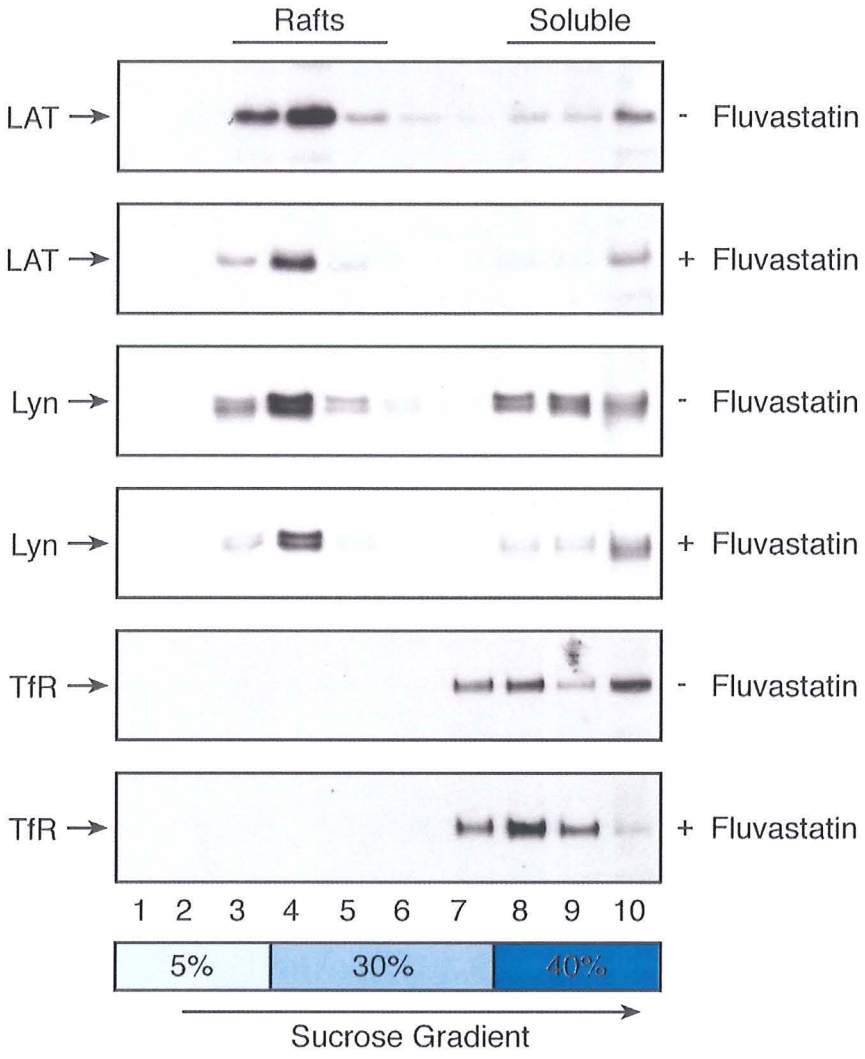


Figure 5.2 Fluvastatin disrupts plasma membrane rafts in resting U937s

Fluvastatin treated and untreated resting U937 cells were lysed in 1% Triton X-100 MNE buffer and subjected to sucrose gradient centrifugation. Fractions of 450µl were taken from the top, numbered 1-10 and western blotted for LAT, Lyn and TfR. This was representative of 3 experiments.

expression levels of FcγRI, FcγRII, FcγRIII and cell binding of FITC conjugated IgG (Table 1). Cells were found to express FcγRI and FcγRII but not FcγRIII. Following treatment with 10μM fluvastatin or 10μM simvastatin for 48 hours, the expression of FcγRI, FcγRII and binding of IgG-FITC were not altered. Therefore, the inhibitory effects of statins on FcγR signalling were not due to reduced cell surface receptor expression. Expression of the human transferrin receptor, used as a control, was similarly unchanged (Figure 5.3).

5.2.3 Induction of Apoptosis in U937 cells

I investigated whether fluvastatin or simvastatin were capable of inducing significant necrosis or apoptosis in U937 cells to exclude these as explanations for the disruption of lipid rafts. Trypan blue staining of cells following statin treatment over the full dose range showed greater than 99% cell viability. Apoptosis was assessed by two methods: morphological examination and caspase activity.

5.2.3.1 Morphological examination

Morphological examination of stained U937 cells following statin treatment was performed in triplicate by counting a minimum of 500 cells per slide. The ability of statins to induce apoptosis of U937 cells was weak and appeared to be dose dependent with 4.6% of cells showing apoptotic nuclei after treatment with 10μM fluvastatin. The corresponding figure for treatment with 5μM simvastatin was 7.6%.

5.2.3.2 Caspase activity

I also used an assay for caspase activity as a marker of apoptosis and this showed consistent results at 5.6% and 6.2% respectively following 10μM fluvastatin and 5μM simvastatin.

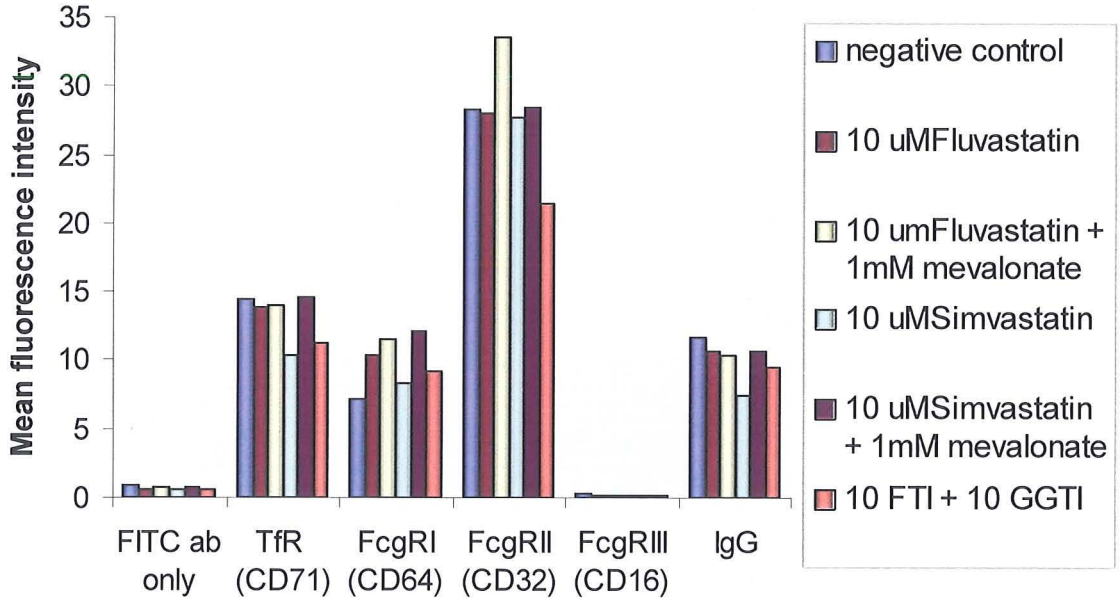


Figure 5.3 Relative Fcγ receptor expression and IgG-FITC binding of U937 cells.

Receptor expression was determined by flow cytometry. Statins or transferase inhibitors did not affect receptor levels. This was representative of 2 experiments.

5.2.4 Effect of a range of statin concentrations on rafts

5.2.4.1 U937 (Figure 5.4)

U937 cells were treated with a range of concentrations of fluvastatin for 48 hours. Disruption of cell rafts by fluvastatin appeared to be dose dependent with concentrations above 0.5μM reducing levels of both LAT and Lyn in raft fractions, with maximal reduction observed at concentrations of 5μM and above (Figure 5.4).

5.2.4.2 Primary human Leukocytes (Figure 5.5)

Primary human leukocytes obtained from a normal volunteer were also treated with a range of fluvastatin concentrations for 48 hours. Although it is not clear from the Western blot (Figure 5.5 A) that primary cells behaved the same as U937s, after densitometry (Figure 5.5 B) and the ratio of rafts to total cell protein calculated, a similar pattern emerged.

5.2.4.3 NK92MI (Figure 5.6)

NK92MI cells were treated with a range of concentrations of fluvastatin or simvastatin for 48 hours. Disruption of cell rafts by either statin appeared to be dose dependent with concentrations above 5μM reduced levels of LAT and Fyn with fluvastatin and LAT and Lyn with simvastatin in raft fractions (Figure 5.6)

5.2.5 Effect of FTI, GGTI and mevalonate rescue on rafts

5.2.5.1 U937 (Figure 5.7)

To investigate whether raft disruption was a result of HMG-CoA reductase inhibition, I examined the effect of mevalonate rescue. U937 cells or were incubated for 48 hours in 10μM fluvastatin to maximally deplete raft fractions of LAT and Lyn (Figure 5.7). Addition of 1mM mevalonate for 12 hours prior to raft fractionation reconstituted levels of Lyn and LAT to resting cell levels. Significantly, incubation of U937 cells with a combination of 10 μM FTI and 10 μM

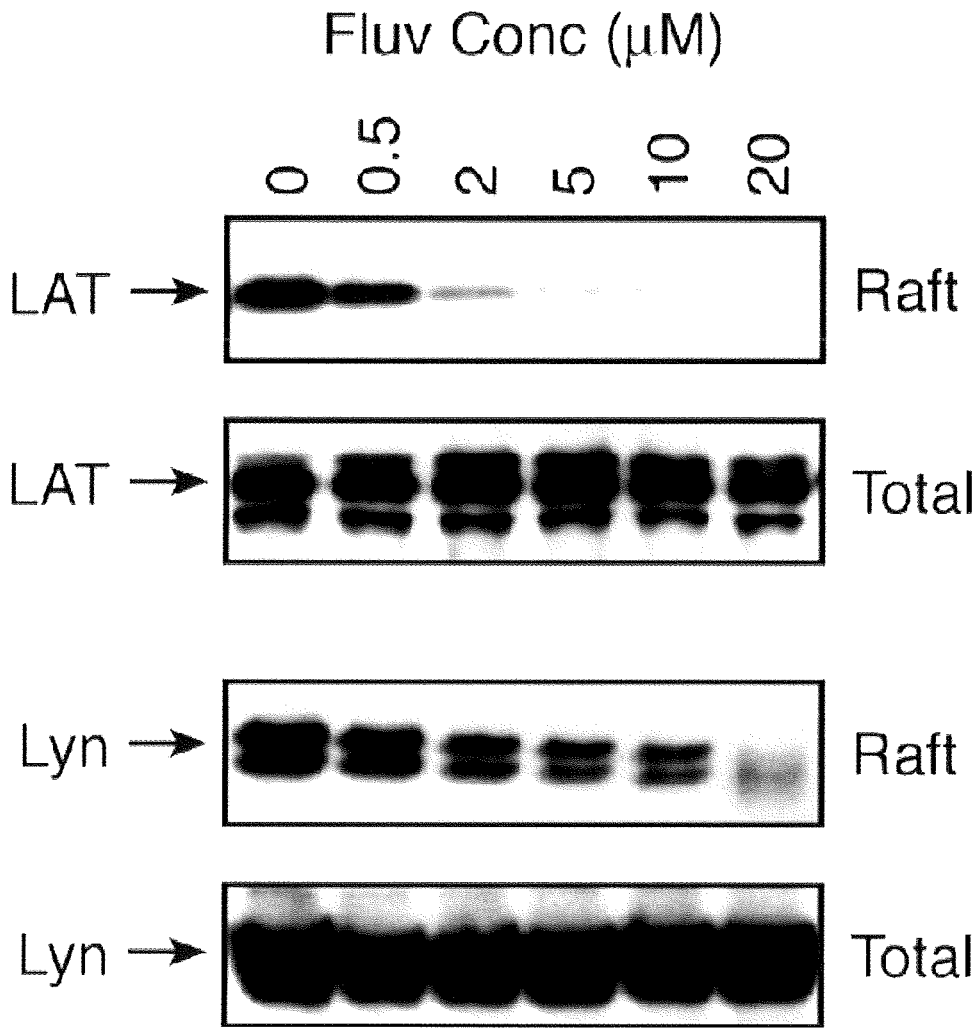


Figure 5.4 A range of fluvastatin concentrations disrupts rafts in U937 cells.

Pooled raft fractions from resting U937 cells treated with increasing concentrations of fluvastatin were western blotted and probed for LAT and Lyn (upper panels). Lower panels compare expression levels in whole cell lysates. This was representative of 2 experiments.

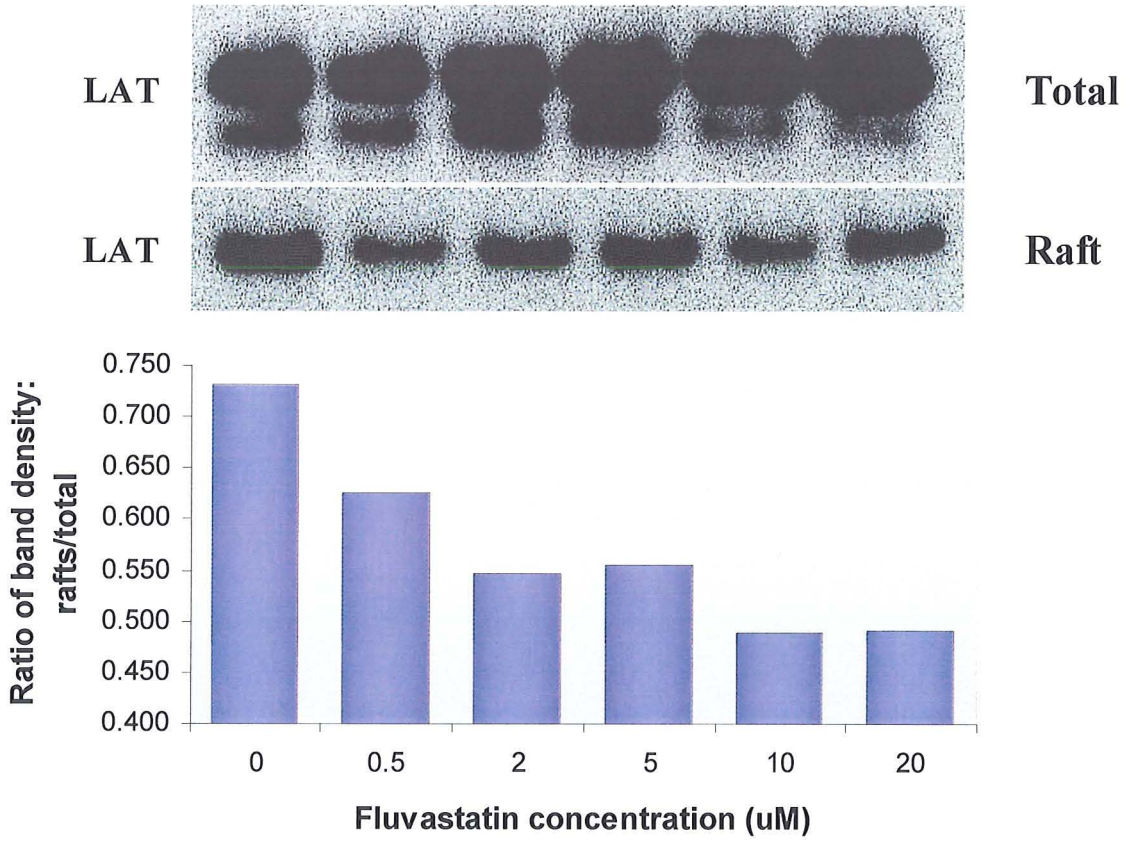


Figure 5.5 A range of fluvastatin concentrations disrupts rafts in primary human leukocytes.

Pooled raft fractions from resting primary human leukocytes treated with increasing concentrations of fluvastatin were western blotted and probed for LAT Densitometry allowed calculation of the ratio of raft LAT levels to whole cell LAT levels to give a comparable reduction in raft levels as U937 cells.

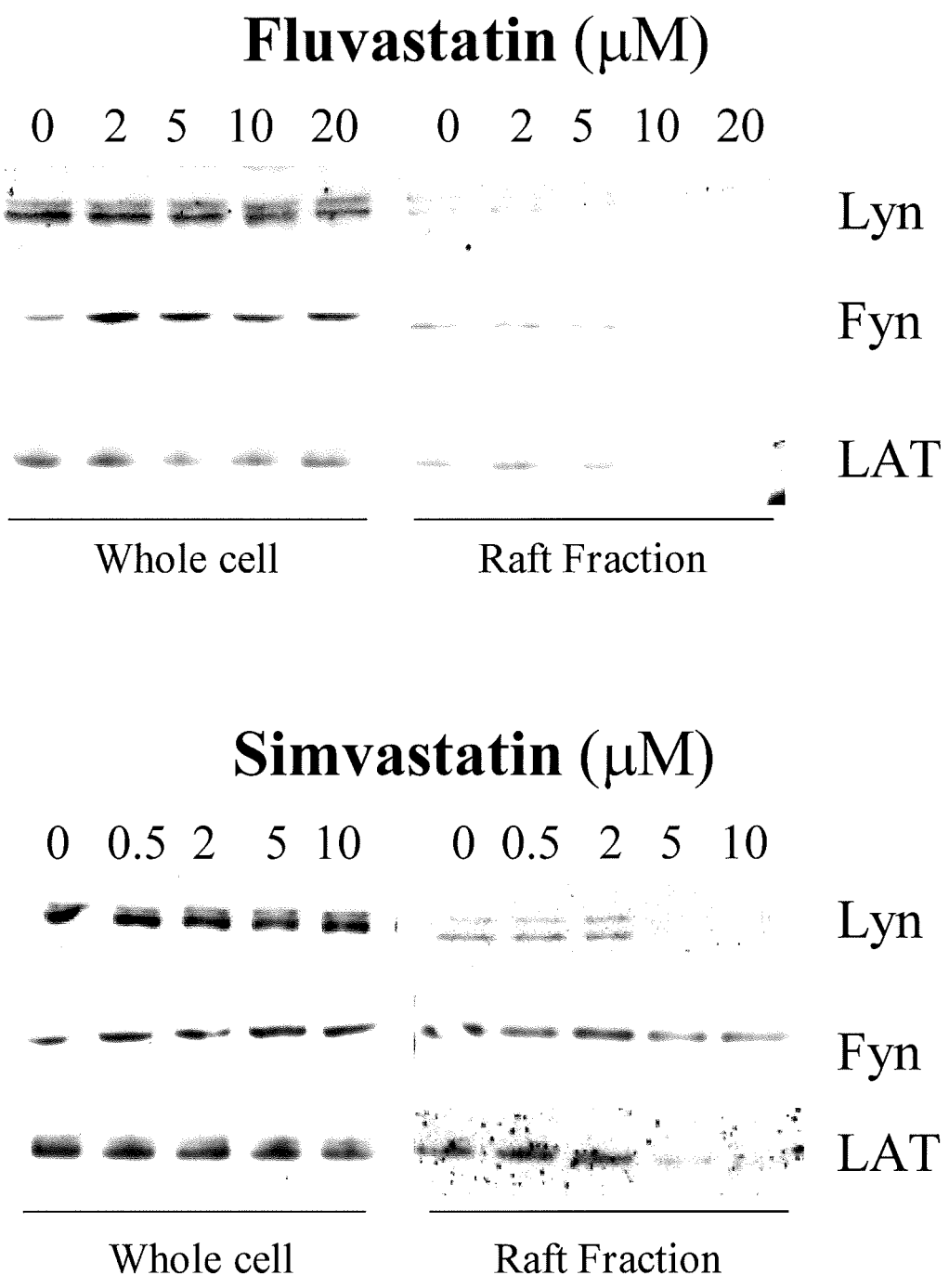


Figure 5.6 A range of fluvastatin and simvastatin concentrations disrupts rafts in NK92MI cells.

Pooled raft fractions from resting NK92MI cells treated with increasing concentrations of fluvastatin or simvastatin were western blotted and probed for Lyn, Fyn and LAT (panels on right). Panels on the left compare expression levels in whole cell lysates.

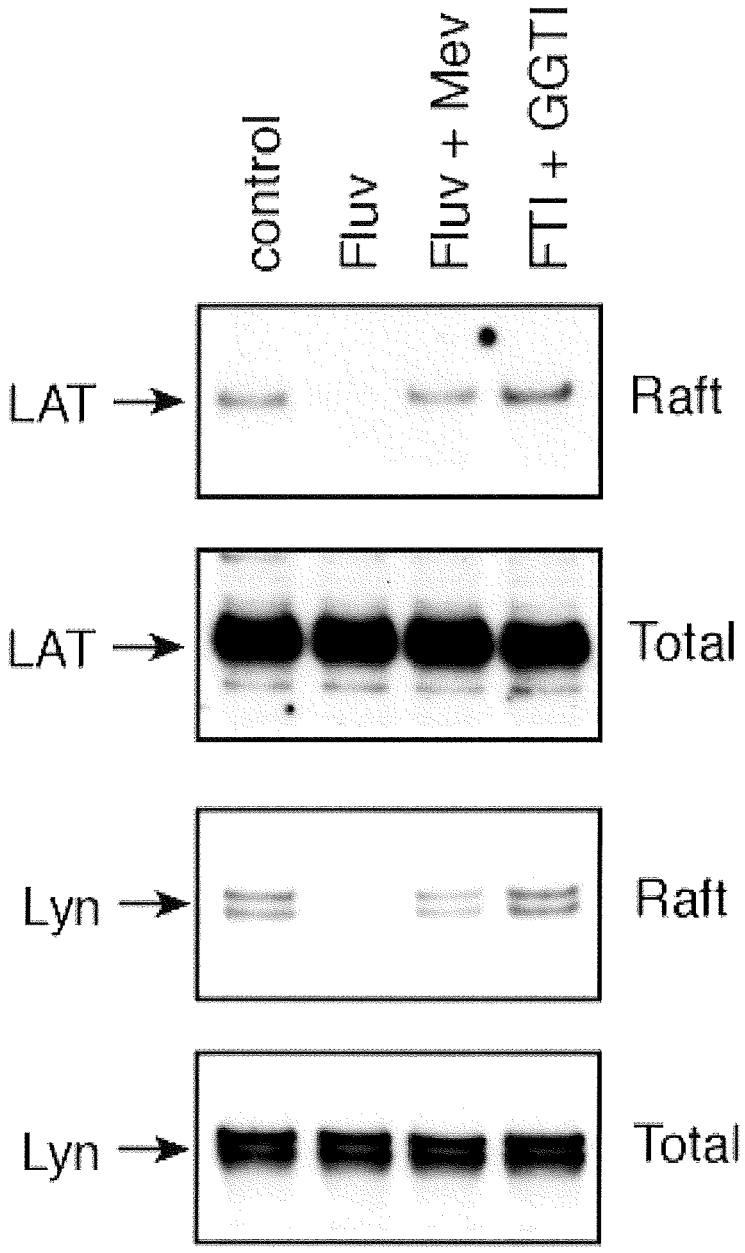


Figure 5.7 Disruption of rafts by fluvastatin in U937 cells was rescued by 1mM mevalonate and unaffected by FTI and GGTI.

Pooled raft fractions from U937 cells treated for 48 hours with 10μM fluvastatin, or 10μM fluvastatin with 1mM mevalonate added in the final 12 hours, were western blotted for LAT and Lyn. The disruption to raft levels by fluvastatin was rescued by the addition of mevalonate. Treatment with 10μM FTI and 10μM GGTI had no effect on raft levels. This was representative of 2 experiments.

GGTI had no measurable effect on either LAT or Lyn raft content. Taken together, these data imply that inhibition of cholesterol synthesis by fluvastatin and not isoprenoid synthesis is responsible for membrane raft disruption.

5.2.5.2 Primary human leukocytes (Figure 5.8)

Primary human leukocytes were also incubated for 48 hours in 10μM fluvastatin and rescued with 12 hours of 1mM mevalonate (Figure 5.8), which was comparable with U937.

5.2.5.3 NK92MI (Figure 5.9)

NK92MI cells were treated in the same way as U937 cells, again with comparable results (Figure 5.9).

5.2.6 Effect of Methyl beta cyclodextrin on rafts (Figures 5.10 and 5.11)

Methyl-β-cyclodextrin (MβCD) is a cyclic oligosaccharide consisting of 7 glucopyranose units, which form a rigid doughnut shape (Figure 5.10). Hydrophobic molecules are incorporated into the cavity by displacing water, rendering the molecule water-soluble. When the water-soluble complex is diluted in a much larger volume of aqueous solvent, the process is reversed, thereby releasing the molecule of interest into the solution. MβCD is efficient in solubilizing cholesterol. At high concentrations (5-100mM), can remove cholesterol at phenomenal rates (300-400%/hour). At lower concentrations, MβCD can serve as cholesterol shuttles, transporting cell membrane cholesterol to serum lipoproteins[314].

NK92MI cells were treated with low (0.5mM) and high (10mM) concentrations of MβCD for 1 hour to assess the effect of cholesterol influx and efflux on rafts. My hypothesis was that cholesterol movement into the membrane might increase raft levels while movement out of the membrane would decrease raft levels. Cholesterol influx with the lower concentration of MβCD did not increase Lyn, Fyn

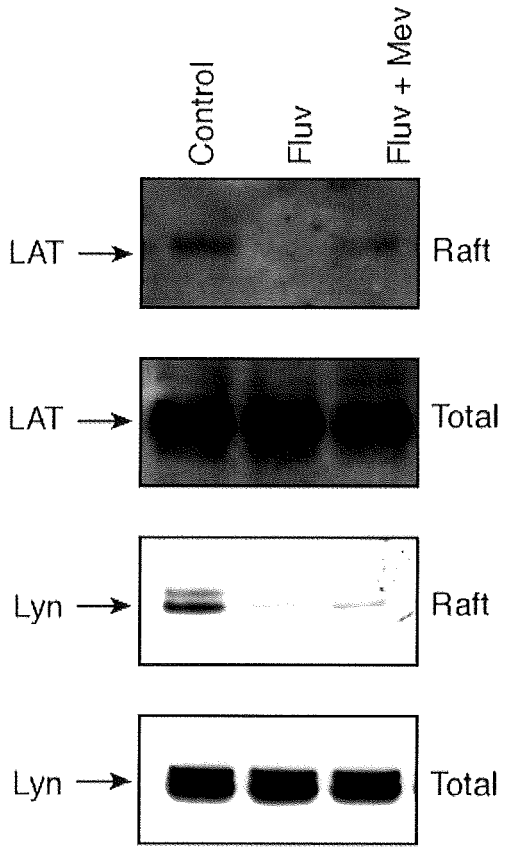


Figure 5.8 Disruption of rafts by fluvastatin in primary human leukocytes was rescued by 1mM mevalonate.

Pooled raft fractions from primary human leukocytes treated for 48 hours with 10μM fluvastatin, or 10μM fluvastatin with 1mM mevalonate added in the final 12 hours, were western blotted for LAT and Lyn. The disruption to raft levels by fluvastatin was rescued by the addition of mevalonate.

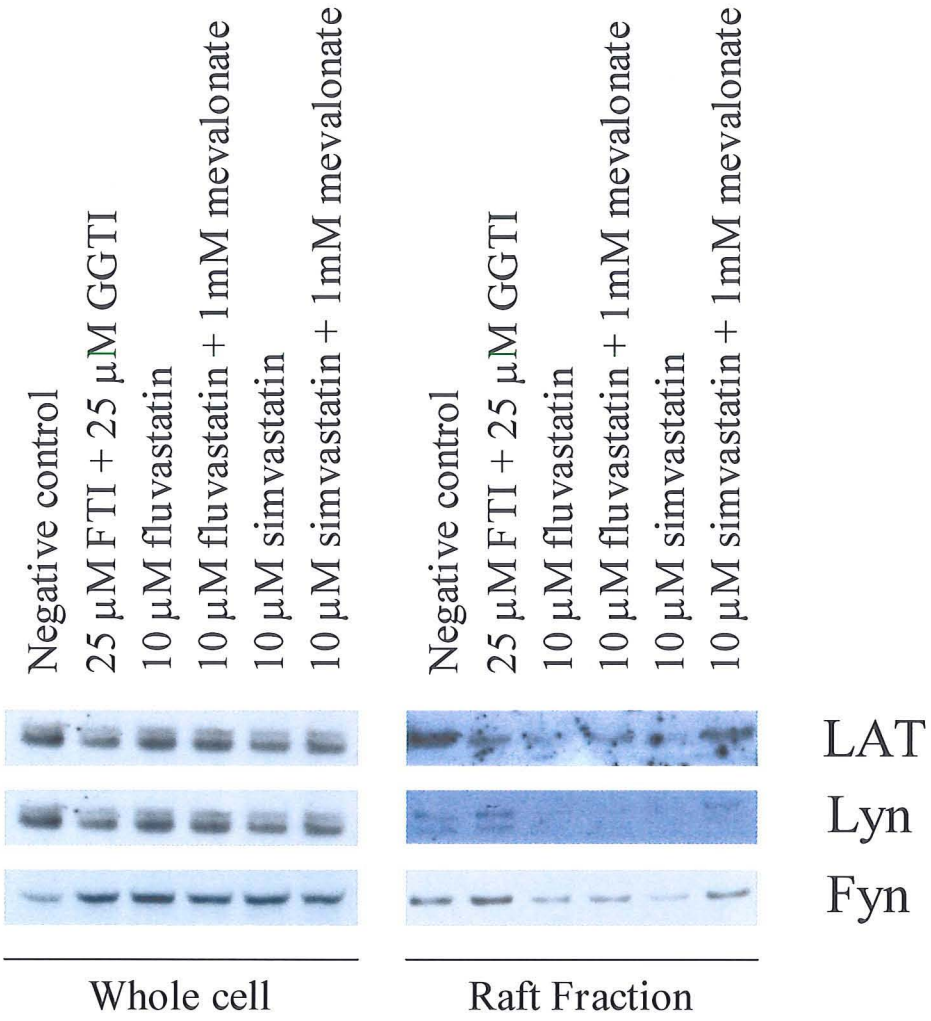


Figure 5.9 FTI and GGTI do not affect rafts and mevalonate rescues statin disruption of rafts

Pooled raft fractions from resting NK92MI cells treated with GGTI plus FTI, fluvastatin or simvastatin were western blotted and probed for LAT, Lyn and Fyn (panels on right). Panels on the left compare expression levels in whole cell lysates.

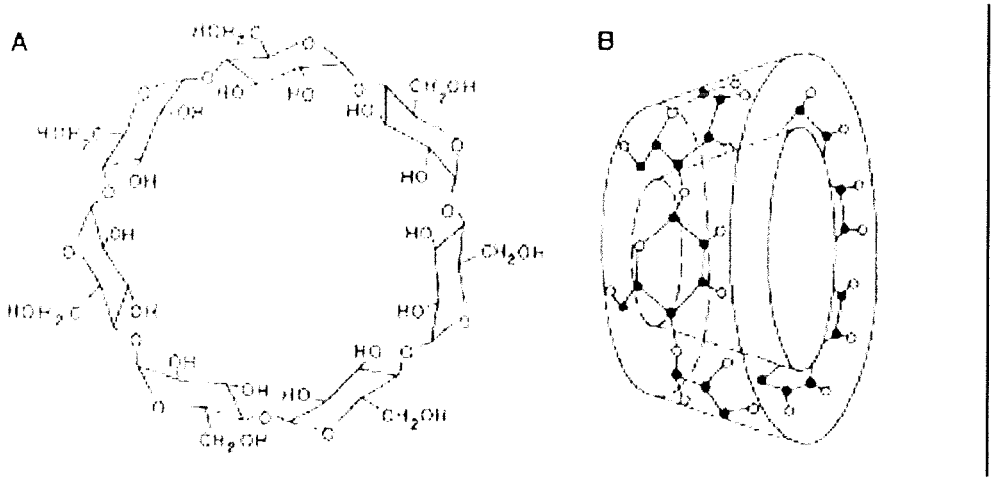


Figure 5.10 The chemical structure (A) and the toroidal shape (B) of the β-cyclodextrin molecule.

7 α-1,4 -linked glucopyranose units form a cone with a hydrophilic outer surface and a lipophilic cavity in the centre.

or LAT levels and therefore had no effect on raft levels. However cholesterol efflux with the higher concentration of MβCD reduced the levels of Lyn, Fyn and LAT and therefore rafts (Figure 5.11).

5.3 Discussion

The mechanisms by which statins exert their effects remains controversial and may be in part independent of changes in serum cholesterol[183, 300]. Current favoured explanations of their mechanism of action have focused on inhibition of isoprenoid biosynthesis. This chapter suggests that inhibition of receptor-mediated cellular responses by statins may also be due to blockade of cholesterol biosynthesis and subsequent disruption of plasma membrane lipid raft function.

Membrane rafts are critical to initiation of signal transduction by many ITAM bearing immune receptors, including FcγR and the B and T cell antigen receptors[279, 315]. Following ligation, receptors translocate into rafts where they co-localise with signal transduction molecules, such as src kinases, adapter proteins and PLC-γ. Since raft structure and function depends on cholesterol, disruption of rafts provides a potential novel mechanism to explain the actions of statins on FcγR signalling. Previous studies have shown that chemical extraction of membrane cholesterol with methyl-β cyclodextrin disrupts lipid rafts and associated receptor mediated signal transduction[279, 315]. In this model system I found that changes in membrane raft content following treatment of cells with statin did not reflect either total cellular or cell supernatant cholesterol levels, and therefore inhibition of cholesterol biosynthesis may reduce cholesterol within specific cellular compartments independently of externally available cholesterol. I showed that reductions in the lipid raft associated signalling proteins LAT, Fyn and Lyn was reduced in a dose dependent manner by low levels of statin (0.5μM) that are representative of *in vivo* concentrations. Disruption of membrane rafts was not seen following treatment of cells with inhibitors of prenylation, supporting the hypothesis that the effect of statins are independent of prenylated proteins.

Cell surface receptors numbers were unaltered by statins, therefore the inhibitory effects of statins on FcγR signalling were not due to reduced cell surface receptor

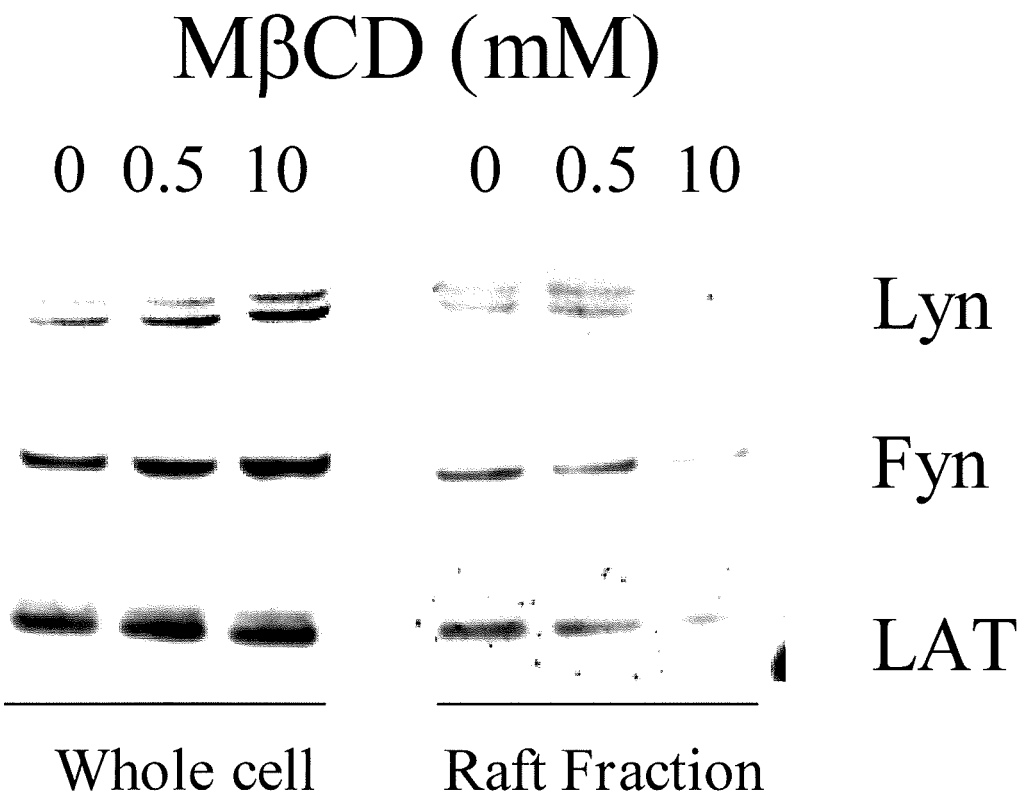


Figure 5.11 High concentration of MβCD disrupts rafts in NK92MI cells.

Pooled raft fractions from resting NK92MI cells treated with MβCD were western blotted and probed for LAT, Lyn and Fyn (panels on right). Panels on the left compare expression levels in whole cell lysates.

expression. The degree of apoptosis induced by statins was minimal and could not account for the changes seen in signal transduction in the whole cell population. Fluvastatin has been reported to enhance apoptosis in vascular smooth muscle cells but only in the presence of exogenous IL-1β[316].

Since other multichain immune recognition receptors (e.g. BCR, TCR) signal through membrane rafts, this mechanism is likely to have broad significance in the immune system and elsewhere[317].

Chapter 6: Functional effects of statins on monocytes

6.1 Introduction

The inflammatory nature of atherosclerotic lesions is well established[128]. These lesions contain high levels of immune complexes, including oxidised low density lipoprotein immune complexes (oxLDL-IC), and macrophages[318]. Monocyte-macrophage Fc receptor activation by immune complexes triggers the production of inflammatory cytokines, metalloproteinases[319] and superoxide generation, an effect that may be inhibited by statins[294, 320, 321]. However, although IC-mediated cell activation has been implicated in the pathogenesis of atherosclerosis, a role for statins in the inhibition of FcγR mediated inflammatory responses had not been investigated, nor had the potential effects of statins on other aspects of mononuclear cell function.

The aim of this chapter was to establish the effect of statins on FcR IC activation in monocytes. I examined the effect of statins on tyrosine phosphorylation plus Erk and p38 signalling pathways (as confirmed in T cells in chapter 4). I then examined the effect of statins on functional responses in the U937 human monocyte cell line[281] by measuring immune complex trafficking and degradation, cytokine release and MMP-1 production. Since tyrosine kinase activation is independent of prenylated proteins, I hypothesised that statins may limit FcγR signalling and function by disrupting membrane rafts (as shown in chapter 5) and therefore reducing release of the inflammatory cytokines and MMP-1, which are found in atherosclerotic lesions. Thus inhibition of prenylation, and more likely disruption of membrane rafts, in monocytes may contribute to the anti-atherosclerotic effects of statins.

6.2 Results

6.2.1 Statins impair Fcγ receptor initiated tyrosine phosphorylation

6.2.1.1 Fluvastatin impairs Fcγ receptor initiated tyrosine phosphorylation (Figure 6.1)

Aggregation of FcγR on U937 cells caused transient tyrosine phosphorylation that peaked at 1-2 minutes and normalised by 10 minutes. Pre-treatment for 48 hours with fluvastatin (10μM) reduced maximal tyrosine phosphorylation by 60% without altering the pattern of phosphorylation (Figure 6.1).

6.2.1.2 Dose dependent inhibition of tyrosine phosphorylation by fluvastatin (Figure 6.2)

U937 cells were cultured for 48 hours in the presence of increasing concentrations of fluvastatin and FcγR crosslinked for 1.5 min. Inhibition of tyrosine phosphorylation was dose dependant (Figure 6.2 A and 6.2 B) and maximal at 5-20μM.

6.2.1.3 Time dependant inhibition of tyrosine phosphorylation by fluvastatin (Figure 6.3)

Inhibition of FcγR responses was also time dependent and required pre-incubation for 48 hours with 10μM fluvastatin, with little inhibition observed after 24 hours (Figure 6.3). To determine whether fluvastatin inhibits FcγR responses via inhibition of HMG-CoA reductase, mevalonate was added to fluvastatin treated cells for the final 12 hours ("mevalonate rescue"). Thus, 1mM mevalonate entirely reversed the effects of fluvastatin on tyrosine phosphorylation.

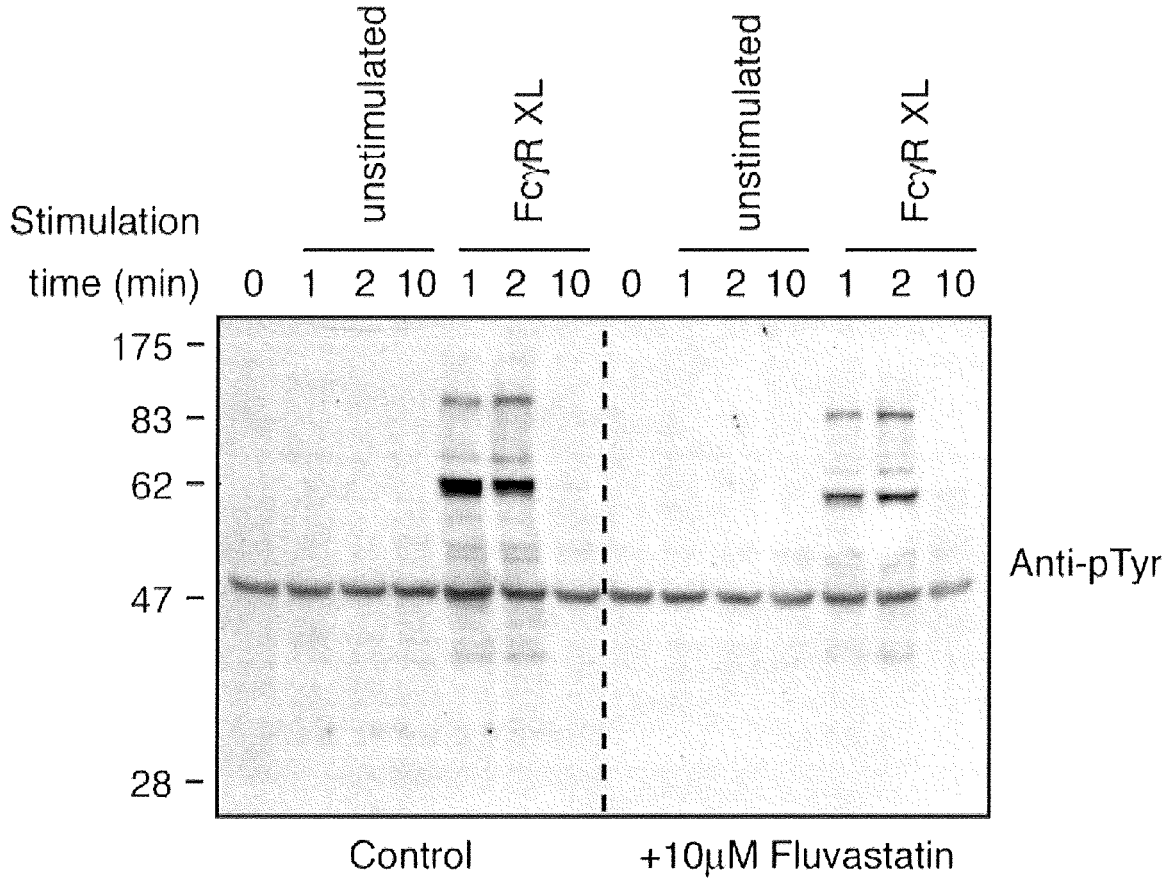


Figure 6.1 Characterisation of tyrosine phosphorylation inhibition by fluvastatin.

U937 cells were cultured for 48 h in control medium or fluvastatin. FcγR were crosslinked with human IgG and goat anti-human IgG at 37°C. Tyrosine phosphorylated proteins were detected by western blotting with anti-phosphotyrosine antibody. Fluvastatin treatment reduced levels of tyrosine phosphorylation.

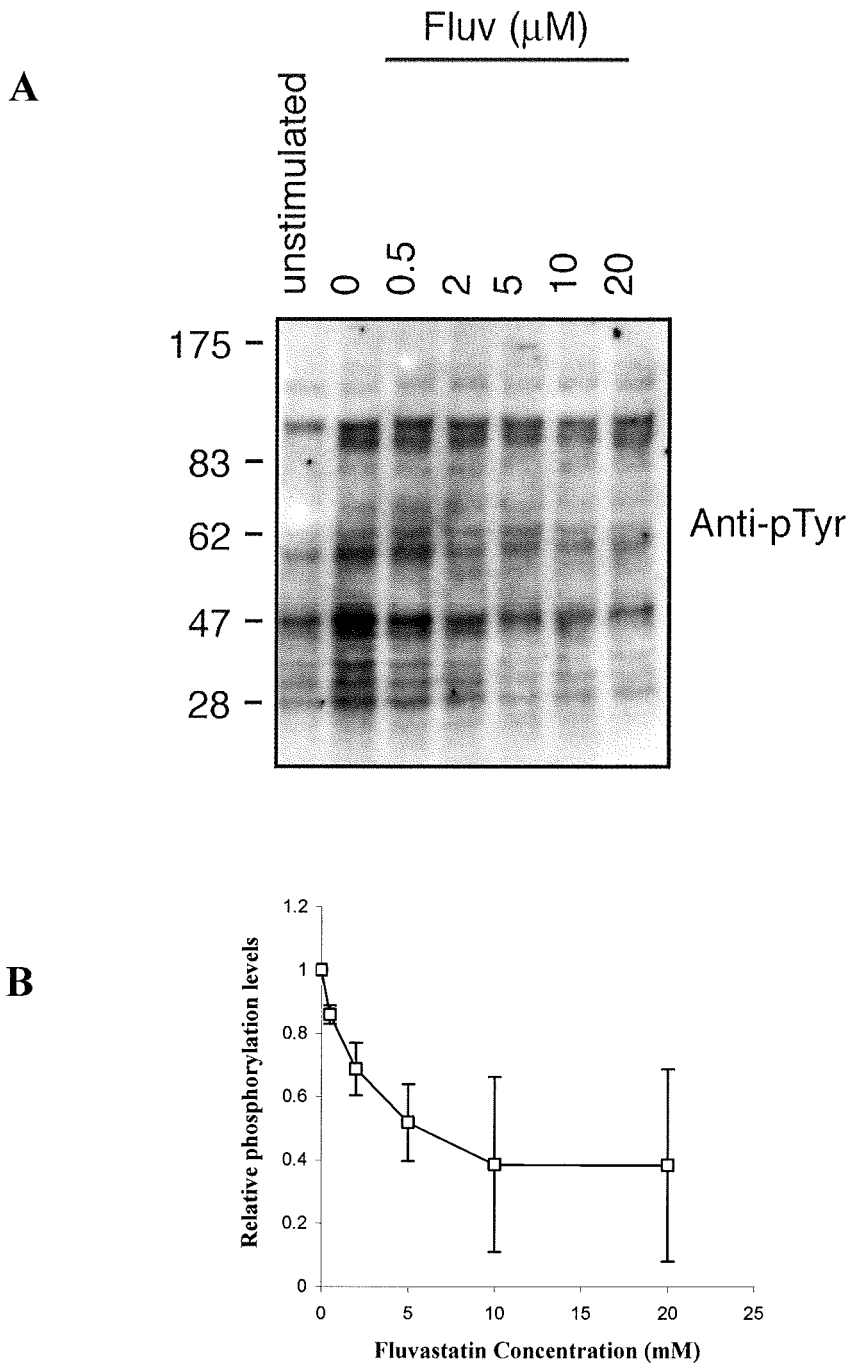


Figure 6.2 Dose dependent inhibition of tyrosine phosphorylation by fluvastatin.

(A) U937 cells were cultured for 48 hours in the presence of increasing concentrations of fluvastatin. FcγR were crosslinked for 1.5 min and western blotted with anti-phosphotyrosine antibody. (B) Blots from three independent experiments were assessed for levels of tyrosine phosphorylation by quantitative densitometry. Data are expressed as the mean (± SE).

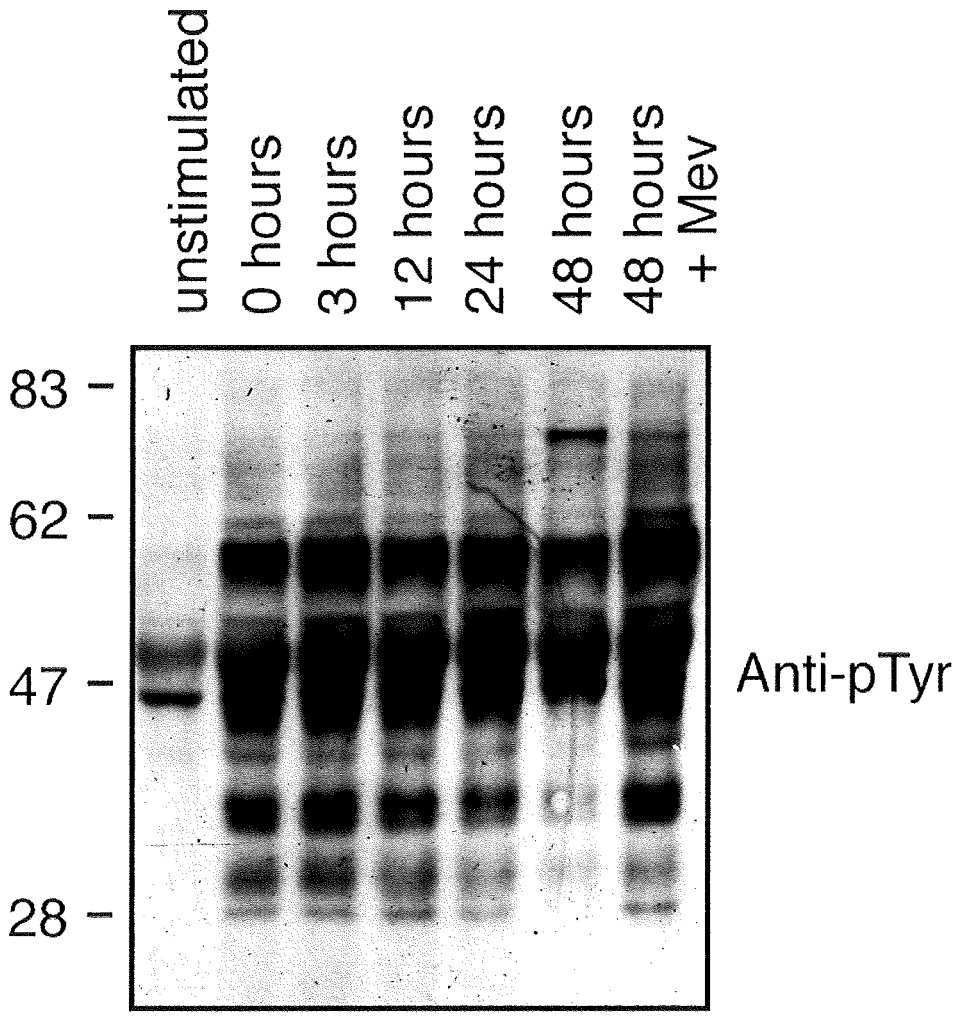


Figure 6.3 Time dependant inhibition of tyrosine phosphorylation by fluvastatin.

U937 cells were cultured with 10μM fluvastatin for the times indicated. In the last lane 1mM mevalonate was added for the final 12 hours. FcγR were crosslinked and western blotted with anti phosphotyrosine antibody.

6.2.1.4 FTI and GGTI have no effect on tyrosine phosphorylation (Figure 6.4)

Tyrosine phosphorylation was unaffected by a combination of FTI and GGTI. Figure 6.4 compared the ineffectiveness of transferase inhibitors with the inhibition by fluvastatin.

6.2.2 Statins impair Fcγ receptor initiated signalling pathways

Downstream signalling pathways p38 and Erk were also inhibited by statins in the same manner as tyrosine phosphorylation.

6.2.2.1 Dose dependent inhibition of signalling pathways by fluvastatin (Figure 6.5)

U937 cells were cultured for 48 hours in the presence of increasing concentrations of fluvastatin and FcγR crosslinked for 1.5 min. Inhibition of p38 and Erk pathways by fluvastatin were dose dependent (Figure 6.5).

6.2.2.2 Dose dependent inhibition of signalling pathways by simvastatin (Figure 6.6)

Inhibition of the p38 and Erk pathways by simvastatin were also dose dependent (Figure 6.6).

6.2.2.3 Time dependant inhibition of signalling pathways by fluvastatin (Figure 6.7)

Inhibition of FcγR responses to p38 was also time dependent and required pre-incubation for 48 hours with 10μM fluvastatin, with little inhibition observed after 24 hours (Figure 6.7).

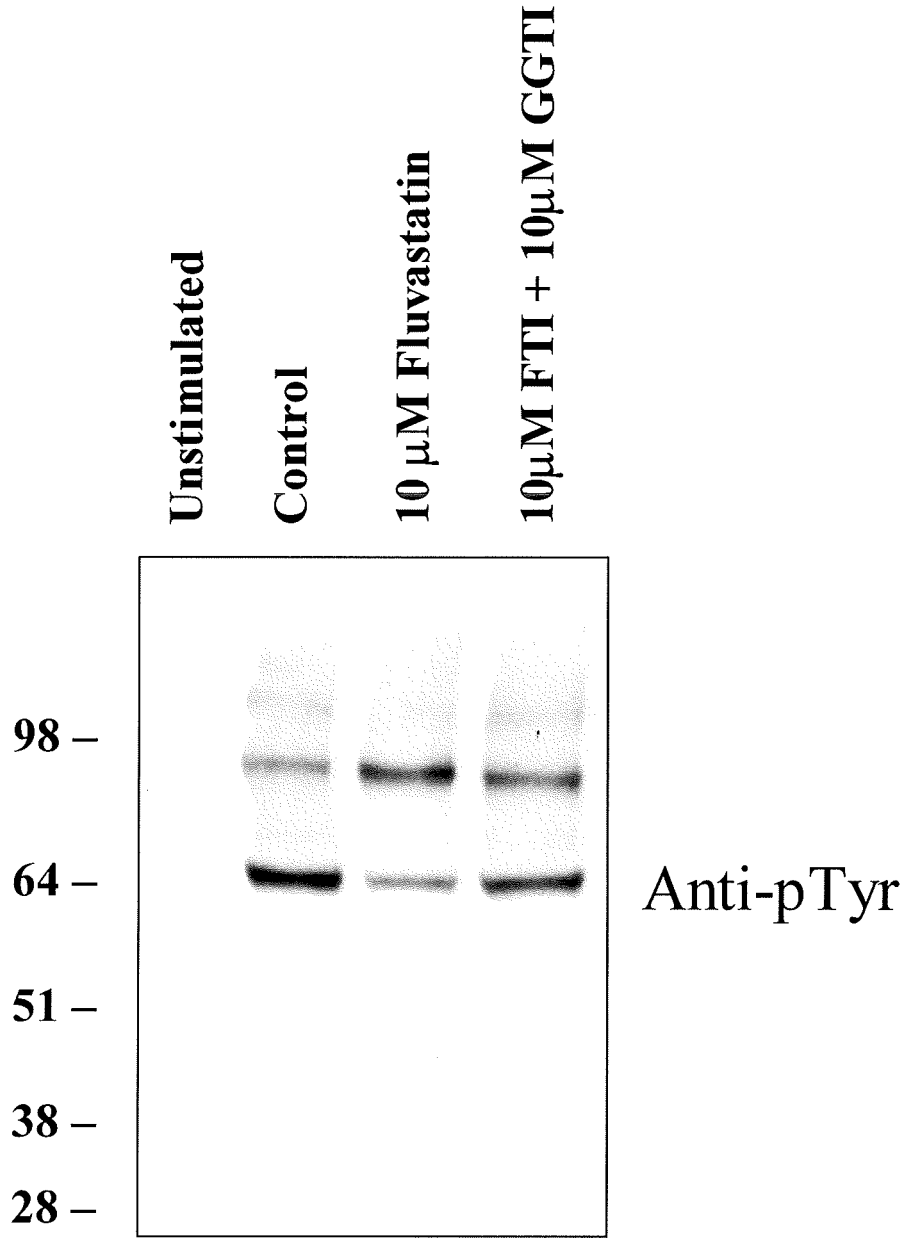


Figure 6.4 No inhibition of tyrosine phosphorylation by FTI and GGTI

U937 cells were cultured for 48 hours in 10μM FTI and 10μM GGTI. FcγR were crosslinked for 1.5 min and western blotted with anti-phosphotyrosine antibody. Fluvastatin was used as an inhibition positive control for comparison.

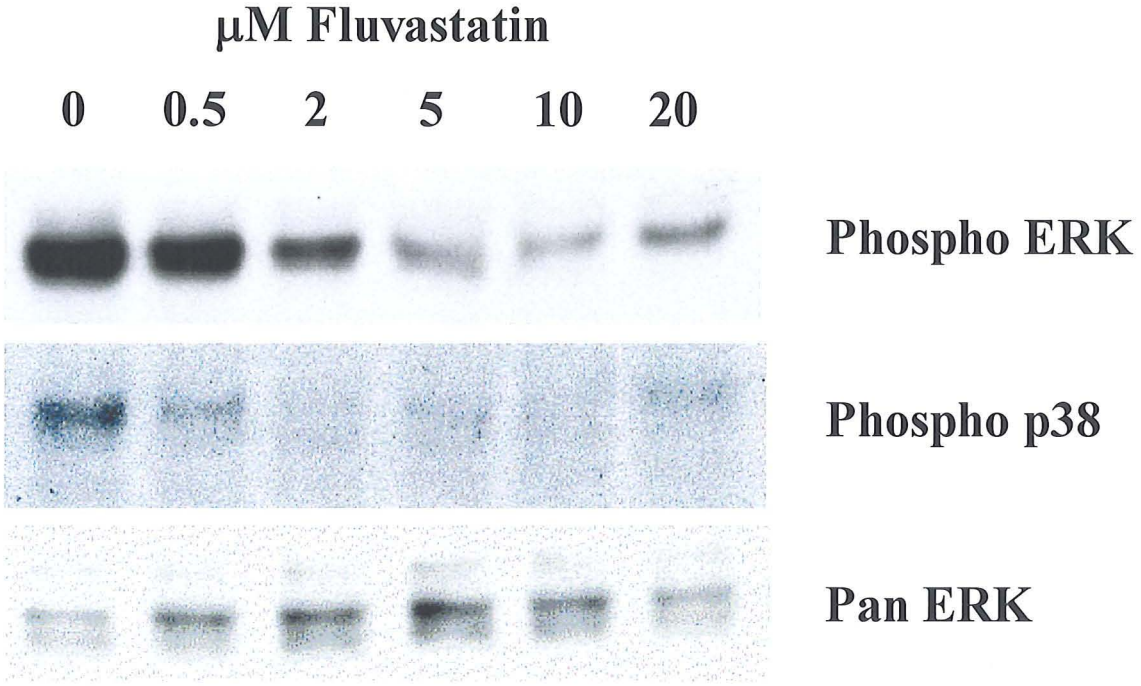


Figure 6.5 Dose dependent inhibition of signalling pathways by fluvastatin.

U937 cells were cultured for 48 hours in the presence of increasing concentrations of fluvastatin. FcγR were crosslinked for 1.5 min and western blotted. ERK and p38 phosphorylation was reduced dose dependently. This was representative of 2 experiments.

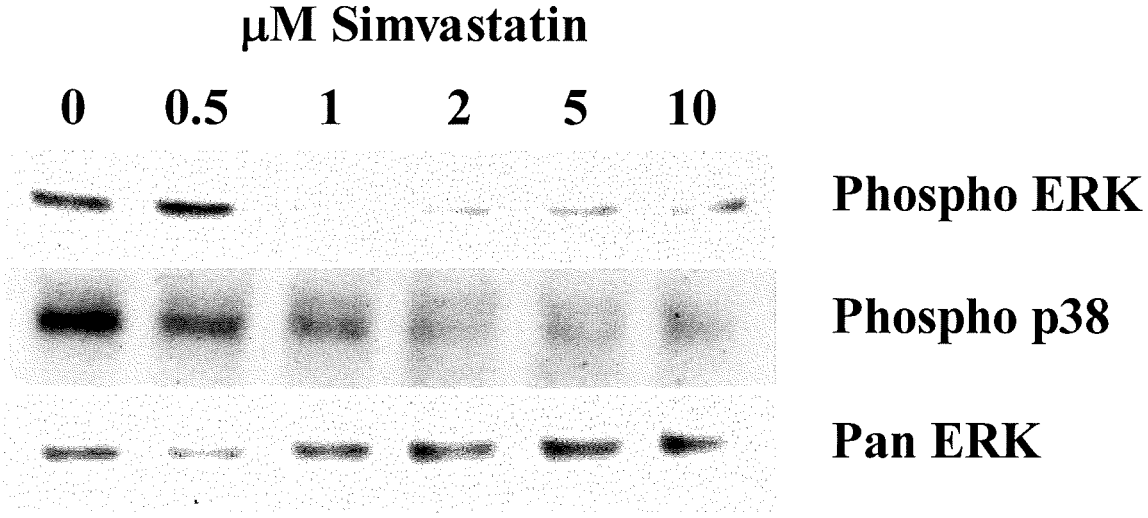


Figure 6.6 Dose dependent inhibition of signalling pathways by simvastatin.

U937 cells were cultured for 48 hours in the presence of increasing concentrations of simvastatin. FcγR were crosslinked for 2 min and western blotted.

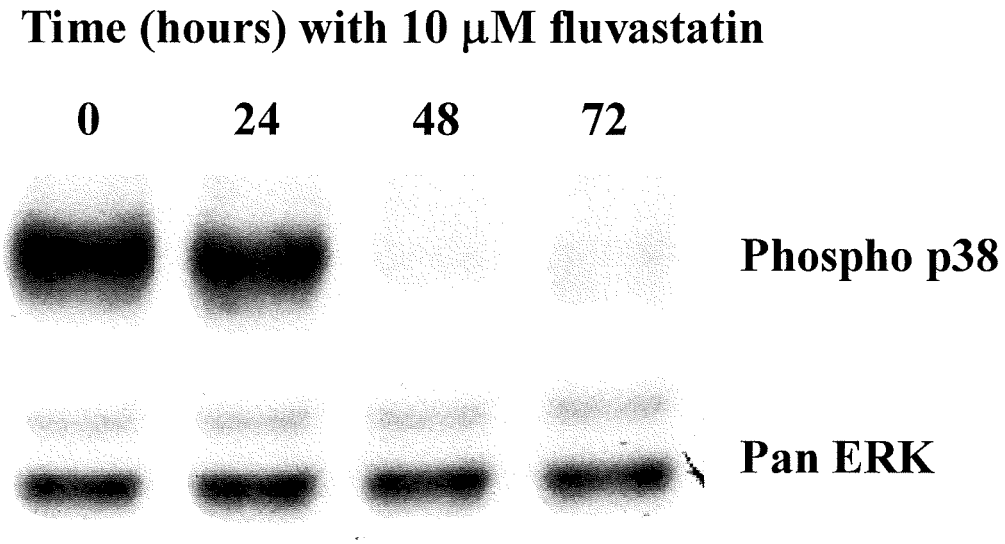


Figure 6.7 Time dependant inhibition of tyrosine phosphorylation by fluvastatin.

U937 cells were cultured with 10μM fluvastatin for the times indicated. FcγR were crosslinked for 1.5 min and western blotted. Pan ERK was used as a loading control. This was representative of 2 experiments.

6.2.2.4 FTI and GGTI have no effect on signalling pathways (Figure 6.8)

P38 and Erk signalling pathways were unaffected by a combination of FTI and GGTI (Figure 6.8). To determine whether fluvastatin inhibits FcγR responses via inhibition of HMG-CoA reductase, mevalonate was added to fluvastatin treated cells for the final 24 hours. 1mM mevalonate entirely reversed the effects of fluvastatin on p38 and Erk signalling pathways.

6.2.3 Statins inhibit immune complex trafficking and degradation

6.2.3.1 10 μM fluvastatin (Figure 6.9)

Internalisation of immune complexes by U937 cells was partially inhibited by 10μM fluvastatin with an approximate 25% reduction in cell-associated radioactivity 15 minutes after crosslinking when compared with control cells (Figure 6.9 A). A similar reduction in internalised counts was seen at all time points examined. In contrast, fluvastatin treatment almost entirely abolished immune complex trafficking (Figure 6.9 B). Thus, in control cells approximately 20% of total internalised radiolabelled immune complexes were degraded within 1 hour of receptor aggregation compared with less than 5% in fluvastatin treated cells.

6.2.3.2 Fluvastatin concentration gradient (Figure 6.10)

Concentrations of fluvastatin as low as 0.5μM significantly inhibited internalisation and trafficking with maximal effect at concentrations greater than 5μM (Figure 6.10).

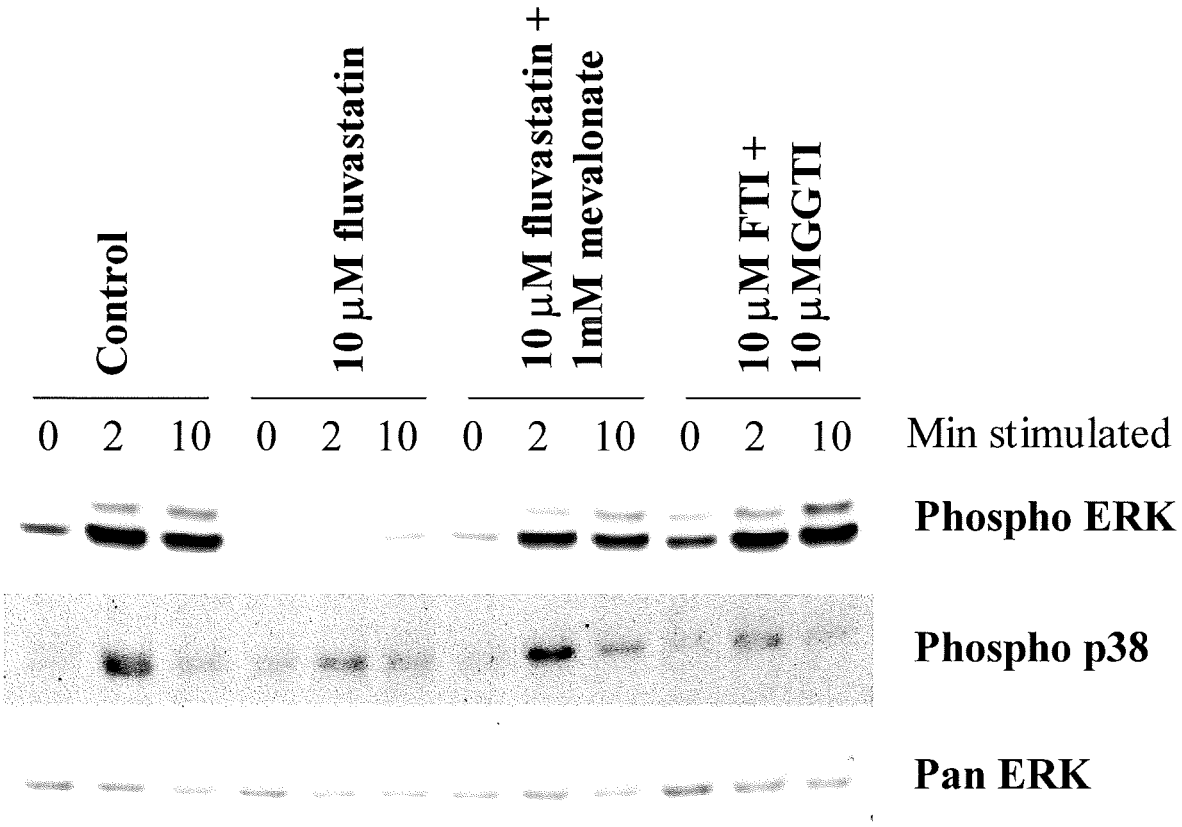


Figure 6.8 No inhibition of signalling pathways by FTI and GGTI

U937 cells were cultured for 48 hours in 10μM FTI and 10μM GGTI. FcγR were crosslinked for the times indicated and western blotted. Fluvastatin was used as an inhibition positive control for comparison (lanes 3-5). In the lanes 7-9, 1mM mevalonate was added for the final 24 hours and reversed the effects of fluvastatin. FTI and GGTI together had no effect on the Erk signalling pathway and very little effect on the p38 signalling pathway.

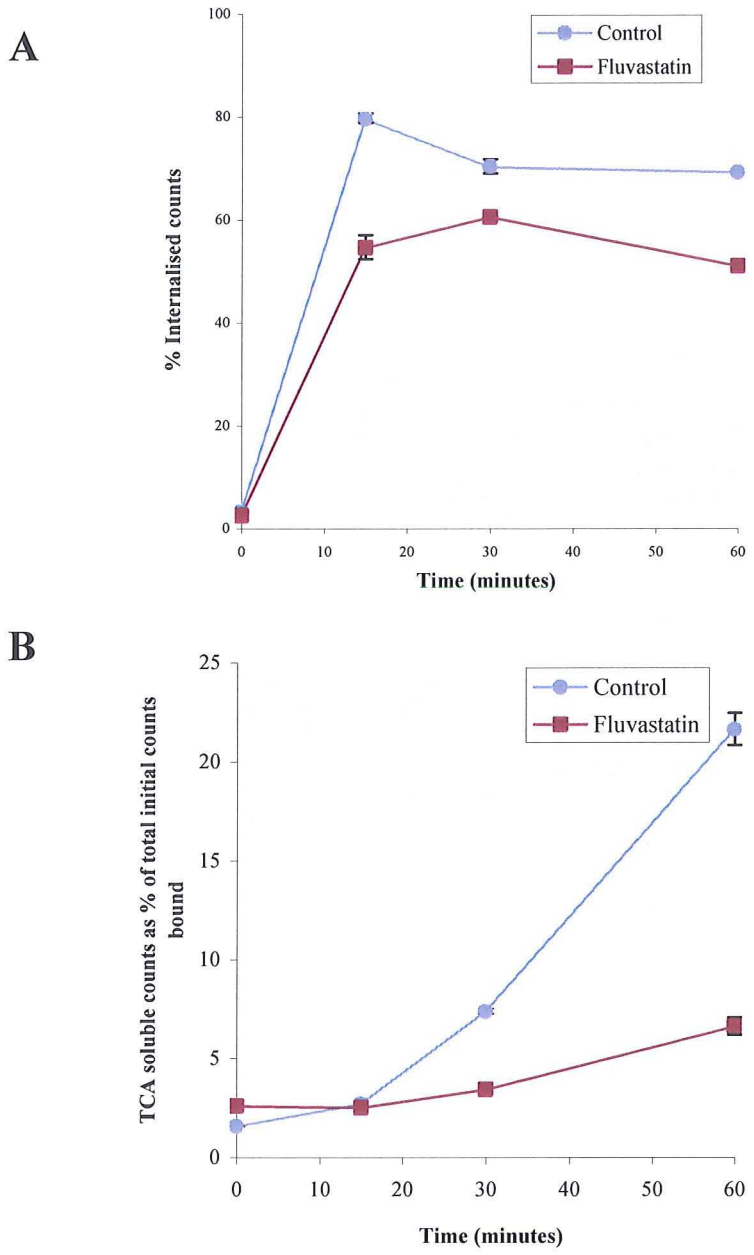


Figure 6.9. FcγR Immune complex internalisation and trafficking was reduced by fluvastatin.

U937 cells treated with 10μM fluvastatin were coated in human IgG and crosslinked with ^{125}I labelled anti-human IgG for the times indicated. (A) Surface bound radiolabelled immune complexes were removed by an acidified PBS (pH 2.0) wash, to leave internalised complexes. (B) Trichloroacetic acid soluble counts in the supernatant were measured and represented immune complexes which had been degraded and trafficked out of the cell. In A and B counts were expressed as percentages of total counts (±SD) and were representative of 3 experiments.

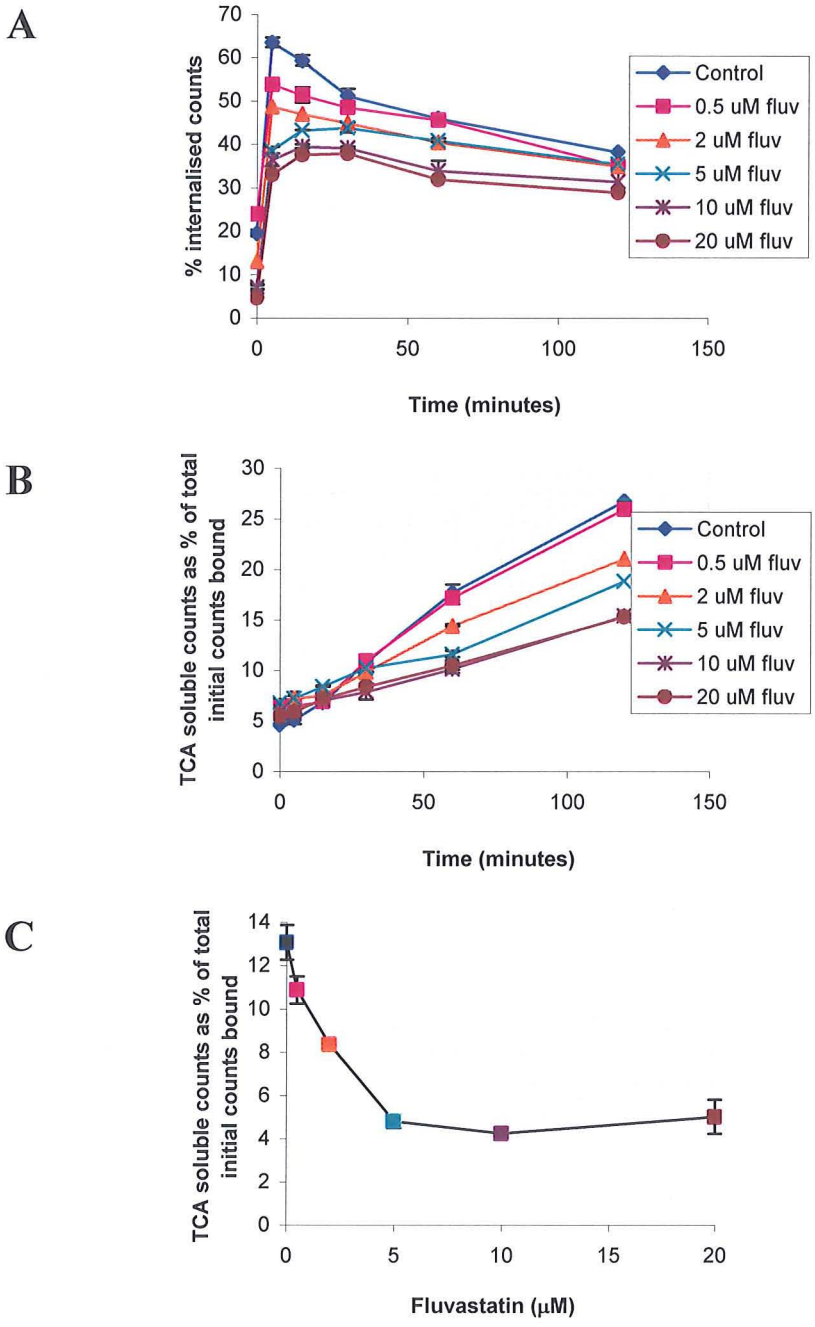


Figure 6.10 Fluvastatin inhibits immune complex trafficking and degradation in a dose dependant manner.

U937 cells were treated with a range of fluvastatin concentrations and crosslinked as above. (A) Represents internalised complexes and (B) represents complexes which had been degraded and trafficked out of the cell. (C) Trichloroacetic acid soluble counts (±SD) from the one hour time point were plotted against fluvastatin concentrations to show the reduction in trafficking with increasing concentration. This was representative of 2 experiments.

6.2.3.3 Simvastatin concentration gradient (Figure 6.11)

Concentrations of simvastatin as low as 0.5μM also significantly inhibited internalisation and trafficking in U937 cells (Figure 6.11).

6.2.3.4 Fluvastatin time course with mevalonate rescue (Figure 6.12)

Internalisation and trafficking was unaffected by short incubation times with fluvastatin. At least 48 hours were required to reduce internalisation and trafficking. The inhibition was completely reversed by addition of 1mM mevalonate 12 hours prior to assays (Figure 6.12).

6.2.3.5 FTI and GGTI (Figure 6.13)

Internalisation and trafficking was unaffected by farnesyl transferase and geranylgeranyl transferase inhibitors (Figure 6.13).

6.2.4 Statins block FcγR mediated release of inflammatory molecules

FcγR mediated release of inflammatory mediators, specifically matrix metalloproteinase-1 (MMP-1) and pro-inflammatory cytokines, may contribute to the progression of atherosclerotic plaques. I therefore investigated the release of MMP-1 and the inflammatory cytokine, IL-6, by U937 cells in response to sustained FcγR activation, using heat aggregated insoluble IgG immune complexes (HA-IgG)[322, 323].

6.2.4.1 Statins inhibit MMP-1 production (Figure 6.14)

U937 cells constitutively produced low levels of MMP-1. Incubation for 24 hours with 100μg/ml HA-IgG, but not with monomeric IgG, resulted in a large increase in MMP-1 release (Figure 6.14). Treatment of cells with 10μM fluvastatin or 10μM

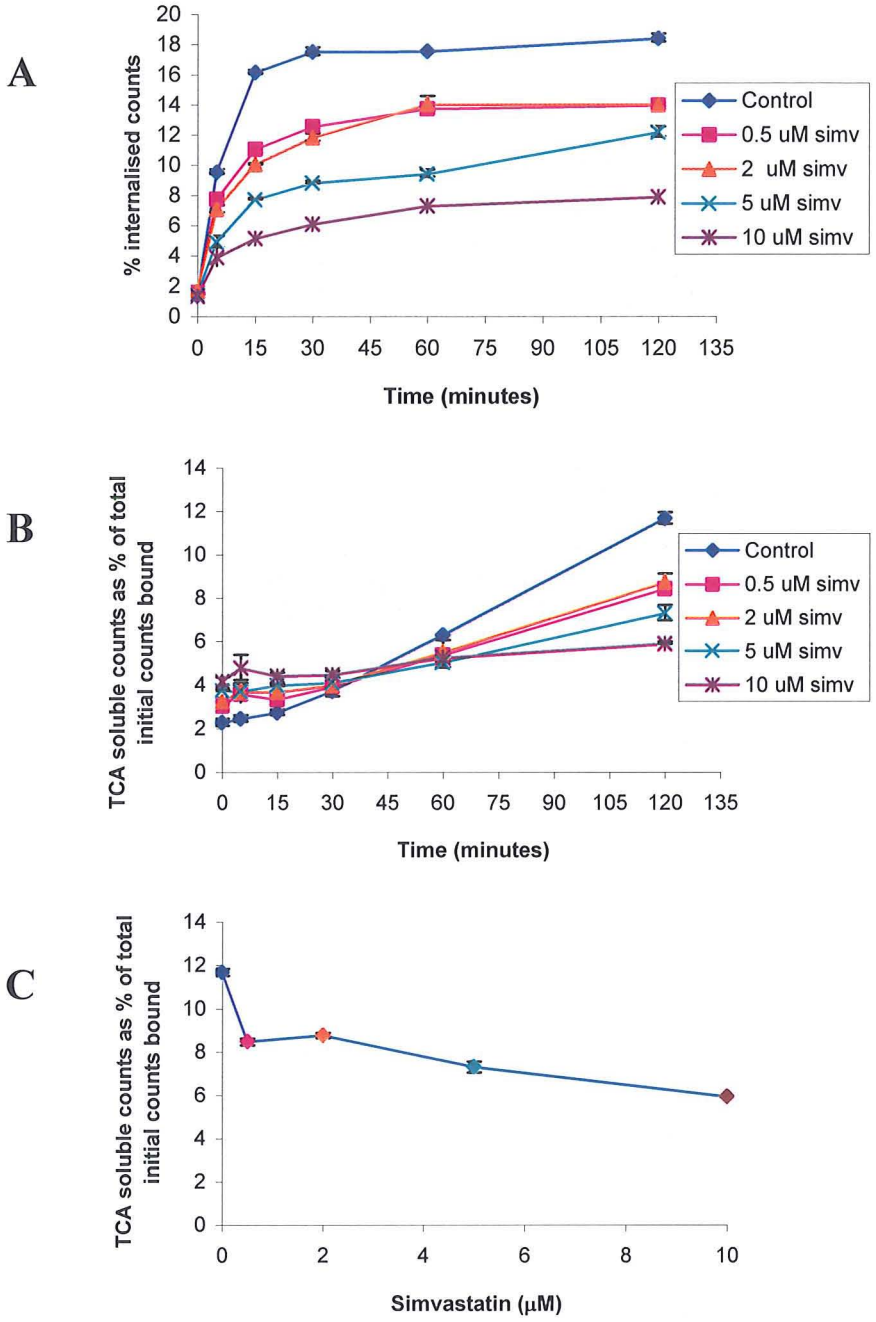


Figure 6.11 Simvastatin inhibits immune complex trafficking and degradation in a dose dependant manner.

U937 cells were treated with a range of simvastatin concentrations and crosslinked as above. (A) Represents internalised complexes and (B) represents complexes which had been degraded and trafficked out of the cell. (C) Trichloroacetic acid soluble counts (±SD) from the two hour time point were plotted against simvastatin concentrations to show the reduction in trafficking with increasing concentration. This was representative of 3 experiments.

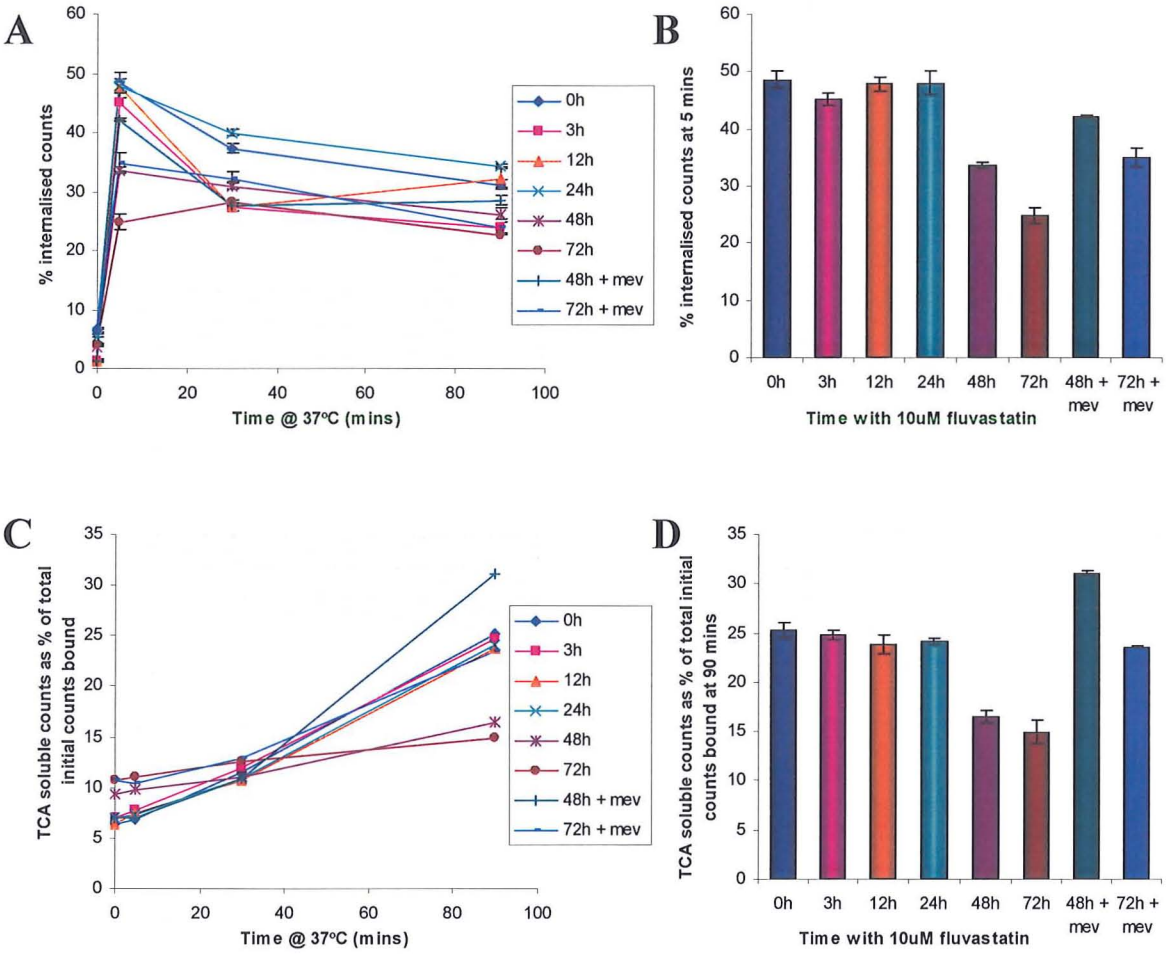


Figure 6.12 Fluvastatin inhibits immune complex trafficking and degradation in a time dependant manner.

U937 cells were treated with 10μM fluvastatin for various times and crosslinked as above. (A) Represents internalised complexes and (B) highlights the five minute time point to show the reduction of internalisation with increasing time in fluvastatin as well as rescue by mevalonate. (C) Represents complexes which had been degraded and trafficked out of the cell. (D) Highlights the trichloroacetic acid soluble counts (±SD) from the 90 minute time point to show the reduction in trafficking with increasing time in fluvastatin as well as rescue by mevalonate. This was an average of 2 experiments.

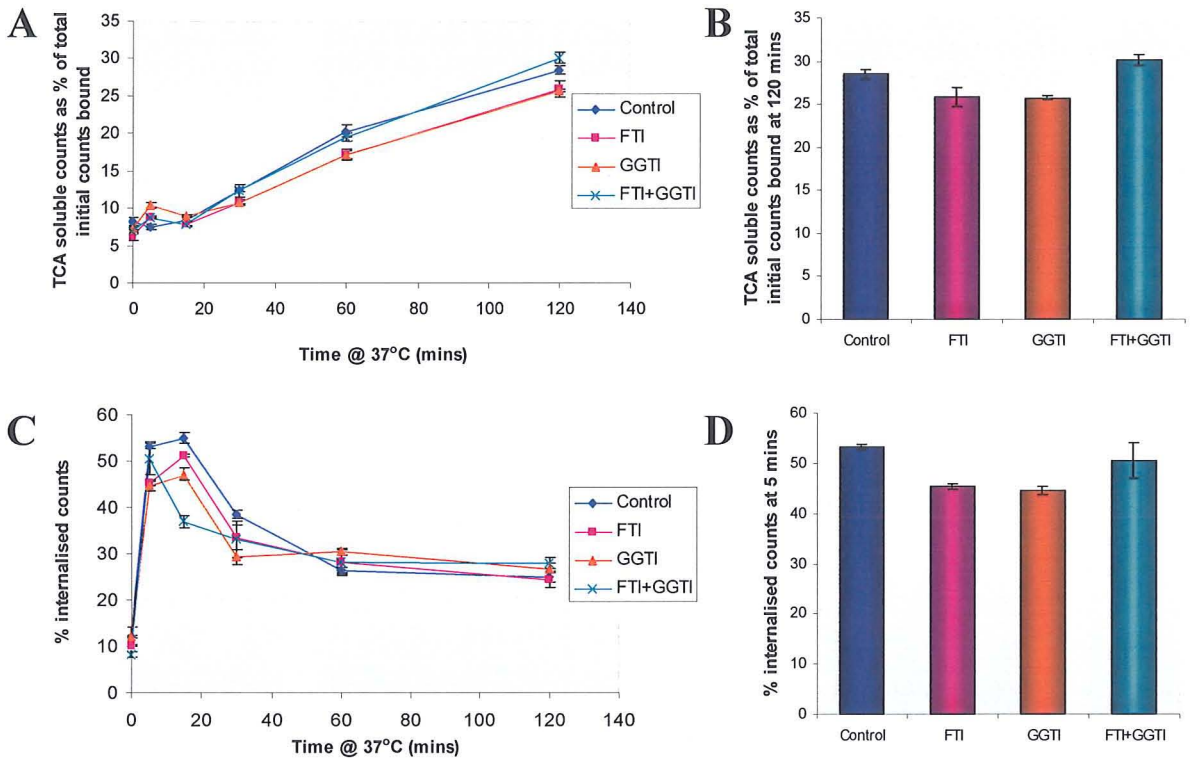


Figure 6.13 FTI and GGTI do not affect immune complex trafficking and degredation.

U937 cells were treated with farnesyl transferase and/or geranylgeranyl transferase inhibitors for 48 hours and crosslinked as before. (A) Represents internalised complexes and (B) compares the five minute time point to highlight the unaffected internalisation. (C) Represents complexes which had been degraded and trafficked out of the cell. (D) Compares the trichloroacetic acid soluble counts (±SD) from the 120 minute time point to show the unaffected trafficking. This was the average of 3 experiments

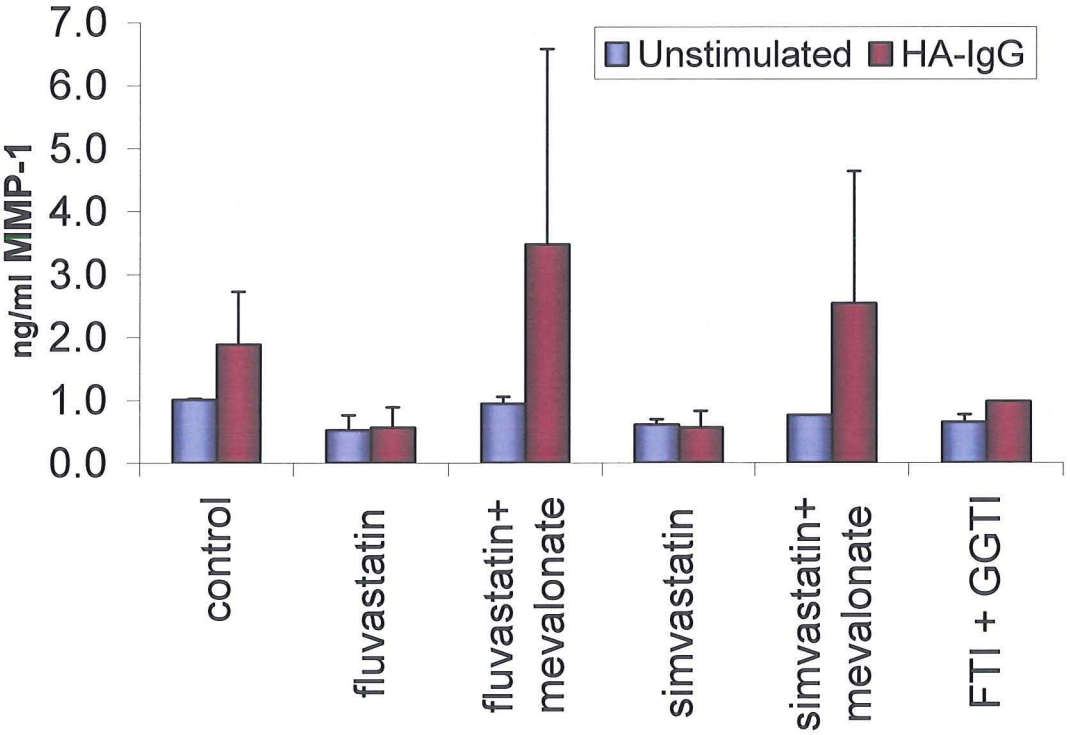


Figure 6.14 Statins block FCγ receptor mediated release of MMP-1.

U937 cells treated for 48 hours and mevalonate for the final 12 hours were stimulated with and without 100μg/ml HA-IgG for 24 hours. Supernatants collected were analysed for MMP-1 release by ELISA. This was the average of 2 experiments (performed in duplicate).

simvastatin for 48 hours reduced constitutive MMP-1 release and abolished the FcγR stimulated MMP-1 response. MMP-1 responses were restored by 1mM mevalonate rescue.

6.2.4.2 *Statins inhibit inflammatory cytokine production*

6.2.4.2.1 Statins and a combination of Farnesyl and geranylgeranyl transferase inhibitors, reduce cytokine production (Figure 6.15)

U937 cells were also found to release increased levels of IL-6, IL-8 and IL-10 in response to HA-IgG, an effect that was abrogated by pre-treatment with 10 μM fluvastatin or 10μM simvastatin. IL-6, IL-8 and IL-10 responses were also restored by 1mM mevalonate rescue (Figure 6.15).

6.2.4.3 *Erk and p38 inhibitors (Figure 6.16)*

As I demonstrated that statins largely abolished coupling of FcγR to the MAPKs, ERK and p38, the dependence of MMP-1 and IL-6 release on these kinases was assessed pharmacologically. Inhibition of the ERK cascade with the MEK inhibitor, 50μM PD98059, entirely abolished both MMP-1 and IL-6 release from U937 cells. In contrast, inhibition of p38 with 5μM SB203580 was found to block IL-6 production but had no effect on MMP-1 release (Figure 6.16).

6.3 Discussion

FcγR mediate several critical functions within the immune system including clearance and disposal of immune complexes, antigen presentation and release of inflammatory mediators. Since atherosclerotic lesions contain macrophages and large amounts of immune complexes such as oxLDL-IC, I was interested to assess the effects of statins on FcγR function using a human monocyte cell line. FcγR-mediated tyrosine phosphorylation was attenuated by fluvastatin and simvastatin at therapeutically comparable concentrations without any alteration in the number of cell surface receptors. Similarly, fluvastatin and simvastatin were observed to

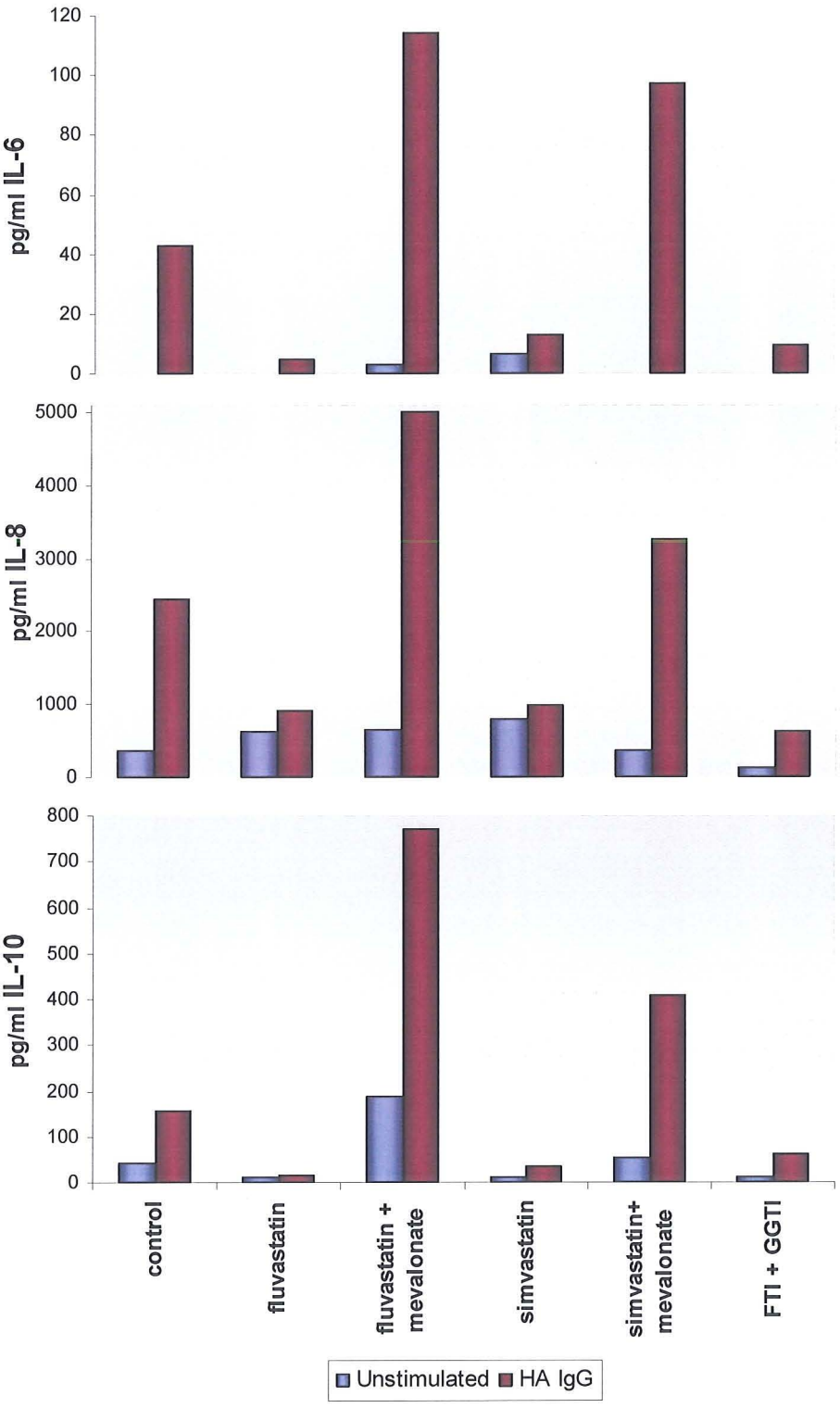


Figure 6.15 Statins block FCγ receptor mediated release of inflammatory cytokines.

U937 cells treated for 48 hours and mevalonate for the final 12 hours were stimulated with and without 100μg/ml HA-IgG for 24 hours. Supernatants collected were analysed for IL-6, IL-8 and IL-10 release by cytometric bead array kit. This was representative of 2 experiments.

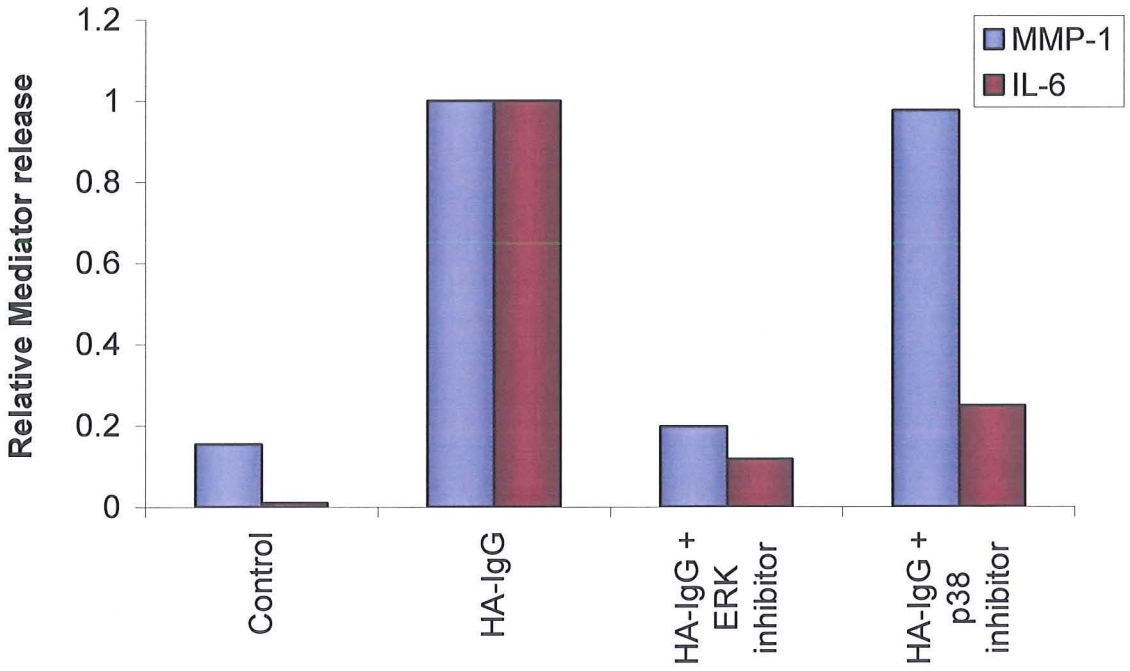


Figure 6.16 ERK inhibitors block release of both MMP-1 and IL-6 whereas p38 inhibitors have no effect on MMP-1 release.

U937 cells were treated with ERK inhibitor (50μM PD98059) or p38 inhibitor (5μM SB203580) for 24 hours during stimulation with HA-IgG. Inhibition of the ERK cascade with ERK inhibitor abolished both MMP-1 and IL-6 release. Inhibition of p38 pathway with p38 inhibitor was found to block IL-6 production but had no effect on MMP-1 release. This was representative of 2 experiments.

cause a large reduction in FcγR-mediated immune complex trafficking and degradation. Inhibition of immune complex internalisation was also seen but was less profound. These effects of statins are of importance as macrophage FcγR-internalised IC can be targeted for degradation and MHC class II antigen presentation to T-cells. The presence of activated CD4+ T-cells and abundant expression of MHC class II antigens within atherosclerotic lesions suggest that specific cellular immune responses, as well as non-specific inflammatory responses, contribute to atherosclerosis[324, 325].

Statins have previously been reported to inhibit production of inflammatory mediators (including MCP-1, MMP-9, TNF-α and IL-8) by monocytes[153, 320, 321]. Inhibition by fluvastatin and simvastatin of FcγR-coupled release of the inflammatory mediators, IL-6, IL-8, IL-10 and MMP-1, and associated inhibition of the MAP kinases ERK and p38, which lie upstream in this pathway of cytokine release, was observed. Classically, coupling of receptors to ERK activation is thought to lie downstream of the farnesylated small GTPase, Ras while p38 is thought to lie downstream of geranylgeranylated Rho family GTPases[301]. However, data suggest that FcγR coupling to ERK is independent of Ras[326, 327]. Specific isoprenyl transferase inhibitors had little or no effect on tyrosine phosphorylation and the ERK pathway, and associated immune complex trafficking and degradation. It is not known whether this is because a Ras independent pathway is involved in trafficking and degradation and prenyl inhibitors would therefore not have an effect or whether it is cholesterol reduction by statins that disrupts rafts and associated signalling pathways. As prenyl inhibitors had some effect on cytokine release, these are likely to be a Ras dependent pathway, which is disrupted by both prenyl inhibition and cholesterol in rafts. Further investigation is required to determine whether statin mediated inhibition of FcγR coupling to these pathways depends on a block in isoprenylation to disruption of membrane raft function through blockade of cholesterol synthesis.

Release of MMP-1 from U937 cells in response to oxLDL-IC has previously been reported [319]. Thus, the observation that fluvastatin or simvastatin abolishes MMP-1 release in response to FcγR stimulation is of particular interest as monocyte MMP-1 has been implicated in the destabilisation of atherosclerotic plaques and subsequent acute coronary events. Inhibition of MMP-1 release could contribute

to the reported plaque stabilising properties of statins. The finding that fluvastatin decreased FcγR mediated IL-6 production in monocytes is particularly pertinent in light of recent clinical data demonstrating that statin therapy results in a significant reduction in systemic IL-6 levels in patients with cardiovascular disease[328].

In conclusion, accumulating evidence indicates a multi-factorial mode of action for statins in the successful reduction of morbidity and mortality in cardiovascular disease. Thus, in addition to lowering LDL-cholesterol levels, statins block leukocyte recruitment to atherosclerotic lesions, inhibit monocyte antigen processing and production of inflammatory mediators and inhibit release of matrix proteases implicated in plaque rupture. To exert these pleiotropic effects statins appear to use multiple mechanisms including inhibition of cholesterol (and cholesterol rich signalling domains) and isoprenoid synthesis. Perhaps by exerting their effects at multiple levels, statins may limit disease progression without inhibiting essential cellular or immunological functions. Overall, the data supports a potential role for Fc receptors and IC in the pathophysiology of atherosclerosis, and identify a potential effect of statins on membrane rafts that may affect other transmembrane receptors and offer novel therapeutic targets in cardiovascular disease.

Chapter 7: Functional effects of statins on Lymphocytes

7.1 Introduction

The cell type for which there is most *in vivo* evidence of statin effect is the natural killer (NK) cell. This is a specialised lymphocyte that targets virus-infected and malignant cells that do not express MHC class I molecules on the cell surface. MHC class I molecules are the ligand for inhibitory receptors on NK cells; thus loss of MHC class renders target cells susceptible to the killing machinery and activating receptors of NK cells[93, 329]. Previously, it was thought that NK cells were predominantly under inhibitory regulation, but it is now known that activation receptors are necessary for cytotoxicity[102, 330]. Recent studies have also shown that NK cell killing is dependent on membrane rafts, where activation receptors are localised, thus offering an explanation for the apparent sensitivity of NK cells to statin therapy[56, 280, 297, 331].

The aim of this chapter was to investigate the effects of statins on modification of membrane rafts and isoprenylation on T cell and NK cell function. I examined the effect of statins on functional responses in primary human T cells by measuring CD3 stimulated proliferation. I then examined the effect of statins on functional responses in the NK92MI human NK cell line by measuring chromium release in a cytotoxicity assay. MβCD and prenyltransferase inhibitors were also utilised to determine whether statins affect mainly raft integrity or prenylation of small G proteins in NK cells. I hypothesised that statins would interrupt signalling pathways by mainly raft disruption to reduce cytotoxicity of NK cells and reduce proliferation of T cells.

7.2 Results

7.2.1 T cell proliferation (Figure 7.1)

Lymphocytes were extracted from normal volunteers and CD3 stimulated T cell proliferation measured by tritiated thymidine incorporation (Figure 7.1). Mevalonate added in the final 18 hours failed to rescue proliferation. However, mevalonate added at the same time as fluvastatin prevented the inhibitory response of the fluvastatin. FTI and GGTI had no effect on T cell proliferation.

7.2.2 Natural killer cells

I sought to elaborate the relative importance of rafts and isoprenylation in vitro, using the immortalised NK cell line - NK-92MI. NK-92MI is an interleukin-2 independent natural killer cell line derived the NK92 cell line by transfection. NK-92 cell line was derived from peripheral blood mononuclear cells from a 50-year-old Caucasian male with rapidly progressive non-Hodgkins's lymphoma.

7.2.2.1 NK cell surface receptors

PCR and FACS were performed to measure some of the known surface receptors of NK cells to ensure comparability of the cell line to primary NK cells.

7.2.2.1.1 PCR (Figure 7.2)

RNA from NK-92MI and U937 cells were extracted using Trizol and reverse transcribed using the SuperScript™ First-Strand Synthesis System for RT-PCR. PCR was performed with various settings optimised for each set of primers (Figure 7.2). NK cells contain mRNA for the classic receptor FCγRIII.

7.2.2.1.2 FACS (Figure 7.3)

NK-92MI and U937 cells were incubated with various antibodies and FACS used to measure receptor protein on the cell surface (Figure 7.3). This confirmed that the

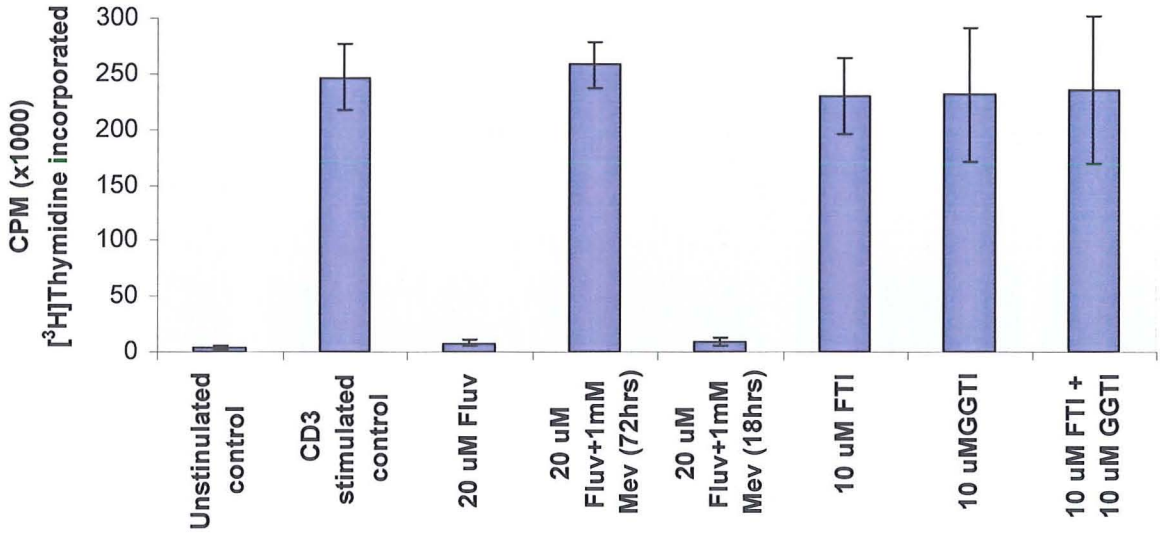


Figure 7.1 Mevalonate rescue of T cell proliferation.

T cells obtained from 4 normal volunteers were stimulated by anti-CD3 ab and the tritiated thymidine incorporated, measured. Mevalonate added at the start (72 hours) prevented fluvastatin exerting an effect. Mevalonate added in the final 18 hours failed to rescue proliferation. FTI and GGTI had no effect on T cell proliferation. This was representative of 2 experiments.

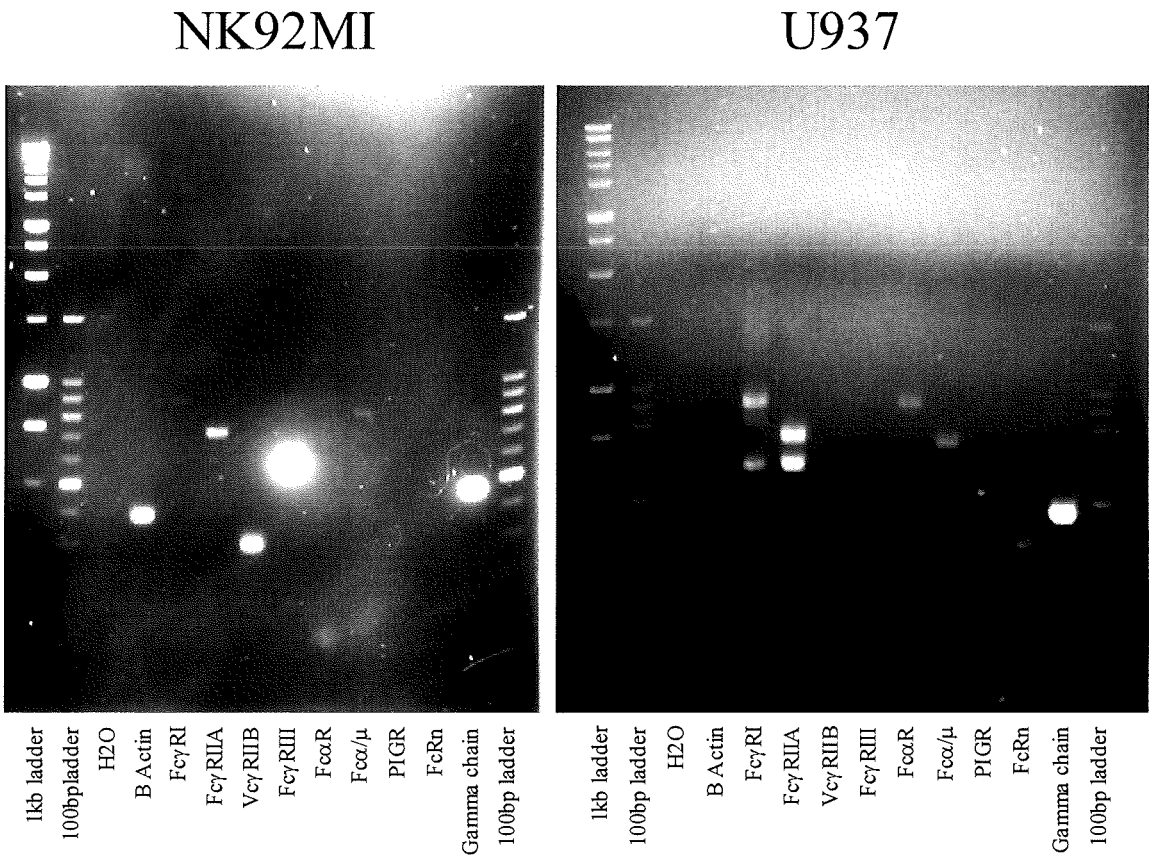


Figure 7.2 PCR of NK cell receptors.

RNA was extracted using Trizol and reverse transcribed using the SuperScript^{tm} First-Strand Synthesis System for RT-PCR. PCR was performed with various settings optimised for each set of primers. U937 cells were used as a PCR control. NK cells contain mRNA for: FcγRI, FcγRIIA, FcγRIIB, FcγRIII, Fcα/μR and the gamma chain.

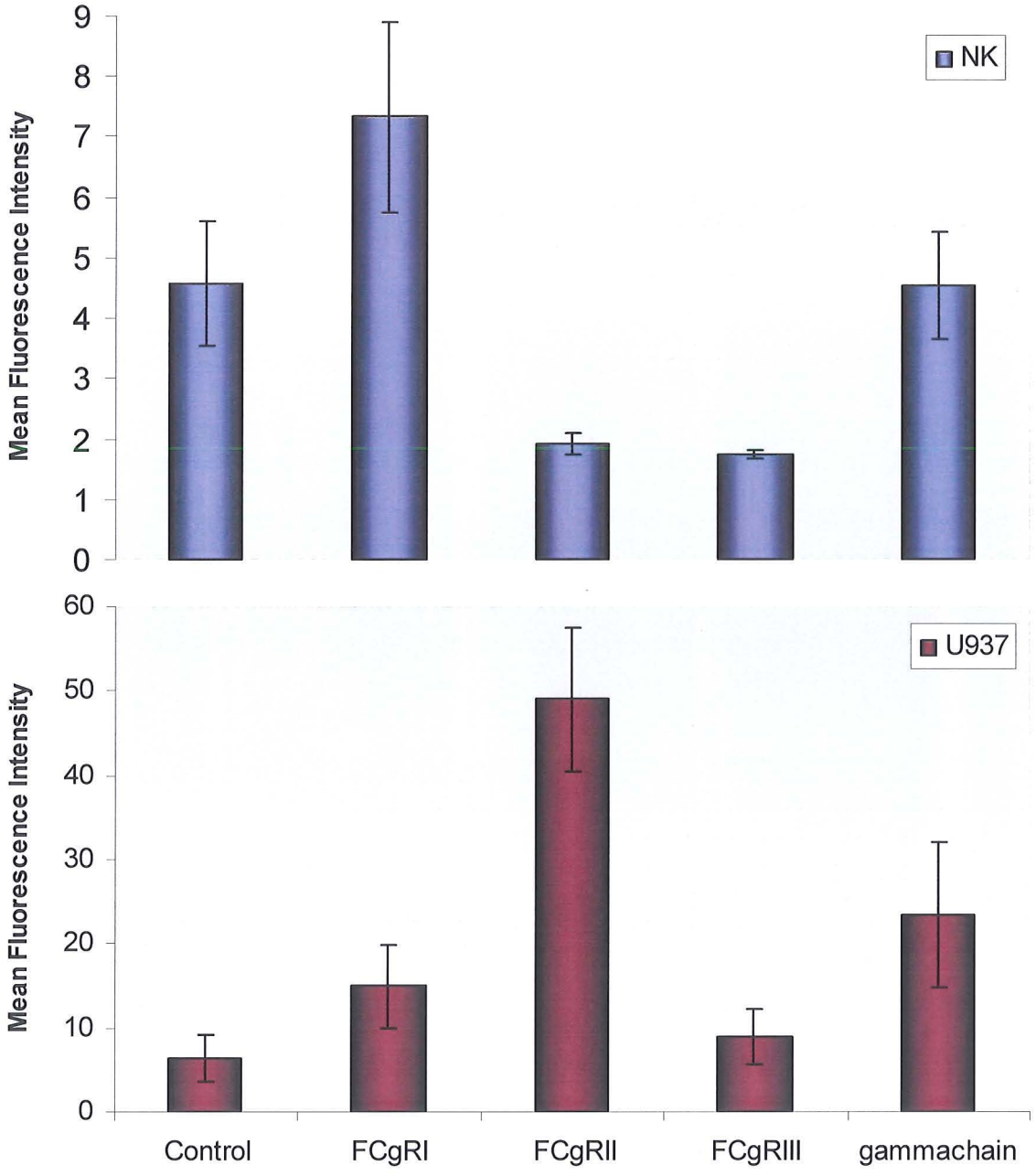


Figure 7.3 FACS of NK cell receptors

NK-92MI and U937 cells were incubated with various antibodies and FACS used to measure receptor protein on the cell surface. Mean fluorescence intensities were plotted. NK cells do not express the classical FcγRIII on their surface. This was the average of 2 experiments.

NK-92MI cell line does not express FcγRIII on its surface even though message is made.

7.2.2.2 NK Proliferation

NK-92 cells were assessed for effects on proliferation by "AlamarBlue" assay.

7.2.2.2.1 Statin concentration gradient (Figure 7.4)

NK-92MI cells were treated with various concentrations of fluvastatin or simvastatin (Figure 7.4). Proliferation was reduced slightly with increasing concentrations of statin.

7.2.2.2.2 Mevalonate rescue (Figure 7.5)

NK-92MI cells were treated with 10μM fluvastatin or simvastatin with 1mM mevalonate added at 24, 48 and 72 hour time points (Figure 7.5). Mevalonate added at the same time as statin prevented any inhibition of proliferation. Mevalonate added 24 hours and 48 hours after statin treatment did not rescue the limited inhibition by statin

7.2.2.2.3 FTI and GGTI (Figure 7.6)

NK-92MI cells were treated with various concentrations of FTI and/or GGTIs, but had no effect on proliferation (Figure 7.6).

7.2.2.3 NK Cytotoxicity

Chromium release assay was performed to assess the effects of cholesterol disruption on NK lysis of ^{51}Chromium labelled K562 target cells. A ratio of 5 NK cells to 1 target cell was used.

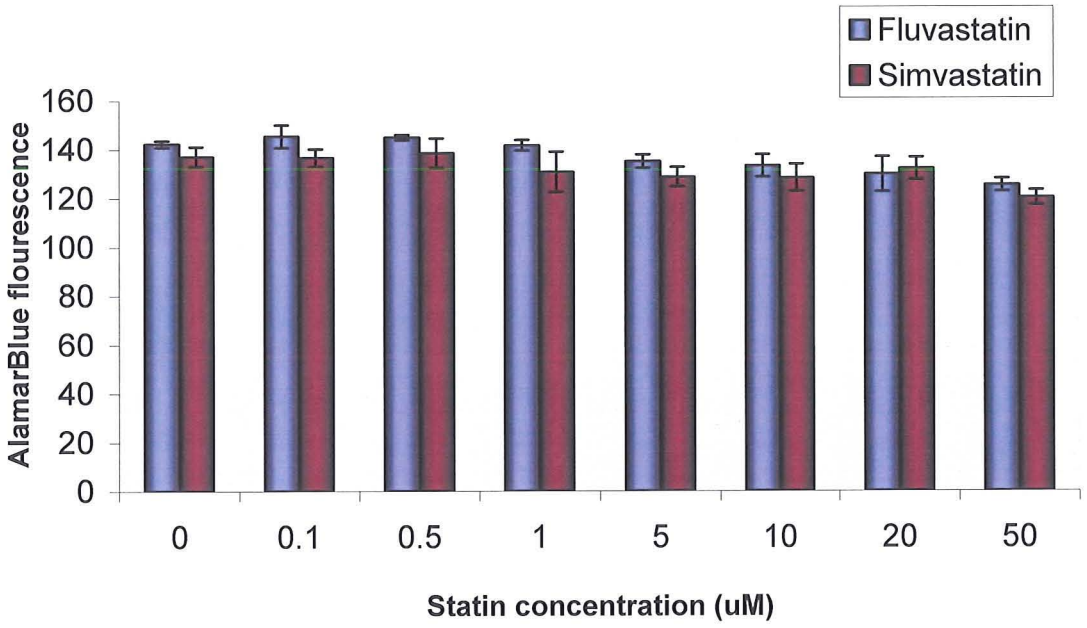


Figure 7.4 Statins minimally reduce proliferation of NK-92MI cells in a dose dependent manner.

NK-92MI cells were treated with increasing concentrations of fluvastatin or simvastatin for 48 hours. Higher concentrations of each statin reduced proliferation slightly. This was an average of 2 experiments performed in replicates if 4.

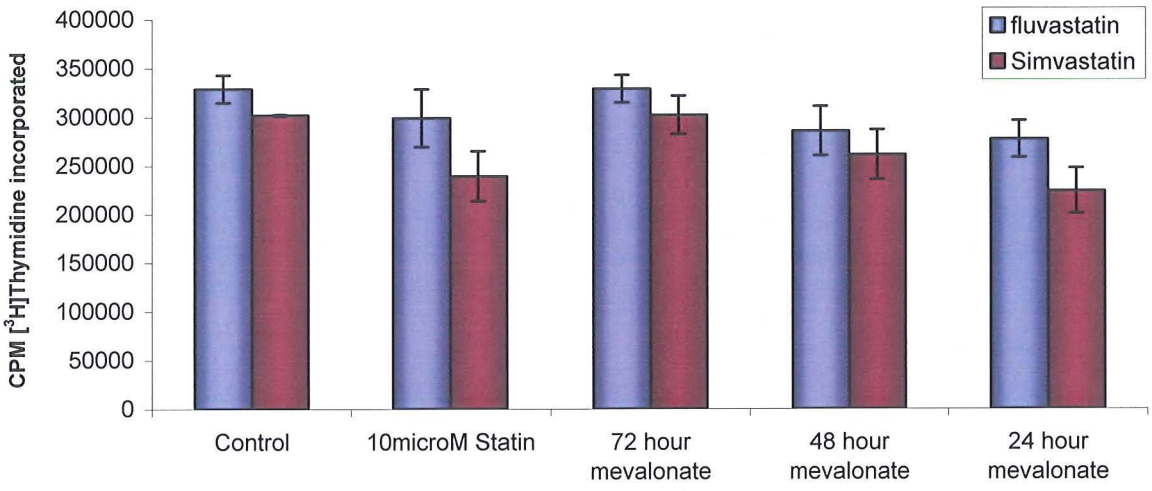


Figure 7.5 Mevalonate rescues inhibition of NK proliferation by statins.

NK-92MI cells were treated with 10μM fluvastatin or simvastatin for 72 hours with 1mM mevalonate added at various time points. Mevalonate added at the same time as statin prevented any inhibition of proliferation. This was representative of 2 experiments performed in replicates of 6.

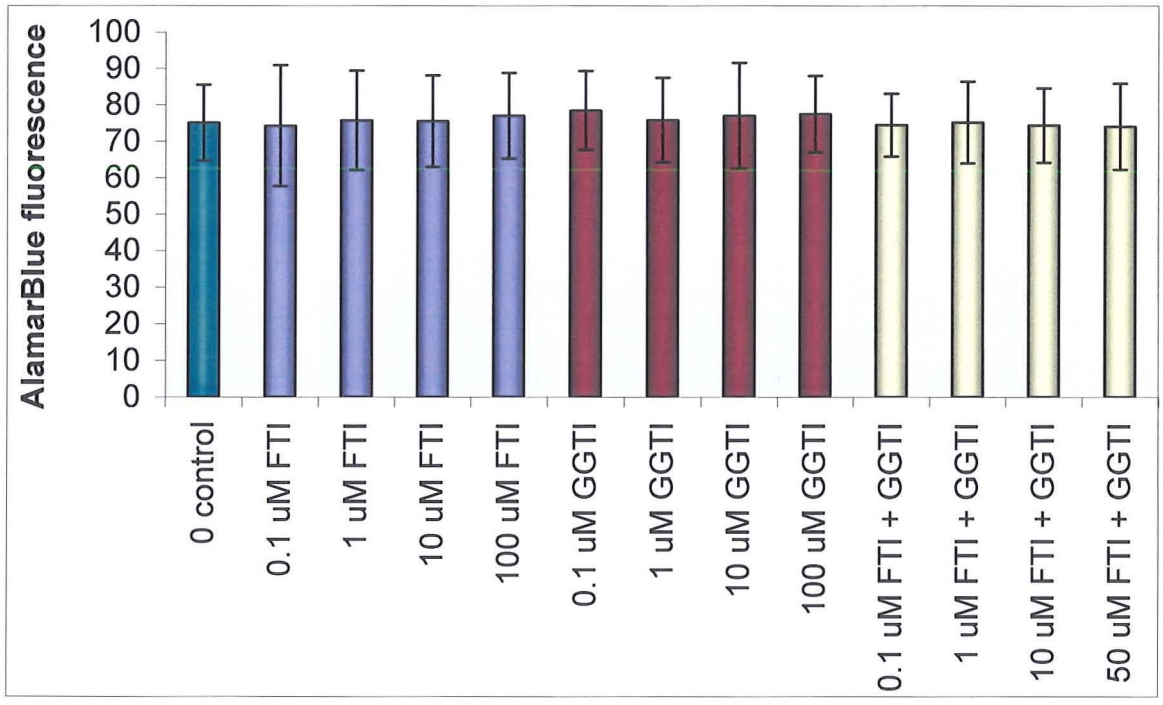


Figure 7.6 FTI and/or GGTI does not affect NK proliferation.

NK-92MI cells were treated with FTI and GGTI in increasing concentrations for 48 hours with no effect. This was the average of 2 experiments performed in replicates of 5.

7.2.2.3.1 Statin concentration gradient (Figure 7.7)

Pre-incubation of NK-92MI cells with either fluvastatin or simvastatin reduced NK killing of K-562 cells in a dose dependent manner (Figure 7.7). Simvastatin was more potent than fluvastatin; both compounds caused 50% inhibition of cytotoxicity at concentrations of 5-10 μM.

7.2.2.3.2 Mevalonate rescue (Figure 7.8)

The effects of statin could be fully reversed by pre-incubation of cells with mevalonate (Figure 3). A minimum of 24 hours pre-incubation was required for full reversal of statin effects; shorter incubations had limited, time-dependent effects (data not shown). (Figure 7.8)

7.2.2.3.3 FTI and GGTI (Figure 7.9)

NK-92MI cells were treated with various concentrations of FTI and/or GGTIs, but had no effect on cytotoxicity (Figure 7.9)

7.2.2.3.4 MβCD (Figure 7.10)

The relationship with membrane cholesterol was investigated further by incubation with MβCD. At high concentrations this facilitates the depletion of membrane cholesterol, associated with reduced NK cell cytotoxicity (Figure 7.10 A). In the presence of cholesterol in the culture media, however, low concentrations increase internalisation of extracellular cholesterol that is likely to explain the increase in NK cell cytotoxicity. The biphasic response of NK cells to MβCD was reduced by omission of serum from the culture medium (Figure 7.10 B).

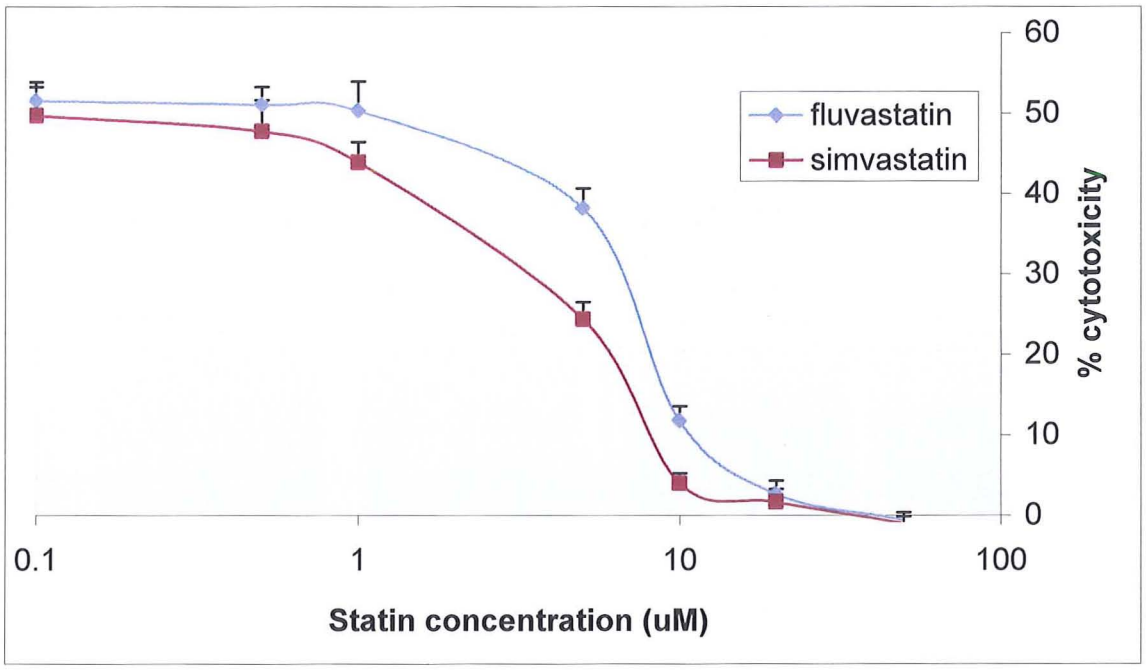


Figure 7.7 Statins reduce NK cytotoxicity in NK-92MI cells in a dose dependent manner.

NK-92 cells were treated with increasing concentrations of fluvastatin or simvastatin for 48 hours. Cell numbers were adjusted to account for proliferation difference and cytotoxicity assay performed in replicates of 6. This was representative of 2 experiments.

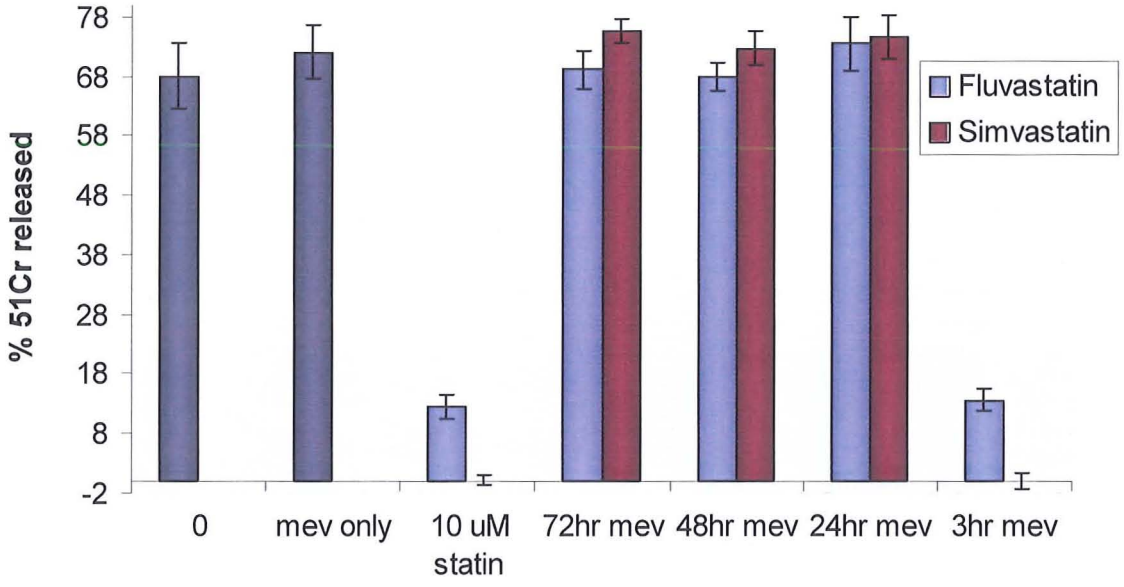


Figure 7.8 Mevalonate rescues statin inhibition of NK cytotoxicity.

NK cells were incubated with 10μM fluvastatin or simvastatin with mevalonate added at various time points. The effect of statin was fully reversed by pre-incubation of cells with mevalonate. A minimum of 24 hours pre-incubation was required for full reversal of statin effects; shorter three hour incubations had no effect. This was representative of 2 experiments performed in replicates of 6.

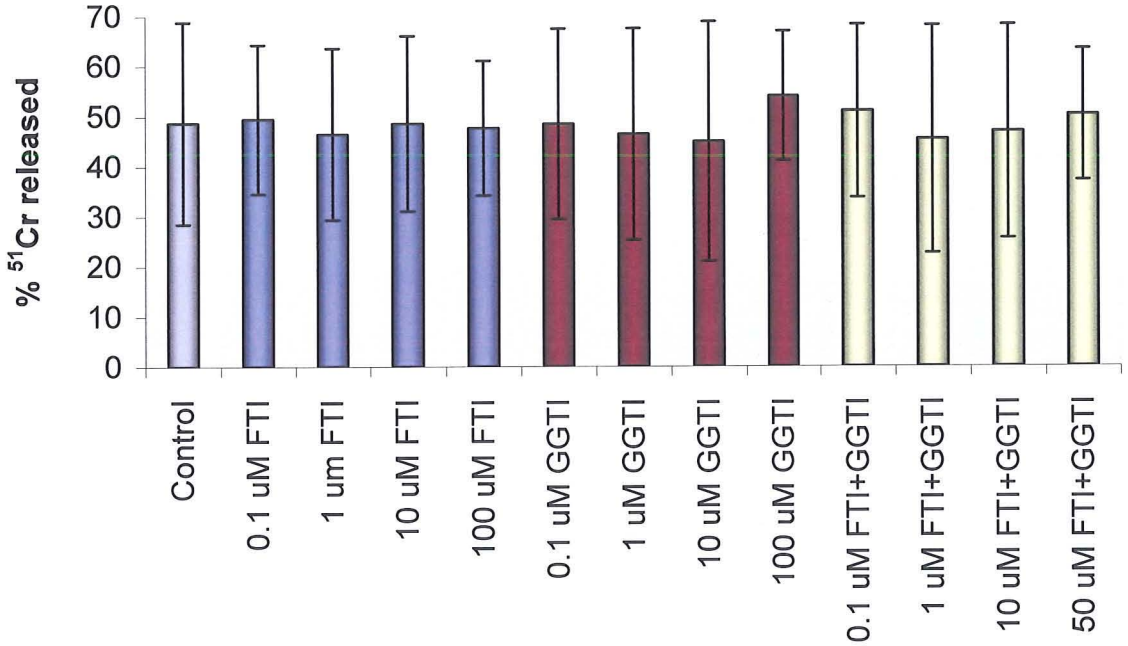


Figure 7.9 FTI and GGTI does not affect NK cytotoxicity in NK-92MI cells.

NK-92MI cells were treated with increasing concentrations of FTI and GGTI for 48 hours with no effect. This was the average of 2 experiments performed in replicates of 6.

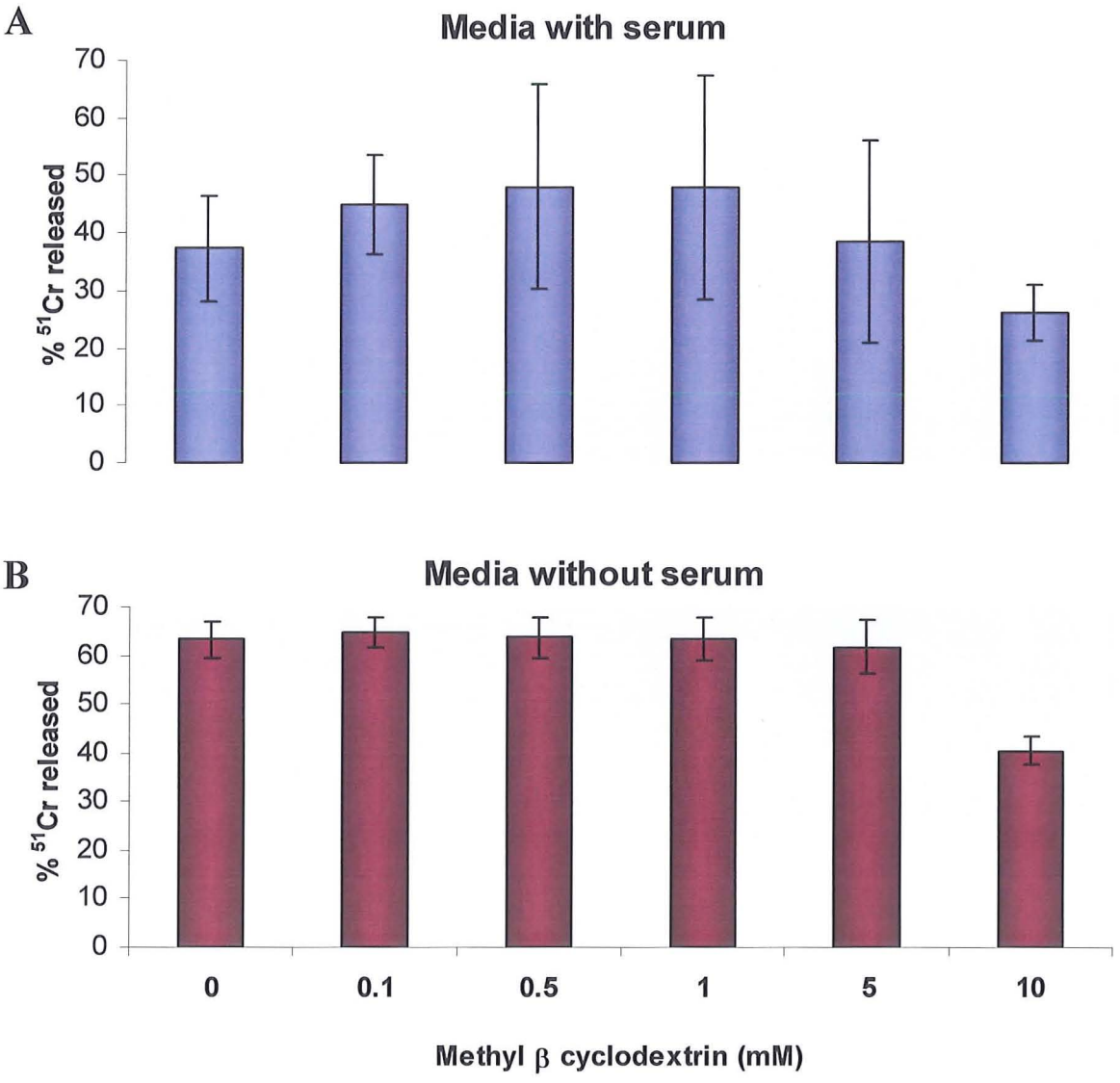


Figure 7.10 High doses of MβCD reduced cytotoxicity of NK-92MI cells and low doses increased cytotoxicity when cultured in media containing cholesterol.

NK-92MI cells were treated with various concentrations of MβCD in culture media with or without FCS for one hour. At high concentrations membrane cholesterol was depleted resulting in reduced NK cytotoxicity. The presence of cholesterol in the culture media, however, increased internalisation of extracellular cholesterol at low concentrations of MβCD and probably caused the increase in NK cell cytotoxicity (A). The increase at low MβCD concentrations was abolished by omission of serum (and therefore extracellular cholesterol) from the culture medium (B).

7.3 Discussion

The effects of statins have been proposed to be independent of changes in circulating cholesterol and to be due to reduction of isoprenoid intermediates in the cholesterol biosynthetic pathway[280, 300]. Isoprenoids play key roles in various aspects of cellular function, specifically the transport and incorporation of lipid insoluble peptides into the cell membrane[46, 332, 333]. Many of these peptides - for example, small G-proteins such as Ras - are involved in membrane signalling and are concentrated within membrane rafts[40, 56, 297, 333]. These signal platforms are dependent on the production of intracellular cholesterol and the recycling of extracellular cholesterol. The prevailing view - that inhibition of isoprenylation explains the pleiotropic effects of statins - is reliant on studies where the inhibitory effects of statins were replaced by the addition of mevalonate, farnesyl pyrophosphate or geranylgeranyl pyrophosphate. However, mevalonate will restore synthesis of all components of the mevalonate pathway distal to HMG-CoA reductase including cholesterol and, since some of the reactions in this pathway are potentially bi-directional, addition of isoprenoids may have a similar effect. Although statins are known to deplete membrane rafts[331, 334] the notion that this may explain the pleiotropic effects of statins has received little attention. The alternative hypothesis – that modification of lipid rafts is the central effect – has found favour amongst nutritionalists to explain the immunomodulatory effects of lipid modifying agents, such as fish oils[297].

A unifying hypothesis would be that membrane and plasma cholesterol (and the density of membrane rafts) may change in parallel. Thus, a treatment that lowers circulating cholesterol is likely to reduce membrane cholesterol and, correspondingly, cell functions that are dependent on membrane cholesterol (and cholesterol rafts). The data provides some preliminary support for this hypothesis.

Potential mechanisms were investigated in a series of *in vitro* studies using an immortalised NK cell line. Depletion of membrane cholesterol using MβCD at high concentrations resulted in reduced NK cell cytotoxicity. Lower concentrations produced a small, reproducible and unexpected increase in NK cell killing. A possible explanation for this is that MβCD increases internalisation of

cholesterol at low concentrations[314] due to its three dimensional structure, and the effect was not seen in the absence of extracellular cholesterol. Exposure to statins had a similar effect that could not be reproduced by inhibitors of farnesyl and geranylgeranyl transferase[332]. Again, this is consistent with an effect mediated by reduction in membrane cholesterol rather than isoprenylation[297]. Western blotting of whole cell membrane and membrane raft fractions were used to determine whether depletion of raft cholesterol was likely (Chapter 5, Figure 5.6 and 5.11). Both statin and MβCD treated cells showed reduced quantities of signal peptides known to be localised in rafts - specifically LAT and Lyn - consistent with reduction in rafts and raft-associated proteins.

NK cells may provide an accessible cell type and the relationship between lipids and immune cell function[297-299] may be applicable to all cells.

Chapter 8: General discussion

The beneficial effects of statin therapy in the treatment of cardiovascular disease are well documented[177, 179, 193, 203]. Although these are likely to be mediated in a large part by the reduction in cholesterol, there is increasing evidence to suggest that inhibition of inflammatory mechanisms may also be important. Some of the beneficial effects of statins have been proposed to be independent of changes in circulating cholesterol and to be due to reduction of isoprenoid intermediates in the cholesterol biosynthetic pathway[280, 300]. Isoprenoids (and isoprenylation) play key roles in various aspects of cellular function, specifically membrane translocation of lipid insoluble peptides into the cell membrane[46, 332, 333]. Many of these peptides, for example Ras and Rho families of GTPases, are involved in membrane signalling and are concentrated within membrane rafts[40, 56, 297, 333]. These signal platforms are dependent on the production of intracellular cholesterol and the recycling of extracellular cholesterol. The common view, that inhibition of isoprenylation explains the pleiotropic effects of statins, is reliant on studies where the inhibitory effects of statins were replaced by the addition of mevalonate, farnesyl pyrophosphate or geranylgeranyl pyrophosphate. However, mevalonate will restore synthesis of all components of the mevalonate pathway distal to HMG-CoA reductase including cholesterol and, since some of the reactions in this pathway are potentially bi-directional, addition of isoprenoids may have a similar effect.

An alternative hypothesis would be that membrane and plasma cholesterol (and the density of membrane rafts) may change in parallel. Thus, a treatment that lowers circulating cholesterol is likely to reduce membrane cholesterol and, correspondingly, cell functions that are dependent on membrane cholesterol (and cholesterol rafts). This thesis provides some preliminary support for this hypothesis and is in keeping with the findings from meta-analysis of clinical studies suggesting that the benefit of statin therapy on cardiovascular outcomes is primarily due to the direct effect of lowering serum cholesterol and hence cell membrane cholesterol[335].

This thesis hypothesised that the pleiotropic effects of statins would reduce lymphocyte functions of T cell proliferation and NK cytotoxicity *in vivo* and *in vitro* and reduce monocyte functions of cytokine release and IC trafficking and degradation. It also aimed to determine the mechanism of immunosuppressive and anti-inflammatory actions of statins by identifying the signalling pathways involved and the mode of disruption whether membrane raft depletion or prenyl inhibition.

The *in vivo* studies of chapter 3 confirmed the benefits of statins in lowering cholesterol and raising HDL. Even in the normal volunteer study, with lipid levels much lower than the majority of patients in clinical trials a significant reduction in LDL cholesterol and increase in HDL cholesterol was observed. *In vivo*, T cell proliferation was reduced in the cardiovascular patients study and statistically insignificantly in the normal volunteer study. The absence of an effect on T cell proliferation is consistent with the absence of infection rates in large-scale trials[177, 179, 193, 203]. Alternatively it could reflect the insensitivity of the assay system or the fact that T cell proliferation is such an important biological response that it may bypass inhibition of a single signalling mechanism.

Natural killer cell function is, however, more sensitive to *in vivo* statin use[250, 285]. A clear reduction in NK cytotoxicity was observed in the normal volunteers, which was reversed by withdrawal from statin therapy. A similar reduction was also obtained in the cardiovascular patients. The changes in cell function paralleled those changes in LDL-cholesterol, reflecting the influence of statin therapy. As with lymphocyte proliferation, there may be differences in sensitivity of the assay making it is easier to detect a reduction in NK cell killing than in lymphocyte proliferation. Alternatively, NK cell activation and killing may be more susceptible to blockade of statin dependent signalling pathways, whether they be dependent on isoprenoids or membrane rafts. Sanni et al[56] suggest that NKG2A inhibitory receptors are excluded from lipid rafts and therefore prevents formation of activation signalling complexes. As activating signals require membrane rafts, disruption of lipid rafts by statins would therefore allow inhibitory receptors to dominate and maintain NK cells in a non-cytotoxic state. The dependence on membrane rafts for activating signals may explain why the *ex vivo* NK cytotoxicity assay was so sensitive to statin treatment.

After showing that statins reduce NK cell cytotoxicity *in vivo*, the possibility that this may reflect an underlying relationship between cholesterol synthesis, membrane cholesterol and cell function was then considered[297]. In the NK study, we found that statin treated patients did not have lower NK cell cytotoxicity than non-statin treated patients but that the overall relationship was with total cholesterol (Chapter 3.10; Table 3.2). The patient selection for this study was not focused on one particular group but was deliberately wide-ranging to provide a spectrum of age, underlying disease and drug therapy to allow comparison of potential influences. The analysis was preliminary and power calculations were not performed, as these were pilot studies. The information gained will require further investigation and be used to power larger, more homogeneous clinical studies. However, the findings concur with the normal volunteer study where there was a close relationship between reduction in NK cell cytotoxicity and lipid levels in normal subjects receiving statins. The meta-analysis by Baigent et al[277] also confirms these findings. The authors concluded that the risk reductions were proportional to the absolute reductions in LDL cholesterol. Thus, rather than being due to the pleiotropic effects of statin treatment, this change may be a consequence of changes in cholesterol levels, both in the circulation and in the cell membrane.

The potential mechanisms were investigated in a series of *in vitro* studies using an immortalised NK cell line. Depletion of membrane cholesterol using MβCD at high concentrations resulted in reduced NK cell cytotoxicity. Exposure to statins had a similar effect that could not be reproduced by inhibitors of farnesyl and geranylgeranyl transferase[332]. Again, this is consistent with an effect mediated by reduction in membrane cholesterol rather than isoprenylation[297]. Western blotting of whole cells and membrane raft fractions were used to determine whether depletion of raft cholesterol was likely. Both statin and MβCD treated cells showed reduced quantities of signal peptides known to be localised in rafts (i.e. LAT and Lyn) consistent with reduction in rafts and raft-associated proteins.

Although these were small-scale, pilot studies that recruited normal subjects and patients over a wide age range, a range of drug therapy and underlying pathology, it poses questions about the relationship between circulating lipids and membrane lipids, and the wider effects on cell membrane structure and signalling. NK cells

may simply provide an accessible cell type and the relationship between lipids and immune cell function[298, 314] may be applicable to all cells.

Monocyte FcγR mediate several critical functions within the immune system including clearance and disposal of immune complexes, antigen presentation, and release of inflammatory mediators, that may be involved in atherosclerosis[336]. Chapter 6 demonstrates that fluvastatin causes a large reduction in FcγR-mediated immune complex trafficking and degradation and, to a lesser extent, IC internalisation. FcγR-mediated tyrosine phosphorylation was attenuated by fluvastatin at therapeutic concentrations without altering the number of receptors. These effects are important as macrophage FcγR-internalised IC can be degraded and MHC class II antigen presented to T-cells. The presence of activated CD4+ T-cells and increased MHC class II expression within atherosclerotic lesions suggest that specific cellular immune responses, as well as non-specific inflammatory responses, contribute to atherosclerosis[325] and then thus be inhibited by statins.

Statins have previously been reported to inhibit production of inflammatory mediators (including MCP-1, MMP-9, TNF-α and IL-8) by monocytes[153, 320, 321]. The FcγR-coupled release of the inflammatory mediators, IL-6 and MMP-1, and associated inhibition of the MAP kinases ERK and p38, which lie upstream in this pathway of cytokine release were inhibited by fluvastatin and simvastatin. Coupling of receptors to ERK activation lies downstream of the farnesylated small GTPase, Ras whilst p38 is downstream of geranyl-geranylated Rho family GTPases[301]. However, recent data suggest that FcγR coupling to ERK is independent of Ras[326] and further investigation is required to determine whether statin mediated inhibition of FcγR coupling to these pathways depends on a block in isoprenylation or to disruption of membrane raft function through blockade of cholesterol synthesis.

Release of MMP-1 from U937 cells in response to oxLDL-IC has previously been reported[319]. Thus, the observation that fluvastatin abolishes MMP-1 release in response to FcγR stimulation is of particular interest as monocyte MMP-1 has been implicated in the destabilisation of atherosclerotic plaques and subsequent coronary events. The finding that fluvastatin decreased FcγR mediated IL-6

production in monocytes is pertinent in light of recent data that statins reduced systemic IL-6 levels in patients with cardiovascular disease[328].

Membrane rafts are critical to initiation of signal transduction by many ITAM bearing immune receptors, including FcγR and the B and T cell antigen receptors[279, 315]. Following ligation, receptors translocate into rafts where they co-localise with signal transduction molecules, such as src kinases, adapter proteins and PLC-γ. Since raft structure and function depends on cholesterol, disruption of rafts provides a potential novel mechanism to explain the actions of statins on FcγR signalling. Raft dependent FcγR mediated signaling is inhibited in a time and dose dependent manner by low levels of fluvastatin (0.5μM) that are representative of *in vivo* concentrations[337], with parallel reductions in the lipid raft associated signaling proteins LAT and Lyn. Disruption of membrane rafts was not seen following treatment of cells with inhibitors of prenylation, supporting the hypothesis that this effect of fluvastatin is independent of prenylated proteins. Since other multichain immune recognition receptors (e.g. BCR, TCR) signal through membrane rafts this mechanism is likely to have broad significance in the immune system and elsewhere[317].

Accumulating evidence indicates a multifactorial mode of action for statins in the reduction of morbidity and mortality from cardiovascular disease. Thus, in addition to lowering LDL-cholesterol levels, statins block leukocyte recruitment to atherosclerotic lesions, reduce NK functions, inhibit monocyte antigen processing and production of inflammatory mediators and inhibit release of matrix proteases implicated in plaque rupture. Multiple mechanisms are likely to underlie these diverse effects including inhibition of cholesterol and isoprenoid synthesis, and disruption of cholesterol rich signalling domains. Statins may confer cardiovascular benefit without inhibiting essential cellular or immunological functions. NK cells have been implicated in atherosclerosis and are known to target cells that do not express HLA class I on the cell surface, including cells that have been infected by viruses[285, 295]. Whether this effect on NK cells contributes to the overall beneficial effects of statin therapy in cardiovascular disease is not clear and will require further investigation. However, the mechanism by which statins inhibit NK cell killing is likely to provide an insight into the signalling mechanisms disrupted by statin therapy and that contribute to their pleiotropic effects in other

cell types[86, 239]. The data also support a potential role for Fc receptors and IC in the pathophysiology of atherosclerosis, and identify a novel effect of fluvastatin on membrane raft function that may also be relevant for other transmembrane receptors in cardiovascular and other diseases.

This thesis had some limitations. Some western blots and cytokine assays were performed twice resulting in small *n* values. It would have been preferable to perform multiple repeats to measure intra and inter assay co-efficients of variations for each assay to increase their reproducibility. However, intra assay variabilities were small and large variabilities of some inter assay values prevented meaningful statistics being performed, although the patterns emerged were comparable.

Work I would like to perform in the future includes development of the measurement of membrane lipid rafts *in vivo*. Although the initial studies (Figures 3.7 and 3.8) were statistically insignificant, improvement of the raft extraction technique and larger numbers would improve the statistical outcome. Large scale clinical studies on groups of patients with differing lipid lowering treatments could be used to determine whether reduction of serum cholesterol is responsible for disruption of lipids in membrane rafts rather than intracellular biosynthesis. NK cells could be used as a model to relate the quantity and quality of lipid rafts, reduced by various lipid lowering treatments, to clinical outcomes or risk of disease. This work could therefore identify a mechanism for the conclusions of Baigent *et al*[277].

8.1 Conclusions

In conclusion, this thesis has demonstrated that statins have a multitude of applications and effects. The pleiotropism of statins due to reduced prenylation was observed *in vitro* by western blot in multiple signalling pathways and confirmed with the use of FT and GGT inhibitors in these pathways. However, the lack of functional effect of FT and GGTIs indicated that prenylation has a lesser impact on the functions of immune effector cells than cholesterol depletion of rafts. The link between plasma cholesterol and raft functions may have wide

varying implications. The comparable results obtained with MβCD indicated that the reduction of cholesterol in the membrane by statins was disrupting rafts and therefore disrupting signalling pathways to a greater extent than prenylation inhibition. It is however likely to be a combination of both processes that contributes to the pleiotropic effect of statins.

References

1. Goldstein JL, Brown MS. Regulation of the mevalonate pathway. *Nature* 1990;343(6257):425-30.
2. Tabernero L, Rodwell VW, Stauffacher CV. Crystal structure of a statin bound to a class II hydroxymethylglutaryl-CoA reductase. *J Biol Chem* 2003;278(22):19933-8.
3. Istvan E. Statin inhibition of HMG-CoA reductase: a 3-dimensional view. *Atheroscler Suppl* 2003;4(1):3-8.
4. Istvan ES, Deisenhofer J. Structural mechanism for statin inhibition of HMG-CoA reductase. *Science* 2001;292(5519):1160-4.
5. Endo A, Kuroda M, Tsujita Y. ML-236A, ML-236B, and ML-236C, new inhibitors of cholesterogenesis produced by Penicillium citrinium. *J Antibiot (Tokyo)* 1976;29(12):1346-8.
6. Blum CB. Comparison of properties of four inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Am J Cardiol* 1994;73(14):3D-11D.
7. Choice of lipid-lowering drugs. *Med Lett Drugs Ther* 1998;40(1042):117-22.
8. Lennernas H, Fager G. Pharmacodynamics and pharmacokinetics of the HMG-CoA reductase inhibitors. Similarities and differences. *Clin Pharmacokinet* 1997;32(5):403-25.
9. Azie NE, Brater DC, Becker PA, Jones DR, Hall SD. The interaction of diltiazem with lovastatin and pravastatin. *Clin Pharmacol Ther* 1998;64(4):369-77.
10. Ichihara K, Satoh K. Disparity between angiographic regression and clinical event rates with hydrophobic statins. *Lancet* 2002;359(9324):2195-8.
11. Zhang FL, Casey PJ. Protein prenylation: molecular mechanisms and functional consequences. *Annu Rev Biochem* 1996;65:241-69.
12. Takai Y, Sasaki T, Matozaki T. Small GTP-binding proteins. *Physiol Rev* 2001;81(1):153-208.
13. Harris CM, Poulter CD. Recent studies of the mechanism of protein prenylation. *Nat Prod Rep* 2000;17(2):137-44.
14. Lobell RB. Prenylation of Ras GTPase superfamily proteins and their function in immunobiology. *Adv Immunol* 1998;68:145-89.
15. Moores SL, Schaber MD, Mosser SD, et al. Sequence dependence of protein isoprenylation. *J Biol Chem* 1991;266(22):14603-10.
16. Sinensky M. Recent advances in the study of prenylated proteins. *Biochim Biophys Acta* 2000;1484(2-3):93-106.
17. Boyartchuk VL, Ashby MN, Rine J. Modulation of Ras and a-factor function by carboxyl-terminal proteolysis. *Science* 1997;275(5307):1796-800.
18. Hancock JF, Cadwallader K, Marshall CJ. Methylation and proteolysis are essential for efficient membrane binding of prenylated p21K-ras(B). *Embo J* 1991;10(3):641-6.

19. Stephenson RC, Clarke S. Identification of a C-terminal protein carboxyl methyltransferase in rat liver membranes utilizing a synthetic farnesyl cysteine-containing peptide substrate. *J Biol Chem* 1990;265(27):16248-54.

20. Rando RR. Chemical biology of isoprenylation/methylation. *Biochem Soc Trans* 1996;24(3):682-87.

21. Whyte DB, Kirschmeier P, Hockenberry TN, et al. K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. *J Biol Chem* 1997;272(22):14459-64.

22. Roskoski R, Jr. Protein prenylation: a pivotal posttranslational process. *Biochem Biophys Res Commun* 2003;303(1):1-7.

23. Armstrong SA, Hannah VC, Goldstein JL, Brown MS. CAAX geranylgeranyl transferase transfers farnesyl as efficiently as geranylgeranyl to RhoB. *J Biol Chem* 1995;270(14):7864-8.

24. Dunphy JT, Linder ME. Signalling functions of protein palmitoylation. *Biochim Biophys Acta* 1998;1436(1-2):245-61.

25. Boutin JA. Myristoylation. *Cell Signal* 1997;9(1):15-35.

26. Hancock JF, Magee AI, Childs JE, Marshall CJ. All ras proteins are polyisoprenylated but only some are palmitoylated. *Cell* 1989;57(7):1167-77.

27. Magee T, Seabra MC. Fatty acylation and prenylation of proteins: what's hot in fat. *Curr Opin Cell Biol* 2005;17(2):190-6.

28. Parton RG, Hancock JF. Lipid rafts and plasma membrane microorganization: insights from Ras. *Trends Cell Biol* 2004;14(3):141-7.

29. Greenwood J, Steinman L, Zamvil SS. Statin therapy and autoimmune disease: from protein prenylation to immunomodulation. *Nat Rev Immunol* 2006;6(5):358-70.

30. Hoffman GR, Nassar N, Cerione RA. Structure of the Rho family GTP-binding protein Cdc42 in complex with the multifunctional regulator RhoGDI. *Cell* 2000;100(3):345-56.

31. DerMardirossian C, Bokoch GM. GDIs: central regulatory molecules in Rho GTPase activation. *Trends Cell Biol* 2005;15(7):356-63.

32. Rossman KL, Der CJ, Sondek J. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol* 2005;6(2):167-80.

33. Liu X, Wang H, Eberstadt M, et al. NMR structure and mutagenesis of the N-terminal Dbl homology domain of the nucleotide exchange factor Trio. *Cell* 1998;95(2):269-77.

34. McTaggart SJ. Isoprenylated proteins. *Cell Mol Life Sci* 2006;63(3):255-67.

35. Donovan S, Shannon KM, Bollag G. GTPase activating proteins: critical regulators of intracellular signaling. *Biochim Biophys Acta* 2002;1602(1):23-45.

36. Bernards A, Settleman J. GAP control: regulating the regulators of small GTPases. *Trends Cell Biol* 2004;14(7):377-85.

37. Benians A, Nobles M, Hosny S, Tinker A. Regulators of G-protein signaling form a quaternary complex with the agonist, receptor, and G-protein. A novel explanation for the acceleration of signaling activation kinetics. *J Biol Chem* 2005;280(14):13383-94.

38. Rajendran L, Simons K. Lipid rafts and membrane dynamics. *J Cell Sci* 2005;118(Pt 6):1099-102.

39. Simons K, Vaz WL. Model systems, lipid rafts, and cell membranes. *Annu Rev Biophys Biomol Struct* 2004;33:269-95.

40. Tuosto L, Parolini I, Schroder S, Sargiacomo M, Lanzavecchia A, Viola A. Organization of plasma membrane functional rafts upon T cell activation. *Eur J Immunol* 2001;31(2):345-9.

41. Dykstra M, Cherukuri A, Sohn HW, Tzeng SJ, Pierce SK. Location is everything: lipid rafts and immune cell signaling. *Annu Rev Immunol* 2003;21:457-81.

42. Kurzchalia TV, Parton RG. Membrane microdomains and caveolae. *Curr Opin Cell Biol* 1999;11(4):424-31.

43. Schroeder RJ, Ahmed SN, Zhu Y, London E, Brown DA. Cholesterol and sphingolipid enhance the Triton X-100 insolubility of glycosylphosphatidylinositol-anchored proteins by promoting the formation of detergent-insoluble ordered membrane domains. *J Biol Chem* 1998;273(2):1150-7.

44. Resh MD. Membrane targeting of lipid modified signal transduction proteins. *Subcell Biochem* 2004;37:217-32.

45. Wang TY, Leventis R, Silvius JR. Partitioning of lipidated peptide sequences into liquid-ordered lipid domains in model and biological membranes. *Biochemistry* 2001;40(43):13031-40.

46. Melkonian KA, Ostermeyer AG, Chen JZ, Roth MG, Brown DA. Role of lipid modifications in targeting proteins to detergent-resistant membrane rafts. Many raft proteins are acylated, while few are prenylated. *J Biol Chem* 1999;274(6):3910-7.

47. Gri G, Molon B, Manes S, Pozzan T, Viola A. The inner side of T cell lipid rafts. *Immunol Lett* 2004;94(3):247-52.

48. Harder T, Engelhardt KR. Membrane domains in lymphocytes - from lipid rafts to protein scaffolds. *Traffic* 2004;5(4):265-75.

49. Sedwick CE, Altman A. Ordered just so: lipid rafts and lymphocyte function. *Sci STKE* 2002;2002(122):RE2.

50. Montixi C, Langlet C, Bernard AM, et al. Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. *Embo J* 1998;17(18):5334-48.

51. Burack WR, Lee KH, Holdorf AD, Dustin ML, Shaw AS. Cutting edge: quantitative imaging of raft accumulation in the immunological synapse. *J Immunol* 2002;169(6):2837-41.

52. Huby RD, Dearman RJ, Kimber I. Intracellular phosphotyrosine induction by major histocompatibility complex class II requires co-aggregation with membrane rafts. *J Biol Chem* 1999;274(32):22591-6.

53. Anderson HA, Hiltbold EM, Roche PA. Concentration of MHC class II molecules in lipid rafts facilitates antigen presentation. *Nat Immunol* 2000;1(2):156-62.

54. Lanier LL. NK cell receptors. *Annu Rev Immunol* 1998;16:359-93.

55. Lou Z, Jevremovic D, Billadeau DD, Leibson PJ. A balance between positive and negative signals in cytotoxic lymphocytes regulates the polarization of lipid rafts during the development of cell-mediated killing. *J Exp Med* 2000;191(2):347-54.

56. Sanni TB, Masilamani M, Kabat J, Coligan JE, Borrego F. Exclusion of lipid rafts and decreased mobility of CD94/NKG2A receptors at the inhibitory NK cell synapse. *Mol Biol Cell* 2004;15(7):3210-23.

57. Abbas AK LA, Pober JS. *Cellular and Molecular Immunology*. 4 ed. Philadelphia: WB Saunders Company, 2000.

58. Gordon S. Macrophages and the Immune Response. In: Paul, editor. *Fundamantel Immunology*. New York: Lippincott-Raven, 1999:533-545.

59. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 2005;5(12):953-64.

60. Harris PE, Ralph P, Gabrilove J, Welte K, Karmali R, Moore MA. Distinct differentiation-inducing activities of gamma-interferon and cytokine factors acting on the human promyelocytic leukemia cell line HL-60. *Cancer Res* 1985;45(7):3090-5.

61. Urzainqui A, Serrador JM, Viedma F, et al. ITAM-based interaction of ERM proteins with Syk mediates signaling by the leukocyte adhesion receptor PSGL-1. *Immunity* 2002;17(4):401-12.

62. Poddar R, Sivasubramanian N, DiBello PM, Robinson K, Jacobsen DW. Homocysteine induces expression and secretion of monocyte chemoattractant protein-1 and interleukin-8 in human aortic endothelial cells: implications for vascular disease. *Circulation* 2001;103(22):2717-23.

63. Frostegard J, Nilsson J, Haegerstrand A, Hamsten A, Wigzell H, Gidlund M. Oxidized low density lipoprotein induces differentiation and adhesion of human monocytes and the monocytic cell line U937. *Proc Natl Acad Sci U S A* 1990;87(3):904-8.

64. Daeron M. Fc receptor biology. *Annu Rev Immunol* 1997;15:203-34.

65. Cambier JC. Antigen and Fc receptor signaling. The awesome power of the immunoreceptor tyrosine-based activation motif (ITAM). *J Immunol* 1995;155(7):3281-5.

66. Dykstra ML, Cherukuri A, Pierce SK. Floating the raft hypothesis for immune receptors: access to rafts controls receptor signaling and trafficking. *Traffic* 2001;2(3):160-6.

67. Kono H, Suzuki T, Yamamoto K, Okada M, Yamamoto T, Honda Z. Spatial raft coalescence represents an initial step in FcgammaR signaling. *J Immunol* 2002;169(1):193-203.

68. Zhang W, Samelson LE. The role of membrane-associated adaptors in T cell receptor signalling. *Semin Immunol* 2000;12(1):35-41.

69. Tridandapani S, Lyden TW, Smith JL, Carter JE, Coggeshall KM, Anderson CL. The adapter protein LAT enhances fcgamma receptor-mediated signal transduction in myeloid cells. *J Biol Chem* 2000;275(27):20480-7.

70. Bradley LM. Migration and T-lymphocyte effector function. *Curr Opin Immunol* 2003;15(3):343-8.

71. Marwali MR, Rey-Ladino J, Dreolini L, Shaw D, Takei F. Membrane cholesterol regulates LFA-1 function and lipid raft heterogeneity. *Blood* 2003;102(1):215-22.

72. Carlos TM, Harlan JM. Leukocyte-endothelial adhesion molecules. *Blood* 1994;84(7):2068-101.

73. Van Seventer GA, Shimizu Y, Horgan KJ, Shaw S. The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells. *J Immunol* 1990;144(12):4579-86.

74. Yusuf-Makagiansar H, Anderson ME, Yakovleva TV, Murray JS, Siahaan TJ. Inhibition of LFA-1/ICAM-1 and VLA-4/VCAM-1 as a therapeutic approach to inflammation and autoimmune diseases. *Med Res Rev* 2002;22(2):146-67.

75. Hogg N, Laschinger M, Giles K, McDowall A. T-cell integrins: more than just sticking points. *J Cell Sci* 2003;116(Pt 23):4695-705.

76. Weitz-Schmidt G, Welzenbach K, Brinkmann V, et al. Statins selectively inhibit leukocyte function antigen-1 by binding to a novel regulatory integrin site. *Nat Med* 2001;7(6):687-92.

77. Weitz-Schmidt G. Lymphocyte function-associated antigen-1 blockade by statins: molecular basis and biological relevance. *Endothelium* 2003;10(1):43-7.

78. Stewart M, Hogg N. Regulation of leukocyte integrin function: affinity vs. avidity. *J Cell Biochem* 1996;61(4):554-61.

79. Leitinger B, Hogg N. The involvement of lipid rafts in the regulation of integrin function. *J Cell Sci* 2002;115(Pt 5):963-72.

80. Shamri R, Grabovsky V, Feigelson SW, Dwir O, Van Kooyk Y, Alon R. Chemokine stimulation of lymphocyte alpha 4 integrin avidity but not of leukocyte function-associated antigen-1 avidity to endothelial ligands under shear flow requires cholesterol membrane rafts. *J Biol Chem* 2002;277(42):40027-35.

81. Wojciak-Stothard B, Williams L, Ridley AJ. Monocyte adhesion and spreading on human endothelial cells is dependent on Rho-regulated receptor clustering. *J Cell Biol* 1999;145(6):1293-307.

82. Vincenti F. New monoclonal antibodies in renal transplantation. *Minerva Urol Nefrol* 2003;55(1):57-66.

83. Pandolfi F, Trentin L, Boyle LA, et al. Expression of cell adhesion molecules in human melanoma cell lines and their role in cytotoxicity mediated by tumor-infiltrating lymphocytes. *Cancer* 1992;69(5):1165-73.

84. Trinchieri G. Biology of natural killer cells. *Adv Immunol* 1989;47:187-376.

85. Zompi S, Colucci F. Anatomy of a murder--signal transduction pathways leading to activation of natural killer cells. *Immunol Lett* 2005;97(1):31-9.

86. Perussia B. Signaling for cytotoxicity. *Nat Immunol* 2000;1(5):372-4.

87. Billadeau DD, Brumbaugh KM, Dick CJ, Schoon RA, Bustelo XR, Leibson PJ. The Vav-Rac1 pathway in cytotoxic lymphocytes regulates the generation of cell-mediated killing. *J Exp Med* 1998;188(3):549-59.

88. Davis DM, Dustin ML. What is the importance of the immunological synapse? *Trends Immunol* 2004;25(6):323-7.

89. Eriksson M, Leitz G, Fallman E, et al. Inhibitory receptors alter natural killer cell interactions with target cells yet allow simultaneous killing of susceptible targets. *J Exp Med* 1999;190(7):1005-12.

90. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol* 2001;22(11):633-40.

91. Riteau B, Barber DF, Long EO. Vav1 phosphorylation is induced by beta2 integrin engagement on natural killer cells upstream of actin cytoskeleton and lipid raft reorganization. *J Exp Med* 2003;198(3):469-74.

92. Colucci F, Schweighoffer E, Tomasello E, et al. Natural cytotoxicity uncoupled from the Syk and ZAP-70 intracellular kinases. *Nat Immunol* 2002;3(3):288-94.

93. Lanier LL. On guard--activating NK cell receptors. *Nat Immunol* 2001;2(1):23-7.

94. Lanier LL, Yu G, Phillips JH. Analysis of Fc gamma RIII (CD16) membrane expression and association with CD3 zeta and Fc epsilon RI-gamma by site-directed mutation. *J Immunol* 1991;146(5):1571-6.

95. Leibson PJ. Signal transduction during natural killer cell activation: inside the mind of a killer. *Immunity* 1997;6(6):655-61.

96. Moretta L, Biassoni R, Bottino C, Mingari MC, Moretta A. Natural killer cells: a mystery no more. *Scand J Immunol* 2002;55(3):229-32.

97. Bauer S, Groh V, Wu J, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 1999;285(5428):727-9.

98. Groh V, Bahram S, Bauer S, Herman A, Beauchamp M, Spies T. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. *Proc Natl Acad Sci U S A* 1996;93(22):12445-50.

99. Welte SA, Sinzger C, Lutz SZ, et al. Selective intracellular retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein. *Eur J Immunol* 2003;33(1):194-203.

100. Backstrom E, Kristensson K, Ljunggren HG. Activation of natural killer cells: underlying molecular mechanisms revealed. *Scand J Immunol* 2004;60(1-2):14-22.

101. Wu J, Song Y, Bakker AB, et al. An activating immunoreceptor complex formed by NKG2D and DAP10. *Science* 1999;285(5428):730-2.

102. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol* 2003;3(10):781-90.

103. Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol* 2005;17(1):1-14.

104. Schmidt KN, Leung B, Kwong M, et al. APC-independent activation of NK cells by the Toll-like receptor 3 agonist double-stranded RNA. *J Immunol* 2004;172(1):138-43.

105. Chuang SS, Kumaresan PR, Mathew PA. 2B4 (CD244)-mediated activation of cytotoxicity and IFN-gamma release in human NK cells involves distinct pathways. *J Immunol* 2001;167(11):6210-6.

106. Tangye SG, Lazetic S, Woollatt E, Sutherland GR, Lanier LL, Phillips JH. Cutting edge: human 2B4, an activating NK cell receptor, recruits the protein tyrosine phosphatase SHP-2 and the adaptor signaling protein SAP. *J Immunol* 1999;162(12):6981-5.

107. Bottino C, Augugliaro R, Castriconi R, et al. Analysis of the molecular mechanism involved in 2B4-mediated NK cell activation: evidence that human 2B4 is physically and functionally associated with the linker for activation of T cells. *Eur J Immunol* 2000;30(12):3718-22.

108. Vitale M, Falco M, Castriconi R, et al. Identification of NKp80, a novel triggering molecule expressed by human NK cells. *Eur J Immunol* 2001;31(1):233-42.

109. Jiang K, Zhong B, Gilvary DL, et al. Pivotal role of phosphoinositide-3 kinase in regulation of cytotoxicity in natural killer cells. *Nat Immunol* 2000;1(5):419-25.

110. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 1986;319(6055):675-8.

111. Gumperz JE, Litwin V, Phillips JH, Lanier LL, Parham P. The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. *J Exp Med* 1995;181(3):1133-44.

112. Yusa S, Catina TL, Campbell KS. SHP-1- and phosphotyrosine-independent inhibitory signaling by a killer cell Ig-like receptor cytoplasmic domain in human NK cells. *J Immunol* 2002;168(10):5047-57.

113. Yusa S, Campbell KS. Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2) can play a direct role in the inhibitory function of killer cell Ig-like receptors in human NK cells. *J Immunol* 2003;170(9):4539-47.

114. Vilches C, Parham P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol* 2002;20:217-51.

115. Llano M, Lee N, Navarro F, et al. HLA-E-bound peptides influence recognition by inhibitory and triggering CD94/NKG2 receptors: preferential response to an HLA-G-derived nonamer. *Eur J Immunol* 1998;28(9):2854-63.

116. Lanier LL. NK cell recognition. *Annu Rev Immunol* 2005;23:225-74.

117. Stebbins CC, Watzl C, Billadeau DD, Leibson PJ, Burshtyn DN, Long EO. Vav1 dephosphorylation by the tyrosine phosphatase SHP-1 as a mechanism for inhibition of cellular cytotoxicity. *Mol Cell Biol* 2003;23(17):6291-9.

118. Screpanti V, Wallin RP, Ljunggren HG, Grandien A. A central role for death receptor-mediated apoptosis in the rejection of tumors by NK cells. *J Immunol* 2001;167(4):2068-73.

119. Street SE, Trapani JA, MacGregor D, Smyth MJ. Suppression of lymphoma and epithelial malignancies effected by interferon gamma. *J Exp Med* 2002;196(1):129-34.

120. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol* 1999;17:189-220.

121. Lauwerys BR, Garot N, Renauld JC, Houssiau FA. Cytokine production and killer activity of NK/T-NK cells derived with IL-2, IL-15, or the combination of IL-12 and IL-18. *J Immunol* 2000;165(4):1847-53.

122. Robertson MJ. Role of chemokines in the biology of natural killer cells. *J Leukoc Biol* 2002;71(2):173-83.

123. Rosenberg HF, Gallin JI. Inflammation. In: Paul, editor. *Fundamental immunology*. 4th ed. New York: Lippincott-Raven, 1999:1051.

124. Libby P. Inflammation and cardiovascular disease mechanisms. *Am J Clin Nutr* 2006;83(2):456S-460S.

125. Falk E, Shah PK, Fuster V. Coronary plaque disruption. *Circulation* 1995;92(3):657-71.

126. Massberg S, Brand K, Gruner S, et al. A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation. *J Exp Med* 2002;196(7):887-96.

127. Horkko S, Binder CJ, Shaw PX, et al. Immunological responses to oxidized LDL. *Free Radic Biol Med* 2000;28(12):1771-9.

128. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation* 2002;105(9):1135-43.

129. Dai G, Kaazempur-Mofrad MR, Natarajan S, et al. Distinct endothelial phenotypes evoked by arterial waveforms derived from atherosclerosis-susceptible and -resistant regions of human vasculature. *Proc Natl Acad Sci U S A* 2004;101(41):14871-6.

130. Peiser L, Mukhopadhyay S, Gordon S. Scavenger receptors in innate immunity. *Curr Opin Immunol* 2002;14(1):123-8.

131. Janeway CA, Jr., Medzhitov R. Innate immune recognition. *Annu Rev Immunol* 2002;20:197-216.

132. Miller YI, Chang MK, Binder CJ, Shaw PX, Witztum JL. Oxidized low density lipoprotein and innate immune receptors. *Curr Opin Lipidol* 2003;14(5):437-45.

133. Mallat Z, Besnard S, Duriez M, et al. Protective role of interleukin-10 in atherosclerosis. *Circ Res* 1999;85(8):e17-24.

134. Robertson AK, Rudling M, Zhou X, Gorelik L, Flavell RA, Hansson GK. Disruption of TGF-beta signaling in T cells accelerates atherosclerosis. *J Clin Invest* 2003;112(9):1342-50.

135. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005;352(16):1685-95.

136. Chang MK, Binder CJ, Miller YI, et al. Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. *J Exp Med* 2004;200(11):1359-70.

137. Palinski W, Miller E, Witztum JL. Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. *Proc Natl Acad Sci U S A* 1995;92(3):821-5.

138. Maron R, Sukhova G, Faria AM, et al. Mucosal administration of heat shock protein-65 decreases atherosclerosis and inflammation in aortic arch of low-density lipoprotein receptor-deficient mice. *Circulation* 2002;106(13):1708-15.

139. Danesh J, Wheeler JG, Hirschfield GM, et al. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *N Engl J Med* 2004;350(14):1387-97.

140. Liuzzo G, Biasucci LM, Rebuzzi AG, et al. Plasma protein acute-phase response in unstable angina is not induced by ischemic injury. *Circulation* 1996;94(10):2373-80.

141. Vlaicu R, Niculescu F, Rus HG, Cristea A. Immunohistochemical localization of the terminal C5b-9 complement complex in human aortic fibrous plaque. *Atherosclerosis* 1985;57(2-3):163-77.

142. Moreno PR, Falk E, Palacios IF, Newell JB, Fuster V, Fallon JT. Macrophage infiltration in acute coronary syndromes. Implications for plaque rupture. *Circulation* 1994;90(2):775-8.

143. van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. *Circulation* 1994;89(1):36-44.

144. Mach F, Schonbeck U, Bonnefoy JY, Pober JS, Libby P. Activation of monocyte/macrophage functions related to acute atheroma complication by ligation of CD40: induction of collagenase, stromelysin, and tissue factor. *Circulation* 1997;96(2):396-9.

145. Amento EP, Ehsani N, Palmer H, Libby P. Cytokines and growth factors positively and negatively regulate interstitial collagen gene expression in human vascular smooth muscle cells. *Arterioscler Thromb* 1991;11(5):1223-30.

146. Jones CB, Sane DC, Herrington DM. Matrix metalloproteinases: a review of their structure and role in acute coronary syndrome. *Cardiovasc Res* 2003;59(4):812-23.

147. Liu J, Sukhova GK, Sun JS, Xu WH, Libby P, Shi GP. Lysosomal cysteine proteases in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2004;24(8):1359-66.

148. Horton DB, Libby P, Schonbeck U. Ligation of CD40 onvascular smooth muscle cells mediates loss of interstitial collagen via matrix metalloproteinase activity. *Ann N Y Acad Sci* 2001;947:329-36.

149. Weber C, Erl W, Weber KS, Weber PC. HMG-CoA reductase inhibitors decrease CD11b expression and CD11b-dependent adhesion of monocytes to endothelium and reduce increased adhesiveness of monocytes isolated from patients with hypercholesterolemia. *J Am Coll Cardiol* 1997;30(5):1212-7.

150. Rasmussen LM, Hansen PR, Nabipour MT, Olesen P, Kristiansen MT, Ledet T. Diverse effects of inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase on the expression of VCAM-1 and E-selectin in endothelial cells. *Biochem J* 2001;360(Pt 2):363-70.

151. Chung HK, Lee IK, Kang H, et al. Statin inhibits interferon-gamma-induced expression of intercellular adhesion molecule-1 (ICAM-1) in vascular endothelial and smooth muscle cells. *Exp Mol Med* 2002;34(6):451-61.

152. Kwak B, Mulhaupt F, Myit S, Mach F. Statins as a newly recognized type of immunomodulator. *Nat Med* 2000;6(12):1399-402.

153. Romano M, Diomede L, Sironi M, et al. Inhibition of monocyte chemotactic protein-1 synthesis by statins. *Lab Invest* 2000;80(7):1095-100.

154. Ito T, Ikeda U, Yamamoto K, Shimada K. Regulation of interleukin-8 expression by HMG-CoA reductase inhibitors in human vascular smooth muscle cells. *Atherosclerosis* 2002;165(1):51-5.

155. Diomede L, Albani D, Sottocorno M, et al. In vivo anti-inflammatory effect of statins is mediated by nonsterol mevalonate products. *Arterioscler Thromb Vasc Biol* 2001;21(8):1327-32.

156. Martin-Ventura JL, Blanco-Colio LM, Gomez-Hernandez A, et al. Intensive treatment with atorvastatin reduces inflammation in mononuclear cells and human atherosclerotic lesions in one month. *Stroke* 2005;36(8):1796-800.

157. Kaneider NC, Reinisch CM, Dunzendorfer S, Meierhofer C, Djanani A, Wiedermann CJ. Induction of apoptosis and inhibition of migration of inflammatory and vascular wall cells by cerivastatin. *Atherosclerosis* 2001;158(1):23-33.

158. Blanco-Colio LM, Villa A, Ortego M, et al. 3-Hydroxy-3-methyl-glutaryl coenzyme A reductase inhibitors, atorvastatin and simvastatin, induce apoptosis of vascular smooth muscle cells by downregulation of Bcl-2 expression and Rho A prenylation. *Atherosclerosis* 2002;161(1):17-26.

159. Guijarro C, Blanco-Colio LM, Ortego M, et al. 3-Hydroxy-3-methylglutaryl coenzyme a reductase and isoprenylation inhibitors induce apoptosis of vascular smooth muscle cells in culture. *Circ Res* 1998;83(5):490-500.

160. Martinet W, Kockx MM. Apoptosis in atherosclerosis: focus on oxidized lipids and inflammation. *Curr Opin Lipidol* 2001;12(5):535-41.

161. Aikawa M, Rabkin E, Sugiyama S, et al. An HMG-CoA reductase inhibitor, cerivastatin, suppresses growth of macrophages expressing matrix metalloproteinases and tissue factor in vivo and in vitro. *Circulation* 2001;103(2):276-83.

162. Ikeda U, Shimpo M, Ohki R, et al. Fluvastatin inhibits matrix metalloproteinase-1 expression in human vascular endothelial cells. *Hypertension* 2000;36(3):325-9.

163. Crisby M, Nordin-Fredriksson G, Shah PK, Yano J, Zhu J, Nilsson J. Pravastatin treatment increases collagen content and decreases lipid content, inflammation, metalloproteinases, and cell death in human carotid plaques: implications for plaque stabilization. *Circulation* 2001;103(7):926-33.

164. Schonbeck U, Libby P. Inflammation, immunity, and HMG-CoA reductase inhibitors: statins as antiinflammatory agents? *Circulation* 2004;109(21 Suppl 1):II18-26.

165. Chase AJ, Bond M, Crook MF, Newby AC. Role of nuclear factor-kappa B activation in metalloproteinase-1, -3, and -9 secretion by human macrophages in vitro and rabbit foam cells produced in vivo. *Arterioscler Thromb Vasc Biol* 2002;22(5):765-71.

166. Eto M, Kozai T, Cosentino F, Joch H, Luscher TF. Statin prevents tissue factor expression in human endothelial cells: role of Rho/Rho-kinase and Akt pathways. *Circulation* 2002;105(15):1756-9.

167. Essig M, Nguyen G, Prie D, Escoubet B, Sraer JD, Friedlander G. 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors increase fibrinolytic activity in rat aortic endothelial cells. Role of geranylgeranylation and Rho proteins. *Circ Res* 1998;83(7):683-90.

168. Alfon J, Royo T, Garcia-Moll X, Badimon L. Platelet deposition on eroded vessel walls at a stenotic shear rate is inhibited by lipid-lowering treatment with atorvastatin. *Arterioscler Thromb Vasc Biol* 1999;19(7):1812-7.

169. Degraeve F, Bolla M, Blaie S, et al. Modulation of COX-2 expression by statins in human aortic smooth muscle cells. Involvement of geranylgeranylated proteins. *J Biol Chem* 2001;276(50):46849-55.

170. Seeger H, Mueck AO, Lippert TH. Fluvastatin increases prostacyclin and decreases endothelin production by human umbilical vein endothelial cells. *Int J Clin Pharmacol Ther* 2000;38(5):270-2.

171. Hoffman R, Brook GJ, Aviram M. Hypolipidemic drugs reduce lipoprotein susceptibility to undergo lipid peroxidation: in vitro and ex vivo studies. *Atherosclerosis* 1992;93(1-2):105-13.

172. Giroux LM, Davignon J, Naruszewicz M. Simvastatin inhibits the oxidation of low-density lipoproteins by activated human monocyte-derived macrophages. *Biochim Biophys Acta* 1993;1165(3):335-8.

173. Aviram M, Hussein O, Rosenblat M, Schlezinger S, Hayek T, Keidar S. Interactions of platelets, macrophages, and lipoproteins in hypercholesterolemia: antiatherogenic effects of HMG-CoA reductase inhibitor therapy. *J Cardiovasc Pharmacol* 1998;31(1):39-45.

174. Aviram M, Rosenblat M, Bisgaier CL, Newton RS. Atorvastatin and gemfibrozil metabolites, but not the parent drugs, are potent antioxidants against lipoprotein oxidation. *Atherosclerosis* 1998;138(2):271-80.

175. Laufs U, La Fata V, Plutzky J, Liao JK. Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. *Circulation* 1998;97(12):1129-35.

176. O'Driscoll G, Green D, Taylor RR. Simvastatin, an HMG-coenzyme A reductase inhibitor, improves endothelial function within 1 month. *Circulation* 1997;95(5):1126-31.

177. TSSSS G. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet* 1994;344(8934):1383-9.

178. Pedersen TR, Olsson AG, Faergeman O, et al. Lipoprotein changes and reduction in the incidence of major coronary heart disease events in the Scandinavian Simvastatin Survival Study (4S). *Circulation* 1998;97(15):1453-60.

179. Shepherd J, Cobbe SM, Ford I, et al. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. *N Engl J Med* 1995;333(20):1301-7.

180. Group WoSCPS. Influence of pravastatin and plasma lipids on clinical events in the West of Scotland Coronary Prevention Study (WOSCOPS). *Circulation* 1998;97(15):1440-5.

181. The Lipid Research Clinics Coronary Primary Prevention Trial results. I. Reduction in incidence of coronary heart disease. *Jama* 1984;251(3):351-64.

182. Buchwald H, Campos CT, Boen JR, Nguyen PA, Williams SE. Disease-free intervals after partial ileal bypass in patients with coronary heart disease and hypercholesterolemia: report from the Program on the Surgical Control of the Hyperlipidemias (POSCH). *J Am Coll Cardiol* 1995;26(2):351-7.

183. Sacks FM, Pfeffer MA, Moye LA, et al. The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators. *N Engl J Med* 1996;335(14):1001-9.

184. Ridker PM, Rifai N, Pfeffer MA, et al. Inflammation, pravastatin, and the risk of coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events (CARE) Investigators. *Circulation* 1998;98(9):839-44.

185. Ridker PM, Rifai N, Pfeffer MA, Sacks F, Braunwald E. Long-term effects of pravastatin on plasma concentration of C-reactive protein. The Cholesterol and Recurrent Events (CARE) Investigators. *Circulation* 1999;100(3):230-5.

186. Group L-tIwPiIDLS. Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. *N Engl J Med* 1998;339(19):1349-57.

187. Downs JR, Clearfield M, Weis S, et al. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study. *Jama* 1998;279(20):1615-22.

188. Downs JR, Clearfield M, Tyroler HA, et al. Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TEXCAPS): additional perspectives on tolerability of long-term treatment with lovastatin. *Am J Cardiol* 2001;87(9):1074-9.

189. Schwartz GG, Olsson AG, Ezekowitz MD, et al. Effects of atorvastatin on early recurrent ischemic events in acute coronary syndromes: the MIRACL study: a randomized controlled trial. *Jama* 2001;285(13):1711-8.

190. Sacks FM. Lipid-lowering therapy in acute coronary syndromes. *Jama* 2001;285(13):1758-60.

191. Pitt B, Waters D, Brown WV, et al. Aggressive lipid-lowering therapy compared with angioplasty in stable coronary artery disease. Atorvastatin versus Revascularization Treatment Investigators. *N Engl J Med* 1999;341(2):70-6.

192. Bucher HC, Hengstler P, Schindler C, Guyatt GH. Percutaneous transluminal coronary angioplasty versus medical treatment for non-acute coronary heart disease: meta-analysis of randomised controlled trials. *Bmj* 2000;321(7253):73-7.

193. Group HPSC. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. *Lancet* 2002;360(9326):7-22.

194. Collins R, Armitage J, Parish S, Sleigh P, Peto R. MRC/BHF Heart Protection Study of cholesterol-lowering with simvastatin in 5963 people with diabetes: a randomised placebo-controlled trial. *Lancet* 2003;361(9374):2005-16.

195. Serruys PW, De Feyter PJ, Benghozi R, Hugenholtz PG, Lesaffre E. The Lescol(R) Intervention Prevention Study (LIPS): a double-blind, placebo-controlled, randomized trial of the long-term effects of fluvastatin after successful transcatheter therapy in patients with coronary heart disease. *Int J Cardiovasc Intervent* 2001;4(4):165-172.

196. Weverling-Rijnsburger AW, Blauw GJ, Lagaay AM, Knook DL, Meinders AE, Westendorp RG. Total cholesterol and risk of mortality in the oldest old. *Lancet* 1997;350(9085):1119-23.

197. Group. AOaCftACR. Major outcomes in moderately hypercholesterolemic, hypertensive patients randomized to pravastatin vs usual care: The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT-LLT). *Jama* 2002;288(23):2998-3007.

198. Pasternak RC. The ALLHAT lipid lowering trial--less is less. *Jama* 2002;288(23):3042-4.

199. Cannon CP, Braunwald E, McCabe CH, et al. Intensive versus moderate lipid lowering with statins after acute coronary syndromes. *N Engl J Med* 2004;350(15):1495-504.

200. de Lemos JA, Blazing MA, Wiviott SD, et al. Early intensive vs a delayed conservative simvastatin strategy in patients with acute coronary syndromes: phase Z of the A to Z trial. *Jama* 2004;292(11):1307-16.

201. Ridker PM, Cannon CP, Morrow D, et al. C-reactive protein levels and outcomes after statin therapy. *N Engl J Med* 2005;352(1):20-8.

202. Nissen SE, Tuzcu EM, Schoenhagen P, et al. Effect of intensive compared with moderate lipid-lowering therapy on progression of coronary atherosclerosis: a randomized controlled trial. *Jama* 2004;291(9):1071-80.

203. Sever PS, Dahlof B, Poulter NR, et al. Prevention of coronary and stroke events with atorvastatin in hypertensive patients who have average or lower-than-average cholesterol concentrations, in the Anglo-Scandinavian Cardiac Outcomes Trial--Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial. *Lancet* 2003;361(9364):1149-58.

204. Nissen SE, Nicholls SJ, Sipahi I, et al. Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: the ASTEROID trial. *Jama* 2006;295(13):1556-65.

205. Goldbourt U, Medalie JH. High density lipoprotein cholesterol and incidence of coronary heart disease--the Israeli Ischemic Heart Disease Study. *Am J Epidemiol* 1979;109(3):296-308.

206. Assmann G, Schulte H. Relation of high-density lipoprotein cholesterol and triglycerides to incidence of atherosclerotic coronary artery disease (the PROCAM experience). Prospective Cardiovascular Munster study. *Am J Cardiol* 1992;70(7):733-7.

207. Eriksson M, Carlson LA, Miettinen TA, Angelin B. Stimulation of fecal steroid excretion after infusion of recombinant proapolipoprotein A-I. Potential reverse cholesterol transport in humans. *Circulation* 1999;100(6):594-8.

208. Aviram M, Hardak E, Vaya J, et al. Human serum paraoxonases (PON1) Q and R selectively decrease lipid peroxides in human coronary and carotid atherosclerotic lesions: PON1 esterase and peroxidase-like activities. *Circulation* 2000;101(21):2510-7.

209. Stremler KE, Stafforini DM, Prescott SM, McIntyre TM. Human plasma platelet-activating factor acetylhydrolase. Oxidatively fragmented phospholipids as substrates. *J Biol Chem* 1991;266(17):11095-103.

210. Mineo C, Yuhanna IS, Quon MJ, Shaul PW. High density lipoprotein-induced endothelial nitric-oxide synthase activation is mediated by Akt and MAP kinases. *J Biol Chem* 2003;278(11):9142-9.

211. Barter PJ. Inhibition of endothelial cell adhesion molecule expression by high density lipoproteins. *Clin Exp Pharmacol Physiol* 1997;24(3-4):286-7.

212. Aviram M, Brook JG. Platelet interaction with high and low density lipoproteins. *Atherosclerosis* 1983;46(3):259-68.

213. Fruchart JC, Duriez P, Staels B. Peroxisome proliferator-activated receptor-alpha activators regulate genes governing lipoprotein metabolism, vascular inflammation and atherosclerosis. *Curr Opin Lipidol* 1999;10(3):245-57.

214. Assmann G, Schulte H. Role of triglycerides in coronary artery disease: lessons from the Prospective Cardiovascular Munster Study. *Am J Cardiol* 1992;70(19):10H-13H.

215. Staels B, Auwerx J. Regulation of apo A-I gene expression by fibrates. *Atherosclerosis* 1998;137 Suppl:S19-23.

216. Vu-Dac N, Schoonjans K, Kosykh V, et al. Fibrates increase human apolipoprotein A-II expression through activation of the peroxisome proliferator-activated receptor. *J Clin Invest* 1995;96(2):741-50.

217. Wang TD, Chen WJ, Lin JW, Cheng CC, Chen MF, Lee YT. Efficacy of fenofibrate and simvastatin on endothelial function and inflammatory markers in patients with combined hyperlipidemia: relations with baseline lipid profiles. *Atherosclerosis* 2003;170(2):315-23.

218. Manninen V, Elo MO, Frick MH, et al. Lipid alterations and decline in the incidence of coronary heart disease in the Helsinki Heart Study. *Jama* 1988;260(5):641-51.

219. Rubins HB, Robins SJ, Collins D, et al. Gemfibrozil for the secondary prevention of coronary heart disease in men with low levels of high-density lipoprotein cholesterol. Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial Study Group. *N Engl J Med* 1999;341(6):410-8.

220. Henderson LM. Niacin. *Annu Rev Nutr* 1983;3:289-307.

221. Tavintharan S, Kashyap ML. The benefits of niacin in atherosclerosis. *Curr Atheroscler Rep* 2001;3(1):74-82.

222. Jin FY, Kamanna VS, Kashyap ML. Niacin accelerates intracellular ApoB degradation by inhibiting triacylglycerol synthesis in human hepatoblastoma (HepG2) cells. *Arterioscler Thromb Vasc Biol* 1999;19(4):1051-9.

223. Sakai T, Kamanna VS, Kashyap ML. Niacin, but not gemfibrozil, selectively increases LP-AI, a cardioprotective subfraction of HDL, in patients with low HDL cholesterol. *Arterioscler Thromb Vasc Biol* 2001;21(11):1783-9.

224. Blankenhorn DH, Nessim SA, Johnson RL, Sanmarco ME, Azen SP, Cashin-Hemphill L. Beneficial effects of combined colestipol-niacin therapy on coronary atherosclerosis and coronary venous bypass grafts. *Jama* 1987;257(23):3233-40.

225. Vogt A, Kassner U, Hostalek U, Steinhagen-Thiessen E. Evaluation of the safety and tolerability of prolonged-release nicotinic acid in a usual care setting: the NAUTILUS study. *Curr Med Res Opin* 2006;22(2):417-25.

226. Taylor AJ, Sullenberger LE, Lee HJ, Lee JK, Grace KA. Arterial Biology for the Investigation of the Treatment Effects of Reducing Cholesterol (ARBITER) 2: a double-blind, placebo-controlled study of extended-release niacin on atherosclerosis progression in secondary prevention patients treated with statins. *Circulation* 2004;110(23):3512-7.

227. Nissen SE, Tsunoda T, Tuzcu EM, et al. Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. *Jama* 2003;290(17):2292-300.

228. Garcia-Calvo M, Lisnock J, Bull HG, et al. The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). *Proc Natl Acad Sci U S A* 2005;102(23):8132-7.

229. Davidson MH, Toth PP. Comparative effects of lipid-lowering therapies. *Prog Cardiovasc Dis* 2004;47(2):73-104.

230. Cell receptors regulate cholesterol : ezetimibe blocks cholesterol absorption. *Cardiovasc J S Afr* 2006;17(2):101.

231. Knopp RH, Gitter H, Truitt T, et al. Effects of ezetimibe, a new cholesterol absorption inhibitor, on plasma lipids in patients with primary hypercholesterolemia. *Eur Heart J* 2003;24(8):729-41.

232. Dujovne CA, Ettinger MP, McNeer JF, et al. Efficacy and safety of a potent new selective cholesterol absorption inhibitor, ezetimibe, in patients with primary hypercholesterolemia. *Am J Cardiol* 2002;90(10):1092-7.

233. Ballantyne CM, Houri J, Notarbartolo A, et al. Effect of ezetimibe coadministered with atorvastatin in 628 patients with primary

hypercholesterolemia: a prospective, randomized, double-blind trial. *Circulation* 2003;107(19):2409-15.

234. Davidson MH, McGarry T, Bettis R, et al. Ezetimibe coadministered with simvastatin in patients with primary hypercholesterolemia. *J Am Coll Cardiol* 2002;40(12):2125-34.

235. Melani L, Mills R, Hassman D, et al. Efficacy and safety of ezetimibe coadministered with pravastatin in patients with primary hypercholesterolemia: a prospective, randomized, double-blind trial. *Eur Heart J* 2003;24(8):717-28.

236. Kerzner B, Corbelli J, Sharp S, et al. Efficacy and safety of ezetimibe coadministered with lovastatin in primary hypercholesterolemia. *Am J Cardiol* 2003;91(4):418-24.

237. Gagne C, Bays HE, Weiss SR, et al. Efficacy and safety of ezetimibe added to ongoing statin therapy for treatment of patients with primary hypercholesterolemia. *Am J Cardiol* 2002;90(10):1084-91.

238. Buchwald H, Boen JR, Nguyen PA, Williams SE, Matts JP. Plasma lipids and cardiovascular risk: a POSCH report. Program on the Surgical Control of the Hyperlipidemias. *Atherosclerosis* 2001;154(1):221-7.

239. Takemoto M, Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. *Arterioscler Thromb Vasc Biol* 2001;21(11):1712-9.

240. Duncan MD, Wilkes DS. Transplant-related immunosuppression: a review of immunosuppression and pulmonary infections. *Proc Am Thorac Soc* 2005;2(5):449-55.

241. David KM, Morris JA, Steffen BJ, Chi-Burris KS, Gotz VP, Gordon RD. Mycophenolate mofetil vs. azathioprine is associated with decreased acute rejection, late acute rejection, and risk for cardiovascular death in renal transplant recipients with pre-transplant diabetes. *Clin Transplant* 2005;19(2):279-85.

242. Ingle GR, Sievers TM, Holt CD. Sirolimus: continuing the evolution of transplant immunosuppression. *Ann Pharmacother* 2000;34(9):1044-55.

243. Woodle ES, Thistlethwaite JR, Jolliffe LK, et al. Humanized OKT3 antibodies: successful transfer of immune modulating properties and idiotype expression. *J Immunol* 1992;148(9):2756-63.

244. Baan CC, Balk AH, van Riemsdijk IC, et al. Anti-CD25 monoclonal antibody therapy affects the death signals of graft-infiltrating cells after clinical heart transplantation. *Transplantation* 2003;75(10):1704-10.

245. Downward J, Graves JD, Warne PH, Rayter S, Cantrell DA. Stimulation of p21ras upon T-cell activation. *Nature* 1990;346(6286):719-23.

246. Schonbeck U, Mach F, Libby P. CD154 (CD40 ligand). *Int J Biochem Cell Biol* 2000;32(7):687-93.

247. Foy TM, Aruffo A, Bajorath J, Buhlmann JE, Noelle RJ. Immune regulation by CD40 and its ligand GP39. *Annu Rev Immunol* 1996;14:591-617.

248. Rose EA, Smith CR, Petrossian GA, Barr ML, Reemtsma K. Humoral immune responses after cardiac transplantation: correlation with fatal rejection and graft atherosclerosis. *Surgery* 1989;106(2):203-7; discussion 207-8.

249. Wenke K, Meiser B, Thiery J, et al. Simvastatin reduces graft vessel disease and mortality after heart transplantation: a four-year randomized trial. *Circulation* 1997;96(5):1398-402.

250. Kobashigawa JA, Katznelson S, Laks H, et al. Effect of pravastatin on outcomes after cardiac transplantation. *N Engl J Med* 1995;333(10):621-7.

251. Kobashigawa JA, Moriguchi JD, Laks H, et al. Ten-year follow-up of a randomized trial of pravastatin in heart transplant patients. *J Heart Lung Transplant* 2005;24(11):1736-40.

252. Katznelson S, Wilkinson AH, Kobashigawa JA, et al. The effect of pravastatin on acute rejection after kidney transplantation--a pilot study. *Transplantation* 1996;61(10):1469-74.

253. Holdaas H, Fellstrom B, Jardine AG, et al. Effect of fluvastatin on cardiac outcomes in renal transplant recipients: a multicentre, randomised, placebo-controlled trial. *Lancet* 2003;361(9374):2024-31.

254. Holdaas H, Jardine AG, Wheeler DC, et al. Effect of fluvastatin on acute renal allograft rejection: a randomized multicenter trial. *Kidney Int* 2001;60(5):1990-7.

255. Jardine A, Holdaas H. Fluvastatin in combination with cyclosporin in renal transplant recipients: a review of clinical and safety experience. *J Clin Pharm Ther* 1999;24(6):397-408.

256. Kobashigawa JA, Kasiske BL. Hyperlipidemia in solid organ transplantation. *Transplantation* 1997;63(3):331-8.

257. Byington RP, Davis BR, Plehn JF, et al. Reduction of stroke events with pravastatin: the Prospective Pravastatin Pooling (PPP) Project. *Circulation* 2001;103(3):387-92.

258. Shepherd J, Blauw GJ, Murphy MB, et al. Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial. *Lancet* 2002;360(9346):1623-30.

259. Colhoun HM, Betteridge DJ, Durrington PN, et al. Primary prevention of cardiovascular disease with atorvastatin in type 2 diabetes in the Collaborative Atorvastatin Diabetes Study (CARDS): multicentre randomised placebo-controlled trial. *Lancet* 2004;364(9435):685-96.

260. Weis M, Heeschen C, Glassford AJ, Cooke JP. Statins have biphasic effects on angiogenesis. *Circulation* 2002;105(6):739-45.

261. Leung BP, Sattar N, Crilly A, et al. A novel anti-inflammatory role for simvastatin in inflammatory arthritis. *J Immunol* 2003;170(3):1524-30.

262. Tikiz C, Utuk O, Pirildar T, et al. Effects of Angiotensin-converting enzyme inhibition and statin treatment on inflammatory markers and endothelial functions in patients with longterm rheumatoid arthritis. *J Rheumatol* 2005;32(11):2095-101.

263. Fisher JE, Rogers MJ, Halasy JM, et al. Alendronate mechanism of action: geranylgeraniol, an intermediate in the mevalonate pathway, prevents inhibition of osteoclast formation, bone resorption, and kinase activation in vitro. *Proc Natl Acad Sci U S A* 1999;96(1):133-8.

264. Mundy GR. Statins and their potential for osteoporosis. *Bone* 2001;29(6):495-7.

265. Jick H, Zornberg GL, Jick SS, Seshadri S, Drachman DA. Statins and the risk of dementia. *Lancet* 2000;356(9242):1627-31.

266. Wolozin B, Kellman W, Ruosseau P, Celesia GG, Siegel G. Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors. *Arch Neurol* 2000;57(10):1439-43.

267. Gottlieb AB. Psoriasis: emerging therapeutic strategies. *Nat Rev Drug Discov* 2005;4(1):19-34.

268. Aronson PJ, Friedman DB. Pharmacologic doses of lovastatin do not predictably affect the course of psoriasis. *Arch Dermatol* 1992;128(1):124.

269. Proksch E. [Antilipemic drug-induced skin manifestations]. *Hautarzt* 1995;46(2):76-80.

270. Proksch E, Holleran WM, Menon GK, Elias PM, Feingold KR. Barrier function regulates epidermal lipid and DNA synthesis. *Br J Dermatol* 1993;128(5):473-82.

271. Zamvil SS, Steinman L. Cholesterol-lowering statins possess anti-inflammatory activity that might be useful for treatment of MS. *Neurology* 2002;59(7):970-1.

272. Neuhaus O, Strasser-Fuchs S, Fazekas F, et al. Statins as immunomodulators: comparison with interferon-beta 1b in MS. *Neurology* 2002;59(7):990-7.

273. Cuzner ML, Gveric D, Strand C, et al. The expression of tissue-type plasminogen activator, matrix metalloproteases and endogenous inhibitors in the central nervous system in multiple sclerosis: comparison of stages in lesion evolution. *J Neuropathol Exp Neurol* 1996;55(12):1194-204.

274. Law M, Rudnicka AR. Statin safety: a systematic review. *Am J Cardiol* 2006;97(8A):52C-60C.

275. Staffa JA, Chang J, Green L. Cerivastatin and reports of fatal rhabdomyolysis. *N Engl J Med* 2002;346(7):539-40.

276. Furberg CD, Pitt B. Withdrawal of cerivastatin from the world market. *Curr Control Trials Cardiovasc Med* 2001;2(5):205-207.

277. Baigent C, Keech A, Kearney PM, et al. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. *Lancet* 2005;366(9493):1267-78.

278. Liao JK. Isoprenoids as mediators of the biological effects of statins. *J Clin Invest* 2002;110(3):285-8.

279. Kono H, Suzuki T, Yamamoto K, Okada M, Yamamoto T, Honda Z. Spatial raft coalescence represents an initial step in Fc gamma R signaling. *J Immunol* 2002;169(1):193-203.

280. Kobashigawa JA. Statins as immunosuppressive agents. *Liver Transpl* 2001;7(6):559-61.

281. Sundstrom C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int J Cancer* 1976;17(5):565-77.

282. Lozzio CB, Lozzio BB. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. *Blood* 1975;45(3):321-34.

283. Lozzio BB, Lozzio CB. Properties and usefulness of the original K-562 human myelogenous leukemia cell line. *Leuk Res* 1979;3(6):363-70.

284. Tam YK, Maki G, Miyagawa B, Hennemann B, Tonn T, Klingemann HG. Characterization of genetically altered, interleukin 2-independent natural killer cell lines suitable for adoptive cellular immunotherapy. *Hum Gene Ther* 1999;10(8):1359-73.

285. Cutts JL, Scallen TJ, Watson J, Bankhurst AD. Role of mevalonic acid in the regulation of natural killer cell cytotoxicity. *J Cell Physiol* 1989;139(3):550-7.

286. Pross HF, Baines MG, Rubin P, Shragge P, Patterson MS. Spontaneous human lymphocyte-mediated cytotoxicity against tumor target cells. IX. The quantitation of natural killer cell activity. *J Clin Immunol* 1981;1(1):51-63.

287. Bonanno E, Tagliafierro G, Carla EC, et al. Synchronized onset of nuclear and cell surface modifications in U937 cells during apoptosis. *Eur J Histochem* 2002;46(1):61-74.

288. Fletcher CD, Barnes JF, Farish E. A rapid semi-micro method for the separation of lipoprotein fractions that uses a benchtop ultracentrifuge. *Clin Chim Acta* 1994;226(1):95-9.

289. Bunce CM, Thick JA, Lord JM, Mills D, Brown G. A rapid procedure for isolating hemopoietic cell nuclei. *Anal Biochem* 1988;175(1):67-73.

290. Divecha N, Banfic H, Irvine RF. The polyphosphoinositide cycle exists in the nuclei of Swiss 3T3 cells under the control of a receptor (for IGF-I) in the plasma membrane, and stimulation of the cycle increases nuclear diacylglycerol and apparently induces translocation of protein kinase C to the nucleus. *Embo J* 1991;10(11):3207-14.

291. Harrison PT, Davis W, Norman JC, Hockaday AR, Allen JM. Binding of monomeric immunoglobulin G triggers Fc gamma RI-mediated endocytosis. *J Biol Chem* 1994;269(39):24396-402.

292. Chakrabarti R, Engleman EG. Interrelationships between mevalonate metabolism and the mitogenic signaling pathway in T lymphocyte proliferation. *J Biol Chem* 1991;266(19):12216-22.

293. Rezaie-Majd A, Prager GW, Bucek RA, et al. Simvastatin reduces the expression of adhesion molecules in circulating monocytes from hypercholesterolemic patients. *Arterioscler Thromb Vasc Biol* 2003;23(3):397-403.

294. Hillyard DZ, Cameron AJ, McIntyre AH, et al. Inhibition of proliferation and signalling mechanisms in human lymphocytes by fluvastatin. *Clin Exp Pharmacol Physiol* 2002;29(8):673-8.

295. Getz GS. Do natural killer cells participate in a killer vascular disease? *Arterioscler Thromb Vasc Biol* 2002;22(8):1251-3.

296. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med* 1999;340(2):115-26.

297. Yaqoob P. Fatty acids as gatekeepers of immune cell regulation. *Trends Immunol* 2003;24(12):639-45.

298. Purasiri P, McKechnie A, Heys SD, Eremin O. Modulation in vitro of human natural cytotoxicity, lymphocyte proliferative response to mitogens and cytokine production by essential fatty acids. *Immunology* 1997;92(2):166-72.

299. Zeyda M, Staffler G, Horejsi V, Waldhausl W, Stulnig TM. LAT displacement from lipid rafts as a molecular mechanism for the inhibition of T cell signaling by polyunsaturated fatty acids. *J Biol Chem* 2002;277(32):28418-23.

300. Maron DJ, Fazio S, Linton MF. Current perspectives on statins. *Circulation* 2000;101(2):207-13.

301. Macara IG, Lounsbury KM, Richards SA, McKiernan C, Bar-Sagi D. The Ras superfamily of GTPases. *Faseb J* 1996;10(5):625-30.

302. Boguski MS, McCormick F. Proteins regulating Ras and its relatives. *Nature* 1993;366(6456):643-54.

303. Seabra MC. Membrane association and targeting of prenylated Ras-like GTPases. *Cell Signal* 1998;10(3):167-72.

304. Lafont V, Ottones F, Liautard J, Favero J. Evidence for a p21(ras)/Raf-1/MEK-1/ERK-2-independent pathway in stimulation of IL-2 gene transcription in human primary T lymphocytes. *J Biol Chem* 1999;274(36):25743-8.

305. Xu S, Khoo S, Dang A, et al. Differential regulation of mitogen-activated protein/ERK kinase (MEK)1 and MEK2 and activation by a Ras-independent mechanism. *Mol Endocrinol* 1997;11(11):1618-25.

306. Lebowitz PF, Prendergast GC. Non-Ras targets of farnesyltransferase inhibitors: focus on Rho. *Oncogene* 1998;17(11 Reviews):1439-45.

307. Bar-Sagi D, Hall A. Ras and Rho GTPases: a family reunion. *Cell* 2000;103(2):227-38.

308. O'Donnell MP, Kasiske BL, Massy ZA, Guijarro C, Swan SK, Keane WF. Isoprenoids and Ras: potential role in chronic rejection. *Kidney Int Suppl* 1995;52:S29-33.

309. Holdaas H, Fellstrom B, Holme I, et al. Effects of fluvastatin on cardiac events in renal transplant patients: ALERT (Assessment of Lescol in Renal Transplantation) study design and baseline data. *J Cardiovasc Risk* 2001;8(2):63-71.

310. Scripture CD, Pieper JA. Clinical pharmacokinetics of fluvastatin. *Clin Pharmacokinet* 2001;40(4):263-81.

311. Faggiotto A, Paoletti R. State-of-the-Art lecture. Statins and blockers of the renin-angiotensin system: vascular protection beyond their primary mode of action. *Hypertension* 1999;34(4 Pt 2):987-96.

312. Robinson MJ, Cobb MH. Mitogen-activated protein kinase pathways. *Curr Opin Cell Biol* 1997;9(2):180-6.

313. Resh MD. Myristylation and palmitylation of Src family members: the fats of the matter. *Cell* 1994;76(3):411-3.

314. Rothblat GH, de la Llera-Moya M, Atger V, Kellner-Weibel G, Williams DL, Phillips MC. Cell cholesterol efflux: integration of old and new observations provides new insights. *J Lipid Res* 1999;40(5):781-96.

315. Kwiatkowska K, Sobota A. The clustered Fcgamma receptor II is recruited to Lyn-containing membrane domains and undergoes phosphorylation in a cholesterol-dependent manner. *Eur J Immunol* 2001;31(4):989-98.

316. Takahashi M, Ogata Y, Okazaki H, et al. Fluvastatin enhances apoptosis in cytokine-stimulated vascular smooth muscle cells. *J Cardiovasc Pharmacol* 2002;39(2):310-7.

317. Cherukuri A, Dykstra M, Pierce SK. Floating the raft hypothesis: lipid rafts play a role in immune cell activation. *Immunity* 2001;14(6):657-60.

318. Salonen JT, Yla-Herttuala S, Yamamoto R, et al. Autoantibody against oxidised LDL and progression of carotid atherosclerosis. *Lancet* 1992;339(8798):883-7.

319. Huang Y, Fleming AJ, Wu S, Virella G, Lopes-Virella MF. Fc-gamma receptor cross-linking by immune complexes induces matrix metalloproteinase-1 in U937 cells via mitogen-activated protein kinase. *Arterioscler Thromb Vasc Biol* 2000;20(12):2533-8.

320. Grip O, Janciauskiene S, Lindgren S. Pravastatin down-regulates inflammatory mediators in human monocytes in vitro. *Eur J Pharmacol* 2000;410(1):83-92.

321. Wong B, Lumma WC, Smith AM, Sisko JT, Wright SD, Cai TQ. Statins suppress THP-1 cell migration and secretion of matrix metalloproteinase 9 by inhibiting geranylgeranylation. *J Leukoc Biol* 2001;69(6):959-62.

322. Alonso A, Bayon Y, Renedo M, Crespo MS. Stimulation of Fc gamma R receptors induces monocyte chemoattractant protein-1 in the human monocytic cell line THP-1 by a mechanism involving I kappa B-alpha degradation and formation of p50/p65 NF-kappa B/Rel complexes. *Int Immunol* 2000;12(4):547-54.

323. Berger S, Ballo H, Stutte HJ. Immune complex-induced interleukin-6, interleukin-10 and prostaglandin secretion by human monocytes: a network of pro- and anti-inflammatory cytokines dependent on the antigen:antibody ratio. *Eur J Immunol* 1996;26(6):1297-301.

324. Hansson GK, Jonasson L, Lojsthed B, Stemme S, Kocher O, Gabbiani G. Localization of T lymphocytes and macrophages in fibrous and complicated human atherosclerotic plaques. *Atherosclerosis* 1988;72(2-3):135-41.

325. Schmitz G, Herr AS, Rothe G. T-lymphocytes and monocytes in atherogenesis. *Herz* 1998;23(3):168-77.

326. Izquierdo M, Leevers SJ, Marshall CJ, Cantrell D. p21ras couples the T cell antigen receptor to extracellular signal-regulated kinase 2 in T lymphocytes. *J Exp Med* 1993;178(4):1199-208.

327. Sanchez-Mejorada G, Rosales C. Fcgamma receptor-mediated mitogen-activated protein kinase activation in monocytes is independent of Ras. *J Biol Chem* 1998;273(42):27610-9.

328. Bickel C, Rupprecht HJ, Blankenberg S, et al. Influence of HMG-CoA reductase inhibitors on markers of coagulation, systemic inflammation and soluble cell adhesion. *Int J Cardiol* 2002;82(1):25-31.

329. Khakoo SI, Brooks CR. MHC class I receptors on natural killer cells: on with the old and in with the new. *Clin Sci (Lond)* 2003;105(2):127-40.

330. Yokoyama WM, Plougastel BF. Immune functions encoded by the natural killer gene complex. *Nat Rev Immunol* 2003;3(4):304-16.

331. Hillyard DZ, Cameron AJ, McDonald KJ, et al. Simvastatin inhibits lymphocyte function in normal subjects and patients with cardiovascular disease. *Atherosclerosis* 2004;175(2):305-13.

332. Massy ZA, Keane WF, Kasiske BL. Inhibition of the mevalonate pathway: benefits beyond cholesterol reduction? *Lancet* 1996;347(8994):102-3.

333. Webb Y, Hermida-Matsumoto L, Resh MD. Inhibition of protein palmitoylation, raft localization, and T cell signaling by 2-bromopalmitate and polyunsaturated fatty acids. *J Biol Chem* 2000;275(1):261-70.

334. Hillyard DZ, Jardine AG, McDonald KJ, Cameron AJ. Fluvastatin inhibits raft dependent Fcgamma receptor signalling in human monocytes. *Atherosclerosis* 2004;172(2):219-28.

335. Robinson JG, Smith B, Maheshwari N, Schrott H. Pleiotropic effects of statins: benefit beyond cholesterol reduction? A meta-regression analysis. *J Am Coll Cardiol* 2005;46(10):1855-62.

336. Pfeiffer JR, Howes PS, Waters MA, et al. Levels of expression of Fcgamma receptor IIA (CD32) are decreased on peripheral blood monocytes in patients with severe atherosclerosis. *Atherosclerosis* 2001;155(1):211-8.

337. Siekmeier R, Lattke P, Mix C, Park JW, Jaross W. Dose dependency of fluvastatin pharmacokinetics in serum determined by reversed phase HPLC. *J Cardiovasc Pharmacol Ther* 2001;6(2):137-45.