Regulation of C5aR expression and function: potential modulation by lipid-lowering drugs

By Elizabeth Palmer

A thesis submitted for the degree of Doctor of Philosophy

Department of Pharmacology
Wales Heart Research Institute
Cardiff University

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Abbreviations

AP	Alternative pathway
ARE	AU-rich elements
ARE-BP	AU-rich element binding protein
Bt_2cAMP	dibutyryl cAMP
bp	Base pairs
C	Complement
C5aR	C5a receptor
C1-Inh	C1-inhibitor
cAMP	Cyclic monophosphate
CAT	Chloramphenicol acetyltransferase
CCP	Complement control protein
CD	Cyclodextrins
ChMβCD	Cholesterol loaded methyl-β-cyclodextrin
COX-2	Cyclooxygenase-2
CP	Classical pathway
DAF	Decay accelerating factor
dATP	2'-deoxyadenosine 5'-triphosphate
dH_2O	De-ionised water
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleosides triphosphates
dsDNA	Double stranded deoxyribonucleic acid
EC	Endothelial cells
E. coli	*Esherichia coli*
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescence protein
ELISA	Enzyme linked immunoabsorbant assay
eNOS	Endothelial nitric oxide synthase
Epacs	Exchange proteins activated by cAMP
ERK	extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorting
fB	Factor B
FCS	Fetal calf serum
fI	Factor I
FITC	Fluorescein isothiocynate
fMLP	Formyl-met-leu-phe
FPP	Farnesyl pyrophosphate
FPR	fMLP receptor
g	Gravity

GAM	Goat anti mouse
gDNA	Genomic deoxyribonucleic acid
GFP	Green fluorescent protein
GGPP	Geranygeranyl-pyrophosphate
GPCR	G protein coupled receptor
GPI	Glycosyl-phosphatidylinositol
GRK	G protein receptor kinases
GTP	Guanosine-5'-triphosphate
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA reductase
hr	Hour
HRP	Horse-radish peroxidase
ICAM	Intracellular adhesion molecule-1
IFNγ	Interferon gamma
Ig	Immunoglobulin
IκB	Inhibitory factor kappa-light-chain-enhancer of activated B cells
IL	Interleukin
I/R	Ischaemic reperfusion
JAK	Janus kinases
JNK	c-Jun N-terminal kinase
kDa	Kilodaltons
K/H	Krebs hepes buffer
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LP	Lectin pathway
LPS	Lipopolysaccharide
M	Molar
MAb	Monoclonal antibody
MAC	Membrane attack complex
mAmp	Milliamp
MAPK	Mitogen activated protein kinase
MBL	mannose-binding lectin
MASP	MBL-associated serine protease
MβCD	Methyl-β-cyclodextrin
MCP	Membrane cofactor protein
MCP-1	Monocyte chemoattractant protein-1
MHC II	Major histocompatibility complex class II
MI	Myocardial infarction
min	Minute(s)
MMP	Matrix metalloproteinase
ml	Millilitre(s)
mM	Millimolar

mRNA	Messenger ribonucleic acid
Mw	Molecular weight
NFAT	Nuclear factor of activate T-cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NF-Y	Nuclear factor Y
nM	Nanomolar
N-terminus	Amino (NH_2) terminus
OD	Optical density
o/n	Overnight
OPD	Orthophenylenediamine
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E2
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-mystrate 13-acetate
PPAR	Peroxisome proliferator-activated receptors
Q-PCR	Quantitative polymerase chain reaction
RAM	Rabbit anti-mouse
ROCK	Rho Kinase
rpm	Revolution per minute
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
s	Seconds
SCAP	SREBP cleavage activator protein
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error mean
SMC	Smooth muscle cell
s/n	Supernatant
SNP	single nucleotide polymorphisms
SREBP	Sterol-regulatory-element-binding protein
STAT	Signal Transducer and Activator of Transcription
STP	Serine, threonine and proline rich domain
TBS	Tris buffered saline
TEMED	N N N' N'-tetramethylethylenediamine
TLR4	Toll-like receptor-4
TNFα	Tumour necrosis factor alpha
Tris	Tris (hydroxmethyl) methyamine

$U937_{C5aR}$	U937 transfected with C5aR
$U937_{CD59\text{-}GPI}$	U937 transfected with GPI anchored CD59
$U937_{CD59\text{-}TM}$	U937 transfected with transmembrane domain MCP CD59
$U937_{EC}$	U937 cell line obtained from European Collection of Cell Culture lines
$U937_{PM}$	U937 cell line obtained from Sheffield University
3'UTR	3 primed untranslated region
µg	Microgram
µl	Microlitre
µM	Micro molar
V	Volt
VSMC	Vascular smooth muscle cells
v/v	Volume to volume
w/v	Weight to volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase
ZA	Zaragozic acid

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Sigma	Poole, Dorset, UK
Stratech	Suffolk, UK
tebu-bio	Cambridgeshire, UK
QIAGEN	Surrey, UK
Welsh Blood Service	Cardiff, UK

Abstracts

Palmer EA, Stott MI, van den Berg CW. Putative CCAAT/NFY and NFAT transcription factor binding sites are important for expression of the C5a receptor in human monocytic U937 cell line. Molecular Immunology 46(2009) 05.193

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Summary

The pro-inflammatory anaphylatoxin C5a exerts its biological actions via the C5a receptor (C5aR), a G-protein-coupled-receptor (GPCR). Cholesterol, a crucial component of biological membranes, has previously been shown to regulate expression and function of numerous GPCRs. As statin therapy is widely used to reduce serum cholesterol levels, it was hypothesised that statins can exert anti-inflammatory effects by down regulation expression and/or function of the C5aR.

This thesis first investigated how basal human C5aR expression was regulated. It was shown that the majority of the -2Kbp promoter region is dispensable for transcription of the C5aR and that the main regulatory domains are localised in the first 200 bp of the promoter region. Furthermore CCAAT and NFAT motifs are important for the transcriptional control of the human C5aR, however the transcription factors which bind to these sites could not be identified.

A model system to measure C5aR expression and function was set up using two pro-monocytic U937 sub-cell lines, which demonstrated that dibutyryl-cyclic-AMP was the best inducer of the C5aR, but only in one of the cell lines. Induction of the C5aR made these cells more responsive to C5a induced intracellular calcium-release and IL-8 and MMP-9 secretion.

Investigating possible effects of cholesterol and modulation of cholesterol on C5aR expression and function showed that statins did not affect expression and function of basal levels of C5aR in monocytes or U937 cells, but reduced induced C5aR expression. Concomitantly C5a induced release of intracellular calcium and secretion of IL-8 reduced, however C5a induced MMP-9 secretion increased. This reduction was due to inhibition of isoprenoid biosynthesis rather than inhibition of cholesterol biosynthesis.

Using sucrose gradient floatation it was shown that the C5aR is unlikely to reside in a lipid raft region of the plasma membrane making it less likely to be susceptible to membrane cholesterol content.

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Regulation of C5aR expression and function: potential modulation by lipid-lowering drugs

Complement activation leads to the release of the potent pro-inflammatory anaphylatoxin C5a, which exerts its biological actions via the C5a receptor (C5aR), a G-protein coupled receptor. Over expression or under regulation of C5a and the C5aR has been implicated in a variety of pathologies, including ischaemia reperfusion injury. Cholesterol, a major risk factor for cardiovascular disease, is a crucial component of biological membranes. Furthermore cholesterol has been shown to regulate expression and function of numerous GPCRs. Statin therapy is the most widely used therapeutic strategy to lower serum cholesterol levels. Evidence suggests that statins also exert beneficial anti-inflammatory effects, thus further improving cardiovascular disease outcome. The principal aim of this thesis was to determine if the C5aR expression and function can be modulated by cholesterol. It is hypothesised that statins can exert anti-inflammatory effects by down regulation of expression and/or function of the receptor for the potent pro-inflammatory anaphylatoxin C5a, C5aR. μl

Chapter 1

General Introduction

1.1 Complement system

Complement (C) is a crucial part of innate immunity. It was identified in the late nineteenth century as a heat labile fraction of serum which complemented the antibody-mediated killing of bacteria. Since then more than 30 soluble and cell surface proteins have been identified, which together interact in a tightly controlled manner to achieve its main physiological functions in host defence against infection, bridging the innate and adaptive immunity and waste disposal (Walport, 2001b, Walport, 2001a).

1.1.1 Complement activation

C activation can be achieved by three pathways: the classical pathway (CP), alternative pathway (AP) and lectin pathway (LP) (figure 1.1).

Activation of the CP is achieved by the recognition subunit C1q of the C1 complex. C1q contains six large globular heads, which bind to the Fc regions of immobilised antigen bound IgG or IgM antibodies. As well as being activated by immune complexes, C1q can also bind other ligands including fragments of cellular and subcellular membranes (e.g. mitochondria membrane), modified host proteins and phospholipids, and C-reactive protein and serum amyloid protein, of the pentraxin family (reviewed in (Kojouharova et al., 2010)).

Activator binding to C1q induces a conformational change that triggers the activation of $C1r_2$, which in turn cleaves $C1s_2$ within the C1 complex.

Activation of the AP allows the rapid and spontaneous deposition of complement C3b on cell surfaces. This is achieved by the spontaneous hydrolysis of C3, which then binds to factor B to form the C3 convertase of the AP following cleavage by factor D. As activation of the AP lacks specificity, host cells are protected from C3b disposition by the expression of complement regulators, such as CR1, DAF and MCP, on their cell surface. However, foreign surfaces are unable to control C3b deposition due to the lack of these regulators, which results in the C3 positive feedback amplification loop.

The LP is the most recently discovered and least understood pathway. This pathway employs germline-encoded pattern recognition receptors, mannose-binding lectin (MBL) and ficolin, which bind to pathogen associated molecular patterns, such as simple carbohydrates and N-acetyl glucosamine groups, present on the pathogen cell surface (Dunkelberger and Song, 2010). Similarly to the CP activation, the LP activation results in the generation of C4bC2a on pathogen cell surfaces, however this is mediated via the MBL-associated serine protease (MASP) complex instead of the C1 complex (reviewed by (Gal et al., 2009)).

More recently it has also been suggested that complement activation could occur by the direct cleavage of either C3 or C5 by serum protease unrelated to complement such as kallikrein and thrombin (Huber-Lang et al., 2006, Dunkelberger and Song, 2010).

1.1.2 The complement cascade

Activation of the complement cascade leads to the proteolytic cleavage of inactive proenzymes to their active form (figure 1.1). Although C activation occurs via three distinct pathways, they all converge at the point of C3 activation by the C3 convertases.

The activation of complexes $C1r_2s_2$, via the CP, or MBL/MASP and ficolin/MASP, via the LP, leads to the cleavage of C4 and C2 to form the C3 convertase C4b2a and released fragments C4a and C2b.

The spontaneous formation of a single C3 convertase ($C3(H_2O)Bb$), via the AP, can lead to C3b deposition on activating surfaces by cleavage of C3. The deposition of C3b can lead to the formation of the predominant C3 convertase, C3bBb, of the AP when deposited in the presence of factor B and factor D. Furthermore this C3 convertase can be stabilised by properdin. Together both these C3 convertases would rapidly activate all C3 and factor B via this positive feedback amplification loop.

The formation of the C3 convertase C4b2a, via the CP or LP, or $C3(H_2O)Bb$ and C3bBb, via the AP, leads to the proteolytic cleavage of C3 to C3b and the released fragment C3a. The addition of C3b to the C3 convertases leads to the formation of the C5 convertases; C4bC3bC2a of the CP and LP and C3bBbC3b of the AP. Both C5 convertases

are able to cleave C5 into C5a and C5b to initiate the terminal pathway. The released fragment, C5a, serves as a potent anaphylatoxin and inflammatory mediator, as does C3a, although to a lesser extent.

1.1.3 The membrane attack complex

The terminal pathway ends by the formation of the transmembrane channel (the membrane attack complex, MAC) within the phospholipid bilayer of the target cell. Following the cleavage of C5, C5b binds to C6 to form C5b-6 and then to C7 to form C5b-7. Binding of C5b-7 to a membrane surface allows C8 to bind and becoming inserted into the membrane forming an unstable pore. Binding of C9 to C5b-8 initiates polymerisation of multiple C9 molecules and incorporation to form the stable C5b-9_n complex. The MAC disrupts the osmotic gradient across the membrane and thus results in a rapid influx of water which leads to swelling and lysis of the cell.

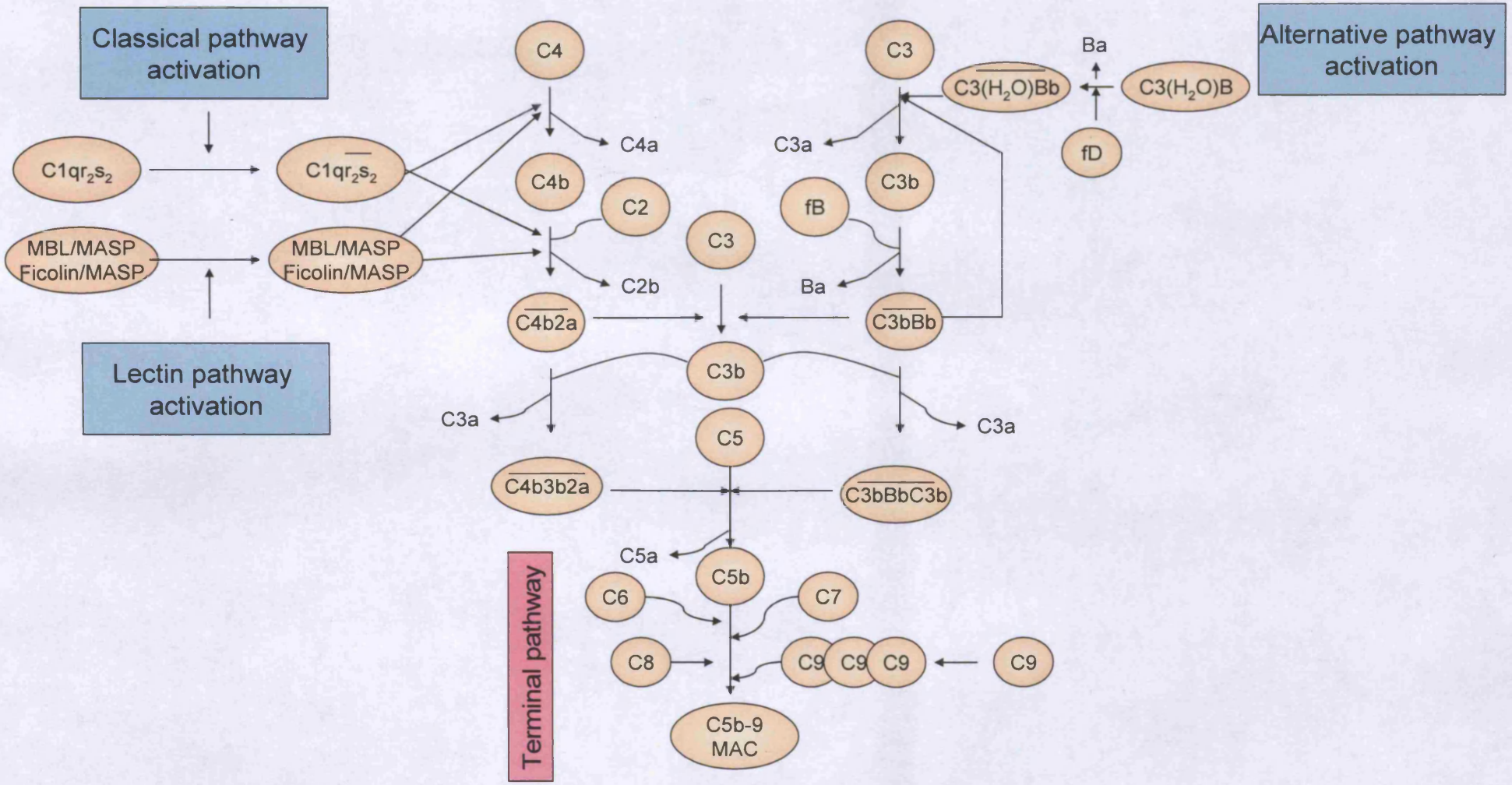


Figure 1.1 The complement cascade. The complement cascade can be activated via three pathways. Activation leads to a series of proteolytic cleavages of individual components and formation of enzymatic complexes which eventually leads to the activation of the terminal pathway and formation of the membrane attack complex. Adapted from S A Law and K B M Reid, Complement in Focus 2nd edition, IRL press.

1.1.4 Biological activities of complement activation

The physiological activities of C can be divided into three main categories; defending the host against infection; acting as an interface between the innate and adaptive immunity; and the clearance of immune complexes and apoptotic cellular waste (Walport, 2001b).

C can defend against bacterial infections by several mechanisms. The opsonisation of bacteria with covalently bound fragments C3b and C4b results in their engulfment and destruction by the phagocytic cells via receptors CR1 and CR3. C can recruit leukocytes towards sites of infection via the pro-inflammatory anaphylatoxins (C3a, C4a and C5a) and their receptors (C3aR and C5aR) present on leukocytes cell surface. The anaphylatoxins can activate leukocytes causing them to increase their cytotoxicity by increasing expression of phagocytic receptors on their cell surface and increasing production of other inflammatory mediators and reactive oxygen metabolites. The anaphylatoxins C3a and C5a and their receptors C3aR and C5aR can cross talk with Toll-like receptors and the coagulation system; therefore further coordinate the innate immune response and preventing the spread of infection by amplifying coagulation (reviewed by (Ricklin et al., 2010)). The formation of the MAC is an essential mechanism for defence against *Neisseria* bacteria infections.

C activation can also serve as an important bridge between the innate and adaptive immunity. One mechanism by which this is achieved is by augmentation of humoral B cell immunity. Expression of the C3 receptors (CR1 and CR2) on B cells and antigen presenting cells (APC) lowers the activation threshold when they encounter antigen opsonised with C3 fragments. Expression of CR2 by follicular dendritic cells is essential for long term B cell memory (Barrington et al., 2002). The anaphylatoxins and their receptors can regulate the humoral B cell response; for instance the C5aR can lower the threshold for Fcγ receptor (FcγR) activation by increasing expression of FcγRI and FcγRIII and down regulating the inhibitory FcγRIIB (Kumar et al., 2006, Shushakova et al., 2002). C can also regulate T cell immunity by its regulators DAF/CD55, MCP/CD46 and CD59, as well as the anaphylatoxins and their receptors (Ricklin et al., 2010). The anaphylatoxins can affect T cell differentiation partly due to their effects on APC but also due to cross talk with the TLR pathway.

The C system can also remove apoptotic cells and immune complexes from tissues via C1q of the CP, as well as opsonisation with C3 and C4 fragments.

1.1.5 Methods of regulating complement activation

As the C cascade involves a series of enzymatic steps its activation can rapidly be amplified and therefore must be tightly regulated to prevent detrimental damage to the host. Regulation is achieved by the expression of membrane bound and fluid phase regulators (figure 1.2), but also by intrinsic mechanisms such as; inactive precursor zymogens need to

and a heavily glycosylated STP rich domain (Caras et al., 1987, Medof et al., 1987). Like MCP, C3b and C4b binding sites are located within the CCP repeats (Law and Reid, 1995).

CR1

Complement receptor 1 (CR1) is an integral membrane glycoprotein which acts as a cofactor for factor I mediating the cleavage of C3b to iC3b and C3f and further cleaving iC3b to C3c and C3dg (Morley and Walport, 2000). Furthermore, CR1 also acts as a cofactor for factor I mediated cleavage of C4b to C4c and C4d. CR1 can also accelerate decay of the C3 and C5 convertases (Medof et al., 1982). CR1 is found on all erythrocytes, B cells, neutrophils, monocytes, a small subset of T cells, follicular dendritic cells and glomerular podocytes (Morley and Walport, 2000).

CD59

CD59 another widely distributed GPI anchored membrane regulator. CD59 can prevent the formation of the MAC by binding to C8 in the C5b-8 complex, thus blocking the binding C9 and formation of the MAC pore (Sugita et al., 1988).

1.1.5.2 Fluid phase regulators

C1 inhibitor

C1-inhibitor (C1-Inh) prevents over activation of the CP and LP. It does so by binding to active C1r and C1s and MASPs causing their displacement from the active complexes (Sim and Reboul, 1981). C1-Inh can also prevent the spontaneous activation of CP by binding reversibly to C1 in fluid phase. Other targets for C1-Inh include factors XIIa, factor Xia, plasmin and kallikrein. The importance of C1-Inh can be seen in hereditary angioneurotic oedema (HANE) where there is a deficiency C1-Inh which is associated with attacks of increased vascular permeability.

Factor I

Factor I is a highly specific serine protease which regulates the C3/C5 convertase. It does so by cleaving the α chain of C4b or C3b using co-factor activities from C4-binding protein (C4bp), CR1, DAF or factor H (Pangburn et al., 1977).

Factor H

Factor H is the main fluid phase regulator of the AP. It has a high affinity for the sialic acid residues attached to glycoproteins present on the host cell membranes compared with the pathogen membrane and thus allows the discrimination between self and non-self. Factor H protects the host from AP activation by attaching to C3b deposited on the host surface and then acting as a cofactor for factor I. The importance of factor H can be observed in fH

deficiencies or impairments where unrestricted AP activation leads to C3 deficiency and predisposes to bacterial infections and conditions such as glomerulonephritis or hemolytic-uremic syndrome (HUS) and age related macular degeneration (AMD) (Ault, 2000, Zipfel et al., 2010).

C4 binding protein

Similar to factor H, C4bp can act as a cofactor for factor I causing the proteolytic inactivation of C4b and C3b. C4bp also contains decay accelerating activity and its principal aim is to decay the C3 convertase (Gigli et al., 1979, Daha and van Es, 1980).

Properdin

Properdin is a positive regulator of C activation via the AP. The binding of properdin to C3b results in (1) resistance to cleavage by factor I, (2) it increases C3b affinity for factor B and (3) it increases stability of the C3 convertase C3bBb (Fearon and Austen, 1975, Smith et al., 1984).

S-protein

S-protein (vitronectin) can prevent the formation of MAC by binding up to three molecules C5b-7 and preventing the complex from binding to the cell surface. S-protein can also prevent C9 polymerisation (Podack et al., 1984).

Clusterin

Similarly to S-protein, clusterin can prevent MAC formation by binding to C5b-7 and preventing the complex from binding to the cell surface (Jenne and Tschopp, 1992).

C8β chain

The C protein C8, which is composed of three non-identical subunits (α, β and γ), can also act as a fluid phase inhibitory protein. In the absence of membranes the C8β chain can bind to soluble SC5b-7 complex, bound by S protein, therefore rendering the complex inactive and membrane insertion cannot take place (Monahan and Sodetz, 1980).

Carboxypeptidase-N

The anaphylatoxins are very potent pro-inflammatory molecules whose activities need to be tightly controlled. The anaphylatoxins C5a, C3a and C4a are readily metabolised to their less active 'des Arg' counter parts by serum and cell surface carboxypeptidase which remove the C-terminal arginine (Bokisch and Muller-Eberhard, 1970). Although C5a des Arg is less capable at binding to the C5aR than C5a, studies have found that C5a des Arg binds with a 10-fold higher affinity to the receptor C5L2 which has led to the suggestion that one function

of C5L2 is to remove circulating C5a des Arg, however, this remains controversial (see section 1.2.6) (Cain and Monk, 2002, Okinaga et al., 2003, Scola et al., 2009).

1.1.6 Complement in pathology

It is essential that the correct balance between C activation and regulation is achieved in order to protect the host from infection and aid the clearance of immune complexes and apoptotic cells, whilst ensuring that the activation is restricted to prevent its deleterious effects to the host (Walport, 2001b, Walport, 2001a). The importance of this balance is observed in pathology where attenuated activation can predispose to infections or to diseases such as systemic lupus erythematosus (SLE) where the delayed clearance of apoptotic cells due to deficiency of CP components triggers autoimmunity (Flierman and Daha, 2007). In contrast, uncontrolled activation of C can lead to excessive inflammation, which has implicated in numerous pathological inflammatory diseases such as rheumatoid arthritis (Guo and Ward, 2005).

1.2 C5a and its receptors

1.2.1 Biological functions of C5a

C5a is the most potent anaphylatoxin generated following C activation, being 20 times more potent than C3a at exerting its pro-inflammatory activities. Following the release of C5a from site of injury, smooth muscle cells lining the blood vessels contract increasing vasodilation and vascular permeability (Ember and Hugli, 1997). Although smooth muscle cell constriction is believed to be due the release of histamine from residential mast cells, secondary to C5a, the identification of C5aR expression on smooth muscle cells suggests a possible direct role (Haviland et al., 1995, Gasque et al., 1998, Zwirner et al., 1999). The released C5a also acts as a potent chemoattractant for neutrophils, monocytes and macrophages (Snyderman et al., 1971, Snyderman et al., 1975, Marder et al., 1985). The binding of C5a to the phagocytes promotes their adhesion to endothelial cells by up-regulating the expression of adhesion molecules on their cell surface, followed by their infiltration and chemotaxis up the C5a concentration gradient towards the site of C activation (Guo et al., 2002). C5a can enhance the phagocytic activities of activated leukocytes by increasing expression of phagocytic receptors and releasing reactive oxygen species, cytokines, chemokines and granule enzyme (Snyderman and Pike, 1984, Goldstein and Weissmann, 1974, Sacks et al., 1978).

In addition to these pro-inflammatory properties C5a also displays some immune modulating functions. For instance it has been proposed that C5a plays an important role in inhalation tolerance regulating Th2 lineage commitment of naïve CD4+ Th lymphocytes

((Kohl et al., 2006); reviewed by (Kohl, 2006)). C5a can also effect T cell differentiation and lineage commitment via cross-talk with the TLR signalling pathways (reviewed (Ricklin et al., 2010, Kohl, 2006)).

The discovery of the C5a receptor, C5aR, expression on non-myeloid cells suggested that the anaphylatoxin may function beyond its immuno-modulating and inflammation functions and roles in tissue regeneration, tissue fibrosis and in brain development have also been suggested (Strey et al., 2003, Mastellos et al., 2001, Hillebrandt et al., 2005, Osaka et al., 1999, Mukherjee and Pasinetti, 2000, Mukherjee and Pasinetti, 2001).

1.2.2 Binding of C5a to the C5aR

C5a binds to the seven transmembrane receptors C5aR and C5L2. Although both receptors are capable of binding to C5a, only the C5aR is functionally coupled to G proteins (Okinaga et al., 2003). Binding of C5a to the C5aR is more complex than other GPCR as binding occurs via two separate domains. The first domain is composed of acidic residues in the N-terminus of the C5aR which interacts with high affinity to the basic residues in the core of C5a (figure 1.3).

Using antibodies raised against N-terminal peptides of the C5aR, two separate studies have demonstrated that blocking the N-terminus inhibits binding of C5a to the C5aR, and anaphylatoxin mediated cellular activation (Oppermann et al., 1993, Morgan et al., 1993). Furthermore, it was suggested that N-terminus of the C5aR, which is rich in negatively charged residues, facilitates the interaction with the positively charged C5a ligand (Oppermann et al., 1993). Removal of the N-terminal residues 2-22, resulted in a 600 fold reduction in affinity of C5a for its receptor, while extending the deletion to residues 30 caused a further 75 fold reduction in C5a binding (DeMartino et al., 1994). Using site directed mutagenesis this study identified key aspartic acid residues (Asp 10, 15, 16, 18, 21 and 27) at the N-terminus as being critical for the high affinity binding of C5a (DeMartino et al., 1994). In contrast another study by Chen and co-workers (1998), using NMR, suggested that residues 21-30 are crucial for the first binding domain of C5a to the receptor (Chen et al., 1998b). Using yeast random saturation mutagenesis which exploits the fact that amino acid residues most critical for receptor function are those that are most resistant to substitution, Hagemann and co-workers found that residues 19-29 remained relatively conserved suggesting their importance for C5a binding, but no single aspartate residue was critical (Hagemann et al., 2006). Chimeric receptors of the human C3aR and C5aR also confirmed that the C5aR N-terminus is required for the high affinity binding of the native C5a ligand, however, the C5a analogue peptide (H-8315) was still able to bind to the receptor, therefore confirming the two step binding model for the human C5aR (Crass et al., 1999). Sulphated tyrosine residues at positions 11 and 14 in the C5aR, have also been identified as critical for

the ability of C5aR to bind C5a and mobilise calcium (Farzan et al., 2001). Although Hagemann and colleagues were unable to support these findings in their yeast random saturation mutagenesis (Hagemann et al., 2006).

The second interaction occurs between the C-terminus of the C5a and a binding pocket formed by the hydrophobic residues in the transmembrane (TM) helices and charged residues at the base of the extracellular loops (ECLs) of the C5aR (Monk et al., 2007). Investigations of the ECLs using random saturation analysis has revealed that both the first and third ECLs are important for receptor activation but not ligand binding. However, ECL1 is more resistant to amino acid substitution than ECL3, suggesting this loop contains more essential amino acid residues (Klco et al., 2006). Investigations of the second ECL revealed that this loop acts as a negative regulator for C5aR activation as the random mutagenesis resulted in ligand independent signalling (Klco et al., 2005).

As well as C5a, several other proteins have been found to bind to the C5aR. S19 ribosomal dimers were first identified as a major monocyte specific chemotactic factor in rheumatoid arthritis synovial tissue (Nishiura et al., 1996). It has since been shown that the S19 ribosomal dimers are liberated by apoptotic HL-60 and AsPC-1 cells and it has therefore been suggested that this monocyte chemotactic factor plays an important role in phagocytic clearance of apoptotic cells (Nishiura et al., 1998, Shibuya et al., 2001). Skp, a periplasmic chaperone protein from *E. coli*, has also been shown to induce chemotactic responses of monocytes and polymorphonuclear leukocytes via the C5aR (Shrestha et al., 2004). Using receptor antagonising experiments Shrestha and co-workers showed that Skp is likely to bind to the C5aR in the same two-step mechanism as C5a and ribosomal S19 (Shrestha et al., 2004). Chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS) efficiently inhibits the activation of neutrophils and monocytes by C5a, and does so by binding to the N-terminal (residues 1-35) of the C5aR (Ippel et al., 2009). Ippel and co-workers found that sulphated tyrosines residues 11 and 14 are essential for the tight binding between C5aR and CHIPS (Ippel et al., 2009).

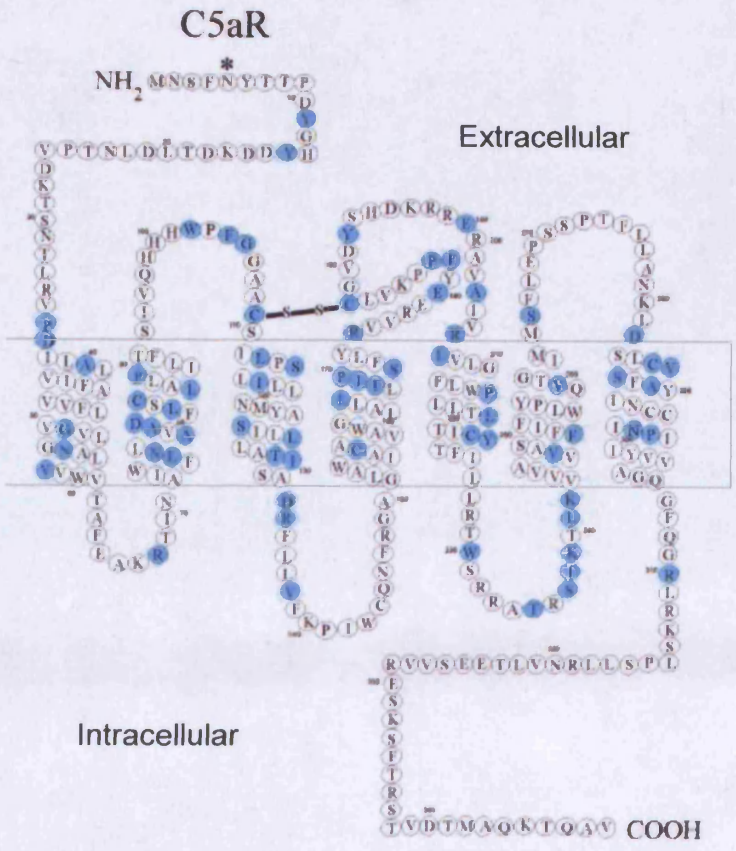


Figure 1.3 Sequence of the C5aR. Taken from Monk et al 2007, blue highlights indicate residues that have been identified by site-directed or random mutagenesis as being important for ligand binding and/or signalling of the C5aR.

1.2.3 Intracellular signalling of the C5aR

G protein coupled receptors activate signalling cascades via a heterotrimeric G protein composed of a Gα, Gβ and Gγ subunits. It is believed that the C5aR is coupled primarily to the pertussis toxin (PTX) sensitive $G\alpha i_2$ G protein; however several studies have also shown that when C5aR is ectopically expressed in COS-7 and human kidney 293 cell lines, the receptor only signals when co-transfected with the PTX insensitive $G\alpha_{16}$ G protein (Vanek et al., 1994, Sheth et al., 1991, Skokowa et al., 2005, Buhl et al., 1993, Amatruda et al., 1993). It has also been shown that in the MonoMac6 cell line, differentiated with dibutyryl cAMP (Bt_2cAMP), to be coupled to PTX insensitive G proteins (Takabayashi et al., 2004).

The G protein subunits bind to the C5aR via the intracellular loops 1 and 2, which act as a selective filter only allowing the correct G proteins to bind to the receptor. These loops also contain essential residues for the activation of the G proteins (Matsumoto et al., 2007a, Matsumoto et al., 2007b). Binding of C5a to the C5aR causes a conformational change which allows Gα-GDP to be exchanged to Gα-GTP by guanine nucleotide exchange factor (Matsumoto et al., 2007b). This exchange causes Gα-GTP to dissociate from the Gβγ

subunits, and then both Gα and Gβγ can transmit the signal to downstream effectors molecules (figure 1.4).

As well as signalling via G-proteins, more recently it has also been suggested that the association of the activated C5aR with β-arrestins, which mediates receptor internalisation (see section 1.2.4.4), also results in the activation of ERK1/2 (Bamberg et al., 2010). Furthermore, it has been suggested that C5L2 can act as a negative intracellular receptor of C5aR/β-arrestin/ERK1/2 signalling via its association to β-arrestins (see section 1.2.6) (Bamberg et al., 2010).

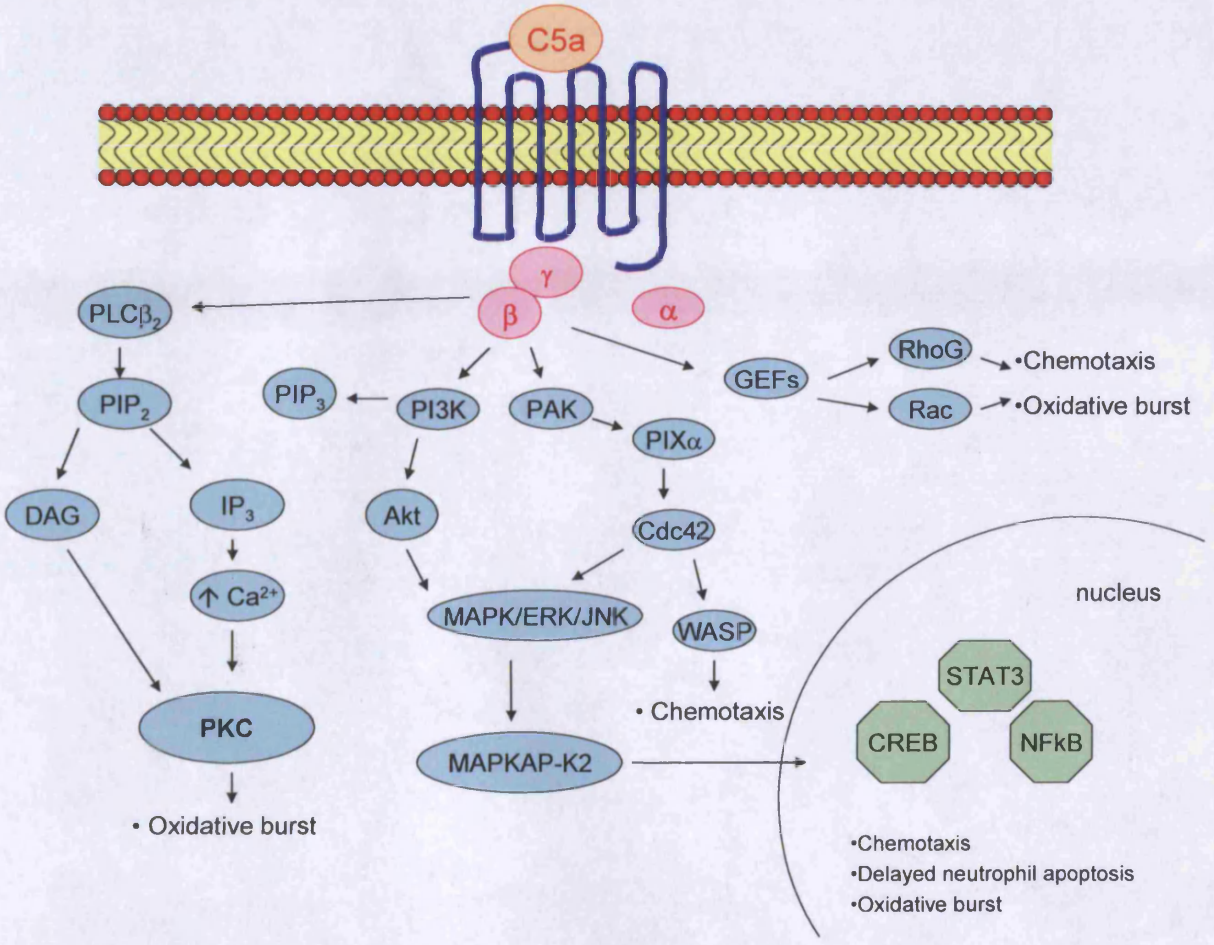


Figure 1.4 Schematic diagram illustrating the down stream signalling mechanisms of the C5aR following binding of C5a (adapted from (Monk et al., 2007, Rabiet et al., 2007)).

1.2.3.1 Activation of phospholipase C β2

Following the binding of C5a to its receptor, phospholipase C β_2 (PLCβ_2) has been shown to be activated by the dissociated Gβγ subunits (Jiang et al., 1996, Camps et al., 1992). Activation of PLCβ_2 results in the hydrolysis of phosphoinositol-4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 diffuses through the cytosol and binds to IP_3 receptors present on the endoplasmic reticulum membrane which in turn causes the release of calcium from the intracellular stores. DAG remains within the plasma

membrane and together the interaction of DAG with protein kinase C (PKC) and rise in intracellular calcium concentrations results in the activation of PKC. PKC activity has been shown to be important for the C5a-induced priming of oxidative burst in phagocytes (Wrann et al., 2007).

1.2.3.2 Activation of phosphoinositol 3-kinase

The dissociated Gβγ subunits have been found activate both p110β and p110γ isoforms of phosphoinositol 3-kinase (PI3K) (Guillermet-Guibert et al., 2008, Stoyanov et al., 1995). PI3K can convert the membrane PIP_2 into phosphoinositol-3,4,5-bisphosphate (PIP_3) (Rabiet et al., 2007). PI3K also activates Akt which phosphorylates p38 MAPK, ERK and c-Jun N-terminal kinase (JNK) and causes C5a induced IL-12 reduction in human monocytes (la Sala et al., 2005). PI3K activity is critical for numerous leukocyte functions. Hirsch and co-workers found that PI3Kγ knockout mice have an impaired respiratory burst and chemotactic activity towards C5a (Hirsch et al., 2000). Another study by Wrann and co-workers has shown that inhibition of the PI3K results in a reduced C5a-induced phagocytic response (Wrann et al., 2007). Convergence of the PI3K/Akt and ERK signalling pathways leads to the activation of cAMP-response element-binding protein (CREB) (Perianayagam et al., 2006). CREB, in turn, modulates the transcription of the anti-apoptotic molecule Bcl2 which has been proposed to be part of the mechanism by which C5a can delay neutrophil apoptosis (Perianayagam et al., 2002, Perianayagam et al., 2004). Signal transducer and activator of transcription (STAT3) has also been shown to become phosphorylated following C5a stimulation. In human erythroleukemia cells this is believed to be achieved via Ras/Raf/MEK/ERK and c-Src/JAK pathways, whereas in neutrophils it is believed to be only achieved by ERK pathway (Lo et al., 2003, Kuroki and O'Flaherty, 1999).

1.2.3.3 Activation of GTP-binding proteins

p21-activated kinase (PAK) has also been shown to be activated in neutrophils following stimulation of the C5aR (Huang et al., 1998). PAKs play an important role in regulation of the motility and cytoskeleton dynamics, as well as in the regulation of transcription factors, such as NFκB, through the MAPK cascade (Bokoch, 2003). A study by Li and colleagues found that the G protein βγ subunits binds PAK1 and with the guanine nucleotide exchange factor PIXα, via PAK-associated. PIXα activates Cdc42, a low molecular weight GTP binding protein part of the Rho family, which in turns activates PAK1. They showed that this Gβγ-PAK1/PIXα/Cdc42 pathway is essential for directional sensing and the persistent directional migration of chemotactic leukocytes (Li et al., 2003). PAK1/PAK2 can activate p38 MAPK, which in turn activates MAKP-activated protein kinase 2 (MAPKAP-K2). Macrophages from mice deficient in MAPKAP-K2 have an impaired chemotaxis towards C5a (Rousseau et al.,

2006). Activated Cdc42 has also been shown to bind to the Wiskott-Aldrich syndrome protein (WASP), which then promotes the association of WASP to the C-terminal cytoplasmic tail of the C5aR (Tardif et al., 2003). Tardif and co-workers suggested that this targeting of WASP to the plasma membrane following C5aR stimulation and Cdc42 activation might be one mechanism by which actin assembly is spatially controlled in cells moving towards C5a.

Activation of other guanine nucleotide exchange factors can lead to the activation of other GTP-binding proteins. pRex1 activation has been shown to activate Rac, another member of the Rho family which can control leukocyte functions such as chemotaxis, phagocytosis and superoxide production (Welch et al., 2002a). RhoG has also been found to become activated in neutrophils following stimulation of the C5aR. RhoG knockout mice have an impaired reactive oxygen species generation in response to C5a (Condliffe et al., 2006).

1.2.4 Regulation of C5aR function

Due to the potent pro-inflammatory nature of C5a-C5aR signalling, a crucial mechanism to limit its excessive activation is to regulate the C5aR function. Regulation of receptor activation can be achieved by receptor oligomerisation, clustering, phosphorylation and desensitization and internalisation.

1.2.4.1 Receptor oligomerisation

Only recently the idea that many GPCR form dimeric structures or higher order oligomeric complexes has become accepted (Milligan et al., 2003). It is believed that receptor oligomerisation may play an important role in receptor biosynthesis and the trafficking of the receptor to the plasma membrane (Rabiet et al., 2007).

A study by Floyd and co-workers found, by fluorescence resonance energy transfer (FRET) analysis, that the C5aR tagged with green fluorescent protein (GFP) forms receptor specific and constitutive dimers *in vivo* and by using *S.cerevisiae* expression system, they showed that this oligomerisation is not dependent on any accessory proteins (Floyd et al., 2003). Subcellular fractionation studies suggest that oligomerisation occurs early in the biosynthesis of the receptor and is important for the transport to the plasma membrane (Rabiet et al., 2007, Floyd et al., 2003, Milligan et al., 2003).

It has been suggested that GPCR dimerisation is likely to occur through association of transmembrane helices 1 and 2 (Geva et al., 2000, Klco et al., 2003). A study by Klco and co-workers investigated the role of the lipid facing residues in C5aR oligomerisation. They found that these residues alone are not essential for oligomerisation and they proposed that it is likely that multiple residues within the transmembrane are responsible for dimerisation. However, they did find that these lipid facing residues are essential for the trafficking of the

C5aR from the endoplasmic reticulum to the plasma membrane (Klco et al., 2009). Although C5aR homodimers do not undergo cross-phosphorylation, the stimulation and phosphorylation of only one monomer is enough to lead to the dimer internalisation (Rabiet et al., 2008). The C5aR has also been shown to form hetero-oligomers with the chemokine receptor 5 (CCR5) (Huttenrauch et al., 2005). In this study they found that G protein receptor kinases (GRK) as well as PKC, promote the cross-phosphorylation of these hetero-oligomers and that β-arrestin then mediated their internalisation.

1.2.4.2 Receptor clustering

Chemotaxis across a chemical gradient induces cell polarisation which results in asymmetrical distribution of the membrane. This asymmetrical distribution of the membrane can cause an enrichment of some chemoattractant receptors at the leading edge towards a point source. A study by Servant and co-workers (1999) found that C5aR forms clusters at the plasma membrane shortly after stimulation with C5a, however, this apparent increase in C5aR concentration reflects the increased relative abundance of plasma membrane rather then preferential accumulation of the receptor at the leading edge (Servant et al., 1999).

1.2.4.3 Receptor phosphorylation

Activation of GPCR leads to their rapid phosphorylation. Phosphorylation can occur by secondary messenger dependent kinases such as PKC or by GRK. Receptor phosphorylation can lead to desensitization of the receptor within milliseconds to minutes of agonist challenge (Pitcher et al., 1998). Desensitization is achieved by the binding of arrestins to the phosphorylated receptor, which causes the uncoupling from the G proteins and receptor internalisation (Rabiet et al., 2007).

The C5aR is primarily phosphorylated on serine residues (Ser-314, Ser-317, Ser-327, Ser-332, Ser-334, Ser-338), as well as threonine residues, all be it to a lesser extent (Giannini et al., 1995, Huttenrauch et al., 2005). Christophe and co-workers found that phosphorylation of either of the two serine pairs (Ser-332/-334 and Ser-334/-338) is a prerequisite for full receptor phosphorylation (Christophe et al., 2000). Phosphorylation of serine residues (Ser-327, Ser-334, Ser-338), allows β-arrestin 1 and 2 to associate with the activated receptor, however, internalisation requires a minimal level of phosphorylation of these key residues (Braun et al., 2003). As well as mediating the uncoupling of G proteins from the receptor, it has also been suggested that bound β-arrestins sterically interfere with further receptor phosphorylation and also protects phosphorylated residues by dephosphorylation by protein phosphatases (Pollok-Kopp et al., 2007). Several studies have shown that mutant C5aRs, which are not able to undergo phosphorylation, are not internalised following agonist binding and undergo a sustained intracellular calcium release

and production of superoxide (Naik et al., 1997, Suvorova et al., 2008, Christophe et al., 2000).

The exact nature of the kinases which phosphorylate the C5aR still remains controversial. The C5aR phosphorylation levels are likely to be regulated by GRK, as well as PKCβ (Langkabel et al., 1999, Pollok-Kopp et al., 2007). Co-expression of GRK 2 and 3 with C5aR in human mast cells, led to the suggestion that these were likely to be the main GRK members involved in phosphorylation of the C5aR (Langkabel et al., 1999). However, when GRK2, as well as GRK6, were over expressed in COS-7 cells transfected with the C5aR, the agonist induced phosphorylation remained unchanged (Milcent et al., 1999).

1.2.4.4 Internalisation, intracellular trafficking and recycling

Following agonist binding some GPCR, but not all, are internalised via β-arrestin-mediated pathway into early endosomes. Internalised receptors can either be recycled back to the plasma membrane or they can be targeted to lysosomes where they are then degraded. A rapid internalisation and recycling process may be critical for receptors whose primary function is to direct cell migration across a chemotactic gradient (Naik et al., 1997).

Within ten minutes of exposure to C5a, the C5aR can be detected within vesicles that cluster in the perinuclear region (Naik et al., 1997). The internalised receptors are rapidly recovered to the plasma membrane. The amount of C5aR re-expressed on the cell surface seems to depend on which cells are being studied; 73% of the C5aR has been found to be re-expressed on the cell surface of neutrophils compared with 90% in the stably transfected rat insulinoma cell line (Suvorova et al., 2005, Naik et al., 1997). Originally it was proposed that C5aR internalisation was mediated in an arrestin, dynamin and clathrin independent manner; however, more recent studies suggest that the C5aR is internalised via the classical clathrin-dependent pathway (Gilbert et al., 2001, Licht et al., 2003, Braun et al., 2003, Suvorova et al., 2005, Huttenrauch et al., 2005). Further investigations are needed in order to define the exact nature of the structural determinants involved in trafficking the C5aR to the lysosomes.

1.2.5 C5aR expression

C5aR expression was originally believed to be restricted to cells of myeloid origin, however it is now known to be expressed by numerous non-myeloid cell types. Summarised in table 1.1 is the non-myeloid cellular distribution of C5aR expression. This table highlights that some evidence for the expression of the C5aR in non-myeloid cells, such as the parenchymal cells of the lungs and kidney, remains controversial with several studies challenging the expression of the C5aR in these cells. A variety of techniques have been employed to detect C5aR; including measuring mRNA by techniques such as RT-PCR,

northern blotting and in situ hybridisation; and measuring protein expression by flow cytometry, western blotting and immunofluorescence. However, traditional techniques of measuring binding of radio labelled C5a are no longer an accurate measure of C5aR expression due to the discovery of the second C5a receptor, C5L2, which can also bind C5a (Cain and Monk, 2002).

C5aR expression has been well documented in the literature to be up-regulated in the myeloblastic cell lines by stimuli including dibutyryl cAMP (Bt_2cAMP), phorbol 12-myristate 13-acetate (PMA) and Interferon-γ (IFN-γ) (table 1.2) (Chenoweth et al., 1984, Rubin et al., 1986, Gavison et al., 1988, Gasque et al., 1998, Burg et al., 1996, Burg et al., 1995). These stimuli are therefore commonly used to investigate the C5aR. Other stimuli, such as forskolin, prostaglandin E2 and isoproterenol when combined with 1,25-$(OH)_2D_3$, IL-1 and TNFα, have also been reported to increase C5aR expression, however, these have been less well characterised (table 1.2) (Rubin et al., 1986, Rubin et al., 1991a, Takabayashi et al., 2004, Gasque et al., 1998).

Despite numerous studies investigating the expression profile of the C5aR, relatively few studies have actually investigated how expression is regulated. Initial studies by Gerard and co-workers found that the gene encoding the human C5aR was located on chromosome 19q13.3 within a chemotactic receptor gene cluster which also encodes the fMLP receptor (FPR) (Gerard et al., 1993). This study characterised the C5aR gene as a having a two exon structure, with the 5' untranslated sequence and initiating methionine codon located within the first exon, which is then separated by a ~9Kbp intron from the second receptor encoding exon (figure 1.5).

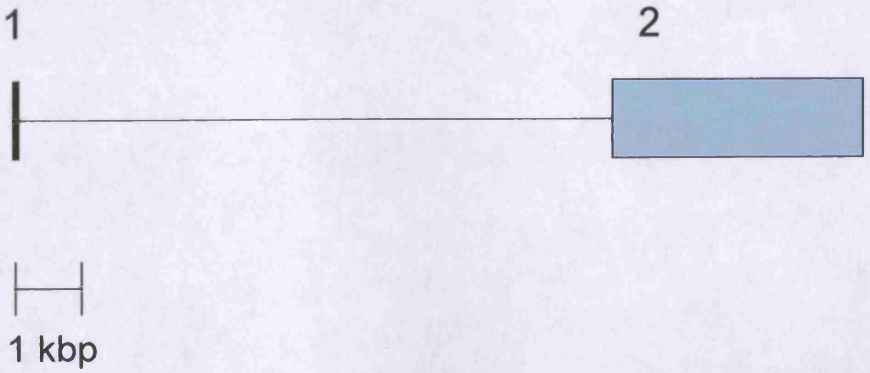


Figure 1.5 Genomic structure of C5aR. Two exons separated by a ~9 kbp intron sequence located between codon 1 and codon 2 (Gerard et al., 1993).

Table 1.1 Non-myeloid distribution of the C5aR. LBS = ligand binding studies (using radio labelled C5a), IC = immunochemistry, WB = western blotting, RT-PCR = reverse transcriptase PCR, FC = flow cytometry, CHX = chemotaxis assay, ISH = *in situ* hybridisation and NB = northern blotting.

Loci	Species	Cell type	Method of detection	C5aR detected	Reference
Circulatory system	Mouse	Microvascular endothelial cells	LBS, IC	Y	(Laudes et al., 2002)
CNS	Human	Microglia, reactive astrocytes	IC, WB, RT-PCR	Y	(Gasque et al., 1995, Gasque et al., 1997)
	Mouse	Neural stem cells	ISH, IC	Y	(O'Barr et al., 2001)
Connective tissue	Human	Synoviocytes	RT-PCR, FC, IC	Y	(Yuan et al., 2003)
Endocrine system	Rat	Anterior pituitary gland and pituitary cell lines	RT-PCR, IC	Y	(Francis et al., 2008)
Immune system	Human	CD3+ and Jurkat T cells	FC, RT-PCR, CHX	Y	(Nataf et al., 1999)
Kidney	Human	Cultured human glomerular mesangial cells	IC,FC,RT-PCR	Y	(Braun and Davis, 1998)
		Tubular epithelial cells	IC,ISH	Y	(Abe et al., 2001a)
		Normal and inflamed renal tubular epithelial cells	IC	N	(Kiafard et al., 2007)
	Mouse	Tubular smooth muscle or endothelial cells	NB,IC	N	(Zwirner et al., 1999)
	Rat	Normal and inflamed renal tubular epithelial cells	IC	N	(Kiafard et al., 2007)
		Normal and inflamed renal tubular epithelial cells	IC	N	(Kiafard et al., 2007)
Liver	Human	HepG2 cell line	NB, LBS	Y	(Haviland et al., 1995)
		Liver parenchymal cells	ISH, IC	Y	(Buchner et al., 1995)
		Liver hepatocytes, bile duct epithelial cells, vascular smooth muscle and endothelial cells	IC	N	(Haviland et al., 1995) (Zwirner et al., 1999)
Lung	Human	Vascular smooth muscle, endothelial cells and bronchial and alveolar epithelial cells	IC	Y	(Haviland et al., 1995)
		Normal and inflamed bronchial epithelial and smooth muscle cells	ISH,IC	Y	(Drouin et al., 2001)
		Inflamed pulmonary epithelial cells	ISH	N	(Fayyazi et al., 1999)
		Bronchial and alveolar epithelial, vascular smooth muscle or	IC	N	(Zwirner et al., 1999)

		endothelial cells			
	Mouse	Normal and inflamed epithelial, endothelial and smooth muscle cells.	IC	N	(Tschernig et al., 2007)
	Rat	Normal and inflamed bronchial epithelial and smooth muscle cells	ISH,IC	Y	(Drouin et al., 2001)
		Normal and inflamed epithelial, endothelial and smooth muscle cells.	IC	N	(Tschernig et al., 2007)
Respiratory tract	Human	Normal or allergic nasal mucosa epithelial, vascular smooth muscle or endothelial cells	IC, WB	N	(Jun et al., 2008)
Skin	Human	Inflamed keratinocytes	ISH	Y	(Fayyazi et al., 1999) (Zwirner et al., 1999)

Table 1.2 Stimuli reported to alter C5aR expression. Abbreviations; LBS = ligand binding studies; CHX chemotaxis assay; FC = flow cytometry; WB = western blotting; NB = northern blotting; RT-PCR = reverse transcriptase PCR; Q-PCR = quantitative PCR; IHC = immunohistochemistry.

	Stimuli	Cell type	Conditions	Methods of detection	Reference
Increase C5aR expression	Cigarette smoke extract	Human bronchial epithelial cells	5% cigarette smoke extract 2h incubation	LBS, FC	(Allen-Gipson et al., 2005)
	Dibutyryl cAMP (Bt_2cAMP)	U937, MM6 and HL-60	72h Incubation 0.5mM Bt_2cAMP	LBS, NB	(Burg et al., 1996, Burg et al., 1995)
		U937	72h Incubation 1mM Bt_2cAMP	LBS, FC	(Chenoweth et al., 1984)
		U937	72h Incubation 10mM Bt_2cAMP	CHX	(Gavison et al., 1988)
		U937	72h Incubation 0.5mM Bt_2cAMP	LBS, NB	(Gerard and Gerard, 1991)
		U937	96h Incubation 1mM Bt_2cAMP	LBS	(Rubin et al., 1986)
		MM6	24h Incubation 0.5mM Bt_2cAMP	LBS, RT-PCR	(Takabayashi et al., 2004)
	Forskolin	U937	72h Incubation 75 uM forskolin	CHX	(Shayo et al., 1997)
		U937	96h Incubation 15 uM forskolin and 10^{-8}M 1,25-$(OH)_2D_3$	LBS	(Rubin et al., 1986, Brodsky et al., 1998)
	Isoproterenol when combined with 1,25-$(OH)_2D_3$	U937	96h Incubation 10^{-7}-10^{-5}M isoproterenol and 10^{-8}M 1,25-(OH)2D3	LBS	(Rubin et al., 1986)
	Prostaglandin E_2 when combined with 1,25-$(OH)_2D_3$	U937	96h Incubation 10nM 1,25-$(OH)_2D_3$ and 0.3uM Prostaglandin E2	LBS	(Rubin et al., 1991a)
	Phorbol 12-myristate 13-acetate (PMA)	U937, MM6 and HL-60	64h Incubation 10nM PMA	LBS, NB	(Burg et al., 1996)
			72h Incubation (THP-1), 48h Incubation (U937	FC, WB,	(Gasque et al., 1995,

		THP-1 and U937 U937	10ng/ml PMA 24h Incubation100nM PMA	RT-PCR NB	Gasque et al., 1998) (Gerard and Gerard, 1991)
	IL-1 (IL-α and IL-1β)	MM6	24h Incubation, 20ng/ml IL-1	LBS	(Takabayashi et al., 2004)
	IL-6	Mouse lung, liver, kidney and heart	During the development of sepsis in mice	IHC, RT-PCR	(Riedemann et al., 2003)
	IL-4 when combined with $1,25\text{-}(OH)_2D_3$	Monocyte-derived DC	7 day Incubation 250U/ml followed by prostaglandin E_2 1ug/ml for 48h	FC,RT-PCR, Q-PCR	(Weinmann et al., 2003)
	IFNγ	U937 U937, HL60 and MM6	48h Incubation 10,100,1000 IU/ml 72h Incubation 1000U/ml rINFγ	FC, RT-PCR LBS	(Gasque et al., 1998) (Burg et al., 1996, Burg et al., 1995)
	TNFα	U937	48h Incubation 10IU/ml TNFα	FC	(Gasque et al., 1998)
Decrease C5aR	IL-4	Human monocytes and monocyte derived DC	24h Incubation 300 U/ml	FC, RT-PCR	(Weinmann et al., 2003, Soruri et al., 2003)

Gerard and colleagues also investigated the promoter activity of a ~800bp fragment, using a chloramphenicol acetyltransferase (CAT) reporter, composed of ~-350bp upstream of the initiating methionine codon, exon 1 and ~450bp of the beginning of the intron, of the human C5aR gene (figure 1.6 A). Their results showed that this ~800bp fragment contained a *cis*-acting element which is responsive to PMA, as treatment of the transfected rat basophilic leukemia (RBL-1) cells with PMA resulted in 2 fold increase in reporter CAT activity and they speculated that an AP-1 site could be responsible for this activity. Gerard and colleagues also found cell-type specific suppressor activity within -346bp and -225bp from the start codon which prevented transcription of the CAT gene when transfected into the neuroblastoma SK-N-SH cell line (Gerard et al., 1993) (figure 1.6 A).

Another study by Hunt and colleagues (2005) investigated the transcriptional regulation of the mouse C5aR gene. In this study they cloned a -2278bp fragment from the transcription start site and created a series of 5'deletions into the pGL2-Basic vector which allowed the promoter activity to be determined by performing luciferase assays (figure 1.6 B). By transfecting these constructs into the mouse macrophage (RAW 264.7) and endothelial (b.End3 and mHEVc) cell lines, which express C5aR mRNA, they found that the majority of the region cloned (-2278bp to -232bp) was dispensable for expression in these cells (Hunt et al., 2005). When they transfected the same constructs into the B16 melanoma cell line, which do not express C5aR mRNA, they found that the -2278bp to -232bp fragments contained a cell type specific suppressor element which resulted in minimal transcriptional activity of the luciferase gene in this cell line. Hunt and colleagues also showed that deletion of fragments between -232bp to -132bp and -132bp to -90bp, resulted in approximately 40% and 80% reduction in promoter activity respectively, in the RAW 264.7, b.End3 and mHEVc cell lines (figure 1.6 B). They identified the transcriptional regulatory site most critical for the promoter activity within the -132bp to -90bp is a putative CCAAT site, which specifically binds nuclear factor-Y (NF-Y). This NF-Y site was also shown to be predominately responsible for LPS induced up regulation of the C5aR, however, there was some contribution from a CP-2 site at position -155bp (Hunt et al., 2005).

Further studies by the same group investigated the transcriptional control mechanisms of the mouse C5aR in glial cells of the central nervous system. They found that the mouse microglial (BV-2) and astrocyte (Ast2.1) cell lines and primary microglial cells contained strong CCAAT box DNA binding activity, however, only weak activity was observed in primary astrocytes. Supershift assays demonstrated that NF-YA protein is critical in formation of this DNA-protein complex. By using the luciferase reporter gene constructs Martin et al. (2007) showed that, contrary to results in murine macrophages, the CCAAT element has little activity in primary astrocytes. They then suggest that C5aR

expression in astrocytes is regulated by additional control regions both 3' and 5' of the CCAAT box (Martin, 2007).

A novel single nucleotide polymorphism (SNP) in the promoter region of the human C5aR at position -245 (T/C) has also been identified (Barnes et al., 2004). This SNP was shown to have a higher frequency in the Afro-Caribbean population compared with the German and Tangier Island population, however, it had no association with asthma or atopy (Barnes et al., 2004). This study also found, using the 2711bp sequence immediately upstream from the 5'UTR of the C5aR gene into the luciferase reporter gene plasmid, that the T to C substitution at position -245bp had no effect of reporter gene activity, when transfected into the U937 cell line, despite an AP-4 motif being disrupted. However, this study did not investigate the effects of this SNP on induced expression (Barnes et al., 2004).

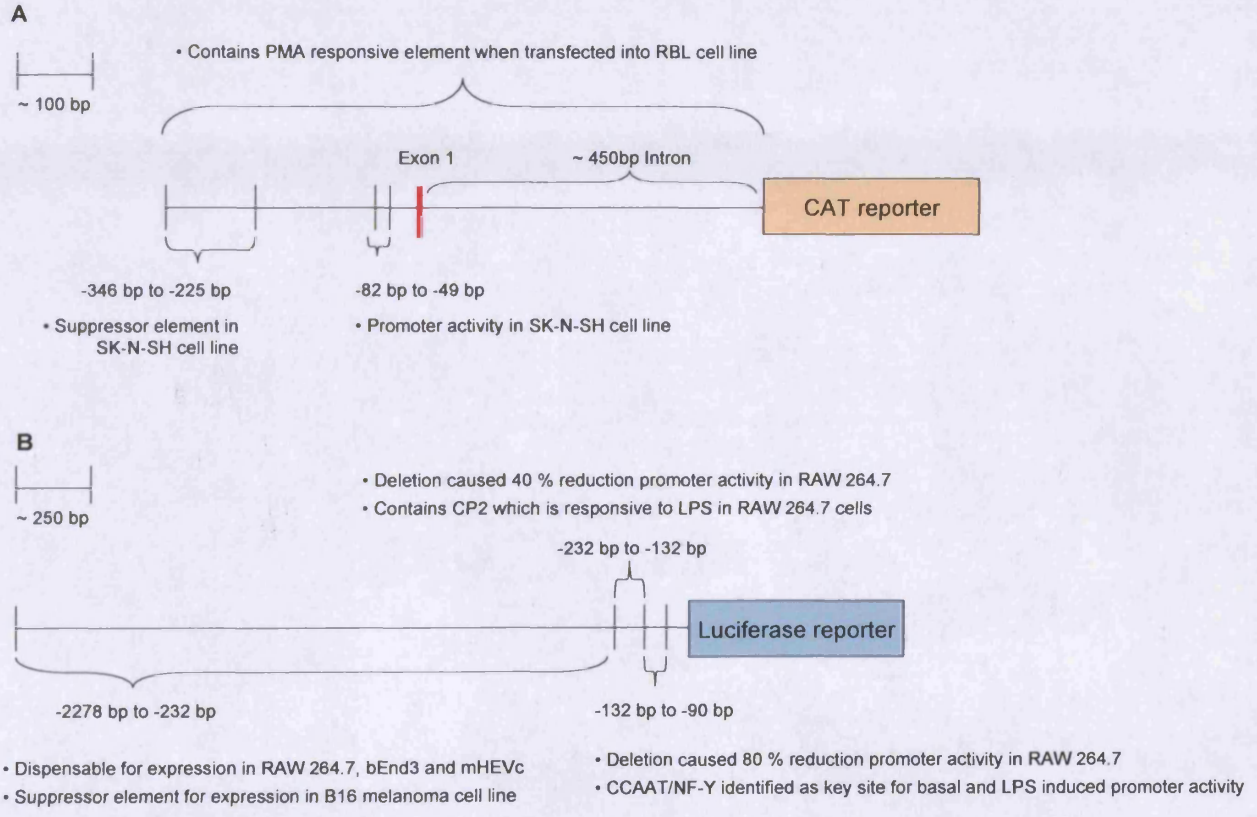


Figure 1.6 Schematic diagram of reporter studies carried out using human C5aR promoter region (A) or mouse C5aR promoter region (B). Results from Gerard et al 1993 (A). Results from Hunt et al 2005 and Martin et al 2007 (B). See text above for full description.

1.2.6 C5L2

As already mentioned, C5a can also bind to the seven transmembrane receptor C5L2, as well as, the C5aR. Although both receptors bind C5a with similar affinities, C5L2 has 10 times higher affinity for C5a des Arg compared with the C5aR (Cain and Monk, 2002). Although both receptors are capable of binding to C5a, only the C5aR is functionally coupled to G proteins (Okinaga et al., 2003). Furthermore, binding of C5a to C5L2 does not induce

MAP-kinase activation, intracellular Ca^{2+} flux, degranulation or chemotaxis; thus further suggesting a non-signalling role (Cain and Monk, 2002, Okinaga et al., 2003). However, the exact role for C5L2 still remains controversial. In rodents, deletion or blockade of the C5L2 receptor was found to enhance the biological activities of C5a suggesting that its role is to limit the pro-inflammatory response to C5a (Gerard et al., 2005, Gao et al., 2005). However a study by Chen and co-workers found that C5L2 deficiency led to a reduction in C5a mediated inflammatory cell infiltration (Chen et al., 2007). Furthermore, in a mouse model of sepsis only blockade of both C5aR and C5L2 improved outcome (Rittirsch et al., 2008). Despite evidence suggesting a more positive regulatory role for C5L2 in C5a signalling in mice, the human C5L2 has been shown to act as negative regulator by removing and degrading active C5a fragments from the extracellular environment when transfected into the rat basophilic leukemia cell line (Scola et al., 2009). In human neutrophils antibody blockade of C5L2 resulted in a dramatic increase in C5a mediated chemotaxis and ERK-phosphorylation but C5aR endocytosis remained unchanged. Bamberg and co-workers also observed that following C5a stimulation both C5aR and C5L2 appeared to translocate to the same compartment, and that both receptors were found to associate with β-arrestins. These findings led the authors to suggest that C5L2 acts as a negatively regulate for C5aR ERK1/2 signalling possibly via β-arrestin pathway in human neutrophils (Bamberg et al., 2010).

C5L2 is co-expressed with C5aR on many cell types including neutrophils, macrophages and immature dendritic cells (Ohno et al., 2000, Chen et al., 2007, Gerard et al., 2005), as well as, in the lung, liver, spleen, brain and heart (Kalant et al., 2005, Gao et al., 2005). Despite being co-expressed with the C5aR, the C5L2 mRNA transcript expression is approximately one-third of that of the C5aR (Gerard et al., 2005), and its actual protein expression is found mainly intracellular compared with the C5aR which is expressed mainly on the cell surface (Bamberg et al., 2010).

1.2.7 C5a and the C5aR in disease

Over production of C5a or increased C5aR expression has been implicated in pathogenesis of many inflammatory conditions, autoimmune and neurodegenerative diseases (table 1.3). Elevated plasma levels of C5a have been noted in humans with sepsis, and is believed to cause neutrophil dysfunction which in turn contributes towards the cytokine storm and multiple organ failure (Guo and Ward, 2005). In cecal ligation and puncture (CLP) induced septic rats neutrophils displayed defective phagocytosis, chemotaxis and NADPH oxidase assembly, which was prevented by blockade of C5a (Huber-Lang et al., 2002). Despite down regulation of C5aR expression in neutrophils, which is probably due to increased internalisation of the receptor following interaction with C5a, Riedemann and co-workers have shown the receptor expression increases in the lungs, liver, kidney and heart in the

mouse CLP septic model, which has been suggested to contribute towards multiple organ failure (Riedemann et al., 2002). In mouse CLP induced sepsis, Rittirsch and co-workers showed that only blockade of both C5aR and C5L2 improved survival rates suggesting that C5L2 may have some signalling role (Rittirsch et al., 2008). For information on the role of C5a/C5aR signalling in atherosclerosis and ischemic reperfusion injury please see sections 1.4.4 and 1.4.5 respectively.

In some instances C5a/C5aR signalling has been shown to have a protective effect initially, however, once an inflammatory response has become established C5a generation exacerbates pathology. For instance in allergic asthma C5a/C5aR signalling can initially have a protective role in the allergen-sensitisation phase by regulating the dendritic cell/T cell interface, however, once allergen inflammation is established it drives Th2 mediated eosinophil and mast cell destruction (Kohl, 2006, Ricklin et al., 2010).

In autoimmune diseases, such as rheumatoid arthritis, C activation initiated by immune complex formation results in a C5a chemotactic gradient which attracts leukocytes to the site of C activation which in turn exacerbates the inflammatory response. Evidence from C5aR-/- mice suggest that the C5a gradient across the synovial joint is crucial for the recruitment and activation of neutrophils and monocytes into the synovium and synovial fluid (Grant et al., 2002). Furthermore, these activated neutrophils secrete pro-inflammatory cytokines TNFα and IL-1β, which recruit additional inflammatory cell subsets and activate residential synovial fibroblasts, and therefore amplifying the inflammatory response and resulting in cartilage and bone degradation (Grant et al., 2002, Woodruff et al., 2002, Weissmann, 2004).

Due to the potentially destructive nature of C5a/C5aR signalling in disease, a C5aR antagonist would have huge therapeutic potential.

Table 1.3 Pathologies associated with over production or under regulation of C5a or increased C5aR expression. Adapted from (Monk et al., 2007).

Disease	Author
Rheumatoid arthritis	(Grant et al., 2002, Woodruff et al., 2002, Neumann et al., 2002, Weissmann, 2004)
Respiratory distress syndrome	(Hammerschmidt et al., 1980)
Inflammatory bowl disease	(Woodruff et al., 2003)
Ischaemia reperfusion injury	(Arumugam et al., 2003, Martin et al., 1988, Proctor et al., 2004, Woodruff et al., 2004, Arumugam et al., 2004a)
Chronic obstructive pulmonary disease	(Marc et al., 2004)
Sepsis	(Huber-Lang et al., 2002)
Asthma and allergy	(Abe et al., 2001b, Baelder et al., 2005, Gerard and Gerard, 2002, Lambrecht, 2006)
Psoriasis	(Kapp et al., 1985)
Atherosclerosis	(Hammerschmidt et al., 1981, Speidl et al., 2005)
Tissue rejection	(Gaca et al., 2006, Gueler et al., 2008)
Glomerulonephritis	(Kondo et al., 2001, Welch et al., 2002b)
Pancreatitis	(Bhatia, 2002)
Multiple sclerosis	(Muller-Ladner et al., 1996)
Neurodegeneration and macular degeneration	(Kijlstra et al., 2005, van Beek et al., 2003, Ager et al., 2010)
Cystic fibrosis	(Fick et al., 1986)
Systemic lupus erythematosus	(Hammerschmidt et al., 1980, Hopkins et al., 1988)
Antiphospholipid syndrome	(Girardi et al., 2003)
Anaphylactic and haemorrhagic shock	(Harkin et al., 2004, Younger et al., 2001)
Burns	(Piccolo et al., 1999)

1.3 Role of cholesterol in GPCR function and expression

Cholesterol is an integral component of biological membranes. Its rigid ring structure allows it to insert between phospholipids present within the lipid bilayer, where it then regulates membrane fluidity and permeability (Pike, 2003). As well as its crucial role in membrane fluidity, cholesterol is now becoming recognised for its importance in the formation of cholesterol rich microdomains termed lipid rafts (Burger et al., 2000, Maxfield and Tabas, 2005). As GPCR are commonly found localised within these lipid raft microdomains or move in or out of such regions following their interaction with their ligand (table 1.4); their function and expression can often be regulated by cholesterol. Discussed below are the different mechanisms by which cholesterol can alter GPCR function and expression within the plasma membrane.

Table 1.4 Summary of the effects of agonist binding on G-protein coupled receptor membrane lipid raft localisation. (Adapted from (Pike, 2003)).

Moves into rafts	**Moves out of rafts**	**Unaffected by agonist**
Angiotensin II type 1 (Ishizaka et al., 1998)	Adenosine A1 (Lasley et al., 2000)	β1-Adrenergic (Rybin et al., 2000)
m2 Muscarinic cholinergic (Feron et al., 1997)	β2-Adrenergic (Rybin et al., 2000)	Endothelin (Chun et al., 1994)
Bradykinin 1 (Sabourin et al., 2002)		Rhodopsin (Seno et al., 2001)
Bradykinin 2 (de Weerd and Leeb-Lundberg, 1997, Haasemann et al., 1998)		
EDG-1 (Igarashi and Michel, 2000)		

1.3.1 Role of cholesterol in GPCR compartmentalisation into lipid rafts

The identification of lipid raft regions within the membrane has allowed the concept of membrane sub-compartmentalisation (Lingwood and Simons, 2010). The compartmentalisation of signalling components into lipid raft or non-lipid raft regions can act as an important regulatory mechanism for the signalling cascade. For instance, the close proximity of signalling components when localised all in one raft would allow a rapid and efficiently signalling mechanism (Pike, 2003). Alternatively it could restrict the signalling flux by physically separating different signalling components which would then be targeted to each other upon activation (Pike, 2003).

Compartmentalisation and targeting of certain receptors to different regions of the plasma membrane can also lead to a polarity of the cell. This cell polarity is crucial for chemoattractant stimulated cells which display noticeable polarised phenotype where lipid

rafts and associated proteins, such as chemokine receptors, are preferentially distributed towards the leading edge (Manes et al., 2001). Several studies have shown that chemotaxis of neutrophils and monocytes towards fMLP is reliant on membrane cholesterol levels (Bath et al., 1991, Dunzendorfer et al., 1997, Wolach et al., 1992). It has been show, using methyl-β-cyclodextrin (MβCD), which depletes membrane of cholesterol, that cholesterol is critical for the clustering and redistribution of the fMLP receptor (FPR), structurally homologous to the C5aR, into lipids rafts during the chemotaxis towards fMLP (Xue et al., 2004). Another study has also shown that although cholesterol was required for polarisation and redistribution of the FPR, cholesterol depletion had no effect on earlier signalling events such as intracellular Ca^{2+} flux (Rose et al., 2008). Furthermore a study by Dunzendorfer and co-workers showed that treatment of neutrophils and monocytes with the statin pravastatin significantly decreased chemotaxis triggered by fMLP (Dunzendorfer et al., 1997). However, a study by Sitrin et al. contradicts these findings and suggests that the FPR is not located in lipid rafts neither does it require intact rafts for its signalling capacity (Sitrin et al., 2006). Although the FPR and the C5aR are often compared in the literature due to their structural similarities, evidence suggests that the C5aR is unlikely to form clusters at the membrane edge towards C5a. As already described in section 1.2.4.2, a study by Servant et al. found that although the C5aR appeared to cluster at the plasma membrane towards the C5a source, this was due to an increase in relative abundance of plasma membrane rather than preferential accumulation of the receptor at the leading edge (Servant et al., 1999). Despite this, a study by Nagao and co-workers found that cholesterol loading of mouse macrophages J774A.1 cells with cholesterol MβCD led to a significant decrease in transmigration towards C5a suggesting that the chemotactic functioning of the receptor is sensitive to membrane cholesterol (Nagao et al., 2007). However, this study did not investigate whether cholesterol loading had any effect on the clustering/membrane compartmentalisation of the C5aR, instead it found that cholesterol loading reduced activation of Rho A, a GTP binding protein involved in downstream signalling of the C5aR (Nagao et al., 2007).

1.3.2 Role of cholesterol on GPCR structural properties

Cholesterol within the plasma membrane can also affect the structural properties of proteins and therefore their function. It does so by either a direct mechanism, where cholesterol can bind directly to the protein, thereby altering its conformation, or indirectly, by influencing the biophysical properties of the membrane lipid bilayer (Burger et al., 2000). The rhodopsin receptor, for example, has been shown to be regulated by cholesterol content in both an indirect and direct manner. Stimulation of this photoreceptor with light induces a conformational change from its inactive to its active form. This change requires the receptor to expand within the plane of the bilayer. In a high-cholesterol environment this transition is

inhibited as the membrane becomes more rigid and therefore signalling is reduced (Albert and Boesze-Battaglia, 2005).

As lipid rafts are rich in cholesterol, GPCRs and their signalling components located in these regions tend to be sensitive to membrane cholesterol levels. For instance, experiments using neutrophils from neonates, which have more fluid plasma membranes due to lower cholesterol to phospholipid ratio, showed that slight rigidification of the plasma membrane using cholesteryl hemisuccinate enhanced the fMLP chemotactic function (Wolach et al., 1992). However, as described above 1.3.1, several studies have shown that membrane cholesterol is crucial for the redistribution of the FPR following its stimulation and to date no published studies have investigated whether cholesterol binds directly to the receptor. As very little is known about the membrane localisation of the C5aR it is difficult to speculate at this stage whether the structural properties of the receptor will be affected either directly or indirectly by cholesterol.

1.3.3 Effects of cholesterol on GPCR internalisation

Following stimulation of GPCR with their ligands many are desensitised by rapid phosphorylation and internalisation of the receptor. Where some GPCR are targeted to lipid raft regions following their activation, these microdomains can then facilitate receptor endocytosis as well as signal transduction (Pike, 2003). Although initially there were conflicting results as to whether C5aR internalisation was mediated by clathrin dependent or independent mechanism, it is now believed that following stimulation of the C5aR with C5a the receptor clusters into clathrin coated pits where it is then internalised in a β-arrestin, dynamin, and clathrin-dependent pathway (Gilbert et al., 2001, Licht et al., 2003, Braun et al., 2003, Suvorova et al., 2005, Huttenrauch et al., 2005). However, as lipid raft dependent endocytosis is characterised by clathrin-independence, as well as cholesterol sensitivity, this would suggest that the C5aR is unlikely to be internalised in a lipid raft dependent manner, however it has not been investigated whether C5aR internalisation is dependent on cholesterol (Lajoie and Nabi, 2010).

Regulating GPCR internalisation by altering membrane cholesterol is another mechanism which cell surface expression can be regulated which will be discussed below in more detail.

1.3.4 Effects of cholesterol on GPCR expression

As cholesterol is a crucial component for maintaining membrane integrity, mammals have developed several feedback regulation mechanisms to sustain cholesterol homeostasis within the cell (see section 1.5) (Goldstein and Brown, 1990). Many genes that are involved in cholesterol metabolism are regulated by sterol-regulatory-element-binding protein

(SREBP), which allows the cell to sense cholesterol levels and regulate gene transcription of target genes accordingly. At high cholesterol levels, SREBP is trapped within the endoplasmic reticulum where it is bound to SREBP cleavage activator protein (SCAP) in its inactive form (Muller-Wieland et al., 1997, Maxfield and Tabas, 2005). Inactivation of SREBP leads to a reduction of cholesterol biosynthesis by reduction in gene transcription of HMG-CoA synthase and HMG-CoA reductase, as well as reducing uptake of LDL-cholesterol via down regulation of the LDL receptor gene. When cholesterol levels are low the SREBP-SCAP complex exits the endoplasmic reticulum and undergoes proteolytic cleavage to release SREBP. SREBP is translocated into the nucleus where it regulates the transcription of many genes such as HMG-CoA reductase, HMG-CoA synthase and LDL receptor gene (Muller-Wieland et al., 1997). Plasma LDL has also been shown to increase expression of the MCP-1 receptor CCR2, a GPCR, in the THP-1 cell line; furthermore its expression is dramatically increased on monocytes from hypercholesterolemic patients (Han et al., 1998). Later investigations found that in a mouse hypercholesterolemia model the CCR2 gene in circulating monocytes is under the transcriptional control of SREBP and PPARγ within the promoter region (Chen et al., 2005, Han et al., 2005).

As cholesterol is an integral part of cellular membranes it can also affect expression of genes which encode cell surface molecules by affecting membrane fluidity and therefore the proper processing and targeting to plasma membrane. For instance simvastatin has been suggested to reduce surface expression of MHC-II molecules by disrupting the cholesterol-containing microdomains which are important for the transport and concentration of MHC-II at the cell surface (Kuipers et al., 2005).

1.4 Atherosclerosis

Atherosclerosis is the primary cause of heart disease in westernised societies. It is a highly complex disease where both environmental and genetic factors play an important role in its susceptibility and progression (table 1.5). Atherosclerosis is an inflammatory disease, initiated by endothelial dysfunction followed by chronic inflammation in the vessel wall (Ross, 1999a). Atherosclerotic lesions are characterised by infiltrating immune cells (mainly monocytes, macrophages, T-cells and mast cells), chronic inflammation, lipid accumulation, cell-death and fibrosis. Unstable lesions may rupture causing thrombotic occlusions of the artery which can lead to serve clinical events such as myocardial infarction or ischaemic stroke (Libby, 2002).

Table 1.5 Risk factors associated with cardiovascular disease. Adapted from (Lusis, 2000).

Genetic risk factors	Environmental risk factors
Elevated LDL/VLDL levels	High fat diet
Reduced levels of HDL	Smoking
Elevated levels of lipoprotein (a)	Lack of exercise
Elevated blood pressure	Infection agents
Elevated levels of homocysteine	C Reactive protein
Family history	
Diabetes and obesity	
Elevated levels of haemostatic factors	
Depression and other behavioural traits	
Gender (male)	
Systemic inflammation	
Metabolic syndrome	

1.4.1 Initiation of atherosclerosis

Early research, which focused on the link between hypercholesterolemia and the development of atherosclerosis, led to the lipid hypothesis model being proposed by Rudolf Virchow in 1856. However, simply reducing serum lipid levels did not prevent the development of the disease. In 1977 the response to injury hypothesis was proposed, which postulated that the lesions of atherosclerosis arise as a result of injury to the arterial endothelium (Ross et al., 1977). In response to endothelium injury, caused by mechanical, chemical or biochemical factors (table 1.5), inflammation and the formation of a fibroproliferative response begins, as a protective mechanism initally, however with time and continuing insult may become excessive (Ross, 1999b).

Endothelium dysfunction, thought to play a large role in the initial, progression and complications of atherosclerosis, manifests itself by abnormal production of vasoactive factors such as decreased nitric oxide production and adhesion molecule expression. Disruption to the endothelial cells leads to an increased permeability that allows LDL to diffuse within the vessel wall which may become retained by its interactions with matrix proteoglycans (Boren et al., 1998). Trapped LDL can become modified by oxidation, lipolysis, proteolysis and aggregation which in turn contributes towards the inflammatory response and foam cell formation (Lusis, 2000). The recruitment of leukocytes, mainly monocytes and T-cells, into the arterial intima represents an essential step in lesion initiation and formation. Experimental evidence has shown that expression of adhesion molecules VCAM-1 and P-selectin are increased on endothelium cells lining aortic plaques in rabbits fed a high cholesterol diet, which is a prerequisite for recruitment of monocytes and T-lymphocytes into the intima (Li et al., 1993, Cybulsky et al., 2001). Animal studies using mice lacking chemokine MCP-1 and its receptor CCR2 have also shown that this

chemotactic gradient is essential for the recruitment of monocytes into the arterial wall (Gu et al., 1998, Boring et al., 1998). A similar role has also been reported for IL-8 (Boisvert et al., 1998). The recruitment of lymphocytes is achieved by a trio of IFNγ inducible chemokines IP-10, Mig and I-TAC (Libby, 2002).

1.4.2 Atherosclerosis progression

Atherosclerosis develops slowly over many years (see figure 1.7 for schematic diagram of the different stages). The first observed stage in atheroma development is the formation of the fatty streak which can be seen in childhood (Stary, 2000). The formation of the fatty streak involves the activation of the recruited monocytes into macrophages by inflammatory cytokines such as TNFα or IFNγ and macrophage colony-stimulating factor MCS-F. Activated macrophages rapidly take up oxidised LDL via scavenger receptors on their cell surface to become foam cells. The importance of scavenger receptors, SR-A and CD36, in formation of foam cells has been demonstrated in mice lacking these receptors, which show a modest reduction in atherosclerotic lesions (Suzuki et al., 1997, Febbraio et al., 2000). The accumulation of lipid-laden foam cells within the early lesion gives the appearance of fatty streak under the microscope hence its name.

Fatty streaks can progress into fibrous plaques by the proliferation and migration of vascular smooth muscle cells (VSMC) into the lesion, which is achieved by the secretion of growth factors and cytokines by macrophages and foam cells. Together the VSMC and leukocytes within the lesion secret extracellular matrix components, which give rise to the fibrous cap.

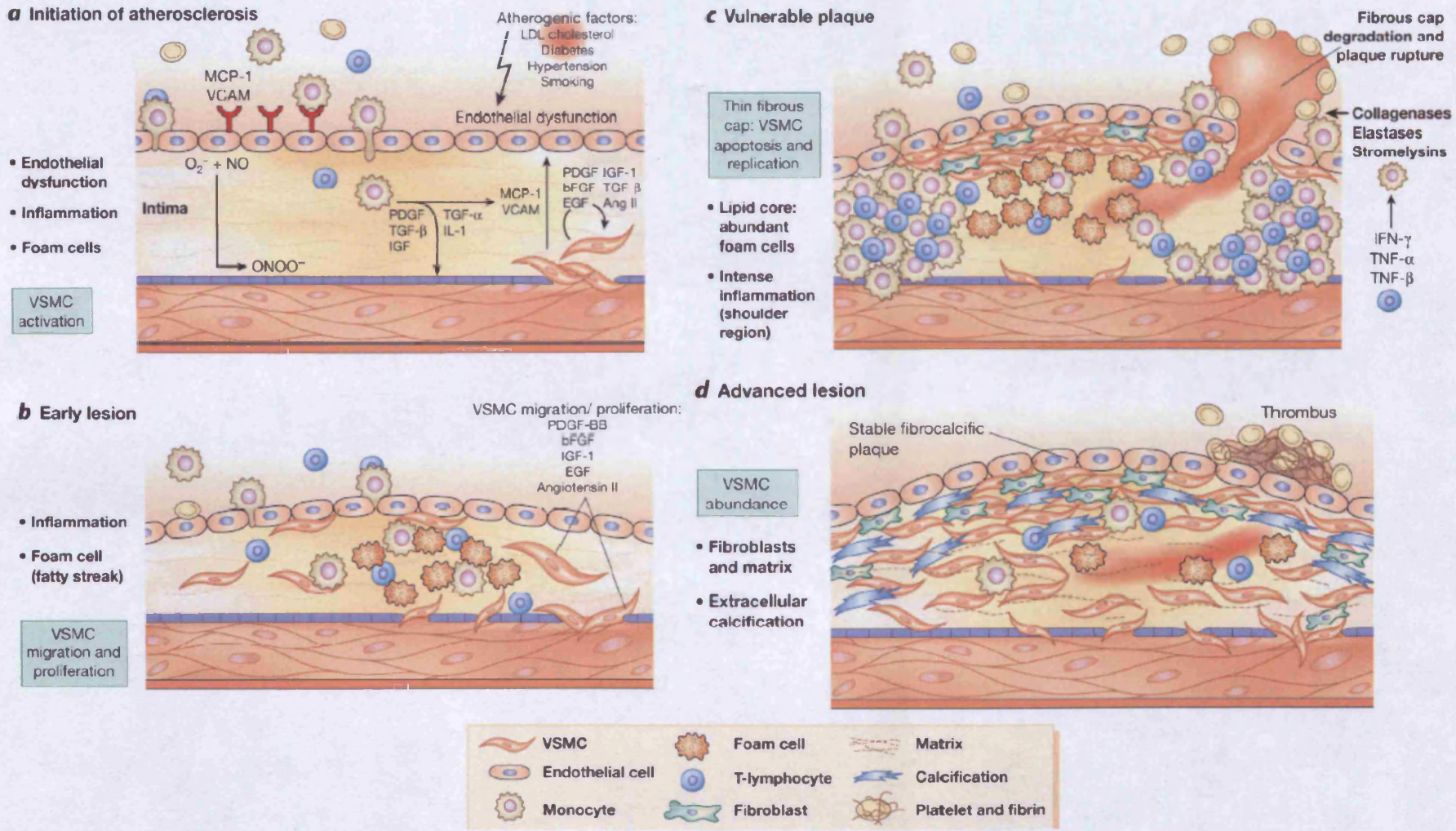


Figure 1.7 The initiation and progression atherosclerosis. The stages of atherosclerotic plaque development: (A) Initiation and response to injury; (B) Early changes in the lesion, macrophage and T lymphocyte invasion, smooth muscle cell migration; (C) Vulnerable plaque lysis ; (D) The complex lesion with smooth muscle cell and collagen forming a fibrous cap. Taken from (Dzau et al., 2002).

1.4.3 Plaque rupture

As the atherosclerotic plaque develops, the lesion becomes overlaid with a fibrotic cap composed of collagen and elastin secreted by the VSMC (Rekhter, 1999). These more complex plaques contain a necrotic core which is rich in lipids and cellular debris from dead cells. At this point the plaques can restrict blood flow through the lumen of the artery; however the greatest danger is of plaque rupture and thrombus formation. Destabilisation of the fibrotic plaque occurs by the continual secretion of pro-inflammatory cytokines and matrix metalloproteinases (MMPs) by activated macrophages, T cells and mast cells, which in turn inhibit the proliferation of the VSMCs and reduce their collagen synthesis. Following plaque rupture prothrombotic lipids spill into the blood pool activating the coagulation cascade. Thrombus formation can cause occlusion of the artery leading to myocardial infarction (MI), ischaemic reperfusion (I/R) injury or ischaemic stoke (Libby, 2002).

1.4.4 Complement in atherosclerosis

Atherosclerosis is a chronic inflammatory disease state, involving inflammation at all stages from initiation to destabilisation. The participation of C in the atherosclerotic process was first proposed over forty years ago by Geertinger and Sorensen who found that C6 deficient rabbits fed on high cholesterol diet had reduced atherosclerosis (Geertinger and Sorensen, 1977). Although the C system has been well recognised for its pro-inflammatory role in atherosclerosis, it is now becoming clear that the C system is likely to have a role in protecting tissue from the accumulation of debris (Haskard et al., 2008).

C activation within atherosclerotic lesions occurs through activation of the CP and the AP (Torzewski et al., 1997, Vlaicu et al., 1985b). Potential triggers for C activation within the lesions include CRP (Torzewski et al., 1998a), auto-antibodies (Vlaicu et al., 1985c, Vlaicu et al., 1985b, Orekhov et al., 1991, Burut et al., 2010) cholesterol crystals (Seifert and Kazatchkine, 1987), apoptotic cells, cellular debris and enzymatic modified LDL (Seifert et al., 1990, Torzewski et al., 1998b, Bhakdi et al., 1999, Klouche et al., 1999, Wieland et al., 1999). Enzymatically modified LDL (E-LDL) represents the most abundant stimulus within the lesions and is capable of activating both the AP and the CP by the presence of circulating auto-antibodies against modified LDL (Seifert et al., 1990, Orekhov et al., 1991, Burut et al., 2010).

Using immunohistochemistry several studies have identified evidence that C components (C1q, C3c, C3d, C4, C9 and Bb) (Niculescu et al., 1987, Vlaicu et al., 1985a, Vlaicu et al., 1985c, Vlaicu et al., 1985b, Vlaicu et al., 1985d), C receptors (CR1, CR3, C3aR and C5aR) (Saito et al., 1992, Oksjoki et al., 2007) and C inhibitory proteins (DAF, factor H, CD59, CR1 and clusterin) are present within atherosclerotic lesions (Niculescu et al., 1990,

Seifert and Hansson, 1989a, Seifert and Hansson, 1989b, Seifert et al., 1992). C activation has also been demonstrated by formation of the terminal pathway complex C5b-9 within atherosclerotic lesions (Vlaicu et al., 1985a). Expression of C5b-9 was found to be higher in advanced fibrotic plaques compared with early lesions and fatty streaks, which led to the suggestion that there is a constant accumulation of these complexes within the lesion (Vlaicu et al., 1985a). In addition C5b-9 has been found to be co-localised with enzymatically modified E-LDL within early lesion, which has been suggested to induce foam cell formation (Seifert et al., 1990, Bhakdi et al., 1995, Torzewski et al., 1998b).

It has been suggested that the function of the C5b-9 complex within the atherosclerotic lesion is unlikely to involve complete lysis of the target cells due to the critical density required to achieve this but instead lower levels of disposition may be important for cellular signalling cascades and stimulate cell activation (Haskard et al., 2008). Several studies have shown that the sublytic assembly of C5b-9 on aortic SMC and endothelial cells induces their activation and proliferation (Niculescu and Rus, 1999). In SMC C5b-9 induced activation of ERK-1, c-Jun, JNK and p38-MAPK, resulting in an increased production of MCP-1, which may be important for the recruitment of monocytes during the initial stages of atherosclerosis (Niculescu and Rus, 1999, Torzewski et al., 1996). Furthermore, it was shown in endothelial cells that C5b-9 induces activation of the PI3K/Akt pathway, which was important for cell cycle progression and proliferation of the endothelial cells (Fosbrink et al., 2006). To protect against formation of the C5b-9 complex on endothelial cells, Kinderlerer and colleagues have found that shear laminar stress can cause up-regulation of CD59 and therefore helps protect endothelial cells from activation (Kinderlerer et al., 2008).

C activation also leads to the generation of the anaphylatoxins C3a and C5a which in turn cause further inflammation (see sections 1.1.4 and 1.2.1). Both receptors C3aR and C5aR have been found to present within the human atherosclerotic plaques but not normal intimas by immunofluorescence and RT-PCR (Oksjoki et al., 2007). Furthermore, C5a has recently been suggested to contribute towards destabilising the lesions by increasing the secretion of MMP-1 and MMP-9 within the plaques (Speidl et al., 2011).

Several experimental models have been used to clarify the role of the C system in vascular injury and atherosclerosis. Results from LdlR-/- C1qA-/- double knockout mice suggest that activation of the CP has an anti-atherogenic role by removing apoptotic cells from early lesions, as these mice had plaques three fold larger and more complex compared to the control mice (Bhatia et al., 2007). Furthermore CP activation has been shown to promote the phagocytosis of modified forms of LDL by human monocytes and monocyte derived macrophages (Fraser and Tenner, 2010). The CP can also be activated by CRP which can bind apoptotic cells and E-LDL aiding their phagocytosis in early lesions, however at high concentrations of E-LDL the protective function of CP activation is overrun by

potentially harmful accumulation of C5b-9 complexes (Bhakdi et al., 2004). Studies involving the LdlR-/- mice crossed with C3-/- mice found that these mice had greater plaque sizes compared with the control animals however the lesions did not advance beyond the fatty streak stage suggesting that a complete C system is essential for maturation of the plaques (Buono et al., 2002, Persson et al., 2004). LdlR-/- crossed with factor B -/- mice showed no significant difference in plaque size suggesting that the AP is not involved in atherogenesis (Persson et al., 2004).

Evidence from experimental animal models suggest that the terminal pathway has a pro-atherogenic role. Initial studies involving C6 deficient hypercholesterolemic rabbits showed that these rabbits contained significantly fewer lesion compared with C6 competent counterparts (Geertinger and Sorensen, 1977, Schmiedt et al., 1998). Similarly ApoE-/- C6-/- mice also showed significantly reduced plaque area and disease severity compared with control counter parts (Lewis et al., 2010). In agreement with the pro-atherogenic role of MAC, several studies have also found that CD59 -/- ApoE-/- mice have earlier death rate and larger plaque area with increased complexity (Lewis et al., 2010, Wu et al., 2009, An et al., 2009). Interestingly a study by Patel and co-workers found C5-/- mice cross bred with ApoE-/- mice displayed no significant change in plaque size at the aortic root, however these studies were performed on mice fed a high fat diet for 22 weeks where disease is more likely to be in advanced stages (Patel et al., 2001).

1.4.5 Complement involvement in myocardial infarction

Following plaque rupture, thrombus formation can lead to occlusion of the artery and thereby causing ischaemic reperfusion (I/R) injury (Entman et al., 1991). The first phase of I/R injury, ischaemic phase, is mediated by anoxic injury caused by decreased mitochondrial ATP generation. The second phase, reperfusion phase, is an inflammatory process which further contributes to tissue injury beyond that generated already in the ischaemic phase (Szeplaki et al., 2009). It is believed that C activation induces cell damage and apoptosis and further amplifies the inflammatory response by up-regulation of adhesion molecules, activation and chemotaxis of polymorphonuclear cells (PMN), secretion of cytokines and reactive oxygen species via the generation of the anaphylatoxins and MAC (Szeplaki et al., 2009).

The C system was first implicated as an important mediator in the inflammatory reperfusion phase of I/R injury in 1971 by Hill and Ward who shown that C activation products were vital for the recruitment of neutrophils into MI of rats (Hill and Ward, 1971). The importance of C activation has since been demonstrated by several studies that have shown that knock down or silencing of C3 is protective against I/R injury (Zhou et al., 2000, Zheng et al., 2006).

Several studies involving the inhibition of C5 cleavage or blockade of C5a using antibodies have been shown to attenuate I/R injury suggesting that generation of the anaphylatoxin C5a and initiation of the terminal pathway are important mediates of C induced injury (Vakeva et al., 1998, Wada et al., 2001, Zhang and Carroll, 2007). Initial trails involving the humanised monoclonal anti-C5 antibody fragment, pexelizumab, which blocks the cleavage of C5 to C5a and C5b (Thomas et al., 1996), suggested that blocking systemic C5 improved survival rates of a patients with coronary bypass grafting but had no effect on survival rates or MI end points (Granger et al., 2003, Verrier et al., 2004, Shernan et al., 2004). Larger phase 3 clinical trails found no significant difference in the end-point of patients with coronary artery bypass receiving pexelizumab compared with placebo, although a reduction in MI and death was observed 30 days postoperative (Verrier et al., 2004, Smith et al., 2006, Testa et al., 2008). However, clinical trials found that pexelizumab had no significant effect on infarct size or clinical outcome in patients with MI (Mahaffey et al., 2003, Armstrong and Granger, 2007, Armstrong et al., 2007). It has been suggested that the difference between these findings is that pexelizumab given prior to surgery may reduce the generalised inflammation process, as observed by reduction in IL-6 and CRP following pexelizumab (Theroux et al., 2005). Whereas following MI local inflammation and injury has become irreversible and C activation is already established, therefore invalidating the beneficial effects of administration of pexelizumab (Testa et al., 2008). Furthermore, Testa and co-workers suggest that following MI penetration of pexelizumab into the affected tissue may be limited due to the microvascular obstruction (Testa et al., 2008). A study by Amsterdam and co-workers involving pre-treatment with monoclonal anti-C5a antibody prior to I/R injury of the descending coronary artery found that the antibody inhibited the neutrophil cytotoxic activity towards C5a but had no effect on MAC formation and neutrophil accumulation and therefore did not reduce infarct size in pigs (Amsterdam et al., 1995). Studies involving C5aR antagonists, such as PMX-53, have shown promising results by reducing I/R injury in rat intestine (Arumugam et al., 2002), kidney (Arumugam et al., 2003), liver (Arumugam et al., 2004b), limb (Woodruff et al., 2004) and mouse intestine (Fleming et al., 2003) following a period of ischemia (reviewed (Monk et al., 2007)).

1.4.6 Therapeutic strategies to lower serum lipid levels

As shown in table 1.5, atherosclerosis is a highly complex disease where both environmental and genetic factors play an important role in its susceptibility and progression. However, one of the primary risk factors associated with the disease is serum cholesterol levels.

Cholesterol is an important component of all biological membranes and is a precursor of bile acid and steroid hormones. Cholesterol can be absorbed from the GI tract or it can be

synthesised by the liver but it circulates through the blood stream bound to lipoproteins (table 1.6). Several epidemiological studies have shown that elevated levels of circulating LDL-cholesterol is a major risk factor for coronary heart disease and has direct relationship with onset of the disease, whereas circulating HDL-cholesterol is inversely proportional (Stamler et al., 1986, LRC-CPPT, 1984). The main strategy employed to reduce serum LDL-cholesterol is statin therapy, which has been shown to reduce both coronary heart disease incidence and mortality by 30 % (NCEP, 2002). Section 1.5 will discuss in more detail how statins lower serum LDL-cholesterol levels.

Another risk factor for atherosclerosis is elevated serum triglycerides, which is commonly associated with other non-lipid and lipid risk factors (NCEP, 2002). Similar to cholesterol, triglycerides can be absorbed from the GI tract or they can be synthesised by the liver and are circulated in the blood bound to lipoproteins VLDL and chylomicrons (table 1.6). Fibrates are commonly given to reduce plasma triglyceride concentration by 30-50% (Barter and Rye, 2006). Fibrates can also increase levels of circulating HDL-cholesterol and occasionally they have been shown to reduce LDL-cholesterol levels (Barter and Rye, 2006). They exert their effects by activating PPARα receptor which is a major transcription factor for the regulation of intracellular and extracellular fatty acid metabolism. PPARα can stimulate lipoprotein lipase which increases the hydrolysis of triglycerides in VLDL or chylomicrons, liberating fatty acids that can be stored as fat or metabolised. PPARα can also stimulate the β-oxidation and degradation of fatty acids (Paumelle and Staels, 2008).

Other lipid lowering therapeutic strategies include bile acid sequestrates, which when taken by mouth sequester bile acids in the intestine and therefore decrease absorption of cholesterol in the GI tract and nicotinic acid, which can reduce hepatic synthesis of triglycerides (NCEP, 2002).

Table 1.6 Major classes of lipoproteins which cholesterol circulate blood stream.

Class	**Apo lipoprotein**
Low density lipoprotein (LDL) Accounts for 60-70% total serum cholesterol	Apo B
High density lipoprotein (HDL) Accounts for 20-30% total serum cholesterol	Apo A- I and –II
Very low density lipoprotein (VLDL) (triglyceride rich) Accounts for 10-15% total serum cholesterol	Apo B-100, Apo C-I, -II and -III and Apo E
Chylomicrons (triglyceride rich)	Apo B-48, Apo C-I, -II and -III and Apo E

1.5 Statins

Statins are the most common drugs given to reduce circulating LDL-cholesterol levels as they have a good safety record and few drug-drug interactions (NCEP, 2002). Statins can lower serum cholesterol levels by two separate mechanisms. Firstly they inhibit HMG-CoA reductase, the rate-limiting step in cholesterol biosynthesis, and secondly they promote clearance of plasma LDL-cholesterol via LDL-receptor mediated endocytosis (NCEP, 2002).

1.5.1 Mevalonate pathway

The mevalonate pathway yields an important precursor, mevalonate, which is essential for generation of isoprenoid intermediates (figure 1.8). As well as being a precursor for cholesterol biosynthesis, these isoprenoid intermediates are also incorporated into other end products such as dolichol, required for glycoprotein synthesis, isopentyl adenine, present in some transfer RNAs, haem A and ubiquinone, which partake in electron transport, isoprenylated proteins, such as small GTPase mainly involved in intracellular signalling pathways; and steroid hormones (Goldstein and Brown, 1990). Thus correct regulation of the mevalonate pathway is crucial so that all cells can precisely synthesise enough mevalonate without the accumulation of potentially toxic metabolites such as cholesterol.

Regulation of the mevalonate pathway is complicated by the fact that cholesterol present in plasma, LDL-cholesterol, can enter the cell by LDL receptor (LDLR) mediated endocytosis (figure 1.8). It is therefore essential that each cell can carefully balance these external and internal sources of sterols well maintaining synthesis of enough mevalonate. Regulation can be achieved by negative sterol feedback loops which can down regulate HMG-CoA synthase and HMG-CoA reductase, as well as LDLR expression (figure 1.8) (Goldstein and Brown, 1990).

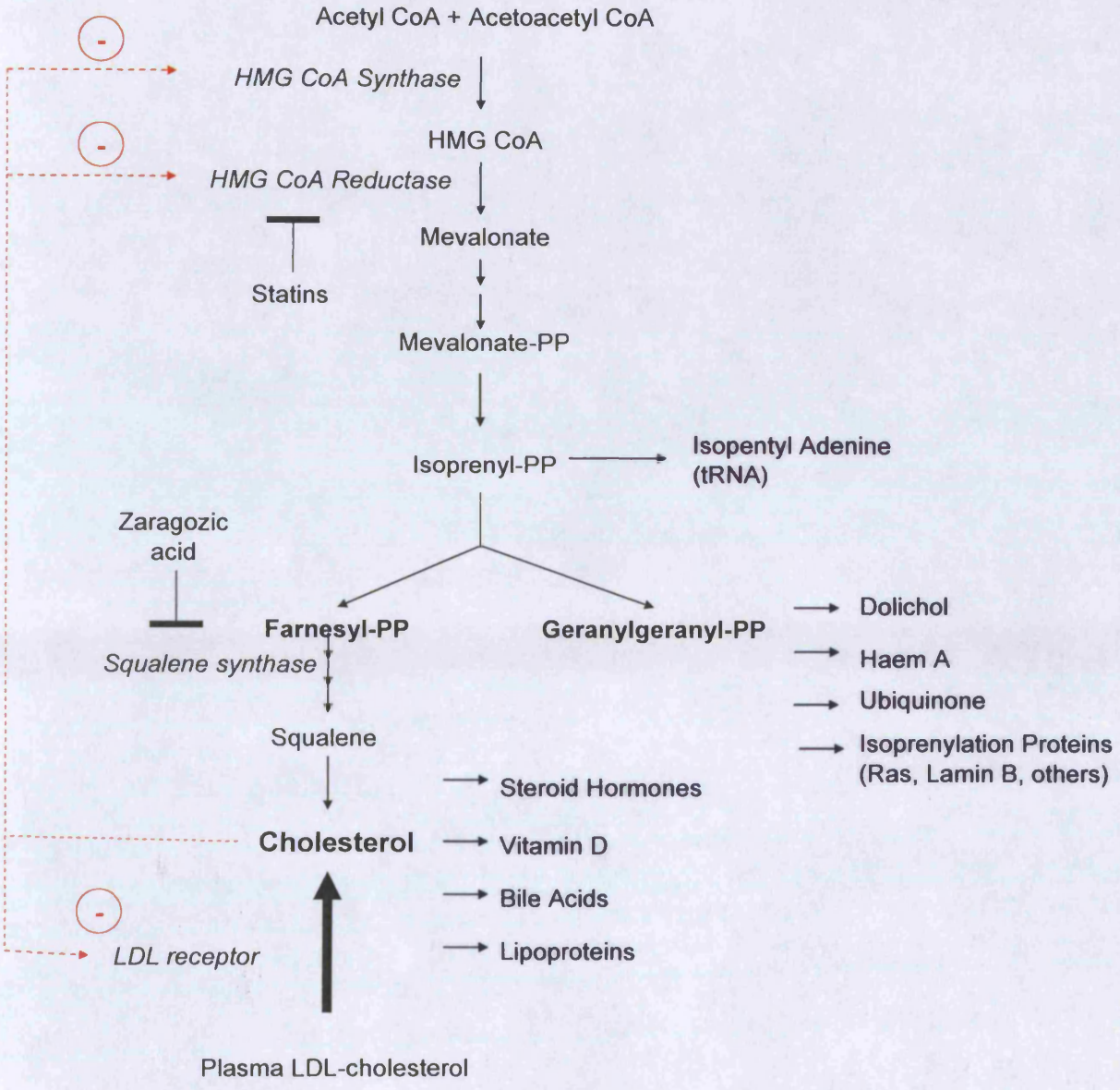


Figure 1.8 Schematic diagram of the mevalonate pathway. The main end product of the mevalonate pathway, cholesterol, can be obtained from two sources: (1) the endogenous pathway by synthesis from acetyl CoA; and (2) the exogenous pathway by endocytosis of circulating LDL-cholesterol via LDLR. End products which incorporate products from the pathway (blue). Regulation of mevalonate is achieved by down regulation of LDLR, HMG-CoA reductase or HMG-CoA synthase via cholesterol mediated negative feedback loops (red). Adapted from (Goldstein and Brown, 1990).

1.5.1 Competitive inhibition of HMG-CoA reductase by statins

By inhibiting HMG-CoA reductase, statins are inhibiting the rate limiting step in cholesterol biosynthesis. The first statin to be identified, mevastatin, was isolated from *Penicillium Citrinium*. Although it was shown to be a potent inhibitor of HMG-CoA reductase, K_i of 1.4 nM, it was also found to have unacceptable hepatocellular toxicity which led to its discontinuation (Endo et al., 1976). Characterisation of another secondary fungi metabolite, isolated from *Aspergilla terreus,* led to the identification of lovastatin, which was found to be a more potent inhibitor of HMG-CoA reductase, K_i 0.6 nM, and reduced cholesterol synthesis in

human studies without the hepatocellular toxicity (Alberts, 1988, Alberts et al., 1980). Since then many other statins have emerged, both naturally occurring and chemically synthesised, and are now commercially available with ranging K_i values 0.1 to 2.3 nM.

All statins have a HMG-CoA like moiety which may be present in an inactive form. These pro-drugs need to undergo enzymatic hydrolysis *in vivo* to their active hydroxy-acid counterparts (Corsini et al., 1995). When active, statins are competitive inhibitors for HMG-CoA reductase and by occupying the HMG binding pocket and part of the binding surface for CoA they inhibit access of the HMG-CoA substrate to the enzyme (Istvan and Deisenhofer, 2001). The inhibitory concentration, K_i, of the statins in the nanomolar range is believed to be achieved by strong favourable van der Waals interactions between the bulky hydrophobic ring structures of the statins and the complementary surface of the HMG-CoA reductase (Istvan and Deisenhofer, 2001). Each of the different type of statins has slightly different interactions with the HMG-CoA reductase which may explain the ranging K_i values. It has also been suggested that the differences in the different statin potencies may be a result of different lipophilicity, with lipophilic statins (such as simvastatin and fluvastatin) being more likely to enter vascular cells by passive diffusion than hydrophobic statins (such as pravastatin and rosuvastatin) which primarily target the liver (Zhou and Liao, 2010). Further still, each statin differs slightly in their pharmacokinetics; for instance simvastatin has a bioavailability of 5%, T_{max} of 1.3-2.4hrs and $T_{1/2}$ of 2-3 hrs, whereas, atorvastatin has a bioavailability of 12%, T_{max} of 2-3hrs and $T_{1/2}$ of 15-30 hrs (reviewed in (Bellosta et al., 2004).

As well as statins, other inhibitors of cholesterol biosynthesis include zaragozic acids. Zaragozic acids, also known as squalestatins, were discovered by two independent groups of scientists as potent squalene synthesis inhibitors (Dawson et al., 1992, Baxter et al., 1992, Bergstrom et al., 1993). These fungal metabolites are believed to inhibit squalene synthase by effectively mimicking the binding of the pS-PP intermediate of sterol synthesis to the enzyme (Bergstrom et al., 1995). Despite being potent inhibitors of squalene synthase, their poor bioavailability after oral administration has meant that these metabolites are not used therapeutically to lower cholesterol but instead are often used experimentally (Amin et al., 1997).

1.5.3 Increased clearance of serum cholesterol by statins

Another mechanism by which statins lower circulating LDL-cholesterol levels is that they can increase expression of LDLRs and therefore increase receptor mediated endocytosis of cholesterol mainly by the liver. This is achieved by the negative feedback loop shown in figure 1.8, where a decrease in intracellular cholesterol levels leads to the proteolytic activation of the transcription factor sterol response element binding protein-2 (SREBP-2) and subsequently to an up regulation of the LDLR gene (Brown and Goldstein, 1980).

1.5.4 Pleiotropic effects of statins

Evidence from several large clinical trials, including CARE, LIPID and HPS, suggested that the cardiovascular benefits of statin therapy did not correlate fully with the magnitude of cholesterol lowering (Sacks et al., 1996, HPS, 2002, LIPID, 1998). Furthermore, statins were also found to reduce the risk of other disease processes, such as transplant-associated artheropathy, that are not associated with elevated lipid levels (Kobashigawa et al., 1995, Wenke et al., 1997). It is now well accepted that as well as reducing serum cholesterol levels, statins can have pleiotropic effects which are independent of cholesterol lowering (Jain and Ridker, 2005). These pleiotropic effects have been the main focus of statin research over the recent decades and are believed to improve coronary heart disease morbidity and mortality by several mechanisms including; improving endothelial function, inhibiting inflammation, reducing oxidative stress, improving plaque stability, and inhibiting pro-thrombotic outcome (table 1.7). It is believed that the majority of these pleiotropic effects are mediated by inhibition of isoprenoid intermediates, farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) which serve as lipid attachments for the post-translational modification, isoprenylation, of a variety of proteins (Zhou and Liao, 2010). However, statins have also been shown to inhibit leukocyte function antigen-1, a member of the β2 integrin family which is involved in lymphocyte recirculation and leukocyte extravasation to sites of inflammation (Weitz-Schmidt et al., 2001). Statins were shown to bind to a novel allosteric site within the LFA-1, which was completely independent of inhibition of the mevalonate pathway (Weitz-Schmidt et al., 2001).

As discussed in section 1.4.4 the C system has been implicated in atherosclerosis at all stages from initiation to destabilisation of the plaque, yet relatively few studies have investigated the effects of statins on C. Mason and co-workers found that incubation of human umbilical cord and aortic EC with atorvastatin and simvastatin, significantly increased DAF expression, which suggests that one of the anti-inflammatory properties of statins is to protect the endothelium from C activation by increasing expression of C regulators on EC surface (Mason et al., 2002). Another study has since shown that DAF expression on monocytes and lymphocytes is significantly lower in patients with hyperlipidemia (Liu et al., 2005). The expression of CD59 has also been shown to be increased on monocytes, lymphocytes and granulocytes in patients receiving atorvastatin therapy (Liu et al., 2005). It has also been proposed that one of the anti-inflammatory properties for the treatment of rheumatoid arthritis with statin therapy is to increase CD59 expression in vascular endothelium under hypoxic conditions (Kinderlerer et al., 2006).

Statins have previously been shown to have no significant effect on *in vitro* C activation by Lappegard and co-workers (2004). In this study they incubated normal human

serum with atorvastatin and pravastatin in the absence or presence of aggregated human IgG (for CP activation) or cobra venom factor (for AP activation) and then measured the amount of complement activation products were generated (Lappegard et al., 2004). Although this study showed that statins did not directly alter C activation *in vitro* it did not investigate what indirect effects statins may have *in vivo* (Lappegard et al., 2004).

As previously described in section 1.4.4, one of the main pro-atherogenic roles of the C system is formation of the C5b-9 complex. A study by Viedt and co-workers found that incubation of VSMC with statins significantly reduced the pro-inflammatory effects of the C5b-9 complex (Viedt et al., 2003). They showed that incubation of VSMC with cerivastatin reduced C5b-9 activation of ERK and transcription factors AP-1 and NFκB, which in turn inhibited IL-6 secretion and cell proliferation (Viedt et al., 2003).

Table 1.7 Pleiotropic mechanisms of statins

Pleiotropic effect	Proposed mechanisms
Improved endothelium function	• Increased bioavailability of NO via up regulation of eNOS expression (Tsao et al., 1994, Laufs et al., 1998, Laufs and Liao, 1998).
Reduced inflammatory and immunological response	• Reduced transmigration of leukocytes by down regulation of adhesion molecules (ICAM-1, P-, E- and L-selectin) on both EC and leukocytes (Niwa et al., 1996). • Reduced secretion of chemokines IL-8 and MCP-1 and expression CCR2 receptor which reduces leukocyte migration (Kothe et al., 2000, Romano et al., 2000, Veillard et al., 2006). • Reduced expression of inflammatory cytokines IL1b, IL-6 and TNFα (Pahan et al., 1997). • Reduced co-stimulatory molecules, IFNγ induced MCH II and CD40 activation and thereby regulating Th1/Th2 commitment (Sadeghi et al., 2001, Townsend et al., 2004, Jasinska et al., 2007, Youssef et al., 2002).
Reduced oxidative stress	• Increased expression antioxidant genes and therefore protects against oxidative injury (Makabe et al., 2010). • Reduced reactive oxygen species generation (Rikitake et al., 2001).
Improved plaque stability	• Reduced MMP expression and secretion (Aikawa et al., 2001, Fukumoto et al., 2001, Sundararaj et al., 2008, Fujimoto et al., 2008).
Reduce pro-thrombotic outcome	• Increased expression of tissue-type plasminogen activator expression (Essig et al., 1998). • Decreased expression of plasminogen activator inhibitor-1 expression (Essig et al., 1998).

To date no published data has investigated what effect, if any, statins may have on the anaphylatoxins and their biological receptors. Due to their potent pro-inflammatory nature, reducing the biological functions of the anaphylatoxins by decreasing expression

and/or reducing functioning of their receptors could have valuable implications in many inflammatory disease states, especially as the strategies to reduce I/R with anti-C5 and other inhibitors are not very successful so far.

1.6 Hypothesis and aims

The potent pro-inflammatory anaphylatoxin C5a exerts its biological actions via the C5aR, a GPCR. Cholesterol, a major risk factor in cardiovascular disease, is a crucial component of biological membranes. Furthermore cholesterol has previously been shown to regulate expression and function of several GPCRs. Statin therapy is the most widely used therapeutic strategy to lower serum cholesterol levels. Evidence suggests that statins also exert beneficial anti-inflammatory effects, thus further improving cardiovascular disease outcome. The principal aim of this thesis was to determine if the C5aR expression and function can be modulated by cholesterol. It is hypothesised that statins can exert anti-inflammatory effects by down regulation of expression and/or function of the receptor for the potent pro-inflammatory anaphylatoxin C5a, C5aR.

The aims of this study are:

- Investigate how C5aR expression is regulated.
- Determine if statins effect C5aR expression, either basal levels or induced expression
- Determine the subcellular plasma membrane localisation of the C5aR
- Investigate whether statins affect the functioning of the C5aR
- Establish a model system in which the above can be measured

Chapter 2

General methods

Chapter 2

General Methods

2.1 Chemicals and reagents

2.1.1 General chemicals and reagents

Human recombinant tumour necrosis factor–α (TNFα) was from PeproTech (TNFSF2). N6,2'-O-Dibutyryladensine 3',5'-cyclic monophosphate sodium salt (Bt_2cAMP), Lipopolysacharide (LPS) *Escherichia coli* (*E. coli*) 0111.B4, Phorbol 12-myristate 13-acetate (PMA) and propidium iodide (PI) were from Sigma. O-phenylene-diamine (OPD) ELISA developing system (S2045) was from Dako (Cambridgeshire, UK). Bovine serum albumin (BSA) was from Fluka (05473). Ultrapure agarose was from Invitrogen. 96-well ELISA microtitre plates were from ICN Pharmaceuticals (7717305) (Basingstoke, UK). BSA (2 mg/ml) standards (23209) were purchased from Pierce (Leicestershire, UK). Human recombinant C5a was from Calbiochem (Nottingham, UK). Fura-2-AM was purchased from Anaspec, Cambridge Bioscience, Cambridge, UK. All other standard laboratory chemicals and reagents were obtained from Sigma (Poole, UK).

2.1.2 Mammalian cell culture reagents

All cell culture RPMI media, penicillin/streptomycin/glutamine, sodium pyruvate, foetal calf serum (FCS) and tissue culture flasks were obtained from Invitrogen (Paisley, UK) unless otherwise stated. Ficoll-Paque™ (17-1440-02) was from GE Healthcare (Buckinghamshire, UK).

2.1.3 Commercial antibodies

Monoclonal mouse anti-C5aR (S5/1), anti-GAPDH (SC-47724) and polyclonal rabbit anti-C5aR (H-100) were from SantaCruz. Primary antibodies anti-α tubulin (DM1A) was from Calbiochem, and anti-intracellular glycophorin C domain (BRGL100) and anti-CD59 (Bric229) were from International Blood Group Reference Laboratory (IBGRL, Bristol, UK).

The following secondary antibodies were used: peroxidase-conjugated rabbit anti-mouse IgG (RAM-HRPO) and goat anti-rabbit IgG (RAM-HRPO) from Stratech (Suffolk, UK). FITC-conjugated goat anti-mouse IgG (GAM-FITC) was from Dako. Goat anti-mouse IgG conjugate with Alexa-647 was from Invitrogen.

2.1.4 Protein biochemistry reagents

All blue precision plus protein standards were from BioRad (161-0373). 30% acrylamide (37.5:1) was from Severn Biotech Ltd (20-2100-10). Supersignal chemiluminescence substrate (ECL) was from Pierce (34080). Hybond nitrocellulose (RPN3032D) was purchased from GE Healthcare Lifesciences.

2.1.5 Molecular biology reagents

Taq polymerase, Q reagent and deoxynucleoside triphosphates (dNTPs) were from Qiagen. 1 Kbp DNA ladder molecular weight markers (10787-018) were from GIBCO and 100 bp markers were from Roche (1721933). Primers were ordered from MWG-Biotech AG.

2.2 Buffers

2.2.1 General buffers

- Phosphate buffered saline (PBS): 145 mM NaCl, 3 mM Na_2HPO_4 and 2.5 mM Na_2HPO_4 in dH_2O, pH 7.4
- FACS buffer: 1 % BSA, 0.01 % NaN_3 in PBS, pH 7.4
- Krebs/Hepes buffer: 1.2 M NaCl, 2.5 M Hepes, 0.48 M KCl, 0.12 M KH_2PO_4, 0.12 M $MgSO_4.7H_2O$, 0.13 M $CaCl_2.2H_2O$, pH 7.4
- Balanced Saline Solution (BSS): 137 mM NaCl, 2.7 mM KCl, 81 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3

2.2.2 Protein biochemistry buffers

- Stacking acrylamide gel buffer: 0.5 M Tris-HCl, 0.4 % (w/v) SDS, pH 6.8.
- Separating acrylamide gel buffer: 1.5 M Tris-HCl, 0.4 % (w/v) SDS, pH 8.8.
- Running buffer: 190 mM glycine, 24.8 mM Tris, 0.5 %(w/v)
- Protein sample buffer (non-reducing): 0.1 M Tris, 10 % (w/v) glycerol, 2 % (w/v) SDS, 0.05 % (w/v) bromophenol blue, pH 6.8.
- Transfer buffer: 26 mM Tris, 192 mM glycine, 20 % (v/v) methanol
- Blocking buffer (for western blotting): 5 % (w/v) fat-free milk (marvel) in PBS/Tween20 (0.1 %).

2.2.3 Molecular biology buffers

- TAE (50 x): 2 M Tris, 2 M acetic acid, 50 mM EDTA, pH 8.5.
- DNA/RNA sample buffer: 50 mM EDTA pH 8.0, 50 % (v/v) glycerol, 0.25 % (w/v) bromophenol blue.

2.3 Methods

2.3.1 General cell culture

U937 were obtained from European Collection of Cell Cultures ($U937_{EC}$) and from Dr Peter Monk Sheffield University, UK ($U937_{PM}$). U937 cells previously transfected with human C5aR ($U937_{C5aR}$) (Kew et al., 1997) were obtained from Dr Eric Prossnitz State University of New York at Stony Brook, USA.

All cell cultures were maintained at 37°C in humidified CO_2 (5%) buffered incubators (Hera cell 150, Heraeus). All U937 cell lines were maintained in complete RPMI 1640 (Invitrogen, 31870-025) media containing 10 % FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 290 μg/ml L-glutamine and 1 mM sodium pyruvate. Depending on cell type, cells were routinely split every 2-3 days as follows: $U937_{EC}$ cells were split approximately 1 in 10, $U937_{PM}$ cells were split approximately 1 in 4 and $U937_{C5aR}$ were split approximately 1 in 8.

2.3.2 Isolation of peripheral blood mononuclear cells

Non-clinical buffy coats were obtained from Welsh Blood Service (Cardiff, UK). Approximately 40 ml of buffy coat was mixed with approximately 150 ml of RPMI. 12 ml of buffy coat was then gently overlaid on 8 ml of RT Ficoll-Paque™ (17-1440-02). The samples were centrifuged for 20 min at 750 g and stopped without breaking. The serum was discarded and the peripheral blood mononuclear cells (PBMC) interface was collected. Two samples were pooled into a new universal tube and resuspended in a total volume of 20 ml RMPI. Samples were centrifuged for 12 min at 370 g with break. The supernatant (s/n) was discarded; cell pellets were gently resuspended in small volume of RPMI and then two samples were pooled and resuspended in a total volume of 20 ml RPMI. The samples were centrifuged again for 10 min at 120 g. The s/n was discarded; cell pellets were gently resuspended in small volume of RPMI and then two samples were pooled and resuspended in a total volume of 20 ml RPMI. The samples were centrifuged for 8 min at 90 g. The s/n was discarded and the cell pellets were resuspended in a total volume of 9 ml of 10 % DMSO, 20 % FCS and 70 % RPMI. 1 ml aliquots (1×10^8 cells) were added to cryovials (Nunc), which were then placed in a Cryol freezing container filled with isopropanol. Following 24 hr in the -80 °C freezer (New Brunswick Scientific), the vials were transferred to liquid N_2 storage containers (Taylor-Wharton). Cells were revived from liquid N_2 as described below in section 2.3.4. For flow cytometry experiments cells were resuspended at 1×10^6 cell/ml and for ELISA cells were resuspended at 1.5×10^4 cell/ml in RPMI supplemented with

15 % FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 290 μg/ml L-glutamine and 1 mM sodium pyruvate.

2.3.3 Isolation of human neutrophils

20 ml of whole blood was collected form healthy volunteers into 200 μl heparin (1000 U/ml, CP Pharmaceuticals UK). The heparinised blood was then mixed with 5 ml 6 % dextran T70 (Pharmacia, 17-0280-07) in BSS and incubated at RT for 40 min without agitation. The upper plasma phase was removed using pasture pipette and centrifuged at 750 g for 1 minute. Supernatant was removed and cell pellet was gently resuspended. The remaining erythrocytes were lysed with 5 ml of sterile H_2O for 30 s and neutralised with 20 ml BSS. The cells were centrifuged again for 1 minute at 750 g. The s/n was discarded and the cell pellet was gently resuspended in 5 ml Krebs/Hepes. The cell suspension was layered over 5ml Ficoll-Paque™ and centrifuged for 25 min at 400g. The upper layers were discarded and cell pellet containing the neutrophils was washed and resuspended in either Krebs/Hepes, PBS or RPMI depending on what experiment they were later used for.

2.3.4 Cryopreservation of cells

Cells growing in log phase were washed twice in sterile 0.9% NaCl. The cells pellets were then resuspended in 1ml filtered sterilised freezing medium (10% (v/v) DMSO, 90% (v/v) FCS) and were then added to cryovials (Nunc). The cryovials were then placed in a Cryol freezing container filled with isopropanol, which provides a constant cooling rate of 1 °C per minute when placed in the -80 °C freezer (New Brunswick Scientific). After 24 hrs in the vials were transferred to liquid N_2 storage containers (Taylor-Wharton) for long term storage.

Cells were revived from liquid N_2 storage by placing the cryovials in a 37 °C water bath until completely thawed. The cells were washed twice in pre-warmed cell culture medium to remove the DMSO, and cultured as normal.

2.3.5 Stimulation of cells in culture

Incubation of cells with reagents was carried out by seeding the cells at a low density (typically 1-2.5 x 10^5 cell/ml) in fresh culture medium containing the required reagents, and culturing at 37 °C with 5 % CO_2 for the required amount of time. When adding a volume of reagent, such as simvastatin, to cells in culture, controls were carried out to ensure uniform incubation volumes by adding the same volume of control buffer or vehicle reagent to all other wells/ flasks.

2.3.6 Flow cytometry

Cells were harvested from culture and washed twice in PBS by centrifugation (1200 rpm for 3 min). The cell number was calculated by resuspended the cell pellet in 3-5 ml of PBS, then 10 µl cell solution was removed and added to a Bright-line cell counting chamber (Sigma) and counted. The cells were centrifuged again for 3 min at 1200 rpm and resuspended at $5x10^6$ cell/ml in FACS buffer. 50 µl of cells was incubated with 50 µl of the primary antibody (1-10 µg/ml) in a round bottomed 96-well microtitre plate. Following incubation for 30 min on ice, the cells were washed twice with 200 µl FACS buffer/well. The cells were incubated for a further 30 min on ice with 50 µl of the fluorescently labelled secondary antibody (typically diluted 1 in 100 with FACS buffer). The cells were washed twice with FACS buffer (200 µl/well) and resuspended in 200 µl 1% paraformaldehyde (diluted in FACS buffer from 4 % stock). Background fluorescence was determined using cells stained with secondary antibody alone, or incubated with an isotype control primary antibody. Cell fluorescence was analysed using a FACSCalibur flow cytometer (BecktonDickinson) within 5 days of fixing. Dead cells were excluded from analysis by their characteristic forward and side scatter. Data was analysed using FlowJo Software. For individual experiments, data is expressed as mean fluorescence intensity (MFI), +/- SEM. For repeat analysis data is expressed as either percentage or relative change compared with the mean control values unless otherwise stated.

2.3.7 SDS-polyacrylamide gel electrophoresis

Acrylamide gels were prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) from stock solutions according to the method of Laemmli (Laemmli, 1970) (see table 2.3.1). The percentage of gel used to separate proteins was dependent on the size of the protein. Gels (1.5 mm thickness for western blotting or 0.75 mm for zymography) were cast using the Hoefer Mighty Small Casters according to manufacturer's instructions. Immediately after pouring the separating gel, the gel was overlaid with 1 ml ethanol to remove any bubbles. Once the gel had polymerised, the ethanol was poured off and the stacking gel poured, using 10 or 15 well combs to create the wells. Once the stacking gel had polymerised the gel was assembled into the Hoefer Mighty Small Gel Electrophoresis apparatus and the running buffer was added to both the upper and lower chambers. When the tank was full the comb was removed and 15 µl-30 µl of sample, mixed 1:1 with sample buffer was added to each well using a Hamilton Microliter syringe (Hamilton, Switzerland). Electrophoresis was carried out at a constant current of 40 mAmp per gel, with continual cooling from passing cold water through the electrophoresis unit, until the pre-stained markers (Bio-Rad) had reached the desired resolution.

Table 2.3.1 SDS-PAGE components

Gel component/ 15 ml	Separating gel (12.5%)	Separating gel (7.5%)	Stacking gel (4%)
Stacking buffer	-	-	1.2 ml
Separating buffer	3.75 ml	3.75 ml	-
30% acrylamide	6.05 ml	5.025 ml	0.675 ml
dH_2O	150 µl	6.06 ml	3.025 ml
10% w/v ammonium persulphate	150 µl	150 µl	50 µl
TEMED	15 µl	15 µl	5 µl

2.3.7.1 Detection of proteins by western blotting

i) Transfer of proteins to nitrocellulose membrane

Following the separation of the sample by SDS-PAGE (as described above), the proteins were then transferred onto nitrocellulose membrane (Hybond, GE Health Care) for western blotting. Gels were first equilibrated for 10 min in transfer buffer. Then a 'sandwich' was created of; 1 thin sponge, 2 pieces of filter paper, nitrocellulose membrane, acrylamide gel, 2 pieces of filter paper and 2 thin sponges, all of which had been pre-soaked in transfer buffer. Once the sandwich had been assembled it was placed into the Hoefer Transfer Unit apparatus, according to the manufacturers instructions, with the membrane placed between the gel and the positive electrode. The proteins were transferred onto the nitrocellulose membrane by electrophoresis in transfer buffer for 1 hr at 100 V with constant cooling by water passing across the base of the apparatus.

ii) Immuno-labelling of protein on nitrocellulose membrane

To prevent non-specific binding of the antibody, the membrane was blocked either over night (o/n) at 4 °C or 1 hr at RT in 25 ml blocking buffer (5 % fat-free milk in PBS/Tween 0.1 %) with constant rolling using Denley Spiramix 10 (Jencons). To enhance the signal for the C5aR both the primary and secondary antibodies were diluted with SignalBoost Immunoreaction kit (Calbiochem) according to manufacturers instructions mixed 1:1 with blocking buffer. The nitrocellulose membrane was then incubated with primary antibody (diluted 1 in 200) at RT for 1 hr. The membrane was washed with 3 x 25 ml PBS/Tween (0.1 %) for 15 min and incubated for another 1 hr at RT with appropriate HRPO-conjugated secondary antibody (diluted 1 in 2000). Membranes were washed 3 x 15 min with PBS/Tween (0.1 %), followed by 2 x 15 min with PBS.

iii) Detection of immuno-labelled proteins

Detection of the immuno-labelled proteins was carried using enhanced chemiluminescence (ECL) system (Pierce, 34080). The ECL system generates light via the oxidation of luminol

in the presence of H_2O_2 by the horse-radish peroxidase conjugated to the secondary antibody. A stable peroxidase solution was mixed with an equal volume of luminol containing buffer, 1 ml of this solution was evenly laid over the nitrocellulose membrane and was incubated for approximately 1 minute. Excess solution was removed as the membrane was placed between two transparent plastic sheets. This was placed in a photographic cassette and exposed to X-ray film for various time periods, ranging from 5s – o/n, depending on the intensity of the chemiluminescence signal.

2.3.8 Ca^{2+} signalling

Intracellular Ca^{2+} release was measured using the technique previously described (Davies et al., 1991). Cells were harvested, washed and resuspended at 1 x 10^7 cell/ml in RPMI. The cells were incubated in the absence or presence of 1 μM Fura-2-AM for 30 min at RT. The cells were washed twice with PBS and then resuspended at 1 x10^7 cell/ml in Krebs/Hepes. Ca^{2+} measurements were performed in a flat bottom 96 well plate using FLUOstar OPTIMA plate reader (BMG Labtech, UK) pre-warmed at 37°C. 100 μl of cells were mixed with 100 μl Krebs/Hepes buffer. Following an equilibration period of approximately 5 min, fluorescence intensity measurements (2 mm orbital scanning) were taken at emission wavelength 510 nm every 5 s following stimulation with excitation wavelength 340 nm, for Ca^{2+} saturated form, and 380 nm, for Ca^{2+} free chelator. The ratiometric measuring between Ca^{2+} bound and unbound forms of the dye significantly reduces the effects of uneven loading, leakage of dye and photobleaching. Measurements were paused while the cells were being stimulated with 5 nM C5a. Following the addition of C5a (10 μl, final concentration 5 nM) the fluorescent intensity measurements were continued immediately. Once the Ca^{2+} levels appeared to return to basal levels with cells were lysed with 10 μl Triton-X100, final concentration 0.5 %, to obtained maximum Ca^{2+} binding to the dye (R_{max}). Once the fluorescence intensity had plateaued, Ca^{2+} ions were chelated by the addition of 40 μl EGTA, final concentration 0.36 M, to give R_{min}. Both the R_{max} and the R_{min} are crucial for the calibration and estimation of the intracellular Ca^{2+} release.

To estimate intracellular calcium release following stimulation of the cells with C5a, the dissociation constant (Kd) needs to be calculated by using equation 1. Once the Kd had been determined this allowed the estimation of the intracellular Ca^{2+} at every 5 s measurement using equation 2. Both equations are from (Al-Mohanna and Hallett, 1988).

Equation 1 $$Kd = \text{Resting } [Ca^{2+}] \frac{(R_{max}-R)}{(R-R_{min})}$$

Resting $[Ca^{2+}]$ = 100 nM

R = Base line fluorescence intensity ratio $F\lambda_1/F\lambda_2$ in which λ_1 (340 nm)

and λ_2 (380 nm) are the fluorescence detection wavelength.

R_{max} = Maximum fluorescence intensity ratio $F\lambda_1/F\lambda_2$ following lysis with Tx100

R_{min} = Fluorescence intensity ratio $F\lambda_1/F\lambda_2$ following chelating ions with EGTA

Equation 2 $$[Ca^{2+}] = Kd \frac{(R_{unknown} - R_{min})}{(R_{max} - R_{unknown})}$$

Kd = Dissociation constant

$R_{unknown}$ = Base line fluorescence intensity ratio $F\lambda_1/F\lambda_2$ in which λ_1 (340 nm) and λ_2 (380 nm) are the fluorescence detection wavelength.

R_{max} = Maximum fluorescence intensity ratio $F\lambda_1/F\lambda_2$ following lysis with Tx100

R_{min} = Fluorescence intensity ratio $F\lambda_1/F\lambda_2$ following chelating ions with EGTA

2.3.9 Propidium iodide exclusion assay

To discriminate between live and dead cells, cells were stained with propidium iodide (PI). PI is a dye that intercalates with DNA and RNA, however is excluded from viable cells due to their non-porous membrane. Once the cells loose their membrane integrity, due to cell death, PI enters the cell and binds to DNA or RNA, thus increasing their fluorescence intensity.

Once the cells had been harvested they were washed twice with PBS. The cells were counted and resuspended at 5 x 10^6 cell/ml in FACS buffer. 50 µl of cells was then mixed with 50 µl FACS buffer containing 5 µg/ml PI, in a 96 well microtitre plate, and incubated on ice for 30 min. The cells were washed twice with 200 µl of FACS buffer and immediately analysed by flow cytometry. Percentage cell death was calculated by gating the PI positive cells and calculating their percentage from the whole population counted.

2.3.10 Bradford assay

Bradford assay was performed to quantify cellular lysate protein concentrations. A BSA standard curve (25-2.5 µg/ml) was made by diluting 1 mg/ml BSA standard (Pierce) with the same sample buffer as cellular lysate, a 0 µg/ml which consisted of sample buffer alone was also included in the standard curve. The samples to be analysed were diluted (typically 1/20 to begin with and then performing doubling dilutions) in the sample buffer to achieve a sample concentration that lied within the standard curve. In triplicate, 100 µl of the samples and BSA standard curve was added to a flat bottom 96-well microtitre plate and 100 µl of RT coomassie protein reagent (BioRad; 1856209). The plate was then incubated at RT for 10 min with gentle shaking. The optical density was measured at 595 nm and sample concentrations were calculated from the standard curve, taking into account dilution factors.

2.3.11 Analysis of DNA by agarose gel electrophoresis

The molecular weight, quality and quantity of DNA fragments was analysed by agarose gel electrophoresis. To make the agarose gel, 0.8-2% (w/v) ultra-pure agarose (Invitrogen) was added to TAE (1x) and heated for 1 minute in a microwave until completely dissolved. The clear solution was allowed to cool to 50 °C after which ethidium bromide was added (0.2 μg/ml final concentration), the solution was mixed, poured into a gel tray and allowed to set at RT. Once the gel was set, it was placed into an electrophoresis tank containing TAE (1x) according to the manufacturers instructions (Mini Sub Cell GT, BioRad). DNA samples (5-10 μl) were mixed with an equal volume of DNA sample buffer and carefully loaded into the wells of the agarose gel. Either 10 Kbp or 1 Kbp DNA molecular size markers were also loaded onto the agarose gel in order to estimate the size of the DNA fragment analysed. Electrophoresis was carried out at a constant voltage (100V) until the dye front had migrated approximately 2/3 through the gel. The gel was visualised on a UV transilluminator lamp and photographed using gel documentation system (BioRad) and Quantity One software (BioRad) for image acquisition.

2.3.12 Statistics

Data was expressed as mean ± standard error of the mean as determined by *n* samples (n = 3 unless otherwise stated) for each condition and analysed using GraphPad Prism software. Statistical analysis was carried out by One-way ANOVA, followed by either Tukey's multicomparison test which compares all pairs of data or Dunnett's test which compares all sets of data with a set control, or Two-way ANOVA followed by Bonferroni post test using confidence intervals of 95 %. Differences were considered significant at values of $p<0.05$.

Chapter 3

Regulation of basal levels of C5aR expression

Chapter 3

Regulation of basal levels of C5aR expression

3.1 Introduction

It was first decided to investigate how basal C5aR expression is regulated in the mammalian monocytic U937 cell line. To achieve this both the transcriptional control mechanisms, as well as, mRNA stability of the C5aR transcripts, will be investigated using EGFP reporter constructs.

3.1.1 Methods of regulating gene expression

There are several steps utilised in eukaryotes to control expression of a gene from transcription to the production of an active protein, figure 3.1.1. Transcription, the first regulation step, allows DNA dependent synthesis of nascent RNA. The nascent RNA is then processed by the addition of the 5'cap, splicing out of introns and 3'cleavage and polyadenylation (Proudfoot et al., 2002). Before the mRNA can be translated into protein, it is first transported into the cytoplasm via the nuclear pore complex. This nuclear-cytoplasm compartmentalisation of both mRNA and proteins required for regulating gene expression is an important regulation mechanism (Reed and Hurt, 2002). Once in the cytoplasm, the mRNA transcripts are either translated into protein, via the ribosome, or targeted for degradation. Following protein synthesis the activity of the protein can further be regulated by post-translational modifications, such as phosphorylation, glycosylation and isoprenylation. Although figure 3.1.1 depicts the stages involved in gene regulation as individual steps, it is known that the protein factors responsible for each step (from transcription to translation) are functionally and sometimes physically connected (Orphanides and Reinberg, 2002). There are no general rules as to the exact mechanism in which a gene may be regulated, as different genes are regulated at different stages. Here the regulation of gene transcription and mRNA stability will be discussed in more detail and their role in regulating the expression of the C5aR will be examined.

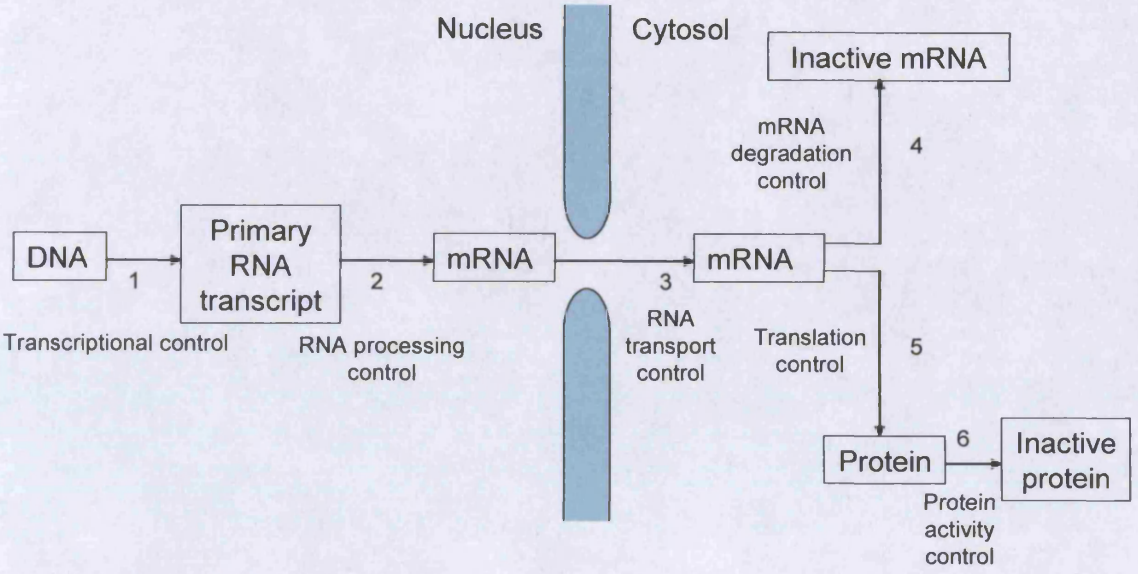


Figure 3.1.1 Schematic diagram illustrating the different control mechanisms for regulating eukaryote gene expression.

3.1.2 Transcription control

Transcription requires both general transcription factors, which initiate transcription, and RNA polymerase II, which catalyzes DNA-dependent synthesis of the nascent RNA. Unlike ubiquitously expressed genes, transcription of specific genes whose proteins define a certain cell lineage or stage in cell cycle or development require promoter specific transcription factors whose activity is tightly controlled (Woychik and Hampsey, 2002).

Transcriptional control can be achieved by regulating the binding of transcription factors to their regulatory elements, as well as, regulating the activity of these factors themselves. Binding of transcription factors to their regulatory elements is greatly influenced by the chromatin structure. Heterochromatin is highly condensed and is composed of untranscribed regions of the genome, whereas euchromatin is more accessible to transcription factors and is composed of transcribed genes (figure 3.1.2) (Richards and Elgin, 2002, Narlikar et al., 2002). The activation of signalling cascades generally ends with the activation of transcription factors. The activity of these factors is regulated by several methods including compartmentalisation and post-translational modifications (Orphanides and Reinberg, 2002). Once activated, these transcription factors bind to their sequence specific DNA elements and with the cooperation of chromatin modifying complexes and other co-regulators, they lead to local chromatin re-modelling which allows access of RNA-polymerase II and the general transcription machinery to the promoter (McKenna and O'Malley, 2002, Narlikar et al., 2002, Orphanides and Reinberg, 2002).

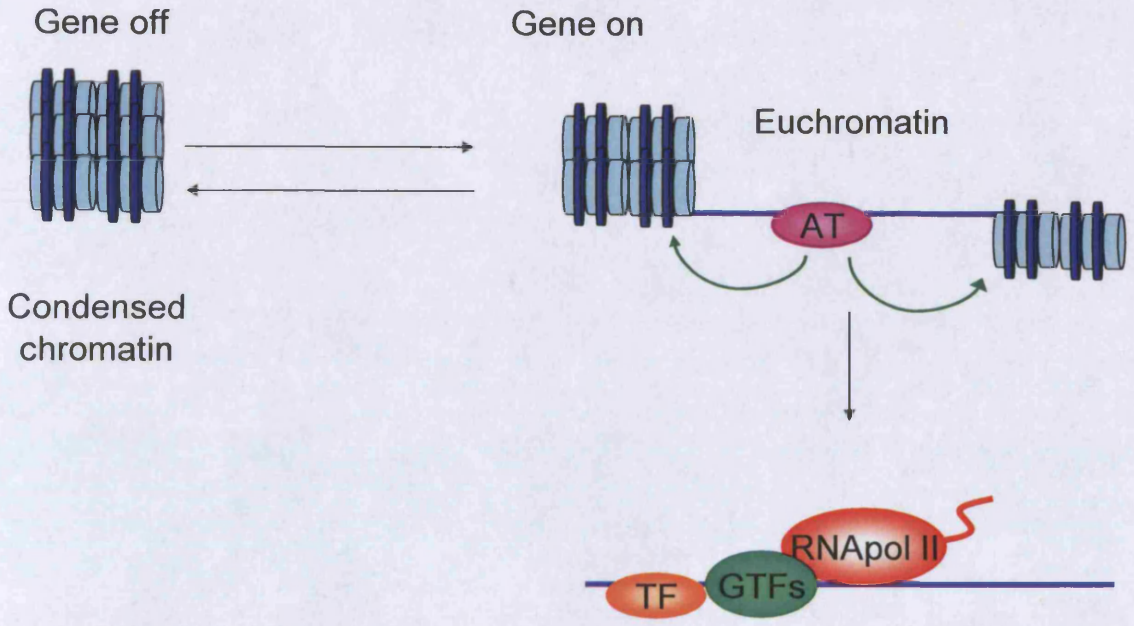


Figure 3.1.2 Schematic diagram illustrating how gene transcription can be regulated. AT = activators of transcription complex which contains chromatin modifying enzymes and co-regulators; TF = transcription factors which binds to regulatory cis-acting elements; GTFs = general transcription factor machinery.

3.1.2.1 Transcriptional regulation of the C5aR gene

The importance of the C5aR has been documented in a variety of pathological conditions (see section 1.2.7), despite this very little is known about the regulation of expression. As previously described in section 1.2.5 several studies have investigated the transcriptional regulation of cell specific, basal levels and induced expression of the C5aR in both human (Gerard et al., 1993) and murine cell lines (Hunt et al., 2005, Martin et al., 2007).

Gerard et al. investigated the transcriptional control of the human C5aR using CAT (chloramphenicol acetyltransferase) reporter construct. The reporter construct contained ~-350bp of the C5aR promoter region, exon 1 and ~450bp of intron 1 (see figure 1.6, Chapter 1) (Gerard et al., 1993). By transiently transfecting the reporter construct into rat basophilic lymphoma (RBL) and the neuroblastoma SK-N-SH cell lines, they found that this construct contained a cell type specific suppressor activity within -346bp and -225bp from the start codon, which prevents the neuroblastoma SK-N-SH cell line from expressing endogenous C5aR (Gerard et al., 1993). They identified a CCAAT site at position -280bp and speculated this may be responsible for the strong suppressor activity in this region. Deletion 5' from -82bp to -49bp resulted in the reporter activity dropping to baseline levels, suggesting that strong promoter activity is present within this region which they speculate could be a putative TATAA motif (Gerard et al., 1993).

As well as investigating how cell specific transcriptional regulation of the C5aR gene is achieved, Gerard and co-workers also investigated regulation of PMA induced C5aR

expression. They found that treatment of the RBL cells, which had been transiently transfected with the reporter construct, when incubated with PMA, doubled the CAT reporter activity. Whereas treatment of the neuroblastoma SK-N-SH cell line with PMA had no effect on the CAT reporter activity. They speculated that an AP-1 site could be responsible for the increased transcriptional activity in the RBL cells, whereas this activity is suppressed in the SK-N-SH cell line (Gerard et al., 1993).

Another group has focused on transcriptional regulation of the mouse C5aR (Hunt et al., 2005, Martin et al., 2007). By using 5' deletions of the mouse C5aR luciferase reporter construct (from -2240bp to +38bp), Hunt and co-workers found that the mouse C5aR promoter region (-2278bp to -232bp) contained a cell type specific suppressor element which prevents the B16 melanoma cell line from expressing C5aR mRNA detected by RT-PCR, whereas, this luciferase reporter promoter construct was active in both the mouse macrophage (RAW 264.7) and endothelial (bEnd.3 and mHEVc) cell lines (Hunt et al., 2005). Furthermore, they found that the majority of the region cloned (-2278bp to -232bp) was dispensable for expression in the RAW 264.7, b.End3 and mHEVc cell lines. Hunt and colleagues also found that deletion of fragments between -232bp to -132bp and -132bp to -90bp, resulted in approximately 40% and 80% reduction in promoter activity respectively, in the RAW 264.7, b.End3 and mHEVc cell lines. They identified the transcriptional regulatory site most critical for the promoter activity within the -132bp to -90bp is a putative CCAAT site, which specifically binds nuclear factor-Y (NF-Y), but not c-Fos or Ets1/2, as determined by supershift assay (Hunt et al., 2005). Interestingly the CCAAT site was shown to be completely conserved between the human and the mouse sequence, however, its role in regulating human C5aR transcription was not investigated (see appendix 9.3 for human and mouse C5aR promoter aligned) (Hunt et al., 2005). This study also investigated the role of other putative sites, including NFκB (-241bp), Octamer (-366bp), AP-4 (-273bp), GATA (-298bp) and CP-2 (-155bp), by site directed mutagenesis these results are summarised in table 3.1.1.

As well as investigating regulation of basal C5aR expression in mouse cell lines, Hunt and co-workers also showed that 10ng/ml LPS increased C5aR expression three fold in the RAW 264.7 cell line. To further investigate whether any of the putative sites mutated by site directed mutagenesis, they incubated the RAW 264.7 cells, which had been transiently transfected with the mutant reporter constructs, with 10ng/ml LPS for 18 hrs and then monitored CAT activity. They found that LPS increased luciferase activity in NFκB, Oct1, AP-4 and GATA mutants, but not CCAAT and CP-2 mutants, suggesting that the putative CCAAT and CP-2 sites are important for LPS induced expression of the mouse C5aR in the RAW 264.7 cell lines (Hunt et al., 2005).

Table 3.1.1 Summary of site directed mutagenesis results of putative transcription factor site in mouse C5aR promoter region published by (Hunt et al., 2005).

Site of mutation	RAW 264.7	b.End3	mHEVc	LPS stimulated in RAW 264.7
CCAAT (-96bp)	Reduced reporter activity	Reduced reporter activity	Reduced reporter activity	Abolished LPS induced activity
CP-2 (-155bp)	Reduced reporter activity	No effect	Reduced reporter activity	Abolished LPS induced activity
NFκB (-241bp)	No effect	No effect	No effect	LPS increased CAT activity
AP-4 (-273bp)	No effect	No effect	Reduced reporter activity	LPS increased CAT activity
GATA (-298bp)	No effect	Increased reporter activity	No effect	LPS increased CAT activity
Oct1 (366bp)	No effect	No effect	No effect	LPS increased CAT activity

The same group has further investigated differences in C5aR transcriptional regulation mechanisms between mouse microglia and astrocytes (Martin et al., 2007). They found that similar to the RAW 264.7 cell line, expression of the C5aR in mouse microglial (BV-2) and astrocyte (Ast2.1) cell line has a strong dependency on a CCAAT motif which was shown to bind NF-Y, but not c-Fos or ETS1/2, by supershift assay (Martin et al., 2007). Whereas they suggest that primary astrocytes are less reliant on the CCAAT motif as only weak activity was observed in the shift assay compared with the astrocyte cell line. Furthermore, they found that transfection of the mouse C5aR promoter reporter constructs into both the astrocyte cell line and the primary astrocytes revealed slightly different dependencies on different regions of the promoter, with deletion from -332bp to -232bp in the cell line resulting in a reduction of luciferase activity compared with an increase in activity observed in the primary astrocytes (Martin et al., 2007). These findings led them to conclude that regulation of the C5aR promoter in primary astrocytes is very different from the previously described mechanism for macrophages or endothelial cells (Martin, 2007, Hunt et al., 2005, Martin et al., 2007).

Several studies have investigated the role of SNPs within the C5 and the C5aR gene, and their association with pro-inflammatory disease states such as asthma (Hasegawa et al., 2004, Barnes et al., 2004, Marceau et al., 1999). A study by Hasegawa and colleagues found 5 SNPs within the C5 gene and they identified a combination of these SNPs within C5a which were protective against childhood and adult asthma (Hasegawa et al., 2004). However, only Barnes and co-workers have investigated the role of SNPs within the promoter region of the human C5aR gene (Barnes et al., 2004). This group found a novel

T/C SNP at position -245bp with the -245C allele having a significantly higher frequency in the Afro-Caribbean population compared with the German and Tangier Island population, however it had no association with asthma or atopy. By creating luciferase reporter constructs of 2711bp region, starting at -1450bp from transcription start site and part of the intro 1, they found that the T to C base substitution, potentially eliminates an AP-4 transcription factor motif, however this had no effect on its promoter activity when transiently transfected into the U937 cell line (Barnes et al., 2004).

3.1.3 mRNA stability

Regulating mRNA stability allows a cell to rapidly respond to changes in internal or external stimuli. It is an important regulatory step for mRNAs whose proteins regulate cell growth and differentiation, such as proto-oncogenes or encodes pro-inflammatory proteins, such as cytokines (Barreau et al., 2005). The stability can be affected by *cis*-acting elements present within different regions of the mRNA (figure 3.1.3).

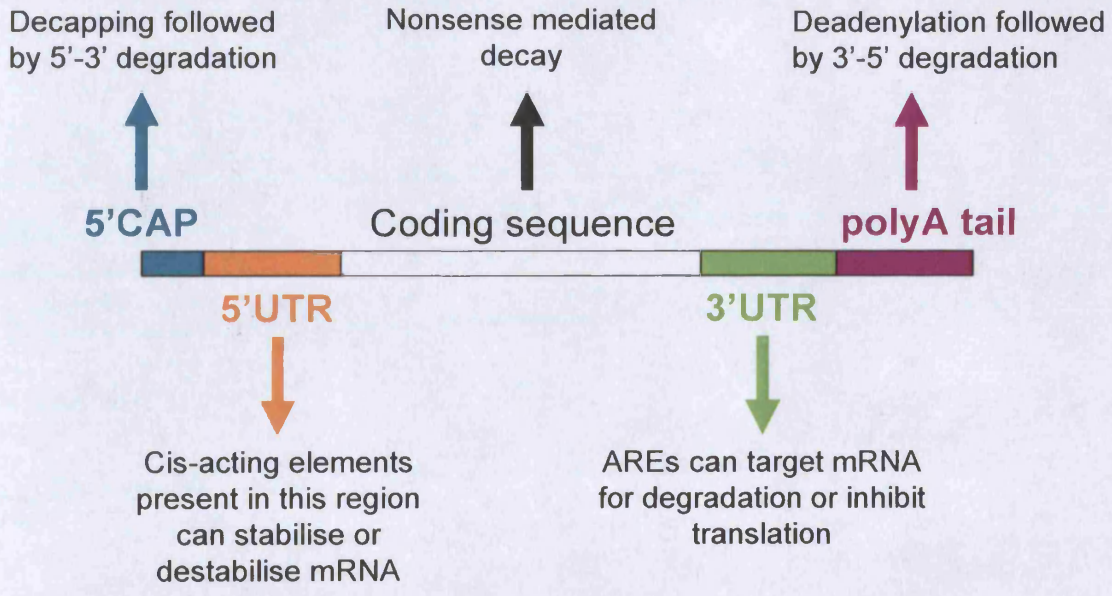


Figure 3.1.3 Schematic diagram of cis-acting elements present within the mRNA which effect its stability.

Both the 5'cap and polyA tail protects the mRNA from degradation (Brennan and Steitz, 2001). Removal of the cap exposes the 5' end to degradation by the 5'-exoribonuclease, Xrn1 (Liu and Kiledjian, 2006). Likewise, the polyA tail also protects the mRNA from 3'-5' endo/exonucleases and it is this polyA shortening which is often the rate limiting step in mRNA turn over (Brennan and Steitz, 2001). Sequences present in the 5'UTR or the coding sequence can also affect mRNA. For example, the 5'UTR of IL-2 mRNA contains a *cis*-acting element which is stabilised by c-Jun N-terminal kinase pathway

(Chen et al., 1998a). MessengerRNA stability can also be affected by the quality of the mRNA. For instance, mRNAs which end prematurely because they have a frameshift or nonsense mutation will be targeted for nonsense-mediated decay as a quality control mechanism (Maquat and Gong, 2009). The 3'UTR region may contain AU-rich elements (AREs), which are the most common *cis*-acting elements that are likely to affect mRNA stability (Chen and Shyu, 1995).

AREs, which are rich in adenosine and uridine, can be classed into three different groups depending on their number and distribution (see table 3.1.2 for examples). ARE-binding proteins (ARE-BPs), which bind to the AREs, and can either destabilise or stabilise the mRNA. The way in which ARE-BP can destabilise the mRNA is by deadenylation and decapping of the mRNA (Barreau et al., 2005). As well as targeting mRNA for degradation, ARE can also reduce protein synthesis by interfering with the translation of the mRNA (Barreau et al., 2005).

Table 3.1.2 Classification of ARE and examples associated ARE-BPs. Taken from Barreau et al 2005.

mRNA Class	Motif	Example of mRNA
I	Multiple dispersed AUUUA motifs, U-rich	*c-myc, c-fos*, IFNγ, p21, Cyclin A, Cyclin B1
II	(A/U)(AUUU)$_n$A (A/U)	TNFα, GM-CSF, IFNa, Cox-2, IL-2, IL-3, IFNβ, VEGF, DAF, C5aR
III	No AUUUA, U-rich region	c-Jun, GLUT1, p53, hsp70, NF-M, GAP-43

3.1.3.1 C5aR mRNA stability

To date it is not known whether the ARE within the C5aR 3'UTR affects mRNA stability. The ARE-mRNA database (ARED), which analysed the distribution of AREs in human mRNA sequences using computational analysis, has shown that the C5aR mRNA contained an ARE which belonged to the class I cluster IV category (section 3.3, figure 3.3.2) (Bakheet et al., 2003) http://brp.kfshrc.edu.sa/ARED). Although this sequence has been identified as a putative ARE it has not been investigated if this ARE has any effect on the C5aR mRNA stability. Further examination of the C5aR 3'UTR sequence also identified a second ARE like motif (see figure 3.3.2). Although it has not been investigated whether either of these AREs play a role in the stabilisation or destabilisation of the C5aR mRNA; several other GPCR, including β2-adrenergic receptor and thyrotropin releasing hormone receptor, mRNAs have been shown to be regulated by such elements (Collins et al 1991). Additionally it has been shown that the MCP-1 receptor, CCR2, and the IL-8 receptor, CXCR2, both contain sequences within their 3'UTR which causes rapid destabilisation of their mRNA following stimulation by LPS (Sica et al., 1997, Saccani et al., 2000, Lloyd et al., 1995,

Sprenger et al., 1994). A study by Xu and co-workers found that stimulation of THP-1 cells with LPS led to the degradation of mRNA via a two step mechanism; firstly deadenylation followed by the rapid destabilisation of the mRNA (Xu et al., 1997). However this study did not investigate the exact nature of the sequences responsible for this activity (Xu et al., 1997).

3.1.4 Methods for studying gene regulation

Several experimental techniques can be used to identify *cis*-acting elements involved in the regulation of expression of a particular gene, as well as identifying the transcription factors which interact with these elements. Discussed below in detail are the experimental techniques which will be used in this study to investigate regulation of C5aR expression.

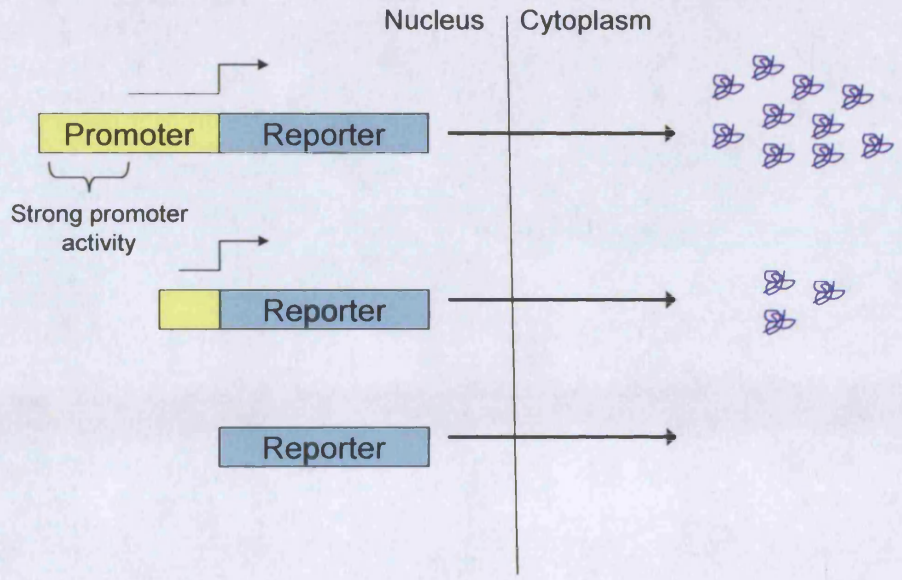
3.1.4.1 Identifying cis-acting elements involved in expression of a particular gene using reporter gene assays

In order to identify potential *cis*-acting elements involved in regulation of gene transcription or mRNA stability using reporter gene assay, genomic clones for the particular region of interest need to be cloned into a reporter vector. By cloning the promoter region of interest upstream of the reporter gene, the extent to which the gene is transcribed is dependent on the regulatory elements present within that promoter (figure 3.1.4). Likewise, by placing the 3'UTR down stream of the reporter gene, which is under the transcriptional control of a strong promoter, the stability of the reporter mRNA is influenced by regulatory elements present within the 3'UTR.

There are several reporter proteins commonly used to investigate gene expression. Table 3.1.3 list common reporter proteins used and their advantages and disadvantages. In this study it was decided to use pEGFP-1 reporter vector, which encodes a variant of wild-type GFP which has been optimised for brighter fluorescence and higher expression in mammalian cells (Cormack et al., 1996). The half life of EGPF is 24 hr, therefore making this vector particularly useful for studying an increase in expression but less suitable for studying decreased in expression. By using this vector, once the cells had been transfected with the reporter constructs, fluorescence can easily be detected by flow cytometry (Gray et al., 2010). The pEGFP-1 vector is a promoter less vector with a multiple cloning site upstream of the EGFP gene, which allows the promoter region of interest to be easily inserted (see section 3.3 for vector map figure). The SV40 3'UTR and polyadenylation sequence is positioned downstream to the EGFP gene in order to direct the proper processing of the 3'end of the EGFP mRNA. In the case of investigating regulation of transcription of C5aR, the ~2Kbp promoter region upstream from the transcription start site will be cloned upstream of the EGFP gene using the multiple cloning site. However to investigate the regulation of

C5aR mRNA, the C5aR 3'UTR will be cloned downstream of the EGFP gene replacing the SV40 3'UTR and polyadenylation signal. For this construct to be transcribed, an EGFP reporter plasmid with the ubiquitous elongation factor 1α (EF1α) promoter placed upstream of the EGFP gene will be used (Gray et al., 2010).

(A) Schematic of 5'deletions



(B) Schematic of unstable 3'UTR

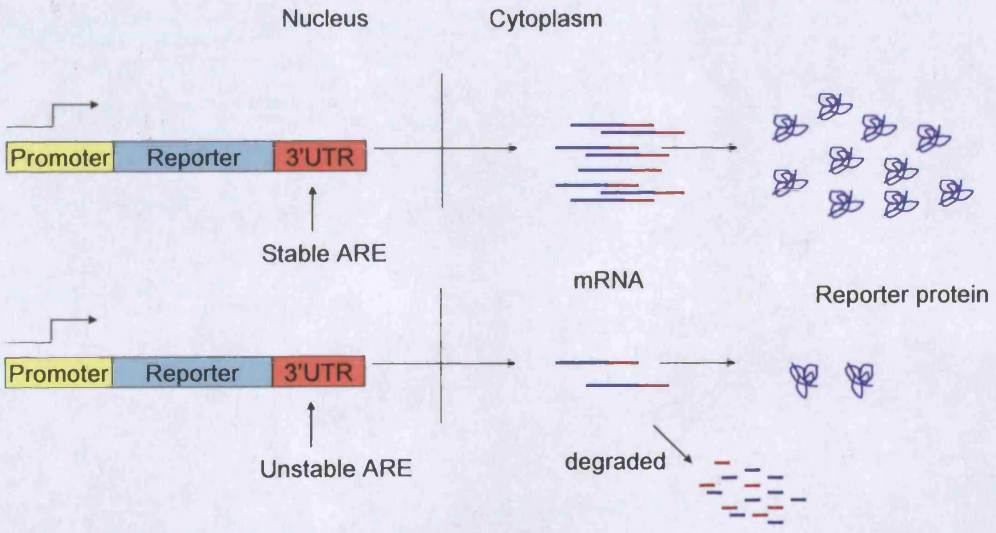


Figure 3.1.4 Schematic of reporter gene assays.

Table 3.1.3 Advantages and disadvantages of different reporter assay. Taken from (Ausubel et al., 2000).

Reporter gene	Assay	Strengths	Weaknesses
Chloramphenicol Acetyltransferase (CAT)	In *vitro* assays only. Assays use either chromatography, differential extraction, fluorescence or immunoassay	• Stable protein • Minimal endogenous activity in mammalian cells	• Assays are time consuming and laborious • Most assays use expensive radioactive substrate • Relatively low sensitivity
Firefly Luciferase	Both in *vitro* and in *vivo* assays available using bio-luminescence	• Non-isotopic • Good sensitivity • Minimal endogenous activity in mammalian cells • Relatively cheap	• Short half life of protein • Require luminometer or scintillation counter
β-Galactosidase	Both in *vitro* and in *vivo* assays available using colorimetric, fluorescence, chemiluminescence and histochemical staining	• Non-isotopic • Various assay formats available • Chemiluminescent assay very sensitive	• Many cell types have high endogenous activity • Require fluorometer or luminometer
Green fluorescent protein	Both in *vivo* and in *vivo* assays using fluorescence microscopy or FACS analysis	• Reporter gene expression and protein localisation in live cells • Assay does not require any additional substrates or co-factors • Quick and easy detection • Fluorescent signal highly resistant to photo bleaching • No apparent toxic effects of GFP expression in bacteria or eukaryotes	• Signal intensity may be too weak for some applications • At high concentrations fluorescence signal may plateau.

To identify regions of interest, the DNA that is cloned into the reporter construct can be manipulated either by creating serial deletions or by incorporating defined mutations within a specific sequence. In this study serial deletions will be created from the 2Kbp promoter region of the C5aR and once transfected into U937 cells changes in EGFP will be detected by flow cytometry. Performing serial deletions of the promoter would identify regions with either strong promoter or repressor activity. From this, with the aid of computational analysis, the role of individual regulatory elements can further be investigated by site directed mutagenesis. To identify what role if any the ARE have on mRNA stability

the sites will be mutated by site directed mutagenesis and changes in EGFP will be monitored by flow cytometry.

In order to monitor EGFP expression *in vivo* the reporter constructs need to be transfected into a eukaryotic cell. There a two types of transfections: transient or stable. Transient transfection does not involve the integration of the constructs into the genome and therefore the DNA introduced is divided during each cell division and expression is eventually lost. However, stable transfection involves the integration of the construct into the genome and is therefore inherited to daughter cells. Although electroporation is commonly used to produce a stable transfection, which incurs a lot of cell death during initial procedure, it allows the sufficient culturing of cells following the procedure to allow the cells to recover. Producing stable transfected cells has several advantages over transient transfections, such as you can produce clonal populations and it allows the culturing of transfected cells with different stimuli for several days. For these reasons it was decided to produce stably transfected cells with the reporter constructs. The cell line which will be used for these studies is the human monocytic U937. This cell line was derived from a patient with histiocytic lymphoma and characterised in 1976 by Sundstrom and Nilsson (Sundstrom and Nilsson, 1976). Since then this cell line has been well characterised for its monocytic like phenotype and its ability to be differentiated to a more macrophage phenotype and up-regulation of the C5aR by stimulation with agents such as PMA and Bt_2cAMP (Sheth et al., 1988, Chenoweth et al., 1984, Rubin et al., 1991a).

3.1.4.2 Identifying transcription factors that interacting with cis-acting elements using electrophoretic mobility shift assays

Electrophoretic mobility shift assay or gel retardation assay was first described by Garner and Revzin in 1981 as a rapid technique that involves separation of free DNA from DNA-protein complexes based on differences in their electrophoretic mobilities in polyacrylamide gels (Garner and Revzin, 1981). Incubation of the labelled DNA probe, typically radio-isotype-labelled or biotinylated, with cellular extract containing the protein, which recognises probe, will create DNA-protein complex that can be separated from unbound probe. The addition of unlabelled competitors to the reaction mixture, in excess, can determine the specificity of the DNA-protein interaction. Additionally, antibodies against the protein in the DNA-protein complex can create a supershift pattern, observed by gel electrophoresis, which can be used to confirm the identity of the protein in the complex. Combining these approaches can identify proteins which bind to the DNA sequence of interest and the specificity of the bond between the DNA probe and the protein.

3.1.5 Aims and hypothesis

The aim of this chapter is to generate promoter and 3'UTR reporter constructs of the C5aR and to investigate how basal levels of expression of human C5aR is regulated in the U937 cell line. Both regulation of gene transcription and mRNA stability will be investigated. It is hypothesised that regulation of human C5aR expression may be similar to that of the mouse due to sequence homology which has already been shown by (Hunt et al., 2005).

3.2 Materials and methods

3.2.1 Chemicals and reagents

3.2.1.1 Molecular biology and cell culture

All general reagents were purchased from Sigma. Phusion High-Fidelity DNA polymerase (F-530) was purchased from Finnzymes. QIAampDNA blood kit (51104), QIAquick PCR Purification Kit (28104), and plasmid purification kits were from Qiagen. Taq polymerase, dNTPs, Q reagent, 25mM $MgCl_2$ pGEM-T easy vector kit, X-Gal and dATP were all from Promega. Restriction enzymes (*Age I, Dpn I, EcoR I, Kpn I, Pst I,Nco I, Xba I, Not I* and *Afl II*), T4 ligase and 100bp ladder were from NEB Biolabs. 1Kbp DNA ladder and UltraPure Agarose were from Invitrogen. Primers were ordered from MWG-Biotech. pEGFP-1 vector was obtained from Clontech. Pfu Turbo DNA Polymerase (600250) was from Stratagene. Electroporation cuvettes were from WebScientific. BigDye 3.1 Terminator cycle sequencing mix was from PE Biosystems. Cyclosporin A and G418 were from Calbiochem. G418 was dissolved in sterile water to a stock concentration of 50 mg/ml, and was subsequently diluted in RPMI to a working concentration of 500 μg/ml. All other reagents were as described in Chapter 2.

Whole cell extract buffer: 10 mM Hepes pH 7.9, 0.4 mM NaCl, 0.5 mM DTT, 0.5 mM PMSF, 5 % glycerol (v/v), 1 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml type I-S soybean trypsin inhibitor.
Cytosolic extract buffer: 10 mM Hepes pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml type I-S soybean trypsin inhibitor.
Nuclear extract buffer: 20 mM Hepes pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25 % glycerol (v/v), 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml type I-S soybean trypsin inhibitor.
Nuclear extract storage buffer: 10 mM Hepes pH 7.9, 20 mM KCl, 0.1 mM EDTA, 0.5 mM PMSF, 1 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml type I-S soybean trypsin inhibitor.
5 x Binding buffer: 50 mM Tris pH 8.0, 750 mM KCl, 2.5 mM EDTA, 0.5 % Triton-X100 (v/v), 40 % glycerol (v/v) and 1 mM DTT.

3.2.1.2 Bacteria and bacterial cell culture

i) Reagents

Bacterial cell strain NM522 was a kind gift from Dr L De'Cruz (Cardiff University). XL1-Blue cells were a kind gift from Dr A Roßbach. Ampicillin and Kanamycin (Invitrogen) were dissolved in dH_2O to a stock solution of 100 mg/ml and 30 mg/ml respectively, filter sterilised and stored at -20°C. X-Gal (20 mg/ml, U120A, Promega) was stored at -20°C.

Resuspension buffer; 50 mM Tris pH 8.0, 10 mM EDTA and 100 μg/ml RNase A. Stored at 4 °C.

Lysis buffer; 200 mM NaOH, 1 % SDS.

Neutralisation buffer; 3 M Potassium acetate, pH adjusted to 5.5 with glacial acetic acid.

ii) Media

LB Medium; 10 g/L bactotryptone, 5 g/L yeast extract and 5 g/L NaCl.

LB-agar plates were prepared by autoclaving LB medium containing 1.5% agar. Appropriate antibiotics, either ampicillin 100 μg/ml or kanamycin 30 μg/ml, were added once cooled to <50°C.

SOC medium; 20 g/L bactotryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 0.18 g/L KCl, 0.95 g/L $MgCl_2$

NZY$^+$ Broth; 10 g/L NZ amine (casein hydrolysate), 5 g/L yeast extract, 5 g/L NaCl, adjust pH to 7.5 and autoclave. Add 12 mM $MgCl_2$, 12 mM $MgSO_4$ and 0.4 % (w/v) glucose filter sterilised.

2-TY medium; 16 g/L bactotryptone, 10 g/L yeast extract and 5 g/L NaCl.

ii) Preparation of chemically competent bacteria

Chemically competent NM522 and XL1-Blue cells were generated using the following protocol. A single isolated colony growing on LB-agar plates was used to inoculate 10 ml of 2-TY media, which was then cultured overnight at 37 °C. The overnight culture (1ml) was used to inoculate 200 ml of 2-TY media and was incubated at 37 °C with vigorous shaking. The optical density at A_{600} of the culture was measured every 30 min until approximately 0.6 A was achieved (app. 2 ½ hrs). The bacteria were chilled on ice for 30 min and centrifuged at 5,000g for 10 min at 4 °C. The cell pellets were resuspended in 10 ml of chilled 0.1M $MgCl_2$ (sterile) and incubated on ice for 30 min. The bacteria were centrifuged at 5,000g for 10 min at 4 °C. The pellets were resuspended in 10 ml of chilled 0.1 M $CaCl_2$ (sterile) and incubated for a further 30 min on ice. The bacteria were centrifuged at 5,000g for 2 min at 4 °C and the pellets were resuspended in 8 ml of chilled 0.1 M $CaCl_2$ with 20% glycerol

(sterile). Aliquots of 80 µl were snap frozen in liquid nitrogen and stored at -80 °C for up to 1 year.

3.2.2 Cloning the human C5aR 5'promoter and 3'UTR into the pEGFP vector

3.2.2.1 Isolating gDNA from U937 cell line

Genomic DNA was isolated from two human monocytic cell lines U937, obtained from European Collection of Animal Cell Cultures U937 ($U937_{ECACC}$) and from Dr Peter Monk, Sheffield University ($U937_{PM}$), using QIAampDNA blood kit following manufacturer's instructions (see below).

5 x 10^6 U937 cells (growing in log phase) were harvested by centrifuging at 1,200 rpm for 3 min and resuspended in 200ul PBS. The cells were lysed by adding 20 µl of QIAGEN protease and 200 µl buffer AL and were then incubating for 10 min at 56 °C. The sample was briefly centrifuged to gather sample to the bottom of the tube. 200 µl ethanol was added to the lysate, mixed by pulse vortexing and the tube was then briefly centrifuged. The mixture was applied to the QIAamp Spin Column without wetting the rim and centrifuged at 7,000 rpm for 1 min. The column was placed into a clean 2 ml collection tube. 500 µl buffer AW2 was applied to the spin column which was then centrifuged for 3 min at 13,000 rpm. To eliminate possible AW2 buffer carry over the spin column was placed in clean 2 ml collection tube and centrifuged for 1 minute at 13,000 rpm. The spin column was placed in a clean 1.5 ml microcentrifuge tube and DNA was eluted in 200 µl buffer AE after incubation for 1 minute at RT followed by centrifuging for 1 minute at 7,000 rpm. The DNA was stored at -20 °C in 10 µl aliquots.

3.2.2.2 PCR amplification of the C5aR promoter and 3'UTR

3.2.2.2.1 PCR of the C5aR 5' promoter

The sequence for the C5aR promoter was obtained from NCBI CoreNucleotide database, GeneBank sequence accession number AC_099491. The reverse primer for the C5aR promoter was designed just 5' of transcription start site and the forward primers were designed up to 2037bp upstream of the transcription start site (see table 3.2.1). All primers were designed with restriction enzyme site overhangs in order to facilitate subcloning from the intermediate pGEM-T vector into the pEGFP vector.

Table 3.2.1 List of primers used for PCR of the C5aR 5' promoter

Fragment size	Sequence (5' to 3')	Restriction enzyme overhang	Annealing temperature
2037bp	Forward – 5'GCGAAT TCCCTTCGCTCA AT TACCGCC 3' Reverse – 5'CGGTACCGTTCTGGTCTCCTGGGCTCCC 3'	*EcoR I* *Kpn I*	71 °C
1524bp	Forward – 5'GCGAATTCAGGGATTCCACTGATTCTAC 3' Reverse – 5'CGGTACCGTTCTGGTCTCCTGGGCTCCC3'	*EcoR I* *Kpn I*	67 °C
1005bp	Forward – 5'GCGAATTCAGCTTCCCAAGTAGCTGGG 3' Reverse – 5'CGGTACCGTTCTGGTCTCCTGGGCTCCC 3'	*EcoR I* *Kpn I*	67 °C
504bp	Forward – 5'GCGAATTCGGCGAAACCCCGTCTTTAC 3' Reverse – 5'CGGTACCGTTCTGGTCTCCTGGGCTCCC 3'	*EcoR I* *Kpn I*	71 °C
200bp	Forward – 5'GCGAATTCCTGGGAGGTGGTTAGAGTCC 3' Reverse – 5'CGGTACCGTTCTGGTCTCCTGGGCTCCC 3'	*EcoR I* *Kpn I*	71 °C
101bp	Forward – 5'GCGAATTCGACGGTCATTTCCTCCCTGCATC 3' Reverse – 5'CGGTACCGTTCTGGTCTCCTGGGCTCCC 3'	*EcoR I* *Kpn I*	71 °C

For the PCR reaction, the following reagents were prepared as shown in the table below.

PCR reaction	
5 x High fidelity GC buffer	10 µl
5 x Q reagent	10 µl
dNTPs (10 mM)	0.5 µl
gDNA	0.5 µl
Forward primer (10 µM)	0.5 µl
Reverse primer (10 µM)	0.5 µl
High fidelity Phusion polymerase	0.25 µl
dH_2O	32.75 µl

The PCR reaction mixes were overlaid with 20ul mineral oil to prevent loss of sample and were then placed in a PCR sprint thermal cycler (Hybaid). The PCR cycle was as detailed below.

Thermal cycling conditions			
Step	**Temperature**	**Time**	**Cycle**
Initial denaturation	98 °C	30 s	1
Denaturation Annealing Extension	98 °C Typically 71 °C 72 °C	10 s 30 s 1 min	35
Final elongation	72 °C	10 min	1

3.2.2.2.2 PCR of the C5aR 3'UTR

The sequence for the C5aR 3'UTR was obtained from NCBI CoreNucleotide database, GeneBank sequence accession number NM_001736. The forward primer for the C5aR 3'UTR was designed just 3' of the stop codon and the reverse primer was designed overlapping the polyadenylation site (see table 3.2.2). Both primers were designed with restriction enzyme site overhangs in order to facilitate from the intermediate pGEM-T vector into the pEGFP vector.

Table 3.2.2 Primers used for PCR of the C5aR 3'UTR

Fragment size	Sequence (5' to 3')	Restriction enzyme overhang	Annealing temperature
1233bp	Forward – 5'CGCGGCCGCGACAGCCTCATGGGCCACTG 3' Reverse – 5'GCGCCTTAAGCAATATTCATTTTTATTTTCCTC 3'	*Not I* *Afl II*	50 °C

Due to differences in the Tm between the forward and reverse primers for the 3'UTR, which could not be avoided, the 3'UTR was amplified using GoTaq Flexi polymerase. For the PCR reaction, the following reagents were prepared as shown in the table below.

PCR reaction	
5 x Colourless GoTaq Flexi buffer	10 µl
5 x Q reagent	10 µl
dNTPs (10 mM)	1 µl
gDNA	1 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
$MgCl_2$ (25 mM)	3 µl
GoTaq Polymerase	0.25 µl
dH_2O	22.75 µl

The PCR reaction mixes were overlaid with 20ul mineral oil to prevent loss of sample and were then placed in a PCR sprint thermal cycler (Hybaid). The PCR cycle was as detailed below.

Thermal cycling conditions			
Step	**Temperature**	**Time**	**Cycle**
Initial denaturation	94 °C	3 min	1
Denaturation	94 °C	30 s	35
Annealing	50 °C	30 s	
Extension	72 °C	1 min	
Final elongation	72 °C	10 min	1

3.2.2.3 Purification of PCR products using QIAquick PCR purification

To remove unwanted enzymes, primers, salts and dNTPs, which may affect downstream applications, the PCR product was purified using QIAquick PCR purification kit following manufacturer's instructions.

5 volumes of buffer PB was added to 1 volume of PCR product. This solution was applied to a QIAquick spin column and collection tube and centrifuged for 1 min at 13,000 rpm. Flow through was discarded and the column was washed with 750 µl of buffer PE and centrifuged for an additional minute. The flow through was discarded and the column was centrifuged for another minute to remove residue ethanol, which can interfere with down

stream applications if not removed completely. The QIAquick column was placed in a clean 1.5 ml collection tube, and the DNA was eluted in 30 µl dH_2O by centrifugation for 1 minute.

3.2.2.4 Cloning the C5aR 5'promoter and 3'UTR into the intermediate pGEM-T vector

In order for the C5aR 5'promoter and 3'UTR to be cloned into the pEGFP vector in the correct orientation they were first cloned into the intermediate cloning vector pGEM-T (Promega). As PCR incorporated different restriction enzyme sites both 5' and 3' of the PCR product (see tables 3.2.1 and 3.2.2) this allows the DNA to be digested out of the pGEM-T vector using the specific enzymes and then to be ligated into the pEGFP vector, which has also been digested with the same enzymes, in the correct orientation.

3.2.2.4.1 Product extension with GoTaq polymerase

As Phusion polymerase produces blunt-end DNA products, to ligate the C5aR 5'promoter fragments into the intermediate pGEM-T vector additional 3' adenosine residues, which are produced by Go Taq polymerase, were required. 4ul of purified PCR product was mixed with 1 µl GoTaq Polymerase (5 u/µl), 2 µl colourless GoTaq Flexi buffer (5 X), 1 µl dATP (2 mM) and 0.6 µl $MgCl_2$ (25 mM). This solution was incubated at 72 °C for 30 min, followed by PCR purification using QIAquick purification kit as described in section 3.2.2.3.

3.2.2.4.2 Ligation of C5aR promoter and 3'UTR into pGEM-T

The addition of the 3' adenosine residues to the PCR fragments provides compatible overhangs for the *pGEM-T* vector which greatly improves the efficiency of ligation of the PCR products into the plasmid vector. Ligation of the C5aR 5' promoter and 3'UTR into the pGEM-T Easy Vector was carried out using Promega kit (A1360) according to manufacture's instructions. The molar ratio of insert: vector was 2:1, for optimum vector insert interactions. The reagents detailed below were all added on ice and then incubated overnight at 4 °C for maximum number of transformants.

Ligation reaction	
pGEM-T Easy Vector (50 ng/µl)	1 µl
Insert DNA	2 µl
2X T4 DNA ligase buffer	5 µl
T4 ligase (3 u/µl)	1 µl
H_2O	1 µl

3.2.2.4.3 Transformation and culture of competent bacteria

Bacterial transformation with recombinant DNA enables replication of the plasmid and thus produces large quantities of DNA. The chemically competent bacterial host strain NM522 were transformed with the ligation reaction mixtures using heat shock method, as described below. Heat shock of chemically competent bacteria that have been prepared with calcium

chloride allows DNA which is bound to the calcium on the surface of the bacterium to be taken up by the competent cell.

After an 80 μl aliquot of competent NM522 had thawed on ice, 3 μl of the ligation mix was gently added and the mixture was then incubated on ice for 30 min. The bacteria received a heat shock at 42 °C for 1 min exactly and were incubated on ice for another 15 min. 500 μl of RT SOC media was added to the bacteria which were then incubated at 37 °C at 225 rpm for 1 hr to allow the bacteria to recover. Aliquots of cells (50 μl and 200 μl) were streaked onto solid support LB-agar plates which contained 100 μg/ml ampicillin and a dry surface coating of 25 μl X-Gal/plate (50 mg/ml). The plates were incubated overnight at 37 °C to allow colonies to grow. As the pGEM-T vector contains an ampicillin resistant gene, only *E. coli* which contains the plasmid will grow on LB ampicillin medium or plates.

3.2.2.4.4 Analysis of recombinant plasmids

Successfully transformed NM522 bacteria with the pGEM-T vector will confer resistance to ampicillin, which allows selective screening. However it is still necessary to screen cells to determine whether they contain plasmid only or plasmid plus insert DNA.

i) Blue/white screening of transformed colonies

Successful cloning of an insert into the pGEM-T vector results in disruption of the lacZ gene which encodes the β-galactosidase enzyme. As a result colonies which contain the DNA insert will appear white, whereas those that do not contain the insert will appear blue.

Using a sterile pipette tip a selection of white colonies and a few blue colonies, to serve as negative control, were streaked onto a new LB-agar plate contain ampicillin (100 μg/ml) and 25 μl X-gal (50mg/ml) using a grid system. The plates were then incubated overnight at 37 °C to allow the colonies grow approximately 2 mm in diameter.

ii) Size screening of DNA insertion in plasmid

In addition to blue/white screening (see above), the size of the plasmid present in the colonies was also analysed as this allows the rapid confirmation of positive colonies as sometimes blue/white screening can produce a false positive (Maniatis et al., 2001). Approximately 2 mm of cells from the selected colonies on the LB-agar plate were placed in a microfuge tube containing 25 μl EDTA (10 mM, pH 8.0) using a sterile pipette tip. 25 μl of fresh lysis buffer (0.2 M NaOH, 0.5 % SDS and 20 % sucrose) was added to each tube, which were then vortexed briefly. Samples were incubated at 70 °C for 5 min after which 12.5 μl of KCl (0.25 M) and 0.25 % w/v bromophenol blue was added. The samples were incubated on ice for 5 min and then centrifuged at 13,000rpm for 5 min at 4 °C. The s/n (30 μl) was analysed on a 0.7 % agarose gel (without ethidium bromide), run at 100 V until the

dye had migrated approximately two thirds down the gel, after which the gel was soaked in an ethidium bromide tank (0.5 μg/ml in dH_2O) for 45 min. DNA bands were then visualised under UV light.

iii) PCR screening of DNA insertion into plasmid

Colonies that had screened positively by size analysis (see above) were also screened by PCR to check for the presence of insert DNA. Approximately 2 mm of cells from the selected colonies on the LB-agar plate were placed in a microfuge tube containing 20 μl sterile H_2O. The samples were then incubated at 99 °C for 10 min followed by incubating on ice for a further 10 min. The samples were centrifuged for 10 min at 13,000 rpm at 4 °C. 2 μl of the s/n was used as the template in a PCR reaction containing forward and reverse primers for either the C5aR promoter region or 3'UTR (see section 3.2.2.2 for details) depending on what insert DNA had been used. Following PCR thermal cycle, as described below, the PCR products were analysed by 1 % agarose gel electrophoresis.

PCR screening reaction	
dH_2O	7.35 μl
5 X Flexi buffer	3 μl
$MgCl_2$ (25 mM)	1 μl
dNTPs (10 mM)	0.4 μl
Go Taq polymerase	0.25 μl
Forward and Reverse primers (10 μM)	0.8 μl

Thermal cycling conditions			
Step	**Temperature**	**Time**	**Cycle**
Initial denaturation	95 °C	5 min	1
Denaturation Annealing Extension	94 °C 60 °C 72 °C	30 s 1 min 2 min	5
Denaturation Annealing Extension	94 °C 60 °C 72 °C	10 s 30 s 1 min	25
Final elongation	72 °C 37 °C	10 min 10 min	1

iv) Isolation of plasmid DNA

Bacteria transformed with plasmid containing insert DNA, was transferred under aseptic conditions from LB-agar plate culture into LB medium (5 ml) containing 100 μg/ml ampicillin. The culture was incubated overnight at 37 °C while constantly shaken at 225 rpm.

Plasmid DNA was isolated and purified according to Qiagen QIAprep Spin Miniprep kit (27104) protocol. The bacteria were harvested from the 5 ml culture by centrifugation for 5 min at 2,000 rpm. The s/n was then discarded and the pellet was resuspended in 250 μl resuspension buffer P1. The bacteria were lysed with 250 μl lysis buffer P2 and mixed by

inverting the tube. The lysis buffer was then neutralised with 350 µl of neutralisation buffer N3 which was mixed immediately and thoroughly by inverting the tube. The sample was centrifuged for 10 min at 13,000 rpm and the s/n applied to a QIAprep spin column. The column was centrifuged further for 60 s at 13,000 rpm and the flow-through was discarded. The column was washed with 500 µl buffer PB by centrifugation for 60 s at 13,000 rpm to remove trace nuclease activity. The flow-through was discarded and the column was washed again by adding 750 µl buffer PE and centrifuging for 60 s at 13,000 rpm. The flow-through was discarded and the column was centrifuged for another 60 s at 13,000 rpm to remove any residual ethanol. The column was placed into a clean 1.5 ml microfuge tube and the DNA was eluted by adding 30 µl sterile H_2O and incubation at RT for 60 s followed by centrifugation for 60 s at 13,000 rpm. The concentration and the purity of the DNA were determined by spectrophotometric analysis.

v) DNA sequencing

Positively screened plasmids that were subsequently purified, underwent sequence analysis using Big Dye 3.1 (PE Biosystems, ABI Prism, 4303152) as in the ABI protocol. The following reagents were added to PCR tubes and overlaid with 20 µl mineral oil.

Big Dye DNA sequencing reaction	
Purified plasmid DNA	0.5ug
Primer (pGEM-T forward or reverse) (10 µM)	0.32 µl
5 X reaction buffer	2 µl
Big Dye 3.1	2 µl
Sterile H_2O	Add to total volume 10 µl

Big Dye thermal cycling conditions			
Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	95 °C	1 min	1
Denaturation	95 °C	10 s	25
Annealing	50 °C	5 s	
Extension	60 °C	4 min	

Following heat cycling, as described above, 10 µl of sterile H_2O was mixed with the PCR tube contents which were pipetted onto a microfilm sheet. The mineral oil residue was retained as the solution moved down the film. The remaining oil free solution was then placed into a clean 0.2 ml microfuge tube and the DNA was precipitated by adding 80 µl of 70 % isopropanol. The mixture was incubated at RT in the dark for 1 hr. The DNA precipitate was then pelleted by centrifuging at 13,000 rpm for 30 min at 4 °C. The s/n was carefully removed and the pellet was washed with 250 µl 70 % isopropanol and centrifuged for another 15 min at 13,000 rpm. The s/n was again discarded and the DNA pellet was

dried by vacuum centrifugation for 20 min then underwent sequence analysis by CBS Facilities, Cardiff University.

3.2.2.5 Subcloning the C5aR 5'promoter and 3'UTR into the pEGFP-1 vector

Following successful cloning of the C5aR 5'promoter fragments and 3'UTR into the intermediate pGEM-T vector these inserts were then subcloned into the pEGFP-1 vector (generated in house (Gray et al., 2010)). As PCR incorporated restriction enzyme sites both 5' and 3' of the PCR products these sites allow the insert to be digested from the pGEM-T vector and into the pEGFP-1 vector in the correct orientation.

3.2.2.5.1 Restriction enzyme digest of the 5'promoter/pGEM-T and pEGFP-1 vector

PCR of the C5aR 5'promoter fragments introduced an *EcoR I* and a *Kpn I* restriction enzyme site 5' and 3' of the PCR fragments respectively, see table 3.2.1. As the pEGFP vector also contained an *EcoR I* and *Kpn I* site within the multiple cloning site both the 5'promoter/pGEM-T vectors and the pEGFP vector can be digested with these restriction enzymes and then ligated together. The resulting product, 5'promoter +pEGFP, will allow the C5aR promoter to drive transcription of the EGFP gene as it is positioned immediately upstream of the gene (see figures 3.3.10 for schematic diagram of constructs generated).

The following reagents were combined; 5 μl BSA (10X), 5 μl buffer 1 (10X, NEB), 1 μl *EcoR I* (10,000 U/ml), 1 μl *Kpn I* (10,000 U/ml), 1 μg 5'promoter/pGEM-T (first reaction mixture) or 1 μg pEGFP (second reaction mixture) and sterile H_2O to a total volume of 50 μl, reagents were then incubated for 1 h at 37 °C. A sample of both restriction enzyme digests were removed and analysed on a 0.8 % agarose gel.

3.2.2.5.2 Generation of the 355bp 5'promoter fragment

An additional 355bp 5'promoter fragment was also created at this stage as the C5aR 5'promoter region contains a *Pst I* site at -355bp downstream of the transcription start site which with the Kpn I site incorporated during PCR will facilitate cloning into the pEGFP-1 vector (figure 3.3.10).

The following reagents were combined; 5 μl BSA (10X), 5 μl buffer 1 (10X, NEB), 1 μl *Pst I* (20,000 U/ml), 1 μl *Kpn I* (10,000 U/ml), 1 μg 2kbp 5'promoter/pGEM-T (first reaction mixture) or 1 μg pEGFP (second reaction mixture) and sterile H_2O to a total volume of 50 μl, reagents were then incubated for 1 hr at 37 °C. A sample of both restriction enzyme digests were removed and analysed on a 0.8 % agarose gel.

3.2.2.5.3 Restriction enzyme digest of the 3'UTR/pGEMT and EF1α + pEGFP-1 vector

In order to identify what effect the C5aR 3'UTR has on mRNA stability the 3'UTR needs to be cloned into the pEGFP-1 vector which also contains a promoter upstream of the EGFP gene.

The powerful elongator factor 1α promoter (EF1α) was previously cloned into the multiple cloning site of the pEGFP-1 vector by Dr Lisa Gray (Dept. Pharmacology, Cardiff University) which will drive transcription of the EGFP gene (Gray et al., 2010). The C5aR 3'UTR was then cloned into the EF1α+pEGFP vector using *Not I* and an *Afl II* restriction enzyme sites which were incorporated during PCR. The C5aR 3'UTR will then replace the SV40 polyadenylation site and therefore direct the proper processing of the EGFP mRNA.

The following reagents were combined; 5 µl BSA (10X), 5 µl buffer 2 (10X, NEB), 1 µl *Not I* (10,000 U/ml), 1 µl *Afl II* (20,000 U/ml), 1 µg 3'UTR/pGEM-T (first reaction mixture) or 1 µg EF1α+pEGFP (second reaction mixture) and sterile H_2O to a total volume of 50 µl, reagents were then incubated for 1 hr at 37 °C. A sample of both restriction enzyme digests were removed and analysed on a 0.8 % agarose gel.

3.2.2.5.4 Preparation of 5'promoter/pGEM-T, 3'UTR/pGEM-T, pEGFP and EF1α+pEGFP DNA for ligation

After successful restriction enzyme digest, the products were precipitated as follows; 10 µl of 5'promoter/pGEM-T or 3'UTR/pGEM-T (first reaction solution) and 2 µl of pEGFP or EF1α + pEGFP (second reaction solution) respectively were mixed with 8 µl sterile H_2O and 80 µl isopropanol (70%), then incubated at RT for 1 hr. The DNA was pelleted by centrifuging at 13,000 rpm for 20 min at 4 °C and washed with 250 µl 70 % ethanol, followed by another centrifugation at 13,000 rpm for 10 min at 4 °C. The s/n was removed and the pellet was dried on a heating block 37 °C for approximately 1 h to remove remain ethanol.

3.2.2.5.5 Ligation of 5'promoter fragments into pEGFP or 3'UTR into EF1α+pEGFP vector

The dried DNA was dissolved in 8 µl sterile H_2O, 1 µl T4 ligase buffer (NEB) and 1 µl T4 ligase (1 U/µl, NEB) and was incubated o/n at 16 °C.

3.2.2.5.6 Analysis of 5'promoter fragments +pEGFP and EF1α+pEGFP+3'UTR constructs

1 µl of the ligation product was used to transform NM522 chemically competent cells by heat shock, as described in section 3.2.2.4.3. This was followed by; plasmid screening by size and PCR, plasmid purification and finally sequencing of the positive colonies (all methods are as described previously). On this occasion sequencing was carried out using a selective forward and reverse primer for pEGFP promoter or 3'UTR region (table 3.2.3).

Table 3.2.3 Primers for pEGFP vector

pEGFP vector primers	Sequence
MCS	Forward –5'-TAG TTA TTA CTA GCG CTA CCG G-3' Reverse –5'-GTG AAC AGC TCC TCG CCC TT-3'
3'UTR	Forward –5'-GCC TGT GCT TCT GCT AGG AT-3' Reverse –5'-GCC GAT TTC GGC CTA TTG GT-3'

Colonies containing correctly sequenced 5'promoter fragments +pEGFP or EF1α +pEGFP + 3'UTR were grown up in 5 ml LB medium at 37 °C o/n then frozen (-80 °C) in 15 % glycerol (in LB medium).

3.2.3 Site directed mutagenesis of potential *cis*-acting elements and AU rich elements

Following the successful cloning of the C5aR promoter fragments into the EGFP reporter vector, the sequence for the 2 Kbp promoter region was entered into Genomatrix MatInspector Software. MatInspector will predict transcription factor binding sites which could potentially control transcription initiation (Cartharius et al., 2005). A total of 292 potential transcription factor binding sites were identified by MatInspector (appendix 9.4), however only three sites were mutated using site directed mutagenesis, NFκB (-238bp to -232bp), CCAAT/NF-Y (-123bp to -119bp) and NFAT (-93bp to -87bp) within the 355bp 5'promoter +pEGFP reporter construct. The ARE database Search Engine identified one AU rich element within the C5aR 3'UTR, 5'-ATGTATTTATTTA-3', which belongs to the class I cluster 5 group (Bakheet et al., 2001, Bakheet et al., 2003). A second potential AU rich element was also identified by the sequence, 5'-TAATTTAAA-3'. Both AREs were mutated by site directed mutagenesis as detailed below.

3.2.3.1 Oligonucleotide-directed mutagenesis

Site directed mutagenesis is a valuable molecular biology technique commonly used in studying gene expression elements. Oligonucleotide-directed mutagenesis is a PCR based method which involves mutant primers annealing to the denatured DNA template and then being extended using a high fidelity DNA polymerase. The parental methylated and hemimethylated DNA is then digested using *Dpn I* endonuclease, leaving the mutant daughter DNA template which is then transformed into competent cells XL-blue cells.

The following reagents were combined; 2 μl reaction buffer (x10), 20 ng dsDNA template (355bp 5'promoter + pEGFP or EF1α + pEGFP + 3'UTR), 125 ng mutant forward primer, 125 ng mutant reverse primer, 0.4 μl dNTPs (10 mM), 4 μl Q reagent (x5), 0.4 μl *Pfu Turbo* and sterile H_2O to a final volume of 20 μl. The mutant primers, each complementary to opposite strands of the vector, were designed in a manner so that they incorporated a new restriction enzyme site at the site of the mutation which will allow easy screening for positive mutants (table below 3.2.4). For schematic diagram of mutants see section 3.3 figures 3.3.15 and 3.3.34).

Table 3.2.4 Primers used for site directed mutagenesis

Primers	Sequences	Restriction enzyme site inserted
NFκB mutant	Forward primer 5'- GGGCCGAGCCTGCACAGGACCATGGTCGGTTTTCCGA-3' Reverse primer 5'- TCGGAAAACCGACCATGGTCCTGTGCAGGCTCGGCCC-3'	*Nco I*
CCAAT mutant	Forward primer 5'- CAGTGCCCAGTGTGCAGACTCTAGAGAGCCCCAGAGAGAAAG-3' Reverse primer 5'- CTTTCTCTCTGGGGCTCTCTAGAGTCTGCACACTGGGCACTG-3'	*Xba I*
NFAT mutant	Forward primer 5'- CCCCAGAGAGAAAGACGGTCCTTAAGGCCCTGCATCTTCCCTTGGGG-3' Reverse primer 5'- CCCCAAGGGAAGATGCAGGGCCTTAAGGACCGTCTTTCTCTCTGGGG-3'	*Afl I*
1st ARE mutant	Forward primer 5'- CTTAAAAAAAATGTACCGGTTTTATGGCAAGTTG-3' Reverse primer 5'- CAACTTGCCATAAAACCGGTACATTTTTTTTAAG-3'	*Age I*
2nd ARE mutant	Forward primer 5'- TAAGCTCTTGTAAGTGAGTTGGTACCAAAAGAAAATTAGGCTGAG-3' Reverse primer 5'- CTCAGCCTAATTTTCTTTTTGGTACCAACTCACTTACAAGAGCTT-3'	*Kpn I*

Extension of the oligonucleotide primers was performed using PCR sprint thermal cycler which produced a mutant plasmid containing staggered nicks. Each reaction mixture was subjected to temperature cycling as detailed below.

Thermal cycling conditions for NFkB, CCAAT and NFAT mutant			
Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	95 °C	30s	1
Denaturation Annealing Extension	95 °C 55 °C 68 °C	30 s 1 min 6 min	18
Hold	4 °C	Hold	1

Thermal cycling conditions for 3'UTR mutants			
Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	95 °C	1 min	1
Denaturation Annealing Extension	95 °C 45 °C 68 °C	30 s 1 min 6 min	18
Hold	4 °C	Hold	1

Following thermal cycling 1 µl of *Dpn I* (10 U/µl) was added to the PCR mixture which was incubated at 37 °C for 2 hr. *Dpn I* endonuclease is specific for methylated and hemimethylated DNA, this means that the parental DNA template, isolated from a dam methylated *E. Coli* strain NM522, which does not contain the desired mutation will be digested and only mutant daughter template will remain. The nicked daughter DNA incorporating the desired mutation was transformed into XL1-blue competent cells, which are able to repair the nicked DNA, using the heat shock method as described in section

3.2.2.4.3. Following transformation, 50 µl and 200 µl aliquots of the cells were streaked onto LB-agar plates which contained 30 µg/ml kanamycin and incubated o/n at 37 °C.

3.2.3.2 Screening mutant plasmids

Successful transformation of XL1-blue bacteria with the pEGFP vector will confer resistance to kanamycin, which allows selective screening. However, it is still necessary to screen cells to determine whether they contain the correct mutation. As the mutant primers incorporated new restriction enzyme sites, the colonies were first screened by restriction enzyme digest and then positive colonies were sent for DNA sequencing analysis.

i) Purifying plasmid DNA

Plasmid DNA was purified from bacterial cells using an alkaline lysis method based on a method developed by Birnboim and Doly (Birnboim and Doly, 1979). A sample of the colonies was selected using a sterile pipette tip and grown o/n in 3 ml of LB-media containing 30 µg/ml kanamycin. 2 ml of the bacteria culture was centrifuged for 5 min at 2,000 rpm and the pellet was resuspended in 100 µl of resuspension buffer. The remaining 1 ml of the bacterial culture was frozen with 15 % glycerol and kept at -80 °C for stock. 200 µl of lysis buffer was added to the resuspended bacterial cells and mix by inversion. The cells were lysed for 5 min at RT before adding 150 µl of neutralisation buffer and mixing by inversion. The samples were centrifuged for 5 min at 13,000 rpm 4 °C. The s/n was recovered and the DNA was purified by performing phenol:chloroform:isoamyl alcohol extraction. An equal volume of phenol:chloroform:isoamyl alcohol was added to the s/n, vortex for 10 s and centrifuged for 1 min at 13,000 rpm. The upper aqueous phase was recovered and placed in a clean 1.5 ml tube. 2.5 times volume of 95 % ethanol was added to the DNA solution which was incubated for 1 h at -20 °C to precipitate DNA. The DNA was pelleted by centrifuging at 13,000 rpm for 5 min at 4 °C. The s/n was discarded and the DNA pellet washed with 200 µl 75 % ethanol and centrifuged at 13,000 rpm for 5 min at 4 °C. The s/n was removed and residue ethanol was allowed to evaporate by air drying pellet o/n. The DNA pellet was dissolved in 50 µl 10 mM Tris pH 8.0.

ii) Screening by restriction enzyme digest

As site directed mutagenesis introduced new restriction enzyme sites, mutant colonies could be identified by performing restriction enzyme digests as described below.

1 µl of purified DNA was mixed with 100 U of the appropriate restriction enzyme and 1 X buffer, see table below. 1 X BSA was also added to some of the reaction mixes and the reaction volume was made up to 10 µl using sterile H_2O. The mixtures were incubated at 37 °C for 2 hr and were analysed on a 1 % agarose gel containing ethidium bromide.

Restriction enzyme	Buffer	BSA
Nco I	NEBuffer 3	-
Xba I	NEBuffer 4	+
Afl II	NEBuffer 4	+
Age I	NEBuffer 1	-
Kpn I	NEBuffer 4	+

iii) Isolation of plasmid DNA and DNA sequencing

Plasmid DNA of positive colonies identified by restriction enzyme digest was subsequently purified using Qiagen QIAprep Spin Miniprep kit and sequenced using Big Dye 3.1, see section 3.2.2.4.4, to confirm the correct mutation had been introduced.

3.2.4 Stable transfection of constructs into the U937 cell line

In order to study the effects of different promoter or 3'UTR reporter constructs on EGFP expression the EGFP reporter constructs were stably transfected into the U937 cell lines by electroporation.

U937 cells growing in log phase were washed three times in cold RPMI and then resuspended at 3 x10^7 cells/ml. 450 µl of cells were added to 10 µg DNA in a 0.4 cm electroporation cuvette which was incubated on ice for 10 min. The cells were electroporated with a Gene Pulser (BioRad) using 0.975 mF and 0.270 V which have been previously optimised in house for optimum transfection yield (Powell et al., 1997). Following electroporation the cells were placed on ice for 10 min and were then placed in 5 ml of cold RPMI for 15 min at RT. The cells were placed in additional 30 ml RPMI supplemented with 10 % FCS, antibiotics and 1.25% DMSO and placed at 37 °C with 5 % CO_2 o/n. The following day the cells were collected by centrifugation for 3 min and 1,200 rpm and resuspended in 20 ml of RPMI selection media containing 500 µg/ml G418, 10 % FCS and antibiotics. Medium was refreshed 2-3 times per week. Approximately 2-3 weeks after transfection EGFP fluorescence was assessed by flow cytometry.

3.2.5 Flow cytometry

Once cells were growing in log phase EGFP fluorescence was monitored by flow cytometry as previously described in section 2.3.6.

To monitor changes in C5aR expression, the cells were stained with monoclonal anti-C5aR (S5/1) primary and GAM-FITC secondary antibody for C5aR staining in the presence of EGFP, as previously described in section 2.3.6.

3.2.6 Electrophoretic mobility shift assay

3.2.6.1 Generation of cellular exact

i) Whole cell extract

Cells growing in log phase were harvested by centrifugation at 1,200 rpm for 3 min and washed with ice cold PBS. The cell suspension in PBS was transferred to an eppendorf and centrifuged for a further 5 min at 10,000 rpm at 4 °C. The s/n was discarded and the cell pellet was freeze thawed in liquid N_2 3 times. The pellet was resuspended in 5 times pellet volume of whole cell extract buffer and pipetted up and down several times. The debris was pelleted by centrifugation for 5 min at 10,000rpm, 4°C and the s/n, containing the whole cell extract, was frozen in liquid nitrogen and stored at -80°C. Prior to use protein concentration was determined by performing a Bradford assay, as described in section 2.3.10

ii) Cytosolic and nuclear extract

Cells growing in log phase were harvested by centrifugation at 1,200 rpm for 3 min and washed with ice cold PBS. The cells were counted and resuspended at 5 $x10^6$ cell/ml in cold cytosolic extract buffer. The cells were pelleted by centrifugation for 1 minute at 13,000 rpm, 4°C. The pellet was resuspended in 50 µl/5 $x10^6$ cells cold cytosolic extract buffer which contained 0.1 % NP-40. The suspension was incubated on ice for 5 min, followed by centrifugation for 5 min at 13,000 rpm, 4°C. The s/n, which contained the cytosolic extract was frozen in liquid nitrogen and placed in -80°C for long term storage. The nuclear pellet was resuspended in 15 µl/5 $x10^6$ cells cold nuclear extract buffer and incubated on ice for a further 15 min. Following centrifugation for 5 min at 13,000rpm, 4°C, the s/n was transferred to a new tube and a further 75 µl/5 $x10^6$ cells cold nuclear extract buffer was added to the nuclear extract and was then frozen in liquid nitrogen and stored at -80°C. Prior to use, protein concentration was determined by performing a Bradford assay, as described in section 2.3.10.

3.2.6.2 Incubation of DNA probe with cellular extract

Prior to incubation of the DNA probe with the cellular extract, the single stranded DNA probes were mixed with an equal quantity of complementary sequence probe and heat inactivated for 5 min at 95°C using PCR machine. The probes were cooled on the bench to allow the complementary sequences to anneal thus forming a double stranded DNA probe. Once cooled the dsDNA probes were kept on ice prior to use.

Table 3.2.5 EMSA probes sequences

Probe	Sequence
CCAAT labelled with biotin	Forward 5'-CCCAGTGTGCAGACCAATGAGAGCCCCAGA-3' biotin Reverse 3'-GGGTCACACGTCTGGTTACTCTCGGGGTCT-5'
CCAAT self	Forward 5'-CCCAGTGTGCAGACCAATGAGAGCCCCAGA-3' Reverse 3'-GGGTCACACGTCTGGTTACTCTCGGGGTCT-5'
CCAAT/NF-Y consensus	Forward 5'-AAGAGATTAACCAATCACGTACGGTCT-3' Reverse 3'-TTCTCTAATTGGTTAGTGCATGCCAGA-5'
CCAAT mutant	Forward 5'-CCCAGTGTGCAGACTCTAGAGAGCCCCAGA-3' Reverse 3'-GGGTCACACGTCTGAGATCTCTCGGGGTCT-5'
NFAT labelled with biotin	Forward 5'-AGAGAAAGACGGTCATTTCCTCCCTGCATCTTCC-3' biotin Reverse 3'-TCTCTTTCTGCCAGTAAAGGAGGGACGTGAAGG-5'
NFAT self	Forward 5'-AGAGAAAGACGGTCATTTCCTCCCTGCATCTTCC-3' Reverse 3'-TCTCTTTCTGCCAGTAAAGGAGGGACGTGAAGG-5'
NFAT consensus	Forward 5'-CGCCCAAAGAGGAAAATTTGTTTCATA-3' Reverse 3'-GCGGGTTTCTCCTTTTAAACAAAGTAT-5'
NFAT mutant	Forward 5'- CCCCAGAGAGAAAGACGGTCCTTAAGGCCCTGCATCTTCCCTTGGGG Reverse 3'- GGGGTCTCTCTTTCTGCCAGGAATTCCGGGACGTAGAAGGGAACCCC

The following were mixed in exact order as described; 8 μg cellular extract, 5 x binding buffer, sterile H_2O (to make total volume 20 μl), 1 μg polydI:dC, 40 pmoles competitor and 40 fmoles biotin-labelled probe. The reaction mixture was incubated at RT for 20 min.

3.2.6.3 Non-denaturing polyacrylamide gel electrophoresis

Following incubation, 20 μl of the reaction mixture was loaded onto a 6 % non-denaturing polyacrylamide gel electrophoresis (see table below), which had been pre-run for 20 min at 100 V. The gel was run for approximately 2 hrs at 100 V, 4 °C in 1 x TBE, until the control lane which contained bromophenol blue within the binding buffer had reached approximately $^2/_3$ down the gel.

6 % non-denaturing polyacrylamide gel	
40 % Acrylamide (29:1)	2.25 ml
5 x TBE	1.5 ml
Sterile H_20	11.085 ml
10 % APS	150 μl
TEMED	15 μl

3.2.6.4 Denaturing polyacrylamide gel electrophoresis

To obtain approximate information regarding the molecular weight of the protein bound to the DNA probe a denaturing SDS-polyacrylamide gel electrophoresis was performed. Following incubation, the reaction mixture was placed in a 96-well microtitre plate and cross-linked twice using the autocross-linking settings on the Stratalinker ® UV Cross Linker, which emits 1200 microjoules of UV. The reaction mixture was then mixed with an equal volume of protein sample buffer, as described in Chapter 2, which contained 0.5 mM DTT. 20 µl of the reaction mixture was loaded onto a 12.5 % separating gel with 6 % stacking gel and run as previously described in 2.3.7. Once the samples had been separated the protein-DNA complexes were transferred to nitrocellulose membrane as described in section 2.3.7 and was then visualised using protocol described in section 3.3.6.6.

3.2.6.5 Transfer Biodyne® Nylon membrane

Protein-DNA binding complexes, which were separated on a non-denaturing polyacrylamide gel, were transferred onto a positively charged Biodyn® Nylon membrane. To do this, the gel was overlaid with the nylon membrane which had been pre-soaked in 1x TBE and sandwiched between 2 pieces of soaked filter paper (Whatman #5) and 1-2 thin sponges, on either side. The gel-membrane 'sandwich' was loaded into a transfer apparatus unit (Hoefer) according to the manufacturer's instructions, with the membrane placed between the gel and the positive electrode. The proteins were transferred onto the nylon membrane by electrophoresis in 1x TBE for 1 hr at 100 V (constantly cooled with water passing across the base apparatus).

3.2.6.6 Detection of biotinylated labelled DNA probe by chemiluminescence

Prior to detecting biotinylated DNA probe using chemiluminescence, the DNA-protein complexes were cross-linked onto the nylon or nitrocellulose (for denaturing PAGE) membrane using Stratalinker ® UV Cross Linker, by placing the membrane facing upwards towards the lamps and using the autocross-linking function which emits 1200 microjoules of UV.

Once the DNA-protein complexes had been cross-linked to the membrane, the blots were developed using Pierce Chemiluminescent Nucleic Acid Detection Kit (89880, Pierce) following manufacturer's instructions. Briefly, the blocking and 4x wash buffer were warmed in a 37 °C water bath until precipitate had dissolved. The membrane was blocked with 15 ml of blocking buffer for 15 min with gentle agitation. 66.7 µl of stabilised streptavidin-horseradish peroxidase conjugate was added to 20 ml of blocking buffer and the blocking buffer was decanted from the membrane and replaced with the conjugated/blocking solution. 1x Wash solution was prepared by adding 25 ml of 4x wash buffer with 75 ml ultrapure water.

Following incubation of the membrane for a further 15 min, the membrane was transferred to a new container and washed 5 times with 20 ml wash solution. The membrane was transferred to another new container and incubated for 5 min with 30 ml of substrate equilibration buffer, with gentle agitation. The substrate working solution was prepared by mixing 3 ml of luminol enhancer solution to 3 ml stable peroxide solution. The membrane was removed from the substrate equilibration buffer and excess buffer was blotting the membrane carefully onto paper towel. The substrate working solution was poured onto the membrane which was incubated for a further 5 min without shaking. The membrane was removed from the working solution and was then placed between two clear plastic sheets avoiding bubbles. The membrane was exposed by placing it in a film cassette and exposing X-ray film for approximately 5 min.

3.3 Results

To determine how C5aR expression is regulated in the mammalian U937 cell line, the C5aR 5'promoter region and 3'UTR were cloned into the pEGFP-1 reporter vector. By placing the C5aR 5'promoter region upstream to the EGFP gene the rate at which EGFP is transcribed, once stably transfected into the U937 cell line, is dependent on the *cis*-acting elements within the promoter region and also the presence of *trans*-acting factors within this cell line. Likewise, by placing the C5aR 3'UTR downstream of the EGFP gene the stability of the EGFP mRNA can be regulated by the presence of AU-rich elements within the 3'UTR.

3.3.1. Cloning the C5aR 5'promoter fragments and 3'UTR into the pEGFP vector

To clone the C5aR 5'promoter region and 3'UTR, gDNA was extracted from the $U937_{EC}$ and $U937_{PM}$ cell lines. Specific primers for the C5aR 5'promoter region and 3'UTR were designed for PCR as shown in figures 3.3.1 and 3.3.2 respectively. PCR of the C5aR 2Kbp promoter region was performed using high fidelity polymerase and separate reactions were performed using gDNA isolated from either $U937_{EC}$ or $U937_{PM}$ as the template DNA (figure 3.3.3). 5'Promoter deletions were then created by PCR using the 2Kbp PCR fragment generated from $U937_{EC}$ gDNA as the template DNA (figure 3.3.4). Restriction enzyme digestion was used to create the 355 bp promoter construct. PCR of the C5aR 3'UTR was performed using GoTaq Flexi polymerase due to differences between the Tm of the primers which could not be prevented (figure 3.3.5). Following PCR the DNA was purified and, where appropriate, the DNA underwent extension with GoTaq polymerase to add additional 3' A residues prior to cloning into pGEM-T. The DNA was ligated into the pGEM-T vector and transformed into chemically competent NM522 using heat shock. Successfully transformed NM522s were screened for the correct insert using both size screening and PCR screening methods. Figure 3.3.6 shows an example of a size screen of colonies that were selected as potential positives. Lane 3 shows a potential positive colony as it has run slower through the gel compared with the samples in lanes 2, 4 and 5. Positive colonies were confirmed by DNA sequence analysis.

Figure 3.3.1 Human C5aR promoter region gDNA sequence (accession number AC_099491). Forward primers as indicated in blue were used to create a series of 5'promoter deletions when paired with the reverse primer indicated in red. Potential transcription factor binding sites which were later mutated using site directed mutagenesis are illustrated in green.

```
        -2035       -2025       -2015       -2005       -1995
          2Kbp forward primer
   1    CCCTTCGCTC ATATTACCGC CTGATCTGTC AGATCAGGGG CGGAATTACA
        GGGAAGCGAG TATAATGGCG GACTAGACAG TCTAGTCCCC GCCTTAATGT
        -1985       -1975       -1965       -1955       -1945
  51    TTCTCATAAG AGCGTGAACC CAGGCCGGGC GCAGTGGCTC AAGCCTGTAA
        AAGAGTATTC TCGCACTTGG GTCCGGCCCG CGTCACCGAG TTCGGACATT
        -1935       -1925       -1915       -1905       -1895
 101    TCCCAGCATT TTGGGAGGCT GAGGCGGGTG GATCACGAGG TCAGGAGTTC
        AGGGTCGTAA AACCCTCCGA CTCCGCCCAC CTAGTGCTCC AGTCCTCAAG
        -1885       -1875       -1865       -1855       -1845
 151    GAGATCAGCC TGGCCAAGAT GGTGAAACCA CGTCTCTACT AAAAATACAA
        CTCTAGTCGG ACCGGTTCTA CCACTTTGGT GCAGAGATGA TTTTTATGTT
        -1835       -1825       -1815       -1805       -1795
 201    AAATTAGCCG GGTGCAGTGG TAGGCACCTG TAGTCCCAGC TACTCGGGAG
        TTTAATCGGC CCACGTCACC ATCCGTGGAC ATCAGGGTCG ATGAGCCCTC
        -1785       -1775       -1765       -1755       -1745
 251    GCTGAGGCAG GAGAATCGCT TGAACCTGGG AGGCGGAGGT TGCAGTGTGC
        CGACTCCGTC CTCTTAGCGA ACTTGGACCC TCCGCCTCCA ACGTCACACG
        -1735       -1725       -1715       -1705       -1695
 301    CGAGATCGTG CCACTGCACT CTAGCCTGGG TGAGAGAGTG AGACTCCATC
        GCTCTAGCAC GGTGACGTGA GATCGGACCC ACTCTCTCAC TCTGAGGTAG
        -1685       -1675       -1665       -1655       -1645
 351    TCAAAAAAAA AAAAAAAAAA AAAAAGAGCA TGAACCCTGT TGTGAACAGC
        AGTTTTTTTT TTTTTTTTTT TTTTTCTCGT ACTTGGGACA ACACTTGTCG
        -1635       -1625       -1615       -1605       -1595
 401    GCATGTGAGG GATCTGGGTT GCAAGCTCCT TATGAGAATC TAATGCCTGA
        CGTACACTCC CTAGACCCAA CGTTCGAGGA ATACTCTTAG ATTACGGACT
        -1585       -1575       -1565       -1555       -1545
 451    TGATCTGTCA CTGTATCCCA TCACCCCCAG ATAGGACCAT CTCGTTACAG
        ACTAGACAGT GACATAGGGT AGTGGGGGTC TATCCTGGTA GAGCAATGTC
        -1535       -1525       -1515       -1505       -1495
                     1.5Kbp forward primer
 501    GAAAACAAGC TCAGGGATTC CACTGATTCT ACATTATGTT GAATTGTATA
        CTTTTGTTCG AGTCCCTAAG GTGACTAAGA TGTAATACAA CTTAACATAT
```

```
        -1485       -1475       -1465       -1455       -1445
 551    ATTATTTCAT TATATATTAC AATGTAACAA TAATAGATGG AGATGGTTGT
        TAATAAAGTA ATATATAATG TTACATTGTT ATTATCTACC TCTACCAACA
        -1435       -1425       -1415       -1405       -1395
 601    GGCTTGTGTA GTTGGGGGAC AGGGTAGCTC CATTTTCTAT TGCCAACTTT
        CCGAACACAT CAACCCCCTG TCCCATCGAG GTAAAAGATA ACGGTTGAAA
        -1385       -1375       -1365       -1355       -1345
 651    ATAACCCTAG AAGGATGATT TTTTTCTTTT CTTTTCTTTT TCTTTTCTTT
        TATTGGGATC TTCCTACTAA AAAAAGAAAA GAAAAGAAAA AGAAAAGAAA
        -1335       -1325       -1315       -1305       -1295
 701    TTTTTTTTTT TGAGATTGAG TCTTGGTGTT GTCAGCCTGG GCTGGAGTGC
        AAAAAAAAAA ACTCTAACTC AGAACCACAA CAGTCGGACC CGACCTCACG
        -1285       -1275       -1265       -1255       -1245
 751    AATGGTGCAA TCTCTGCTCA CTGCAACCTC CGCCTCCTGG GTTCCAGCAA
        TTACCACGTT AGAGACGAGT GACGTTGGAG GCGGAGGACC CAAGGTCGTT
        -1235       -1225       -1215       -1205       -1195
 801    TTCTCCTGCC TCAGCCTTCT GAGTAGCTGA GATTACAGGC ACCCACCACC
        AAGAGGACGG AGTCGGAAGA CTCATCGACT CTAATGTCCG TGGGTGGTGG
        -1185       -1175       -1165       -1155       -1145
 851    ACGCCTGGCT AATTTTTGTA TTTTTAGTAG AGATGGGGTT TCACCATATT
        TGCGGACCGA TTAAAAACAT AAAAATCATC TCTACCCCAA AGTGGTATAA
        -1135       -1125       -1115       -1105       -1095
 901    GGCCAGGCTG GTGACGATGG TTTTTTTTTT TTGAGACAGA GTCTTGCTTT
        CCGGTCCGAC CACTGCTACC AAAAAAAAAA AACTCTGTCT CAGAACGAAA
        -1085       -1075       -1065       -1055       -1045
 951    GTTGCCCAGG CTGGAGTGCA GTGGCGCAAT CTTGGCTCAC TGCAACCTCT
        CAACGGGTCC GACCTCACGT CACCGCGTTA GAACCGAGTG ACGTTGGAGA
        -1035       -1025       -1015       -1005       -995
                                                  1Kbp forward primer
1001    GCCTCCCAGG TTTAAGCAAT TCTCCTGCCT CAGCTTCCCA AGTAGCTGGG
        CGGAGGGTCC AAATTCGTTA AGAGGACGGA GTCGAAGGGT TCATCGACCC
        -985      -975        -965        -955        -945
1051    ATTACAGGCA CCTGCCACCA CACCTGGGTA ATTTTTGTAT TTTTAGTAGA
        TAATGTCCGT GGACGGTGGT GTGGACCCAT TAAAAACATA AAAATCATCT
        -935        -925        -915        -905        -895
1101    GACAGGGTTT CACCATGTTG GCCAGACTCG TCTTGAACTC CTGACCTCAA
        CTGTCCCAAA GTGGTACAAC CGGTCTGAGC AGAACTTGAG GACTGGAGTT
        -885        -875        -865        -855        -845
1151    GTGATTCATT TGCCTCAGCC TCCCAAAGTG CTGGGATTAT AGGTGTGAGC
        CACTAAGTAA ACGGAGTCGG AGGGTTTCAC GACCCTAATA TCCACACTCG
```

```
        -835        -825        -815        -805        -795
1201    CATTGCACCT GGCCCTAGAA GGAAGATTTA ACAGTAAAAT ATACTTAAAT
        GTAACGTGGA CCGGGATCTT CCTTCTAAAT TGTCATTTTA TATGAATTTA
        -785        -775        -765        -755        -745
1251    GCTGGGCTAC CTCCGCAGAC TAGCTCAGCC TCTTTGGGTC TCAAATCAGC
        CGACCCGATG GAGGCGTCTG ATCGAGTCGG AGAAACCCAG AGTTTAGTCG
        -735        -725        -715        -705        -695
1301    CTGGCCGGGA GGCTGAGTGG AGGTTACAGT CTCTGCTTTC TTGAATCCCA
        GACCGGCCCT CCGACTCACC TCCAATGTCA GAGACGAAAG AACTTAGGGT
        -685        -675        -665        -655        -645
1351    AGGGAGGGGT TTAAGCCAAC TGCATTCCTG AGCTTTTGCC CAGAAGGCTG
        TCCCTCCCCA AATTCGGTTG ACGTAAGGAC TCGAAAACGG GTCTTCCGAC
        -635        -625        -615        -605        -595
1401    GCGTCTTAGG AGGAAAGAGA TAGATGGCTC CAGGCCGGGC GCAGTGGCTC
        CGCAGAATCC TCCTTTCTCT ATCTACCGAG GTCCGGCCCG CGTCACCGAG
        -585        -575        -565        -555        -545
1451    ACACTTGTAA TTCTAGCACT TTGGGAGGTT GAGGTGGGTG ATTGCCTGAG
        TGTGAACATT AAGATCGTGA AACCCTCCAA CTCCACCCAC TAACGGACTC
        -535        -525        -515        -505        -495
                                                  500bp forward primer
1501    CTCAGGAGTT TGAGTCCAGC CTGGCCAACA TGGCGAAACC CCGTCTTTAC
        GAGTCCTCAA ACTCAGGTCG GACCGGTTGT ACCGCTTTGG GGCAGAAATG
        -485        -475        -465        -455        -445
1551    TAAAAATACA AAAAATTAGC CGGGGGTGGT GACACACACG TGTAATCCCA
        ATTTTTATGT TTTTTAATCG GCCCCCACCA CTGTGTGTGC ACATTAGGGT
        -435        -425        -415        -405        -395
1601    GCTACTCGGG AGGCTGAGGC AGGAGAAGTG CTTGAACCCA GGAGGCAGAG
        CGATGAGCCC TCCGACTCCG TCCTCTTCAC GAACTTGGGT CCTCCGTCTC
        -385        -375        -365        -355        -345
                                             PstI used for 355bp fragment
                                             ~~~~~~
1651    GTTACAGAGA GCCGAGATTG CACCACTCCA CTGCAGCCTG GGCGACACAG
        CAATGTCTCT CGGCTCTAAC GTGGTGAGGT GACGTCGGAC CCGCTGTGTC
        -335        -325        -315        -305        -295
1701    CAAGACTCTA TCAAAAAAAA AAAAAAAAAA ATGAGAGAGA AGAGATGGCC
        GTTCTGAGAT AGTTTTTTTT TTTTTTTTTT TACTCTCTCT TCTCTACCGG
        -285        -275        -265        -255        -245
                                                       NFkB site
1751    CCAAATAGGG AAACCAAGGC CAGGAGAGGG GCCGAGCCTG CACAGGAGCT
        GGTTTATCCC TTTGGTTCCG GTCCTCTCCC CGGCTCGGAC GTGTCCTCGA
```


-235 -225 -215 -205 -195
200bp forward primer
1801 TCCTCGGTTT TCCGAGCGCC GGCCCCCCTT CTCTGCCTGG GAGGAGGTGG
AGGAGCCAAA AGGCTCGCGG CCGGGGGGAA GAGACGGACC CTCCTCCACC
-185 -175 -165 -155 -145
1851 TTAGAGTCCC CTGGGTGTGT GCCCCGCAGA GGGAGCTCTG GCCTCAGTGC
AATCTCAGGG GACCCACACA CGGGGCGTCT CCCTCGAGAC CGGAGTCACG
-135 -125 -115 -105 -95
CCAAT binding factors (NFY) 100bp forward primer
1901 CCAGTGTGCA GACCAATGAG AGCCCCAGAG AGAAAGACGG TCATTTCCTC
GGTCACACGT CTGGTTACTC TCGGGGTCTC TCTTTCTGCC AGTAAAGGAG
Nuclear factor of activate T-cells
-85 -75 -65 -55 -45
1951 CCTGCATCTT CCCTTGGGGC TTTAAAAACC ACAGCCCTTG GGCAGGAGGG
GGACGTAGAA GGGAACCCCG AAATTTTTGG TGTCGGGAAC CCGTCCTCCC
-35 -25 -15 -5
Start codon
2001 ACCTTCGATC CTCGGGGAGC CCAGGAGACC AGAACATGGT GAGTCTCGAA
TGGAAGCTAG GAGCCCCTCG GGTCCTCTGG TCTTGTACCA CTCAGAGCTT
Reverse primer

Figure 3.3.2 Human C5aR 3'UTR cDNA sequence. Primers used for PCR are highlighted in blue for the forward primer and red for the reverse primer. The two AU rich elements (ARE) are highlighted in green and the polyadenylation signal is underlined.

Forward primer

```
   1  GCGACAGCCT CATGGGCCAC TGTGGCCCGA TGTCCCCTTC CTTCCCGGCC
  51  ATTCTCCCTC TTGTTTTCAC TTCACTTTTC GTGGGATGGT GTTACCTTAG
 101  CTAACTAACT CTCCTCCATG TTGCCTGTCT TTCCCAGACT TGTCCCTCCT
 151  TTTCCAGCGG GACTCTTCTC ATCCTTCCTC ATTTGCAAGG TGAACACTTC
 201  CTTCTAGGGA GCACCCTCCC ACCCCCCACC CCCCCCACAC ACACCATCTT
 251  TCCATCCCAG GCTTTTGAAA AACAAACAGA AACCCGTGTA TCTGGGATAT
 301  TTCCATATGG CAATAGGTGT GAACAGGGAA CTCAGAATAC AGACAAGTAG
                              1st ARE
 351  AAAGATTCTC GCTTAAAAAA AATGTATTTA TTTTATGGCA AGTTGGAAAA
 401  TATGTAACTG GAATCTCAAA AGTTCTTTGG GACAAAACAG AAGTCCATGG
                              2nd ARE
 451  AGTTATCTAA GCTCTTGTAA GTGAGTTAAT TTAAAAAAGA AAATTAGGCT
 501  GAGAGCAGTG GCTCACGCCT GTAATCCCAG AACTTTGGGA GGCTAAGGTG
 551  GGTGGATCAC CTGAGGTCAA GAGTTCCAGA CCAGGCTGGC CAGCATGGTG
 601  AAACCCCGTC TGTACTAAAA ATACAAAAAA TTAACTGGGC ATGGTAGTGG
 651  GTGCCTGTAA TCCCAGCTAC TTGGGAGGCT GAGGTGGGAG AATTGCTCGA
 701  ACTTGGAGGT GGAGGTTGTG GTGAGCCATG ATCGCACCAC TGCACTCTAG
 751  CCTGGGTGAC CGAGGGAGGC TCTGTCTCAA AAGCAAAGCA AAAACAAAAA
 801  CAAAAACACC TAAAAAACCT GCAGTTTTGT TTGTACTTTG TTTTTAAATT
 851  ATGCTTTCTA TTTTGAGATC ATTGCAAACT CAACACAATT GTAAGTAATG
 901  ATACAGAGGG ATCTTGTGTA CCCTTCACCC AGCCTCCCCC AATGGCAACA
 951  TCTTGCAAAA CTACAATGTA GTCTCATAAC CAGGATATTG ACATTGATAC
1001  AGTGAAGATA CAGGACATTC TCATCACCAC AGGGATCCCC AGGATGCCCA
1051  CTTCCCTCCA CCCCCACACC CCAGCCGTGT CCCTAACCCC TGGCAACCAG
1101  GAATCCACTC TCCATTTCTA TAATGTTGTC ATTTCAAGAA TGTTATTCAA
1151  TGGAATCATA TAGTATGTAA CCTGTTTTGA GCTTAAAAAA AAAGTATACA
                        Reverse primer
1201  TGACTTTAAT GAGGAAAATA AAAATGAATA TTG
                  Polyadenylation signal
```

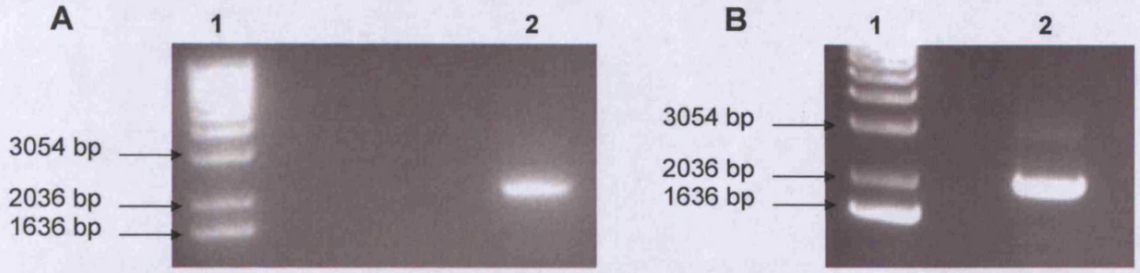


Figure 3.3.3 PCR of the C5aR 2Kbp 5'promoter region. Genomic DNA isolated from $U937_{EC}$ (A) or $U937_{PM}$ (B) was used as a template for PCR amplification of the C5aR 2Kbp 5'promoter region. To determine the approximate molecular weight the PCR product were analysed by agarose (1 %) gel electrophoresis. (A) Lane 1 molecular weight markers and lane 2 PCR using C5aR 2Kbp 5'promoter specific primers. (B) Lane 1 molecular weight markers and lane 2 PCR using C5aR 2Kbp specific primers.

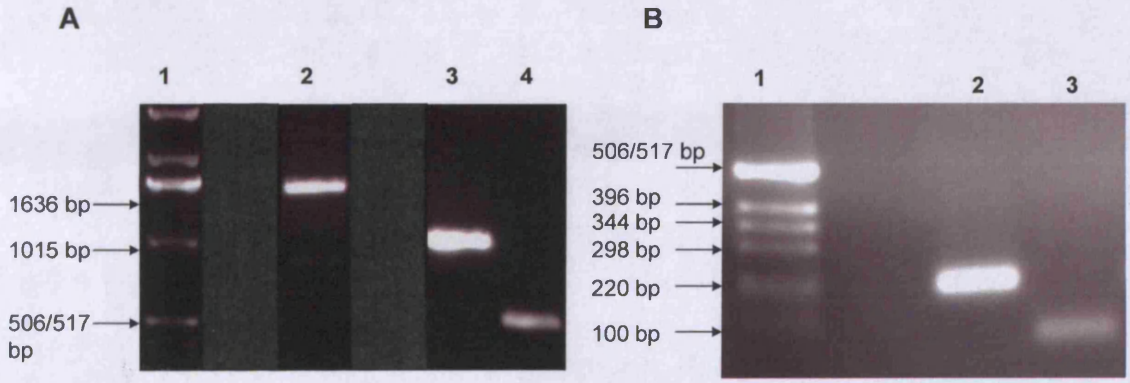


Figure 3.3.4 PCR of the 5'promoter deletion fragments. The 5'promoter deletions were generated by PCR using the 2 Kbp 5'promoter fragment from $U937_{EC}$ as the template. To determine the approximate molecular weights, PCR products were analysed by agarose (1 %) gel electrophoresis. (A) lane 1, molecular weight markers; lane 2, PCR using 1.5 Kbp 5' promoter specific primers; lane 3, PCR using 1 Kbp 5'promoter specific primers; lane 4, PCR using 500 bp 5'promoter specific primers. (B) Lane 1, molecular weight markers; lane 2, 200 bp 5'promoter specific primers; lane 3, 100 bp 5'promoter specific primers.

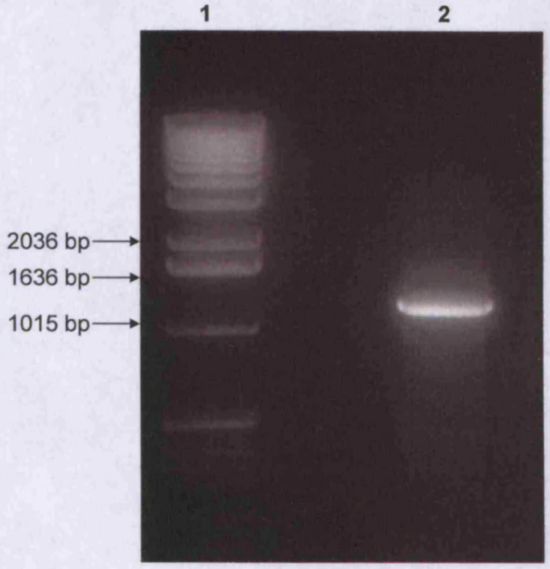


Figure 3.3.5 PCR of the C5aR 3'UTR. $U937_{EC}$ gDNA was used as a template for PCR amplification of the C5aR 3'UTR region. To determine the approximate molecular weight the PCR product were analysed by agarose (1 %) gel electrophoresis; lane 1, molecular weight markers; lane 2, PCR using C5aR 3'UTR specific primers.

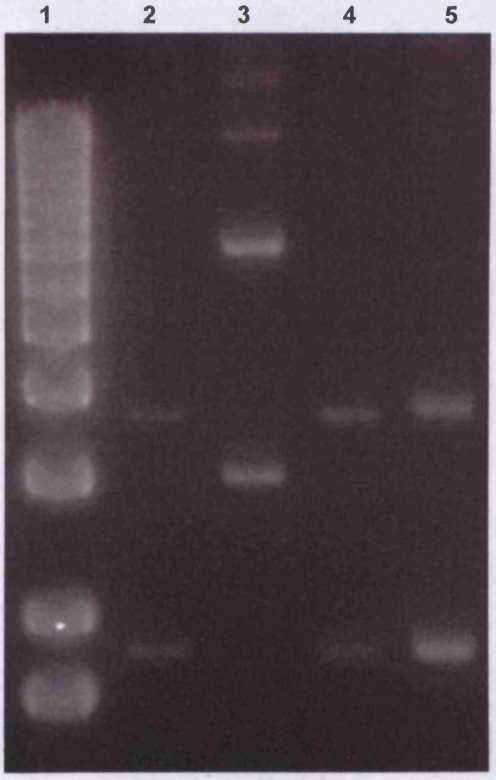


Figure 3.3.6 Size screening for potentially positive colonies. Plasmid DNA was isolated from potential 2Kbp 5'promoter/pGEM-T colonies using the alkaline lysis method as described in section 3.2.2.4.4. To identify potential positive colonies, plasmid DNA was separated by agarose (0.7 %) gel electrophoresis; lane 1, molecular weight markers; lanes 2, 4 and 5, show colonies with just the pGEM-T vector; lane 3, successful 2Kbp 5'promoter/pGEM-T colony.

In order to obtain EGFP reporter constructs that could then be transfected into the U937 cell lines, the C5aR 5'promoter fragments or 3'UTR were subcloned into the pEGFP-1 or EF1α+pEGFP-1 vectors respectively using restriction enzymes sites which were incorporated during PCR (see tables 3.2.1 and 3.2.2 for full details). Figure 3.3.7 shows both the pGEM-T and pEGFP-1 vector systems used for cloning and important restriction enzyme sites. Figure 3.3.8 shows in lane 2 the 2Kbp 5'promoter has been successfully excised from 2kbp 5'promoter/pGEM-T, in lane 3 successfully digested pEGFP-1 vector. NM522 bacteria were transformed with the 5'promoter+pEGFP or EF1a+pEGFP+3'UTR constructs. Colonies resistant to kanamycin were screened by size and PCR. Figure 3.3.9 shows an example of a PCR screen of colonies which were being screened for the 2 Kbp 5'promoter fragment; lane 2 shows a positive colony as a band was seen at approximately 2 Kbp; lane 1 shows a negative colony which contains a smaller insert. Positive colonies were confirmed by sequence analysis, which were then aligned with published sequences.

Detailed in appendix 9.1 are the full sequences for the 5'promoter fragments generated using gDNA from $U937_{EC}$. Appendix 9.2 shows the consensus 2 Kbp promoter sequence from $U937_{EC}$ and $U937_{PM}$ aligned with the human sequence already published by Gerard and colleagues and GeneBank AC_099491, as well as, the chimpanzee promoter sequence.

Seven base pair differences were found between the $U937_{EC}$ and $U937_{PM}$ 5'promoter sequences (table 3.3.1). Three of these differences (-1663bp, -1662 and -1661bp) are additional adenosine residues present in the $U937_{PM}$ and chimpanzee sequence but not the $U937_{EC}$. This region of the promoter contains 25 adenosine residues repeated, which could potentially result in errors during cloning procedures. However, as they are also present within the chimpanzee sequence this suggests they could be insertions/deletions. The $U937_{EC}$ sequence has a C insertion at position -730bp and a G to A substitution at position -464bp, which are unique to this cell line suggesting that this could be due to errors during cloning or sequencing (table 3.3.1). However both these differences resulted in an insertation or deletion of a new putative *cis*-acting element compared with the $U937_{PM}$ cells as predicted by MatInspector (table 3.3.2). The C insertion at position -730bp resulted in a new putative site, E2F-myc activator/cell cycle regulator, being present in the $U937_{EC}$ cells promoter but absent in the $U937_{PM}$ promoter. Furthermore, the matrix similarity, which is the similarity with the consensus neighbouring residues (not key residues), altered slightly in the putative neuron-restrictive silencer factor between the $U937_{EC}$ and $U937_{PM}$ promoter (table 3.3.2). The -464 G/A substitution resulted in the putative zinc binding proteins factor sites being absent in the $U937_{EC}$ promoter compared with the $U937_{PM}$ (table 3.3.2). Several other substitutions (A/G -464bp, A/G -396bp and T/C -253bp) have been previously been identified as SNPs with their unique SNP identification number listed in table 3.3.2. Both the -396bp

(A/G) and -253bp (T/C) substitution resulted in a reduced core similarity in two putative sites, activator protein 2 and MAF and AP1 related factors, between the two promoters (table 3.3.2). However these differences indentified by MatInspector do not necessarily equate to changes in promoter activity as these sites are only predicted sites. Shown in appendix 9.5 are the sequence alignments for the C5aR 3'UTR region. There are 8bp differences between the $U937_{EC}$ and $U937_{PM}$ 3'UTR, 7 of these differences are unique to either one of these sequences. Shown in figure 3.3.10 is a schematic diagram of the C5aR 5' promoter and 3'UTR EGFP reporter constructs generated.

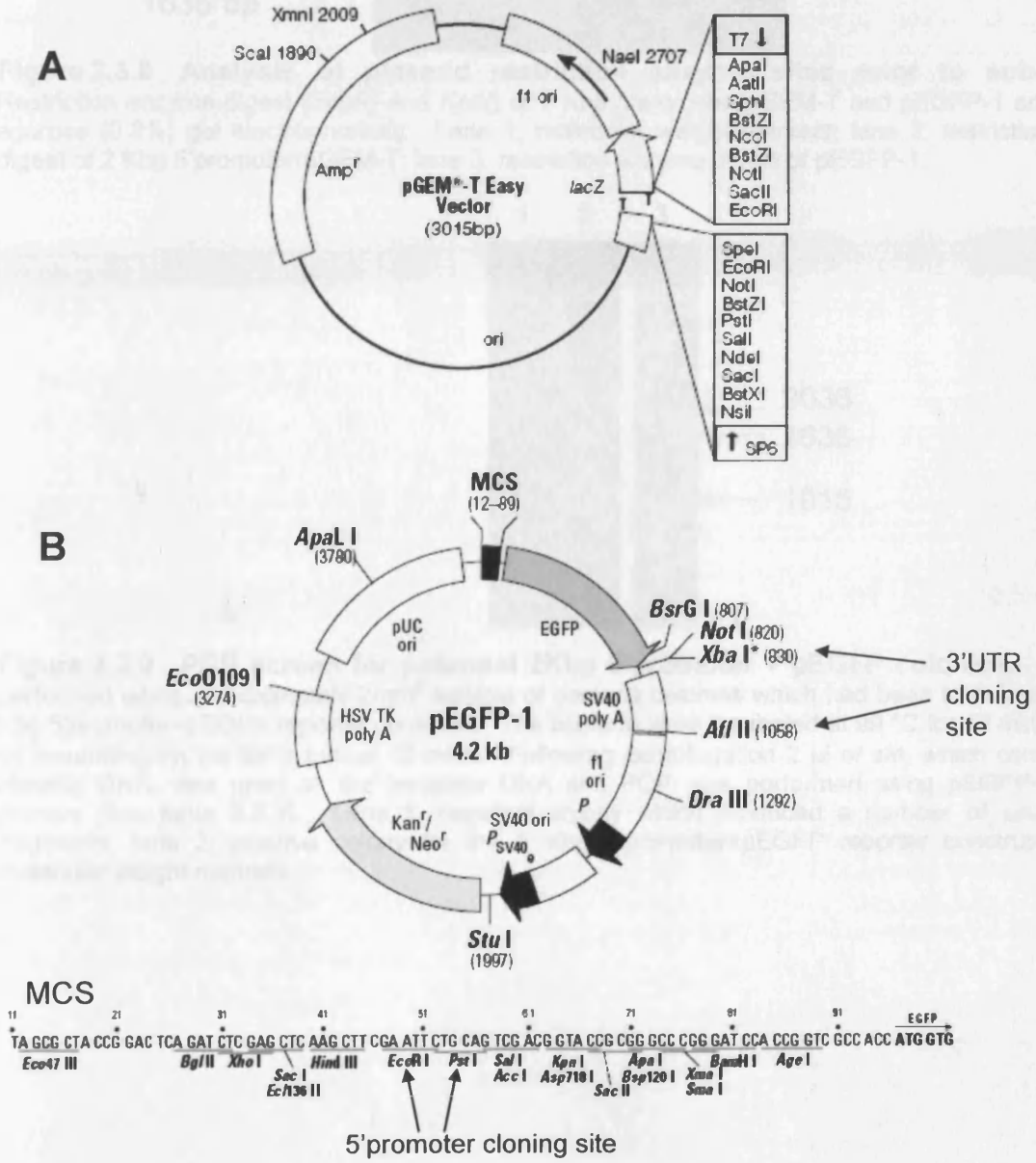


Figure 3.3.7 Plasmid maps. pGEM-T Easy vector (A) and pEGFP-1 (B) vector maps with reference points.

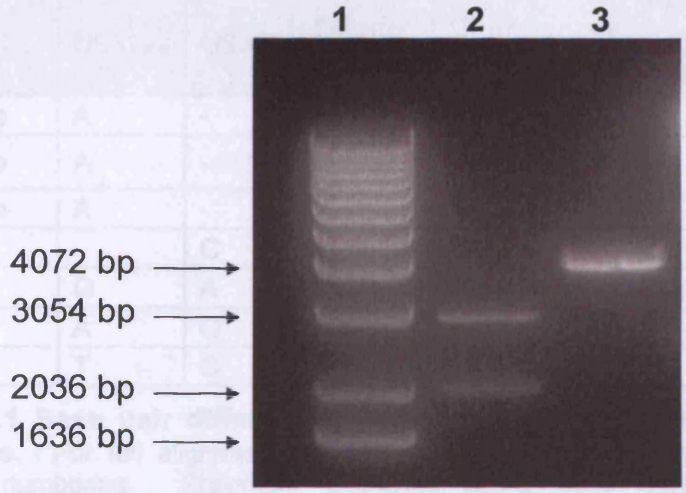


Figure 3.3.8 Analysis of plasmid restriction enzyme sites prior to sub-cloning. Restriction enzyme digest (*EcoRI* and *KpnI*) of 2 Kbp 5'promoter/pGEM-T and pEGFP-1 analysed by agarose (0.8%) gel electrophoresis. Lane 1, molecular weight markers; lane 2, restriction enzyme digest of 2 Kbp 5'promoter/pGEM-T; lane 3, restriction enzyme digest of pEGFP-1.

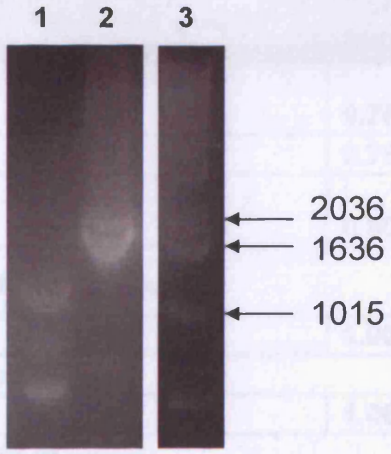


Figure 3.3.9 PCR screen for potential 2Kbp 5'promoter + pEGFP colonies. PCR was performed using approximately 2mm^2 sample of bacteria colonies which had been transformed with 2 Kbp 5'promoter+pEGFP reporter construct. The bacteria were incubated at 99 °C for 10 mins followed by incubating on ice for a further 10 mins. Following centrifugation 2 µl of s/n, which contained the plasmid DNA, was used as the template DNA and PCR was performed using pEGFP-1 specific primers (see table 3.2.3). Lane 1, negative colony which produced a number of smaller DNA fragments; lane 2, positive colony for the 2 Kbp 5'promoter+pEGFP reporter construct; lane 3, molecular weight markers.

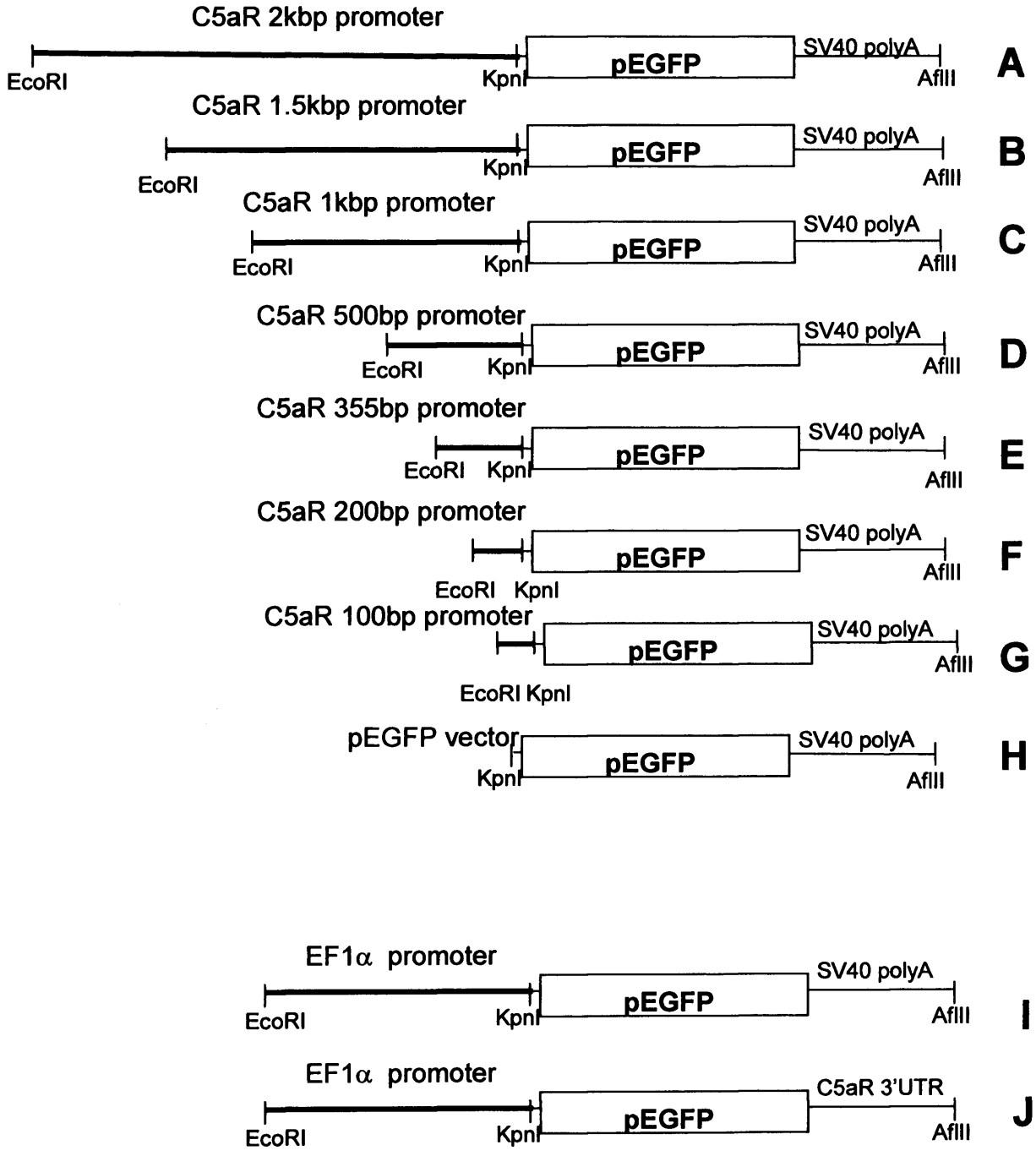
Position	$U937_{PM}$	$U937_{EC}$	Gene bank	Chimpanzee sequence	Published by (Gerard et al., 1993)	SNPs previously identified
-1663 bp	A	-	-	A		
-1662 bp	A	-	-	A		
-1661 bp	A	-	-	A		
-730 bp	-	C	-	-		
-464 bp	G	A	G	G		rs60925053
-396 bp	A	G	A	G		rs59259352
-253 bp	T	C	C	C	T	rs10404456

Table 3.3.1 Base pair differences between the $U937_{EC}$ and $U937_{PM}$ 2 Kbp promoter sequences. For full alignment see appendix 9.2. Position number is based on the $U937_{EC}$ sequence numbering. Previously published SNPs were identified using blastn suite-SNP (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_SPEC=SNP&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on).

	Position		Strand	Core Similarities	Matrix Similarities
	From	to			
Neuron-restrictive silencer factor	**-738**	**-718**	**-**	**0.782**	**0.707**
	-738	**-718**	**-**	**0.782**	**0.721**
E2F-myc activator/cell cycle regulator	**-737**	**-721**	**+**	**0.809**	**0.859**
	Absent in $U937_{PM}$				
Zinc binding protein factors	**-474**	**-452**	**-**	**1.000**	**0.925**
	Absent in $U937_{EC}$				
Zinc binding protein factors	**-471**	**-449**	**-**	**1.000**	**0.943**
	Absent in $U937_{EC}$				
Activator protein 2	**-469**	**-455**	**+**	**0.881**	**0.925**
	-469	**-455**	**+**	**0.830**	**0.912**
MAF and AP1 related factors	**-258**	**-238**	**+**	**0.875**	**0.948**
	-258	**-238**	**+**	**0.750**	**0.905**

Table 3.3.2 Putative sites which differ between the $U937_{EC}$ (in green) and $U937_{PM}$ (in blue). Core similarity is the similarity between the core residues of the consensus sequences for the putative sites. Matrix similarities are the similarities between the surrounding residues which are less critical for transcription factor binding.

Figure 3.3.10 Schematic diagram of C5aR 5'promoter and 3'UTR EGFP reporter constructs. The C5aR 5'promoter region was cloned upstream of the EGFP gene, where as the 3'UTR was placed down stream. (A) C5aR 2 Kbp 5'promoter fragment; (B) C5aR 1.5 Kbp 5'promoter fragment; (C) C5aR 1 Kbp 5'promoter fragment; (D) C5aR 500 bp 5'promoter fragment; (E) C5aR 355 bp 5'promoter fragment; (F) C5aR 200 bp 5'promoter fragment; (G) C5aR 100 bp 5'promoter fragment; (H) promoter-less pEGFP vector, negative control; (I) EF1α promoter + pEGFP + SV40; (J) EF1a promoter + pEGFP + C5aR 3'UTR.



3.3.2 The majority of the 2Kbp 5'promoter is dispensable for expression

By placing the C5aR 2Kbp 5'promoter region upstream of the EGFP cDNA, the rate at which EGFP is transcribed is dependent on the *cis*-acting elements present within this promoter. EGFP was chosen as it allows changes in transcription activity and therefore EGFP expression to be easily detected by flow cytometry. Transfection of the 2Kbp 5'promoter EGFP reporter constructs into the mammalian U937 cell lines will allow us to investigate how the expression of the C5aR is regulated in this cell line. Subsequent deletion analysis can then identify regions within the promoter that contain strong promoter or suppressor activity which together with Genomatix MatInspector Software can be used to predict potential transcription factor binding sites.

The 5'promoter+pEGFP reporter constructs were stably transfected into the U937 cell line using electroporation. Following electroporation the cells were placed in complete RPMI media with 1.25% DMSO for 24 h and then in 500 μg/ml G418 selection media for 2-3 weeks. DMSO has previously shown to improve transfection efficiency in HL-60, TR146, Cos-7 and L132 cell lines, and was applied to these experiments as it reduced the time needed for the U937 cells to recover following electroporation from 4 weeks to 2-3 weeks, data not shown (Melkonyan et al., 1996). Once the cells had recovered and were growing in log phase, EGFP fluorescence was analysed by flow cytometry. Figure 3.3.11 shows a typical histogram plot of U937 cells transfected with an EGFP reporter construct. The histogram plot shows two different fluorescent cell populations, an EGFP negative and an EGFP positive population. The EGFP negative population was always present, even after 2-3 weeks in G418 selection media which killed the non-transfected U937 control cells. These results suggest that the EGFP negative population expresses the neomycin-resistance cassette but not the EGFP gene. Stable cell lines are generated when the exogenous DNA aligns with the chromosomal DNA of the cell and a homologous recombination event occurs which results in the exogenous DNA being inserted into the chromosomal DNA. It is therefore possible that during the homologous recombination only the neomycin resistance gene is inserted into the chromosomal DNA of these cells and hence their G418 resistance but EGFP negative nature. Alternatively the recombination may have occurred within the EGFP coding region and therefore disrupting the EGFP gene. When analysing the effects of different reporter constructs on EGFP fluorescence only the EGFP positive cells was measured from multiple transfection typically n=4.

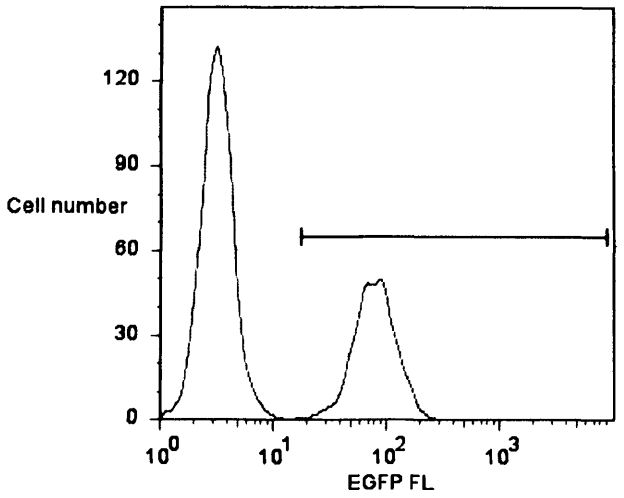


Figure 3.3.11 Histogram plot of $U937_{EC}$ which have been transfected with the 2 Kbp 5'promoter + pEGFP reporter construct. Following transfection of $U937_{EC}$ cell line with the 2 Kbp 5'promoter+pEGFP reporter construct, the cells were run on the flow cytometer and EGFP fluorescence was measured using FL1. The histogram plot shows two different fluorescent cell populations.

The 2Kbp 5'promoter constructs generated using either gDNA from $U937_{EC}$ (2Kbp(**EC**)+pEGFP) or $U937_{PM}$ (2Kbp(**PM**)+pEGFP) were transfected into both cell lines and changes in EGFP fluorescence was monitored by flow cytometry. Figure 3.3.12 shows that there was no significant difference in promoter activity between the two reporter constructs, which suggests that there is no detectable difference in the transcriptional activity between the two 2Kbp promoter regions cloned. Figure 3.3.12 also shows that there was no significant difference between the cell lines used, which suggests that both cells contain the necessary transcription factors to drive transcription.

When the 5'promoter deletion fragments were transfected into the $U937_{EC}$ cell line, it was shown that the majority of the -2 Kbp 5'promoter is dispensable for basal expression (figure 3.3.13). Deletions from -1.5Kbp to -1Kbp and -1Kbp to -500bp both resulted in a slight, although not statistically significant, increase in EGFP fluorescence, which suggest that some suppressor elements might be deleted. Figure 3.3.13 shows that a significant decrease in EGFP expression was observed between the -1Kbp and -500bp and the -100bp fragment. Although deletion from -200bp to -100bp resulted in a decrease in promoter activity this was not statistically significant. The figure also shows that the -100bp fragment still contained double the promoter activity compared with the pEGFP control vector, which suggest that this fragment still contains some promoter activity.

Transfection of the $U937_{PM}$ cell line with 5'promoter deletion reporter constructs produced similar pattern as the $U937_{EC}$ cell line transfected with the same constructs (figure 3.3.14). As shown in figure 3.3.14 there was a statistically significant increase in EGFP fluorescence between the -2Kbp fragment and the -500bp, -355bp and 200bp fragments

suggesting there may be some suppressor activity between -2Kbp and -500bp. The -100bp fragment displayed significantly less EGFP fluorescence compared with the -500bp and the -355bp fragments suggesting that some promoter activity was deleted between these regions (figure 3.3.14). However, deletion between the -200bp and the -100bp fragment failed to produce a statistical significance despite a reduction in EGFP fluorescence being observed (figure 3.3.14).

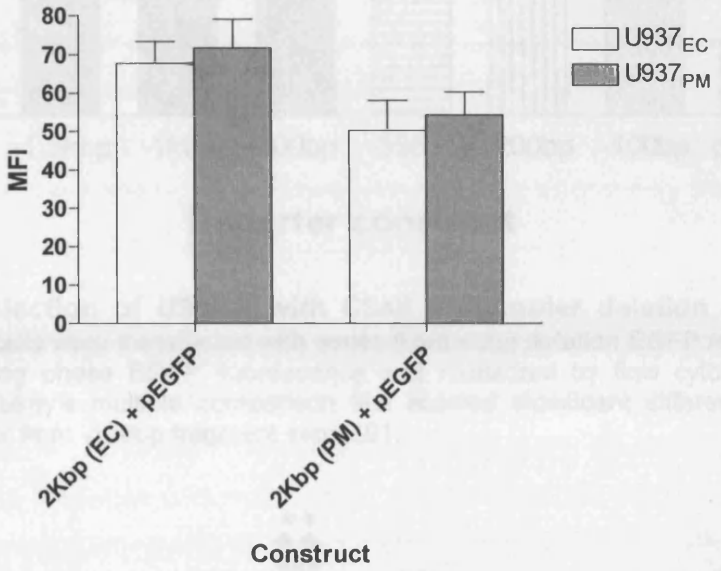


Figure 3.3.12 Comparison of EGFP fluorescence in both U937 cell lines transfected with the 2kbp 5'promoter+pEGFP reporter constructs generated from both gDNA templates. $U937_{EC}$ and $U937_{PM}$ cells were transfected with either 2Kbp (**EC**) or 2Kbp (**PM**) + pEGFP reporter construct. Once the cells were growing in log phase EGFP fluorescence was monitored by flow cytometry. Two-way ANOVA performed on the promoter EGFP report constructs showed no significant difference between the cell line used or the 2Kbp promoter EGFP construct transfected.

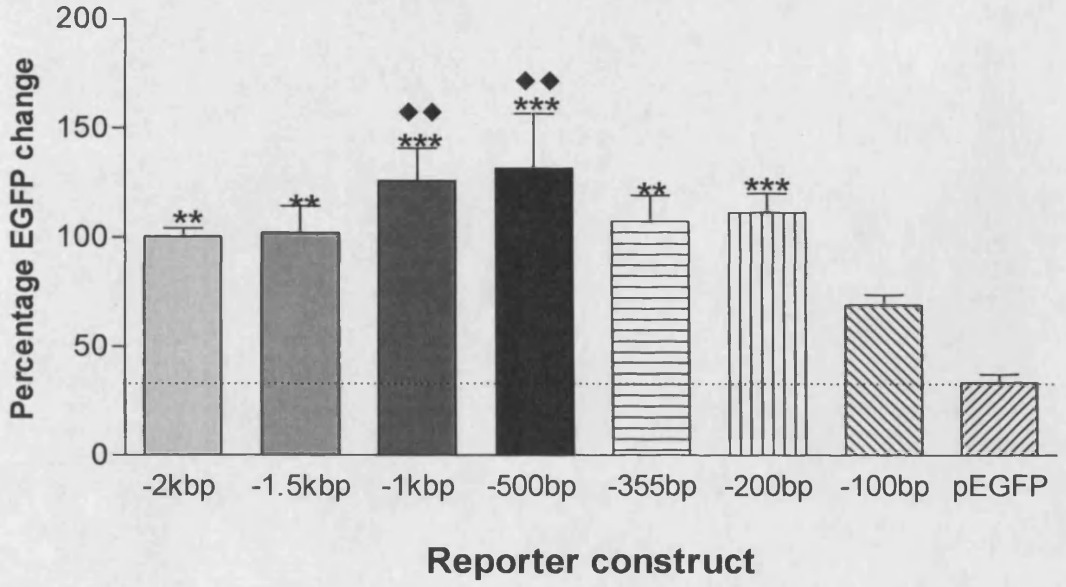


Figure 3.3.13 Transfection of $U937_{EC}$ with C5aR 5'promoter deletion EGFP reporter constructs. $U937_{EC}$ cells were transfected with series 5'promoter deletion EGFP reporter constructs and once growing in log phase EGFP fluorescence was monitored by flow cytometry. One-way ANOVA followed by Tukey's multiple comparison test showed significant difference from pEGFP **p<0.01 and p<0.001 or from -100bp fragment ♦♦p<0.01.

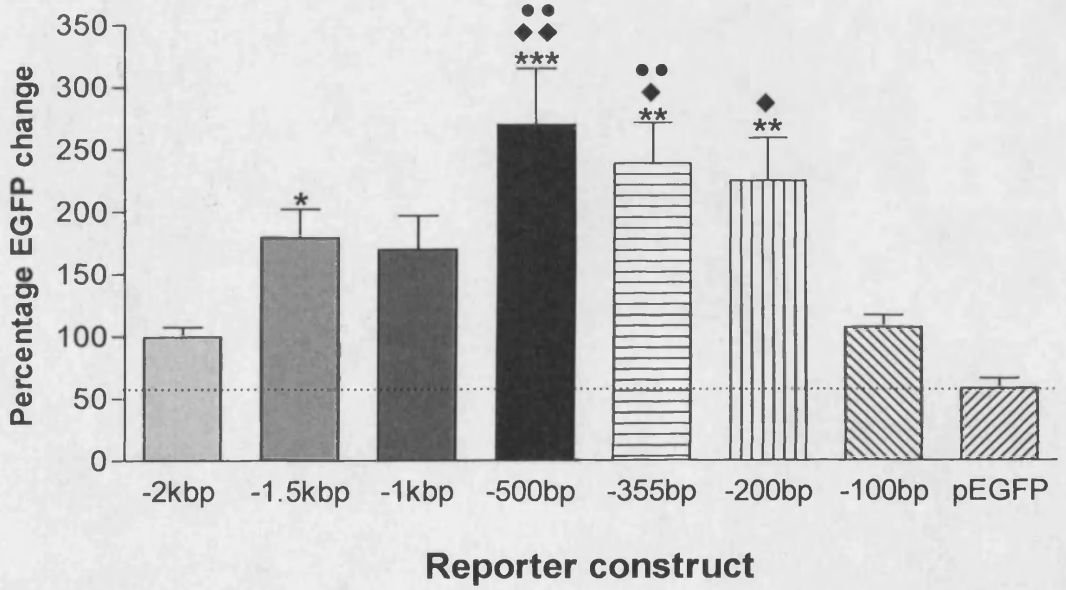


Figure 3.3.14 Transfection of $U937_{PM}$ with C5aR 5'promoter deletion EGFP reporter constructs. $U937_{PM}$ cells were transfected with series 5'promoter deletion EGFP reporter constructs and once growing in log phase EGFP fluorescence was monitored by flow cytometry. One-way ANOVA followed by Tukey's multiple comparison test showed significant difference; to -2 Kbp fragment ♦p<0.05, ♦♦p<0.01; to -100bp fragment •p<0.05, ••<p<0.01; to pEGFP *p<0.05, **p<0.01, ***p<0.0001

3.3.3 Putative CCAAT/NF-Y and NFAT binding sites are important promoter elements for transcription control of EGFP in the -355bp+pEGFP reporter construct

As well as using the promoter deletions to identify regions of promoter or suppressor activity, the -2Kbp promoter sequence from both cell lines were entered into Genomatrix MatInspector Software, which can be used to predict potential transcription factor binding sites, in each case a total of 292 putative sites were identified (appendix 9.4).

To determine which *cis*-acting elements are important for transcription, potential transcription factor binding sites were mutated using site directed mutagenesis. Site directed mutagenesis allows us to introduce specific mutations within the core sequence of potential *cis*-acting elements which will then prevent the necessary transcription factor from recognising the site. If the transcription factor binding site is important for transcription, then mutating the core sequence should prevent transcription.

Three potential *cis*-acting elements, NFκB (-238bp to -232bp), CCAAT/NF-Y (-123bp to -119bp) and NFAT (-93bp to -87bp) were mutated within the -355bp 5'promoter+pEGFP reporter construct (figure 3.3.15). The putative NFκB site was mutated as this transcription factor regulates expression of genes whose proteins encode pro-inflammatory molecules or involved in the immune response (Hoffmann et al., 2006). Although this site was mutated by Hunt and co-workers and was shown to have no effect on C5aR expression in the mouse, the human and the mouse sequence in this region show less homology (appendix 9.3) (Hunt et al., 2005). Furthermore, statins have been shown to interfere with NFκB activation and therefore regulate the expression of other genes including MCP-1 and IL-6 (see Chapter 5 for full details) (Veillard et al., 2006, Ortego et al., 1999, Massy et al., 2000, Guijarro et al., 1996). It was decided to mutate the CCAAT site as this site has been previously been shown to be an important site in regulation of the mouse C5aR expression and there is great homology between the mouse and human sequence within this region of the promoter (appendix 9.3)(Hunt et al., 2005, Martin, 2007). The decision to mutate the putative NFAT site was based on induced expression of the C5aR. Previously NFAT has been shown to regulate induced expression of several cytokines, cell surface GPCRs, including CCR2, FcγRs and thrombin receptor (see table 3.4.2 for full details), however its role in C5aR expression has not been investigated.

Site directed mutagenesis of each putative element was designed to incorporate a new restriction enzyme site, which would allow easy screening of potential mutants prior to sequencing. Figures 3.3.16, 3.3.17 and 3.3.18 each show the restriction enzyme digest and the sequencing results confirming the mutated NFκB, CCAAT/NF-Y and NFAT sites respectively. Following transfection of these mutant-constructs into the $U937_{EC}$ cell line

changes in EGFP fluorescence were monitored by flow cytometry. Figure 3.3.19 shows that mutating either putative CCAAT/NF-Y or NFAT in the -355bp promoter fragment resulted in the EGFP fluorescence being barely above the background pEGFP fluorescence, suggesting that both sites play a key role in transcription of the C5aR gene. Figure 3.3.19 also shows that some promoter activity was also attributed to the putative NFκB site, as site directed mutagenesis of this site significant reduced EGFP fluorescence compared to the wt -355bp fragment.

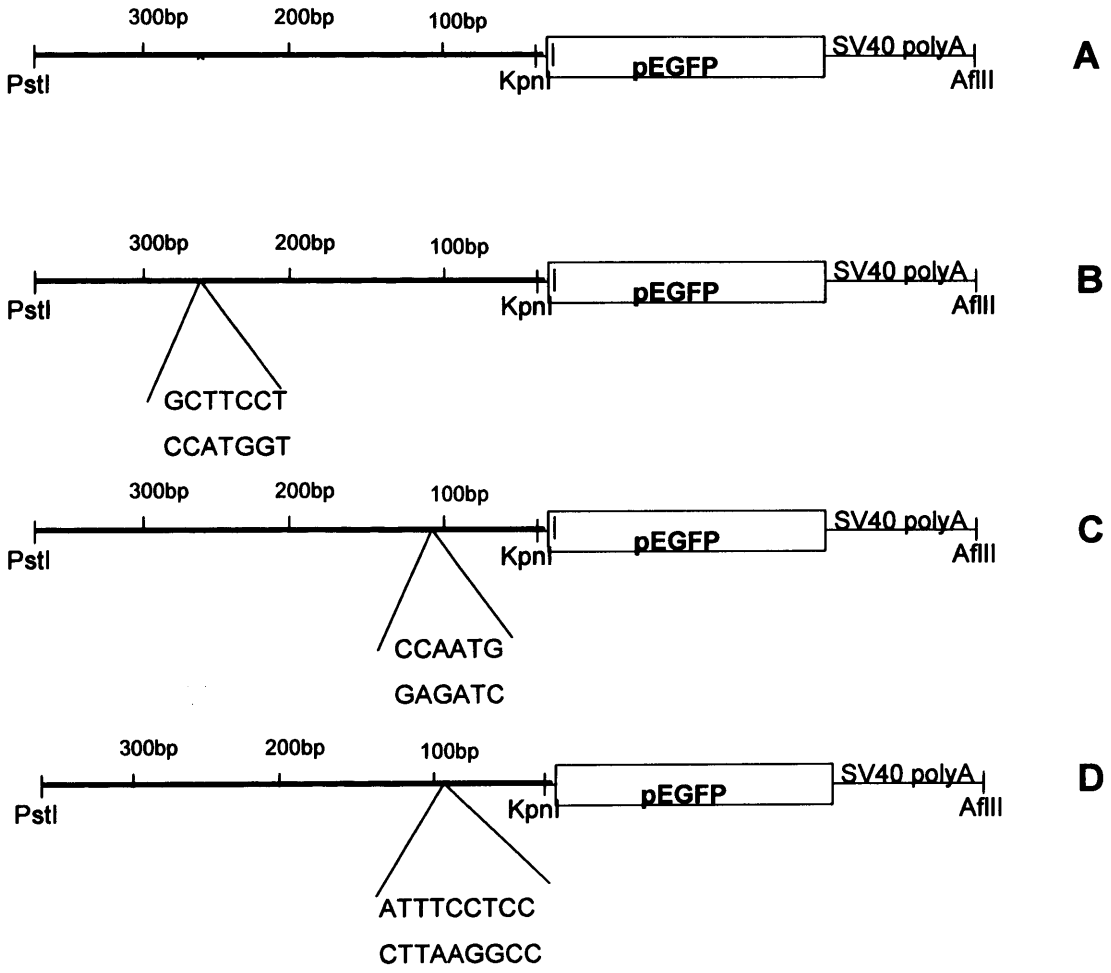


Figure 3.3.15 Schematic diagram illustrating the location of each of the putative site mutated by site directed mutagenesis. (A) Wild type -355bp + pEGFP generated from $U937_{EC}$; (B) NFκB site; (C) CCAAT/NF-Y site; (D) NFAT site. Sequences in black are the wild type sequences, whereas in blue is the sequence following site directed mutagenesis.

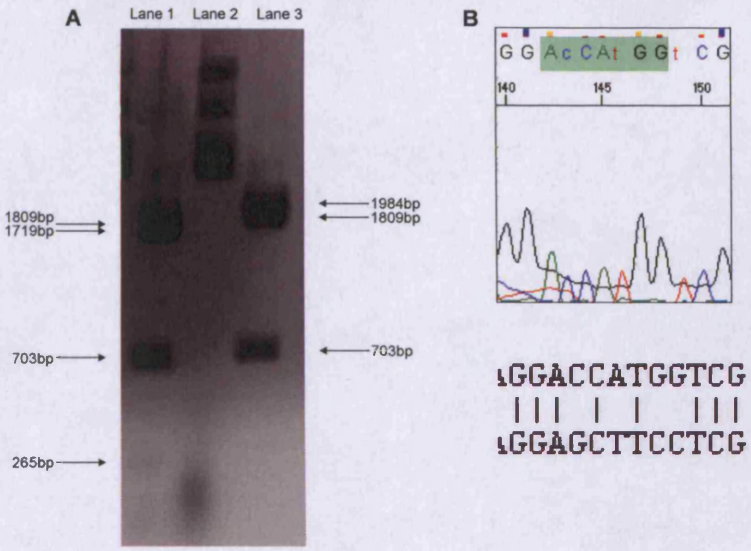


Figure 3.3.16 Screening for 355bp NFκB mutant. Potential colonies were first screened by restriction enzyme digest (A). Lane 1 shows a positive 355bp 5'promoter NFkB mutant colony as the plasmid DNA has been cut four times by Nco I into fragments 1809bp, 1719bp, 703bp and 265bp. Lane 3 shows the wild type 355bp 5'promoter+pEGFP construct which has been cut three times with Nco I; lane 2 shows undigested DNA. (B) To confirm the correct mutation had been introduced the 355bp NFkB mutant DNA was sequenced. Sequence alignment shows wild type sequence bottom and mutated sequence top.

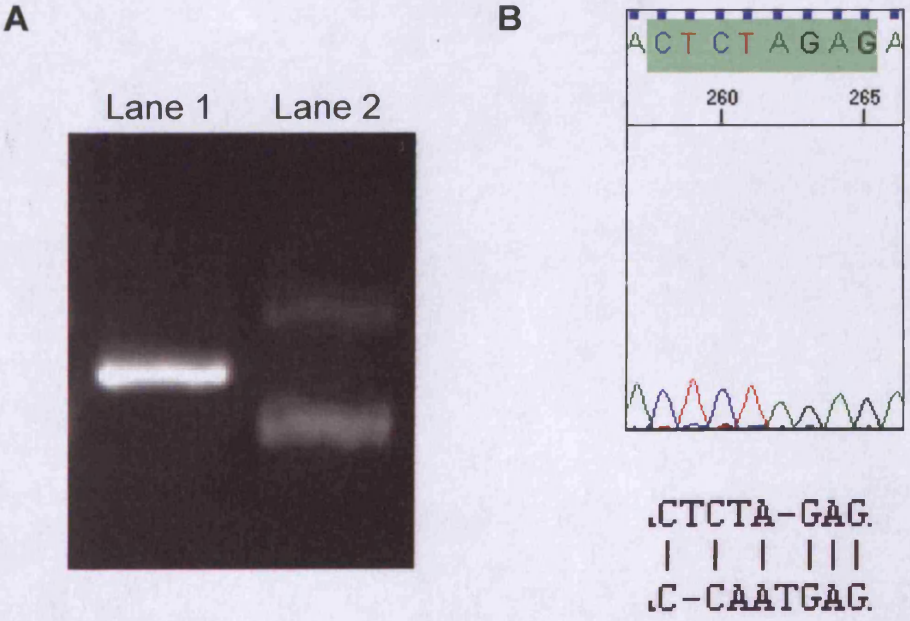


Figure 3.3.17 Screening for 355bp 5'promoter CCAAT/NF-Y mutant. Potential colonies were first screened by restriction enzyme digest (A). Lane 1 shows a positive 355bp 5'promoter NFAT mutant colony as the plasmid DNA has been cut once by Xba I into a 4496bp fragment; lane 2 the wild type 355bp 5'promoter+pEGFP construct which did not get cleaved by Xba I. (B) To confirm that the correct mutation had been introduced the 355bp NFkB mutant DNA was sequenced. Sequence alignment shows wild type sequence bottom and mutated sequence top.

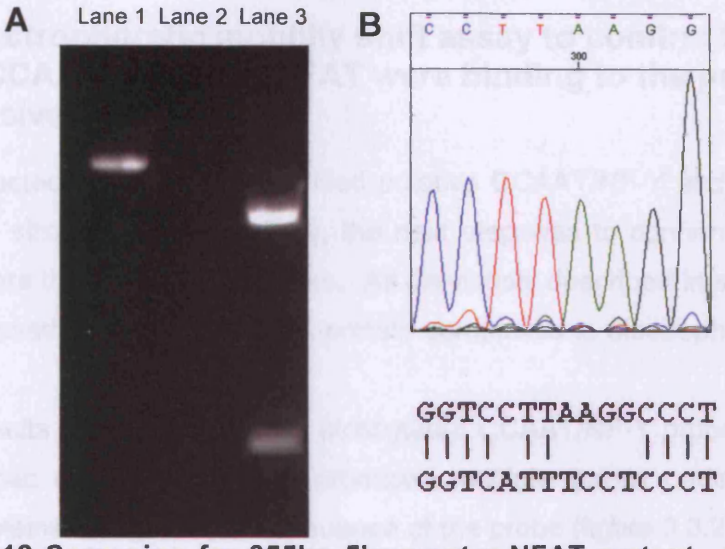


Figure 3.3.18 Screening for 355bp 5'promoter NFAT mutant. Potential colonies were first screened by restriction enzyme digest (A). Shown in lane 1 is the wild type 355bp 5'promoter+pEGFP construct which has been cut once with Afl II; lane 3 shows a positive 355bp 5'promoter NFAT mutant colony as the plasmid DNA has been cut twice by Afl II into a 3414bp and 1082bp fragments. (B) To confirm the correct mutation had been introduced the 355bp NFkB mutant DNA was sequenced. Sequence alignment shows wild type sequence bottom and mutated sequence top.

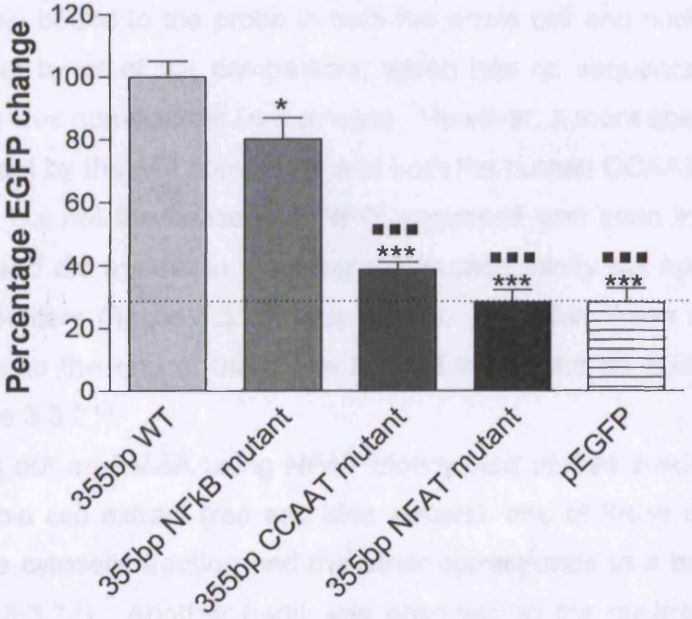


Figure 3.3.19 Site directed mutagenesis of putative CCAAT/NF-Y or NFAT cis-acting elements abolished transcription control in -355bp+pEGFP reporter construct. Mutant -355bp promoter construct were transfected into the $U937_{EC}$ cell line and once cells were growing in log phase EGFP fluorescence was monitored by flow cytometry. One-way ANOVA followed by Tukey's multiple comparison test show significant difference, as marked by asterisk, with the WT -355bp construct, * $p<0.05$, *** $p<0.001$, and with the NFκB , ▪▪▪ $p<0.001$, n = 4.

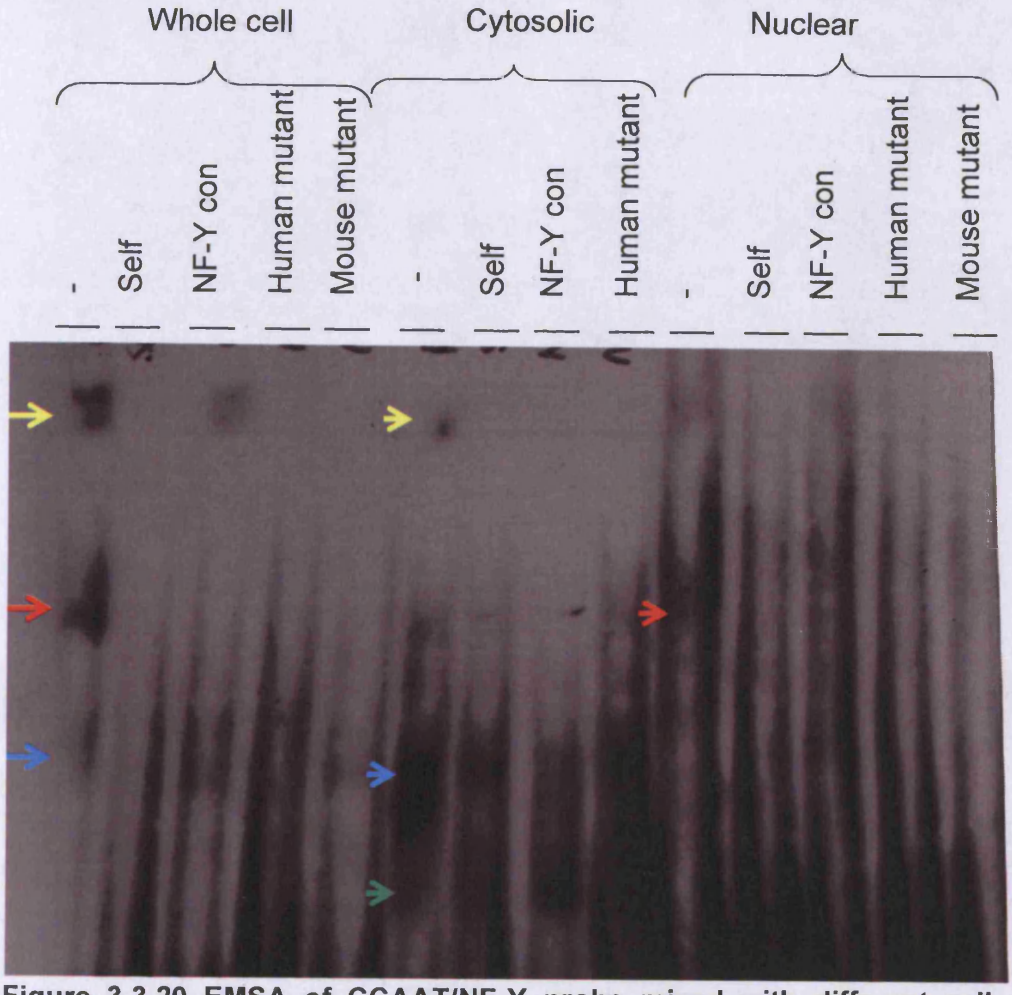


Figure 3.3.20 EMSA of CCAAT/NF-Y probe mixed with different cell extracts from U937$_{EC}$ cell line. Whole cell, cytosolic or nuclear extract were mixed with the biotinylated CCAAT/NF-Y probe in the absence or presence of competitor (see table 3.2.5 for probe sequences). Samples were run on a 6% non-denaturing PAGE. Yellow and blue arrows indicate bands present in both whole cell and cytosolic lysate. Red arrow indicates band appearing in both whole cell and nuclear lysate. Green arrow indicates band appearing in cytosolic lysate only.

NF-Y Consensus	AAGAGATTAACCAATCACGTACGGTCT
Human C5aR CCAAT	CCCAGTGTGCAGACCAATGAGAGCCCCAGA
Human CCAAT mutant	CCCAGTGTGACGACTCTAGAGAGCCCCAGA
Mouse CCAAT mutant	CCCAGTGTGCAGACCGCAGAGAGCCCCAGA

Figure 3.3.21 CCAAT/NF-Y probes aligned. Mouse CCAAT mutant was the same sequence as the mutant probe used by (Hunt et al., 2005).

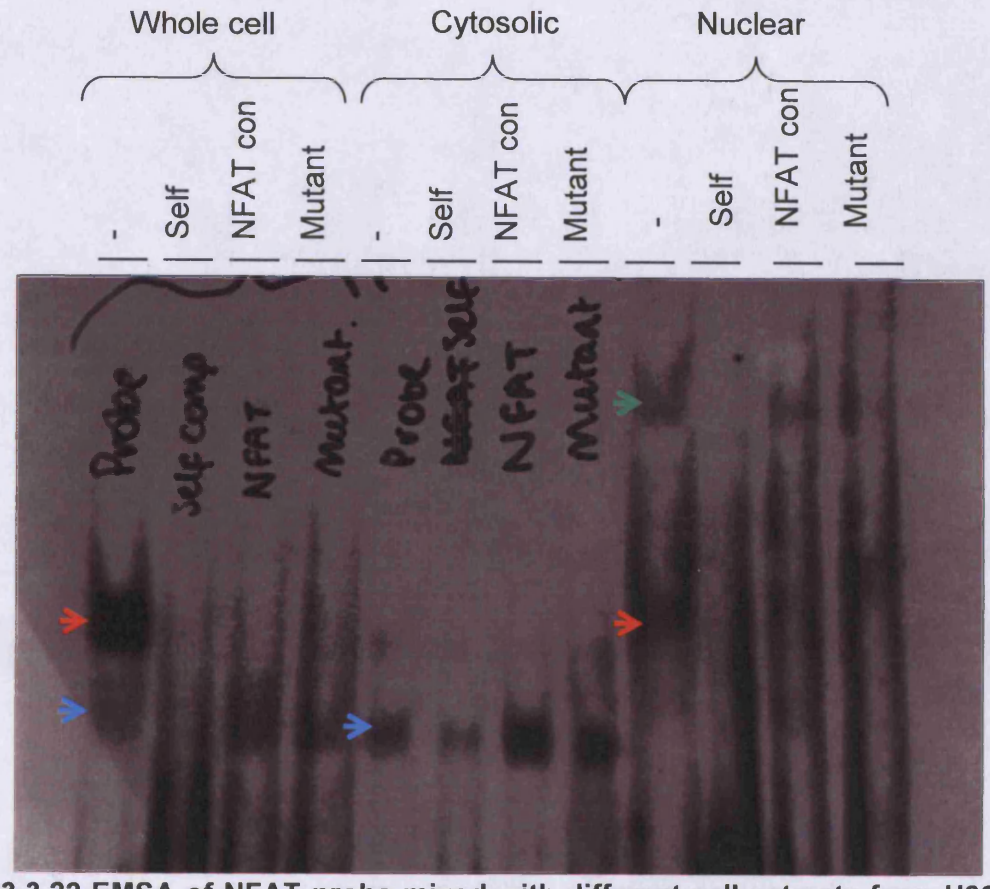


Figure 3.3.22 EMSA of NFAT probe mixed with different cell extracts from U937$_{EC}$ cell line. Whole cell, cytosolic or nuclear extract were mixed with the biotinylated CCAAT-NFY probe in the absence or presence of competitor (see table 3.2.5 for probe sequences). Samples were run on a 6% non-denaturing PAGE.

NFAT mutant	CCCCAGAGAGAAAGACGGTC CTTAAGGCCCTGCATCTTCCCTTGGGG
C5aR NFAT	AGAGAAAGACGGTCATT TCCTCCCTGCATCTTCC
NFAT consensus	ATACTTTCAAATTTCCTCTTTGGGCG

Figure 3.3.23 NFAT probes aligned.

Comparison of CCAAT/NF-Y EMSA using either $U937_{EC}$ or $U937_{PM}$ whole cell extract found that both extracts produced three similar bands (figure 3.3.24 A, indicated by yellow, red and blue arrows). In both cases the two lower bands were competed out by all of the competitors (red and blue arrows), whereas, the higher band was competed out by the self and the CCAAT/NF-Y mutant but not the NF-Y consensus (yellow arrow)(figure 3.3.24 A). The NFAT EMSA produced similar results to that of the CCAAT/NF-Y EMSA in the sense that three bands were produced (figure 3.3.24 B, indicated by yellow, red and blue arrows). However, in the NFAT EMSA the lower band (blue arrow) was not competed out by all the probes, instead it was only competed out by the NFAT mutant. The higher band (yellow arrow) was again competed out by the NFAT self and the NFAT mutant competitors but not by the NFAT consensus and the middle band (red arrow) was competed out by all the competitors (figure 3.3.24 B).

As in both the CCAAT/NF-Y and the NFAT EMSA there is a higher band (yellow arrow), which is competed out by self competitors and the mutants but not by either the NF-Y consensus or the NFAT consensus it was decided to run both CCAAT/NF-Y and NFAT EMSAs alongside each other. As shown in figure 3.3.25 when both the CCAAT/NF-Y and the NFAT biotinylated probe were mixed with whole cell extract from $U937_{EC}$ there appears to be a similar higher band (yellow arrow) being produced by both probes bound to protein. The CCAAT/NF-Y biotinylated probe in this experiment was competed out by all the probes, however this was unique to this one experiment, whereas the NFAT biotinylated probes was competed out by the self, NFAT and the CCAAT mutant but not the NFAT consensus (figure 3.3.25). This same experiment was performed alongside a denaturing EMSA were the samples had been cross-linked prior to running samples on a 12.5% SDS-PAGE. The denaturing EMSA showed the same results with the competitors as the normal EMSA as well as being able to give more information on the size of the proteins bound to the probe (figure 3.3.26, as indicated by red arrows). As shown in figure 3.3.26 the denaturing EMSA for both the CCAAT/NF-Y and the NFAT biotinylated probes produced two bands which were approximately 80 kDa and 110 kDa (indicated by red arrows). These observed Mw bands are consistent with Mr for NFAT, range from 83 to 136 kDa (Lyakh et al., 1997). However due to the high background of the blot these bands are quite difficult to see. These results taken together suggest that the protein which binds to both these probes is likely to be the same protein and is unlikely to be either NF-Y or NFAT as it has a tendency not to be competed out by either of these consensus sequences.

As shown in figure 3.3.27 both the putative CCAAT/NF-Y and NFAT sites are in very close proximity and in the case of the mutant probes they overlap sequences. For this reason new shorter probes, which had no overlapping sequences between the CCAAT/NF-Y or the NFAT probes, were designed and the EMSAs were repeated (figure 3.3.28). Figure

3.3.29 shows that repeating both the CCAAT/NF-Y and the NFAT EMSA with the short probes drastically reduced the amount the number of proteins binding to the probe. A consistent band was seen in multiple experiments using the NFAT biotinylated probe however this was competed out by all the competitors, suggesting either non-specific binding or a very weak interaction between the DNA probe and the protein. The shorter CCAAT biotinylated probe also exhibited protein binding however this was less consistent than the NFAT results but was still competed out by all the competitors (figure 3.3.29).

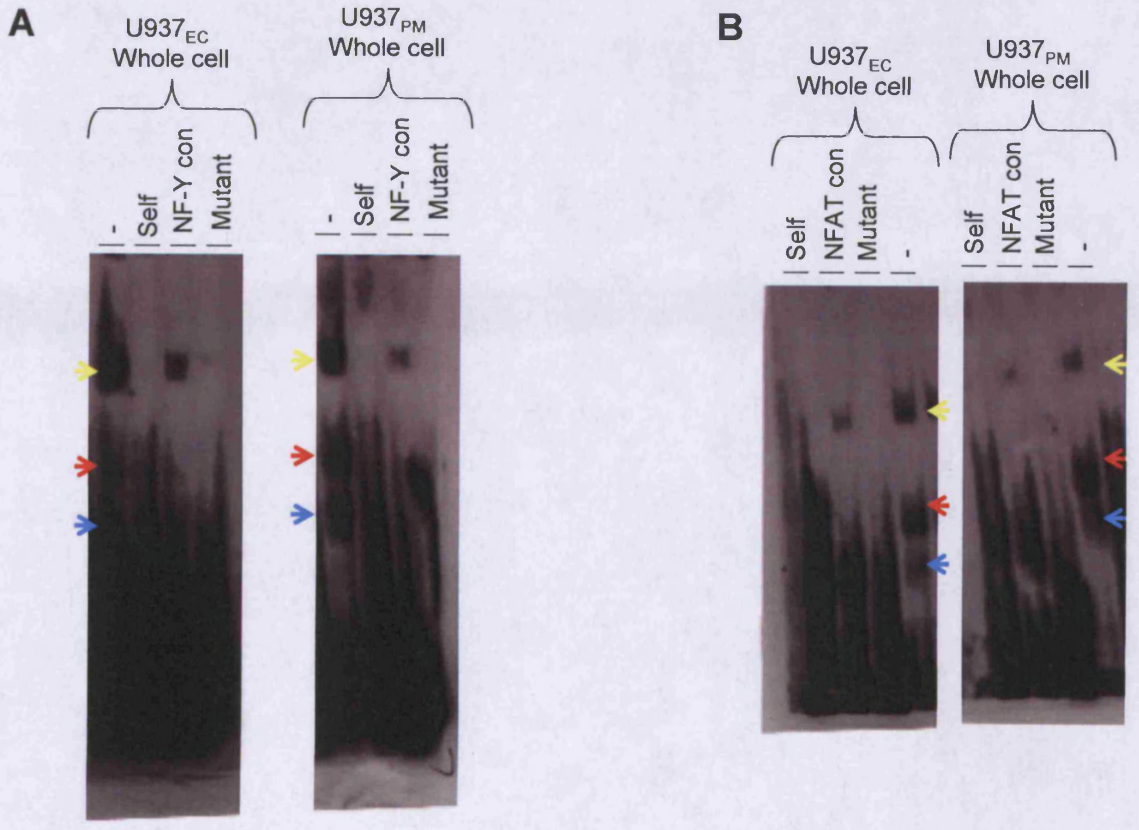


Figure 3.3.24 EMSA of CCAAT/NF-Y or NFAT probe with lysate from either $U937_{PM}$ or $U937_{EC}$. Whole cell lysate from either $U937_{EC}$ or $U937_{PM}$ was incubated with (A) CCAAT/NF-Y probe or (B) NFAT probe. Samples were run on a 6% non-denaturing PAGE.

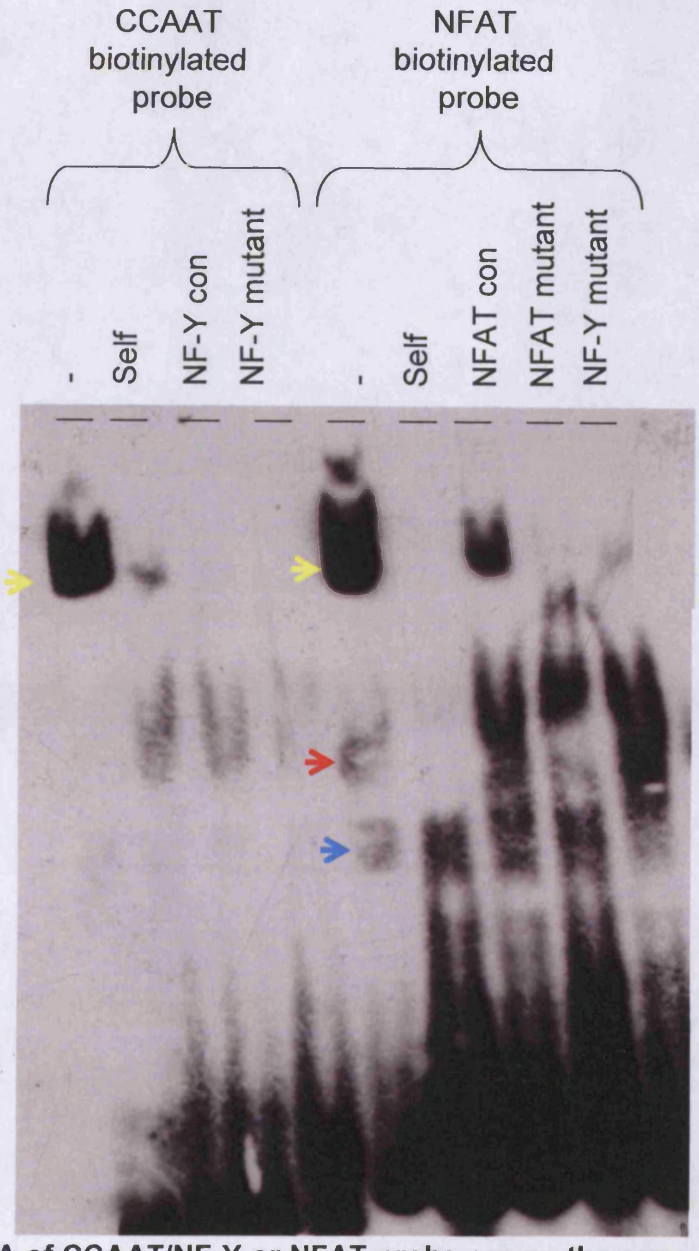


Figure 3.3.25 EMSA of CCAAT/NF-Y or NFAT probe run on the same gel. Nuclear extract from $U937_{EC}$ was incubated with either CCAAT/NF-Y or NFAT biotinylated probe in the absence or presence of different competitors. Samples were run on a 6% non-denaturing PAGE.

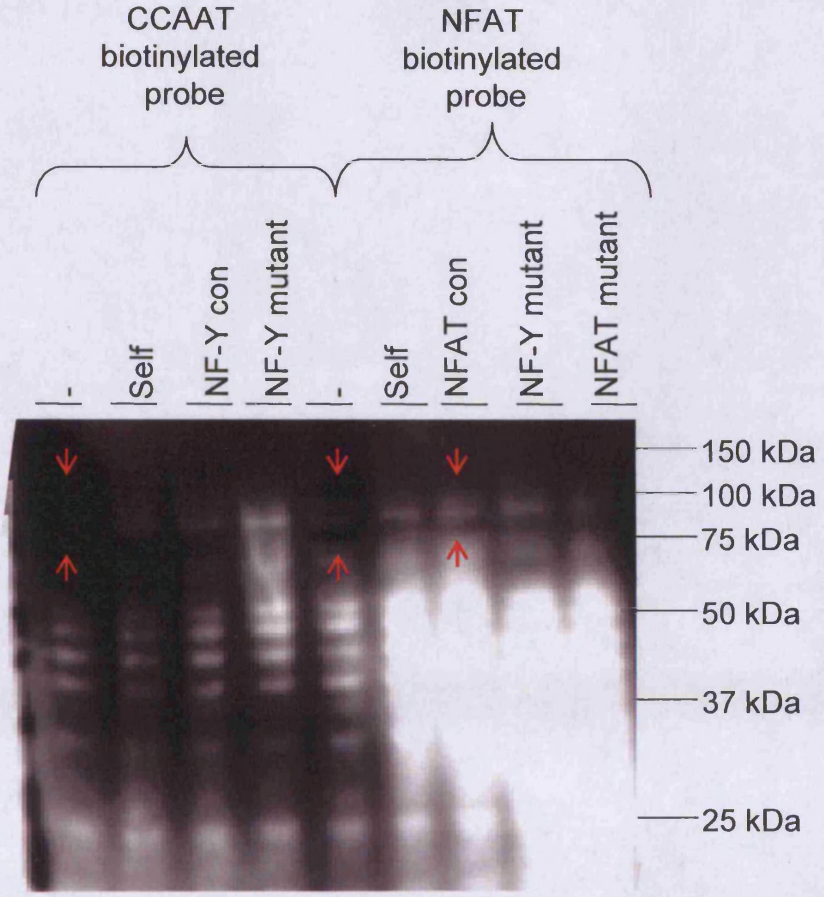


Figure 3.3.26 Denaturing EMSA. Nuclear extract from $U937_{EC}$ was incubated with either CCAAT/NF-Y or NFAT biotinylated probe in the absence or presence of different competitors. Samples were run on a 12.5% SDS-PAGE. Arrows indicate bands produced from proteins binding to the biotinylated probes, unfortunately the background is very high and has interfered with the scanning.

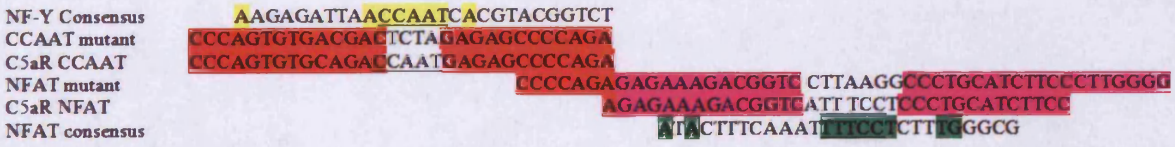


Figure 3.3.27 Probe sequence alignments. Underlined sequences show key residues for transcription factor binding. Highlighted sequences show sequence homology.

C5aR CCAAT/NF-Y	CAGACCAATGAGAGC
CCAAT mutant	CAGACTCTAGAGAGC
CCAAT/NF-Y consensus	TTAACCAATCACGTA

C5aR NFAT	GGTCATTTCCTCCCTG
NFAT mutant	GGTCCTTAAGGCCCTG
NFAT consensus	CAAATTTCCTCTTTG

Figure 3.3.28 Shorter EMSA probes aligned

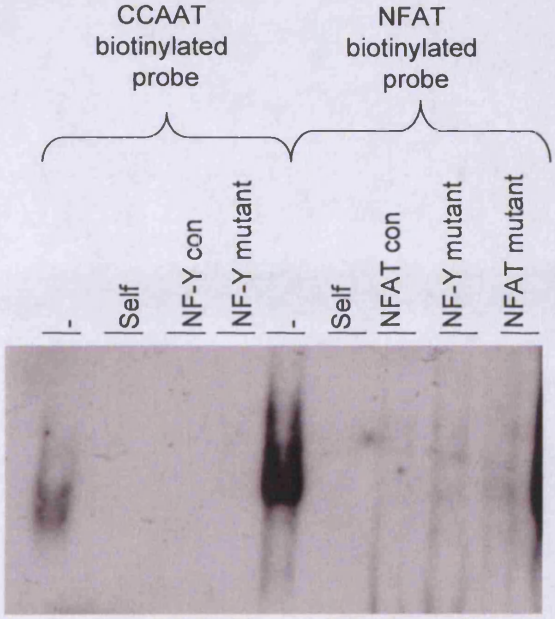


Figure 3.3.29 EMSA with shorter CCAAT and NFAT probes. Nuclear extract from $U937_{EC}$ was incubated with either CCAAT/NF-Y or NFAT biotinylated probe in the absence or presence of different competitors. Samples were run on a 6% non-denaturing PAGE.

As the EMSAs were unable to determine the exact *trans*-acting factors binding to the regions of interest it was decided to incubate the cells with cyclosporin A. Cyclosporin A inhibits calcineurin activity, which normally dephosphorylates NFAT, so that it can be transported to the nucleus. Thus if NFAT plays an important role in regulating C5aR expression, incubation with cyclosporin A should reduce C5aR expression.

As shown in figure 3.3.30 incubation of $U937_{EC}$ with 10 μg/ml cyclosporin A significantly reduced C5aR expression compared with the control, however this decrease was less than what was anticipated as previously mutating the NFAT site abolished the promoter activity (figure 3.3.19). At this concentration, however, there was a reduced cell number following incubation for 2 days (data not shown), suggesting that this concentration may have anti-proliferative effects.

When the -355bp+pEGFP reporter constructs were incubated with 10 μg/ml cyclosporin A, both the wild type construct and the NFkB mutant showed a decrease in EGFP fluorescence compared with the control, although this was not statistically significant. The EGFP fluorescence remained unchanged in both the NFAT and CCAAT mutants following incubation with cyclosporin A (figure 3.3.31).

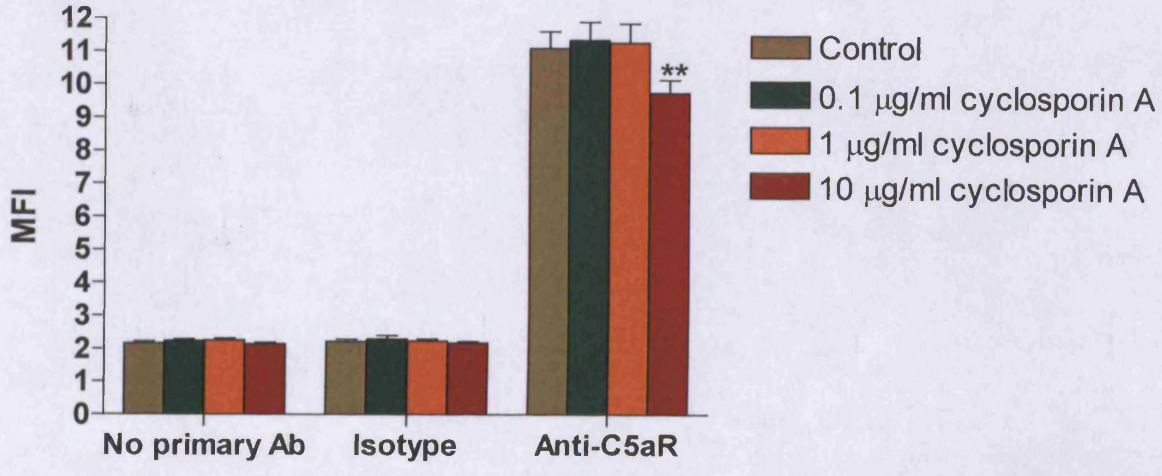


Figure 3.3.30 Effects of cyclosporin A on C5aR expression. Following incubation of $U937_{EC}$ for 2 days with increasing concentration of cyclosporin A, C5aR expression was detected by flow cytometry using anti-C5aR (S5/1) followed by goat anti-mouse-FITC. IgG_1 isotype control (anti-glycophorin) was used to measure non-specific binding via the Fc receptors. One-way ANOVA followed by Tukey's post test shows significant difference to the control, ** $p < 0.01$.

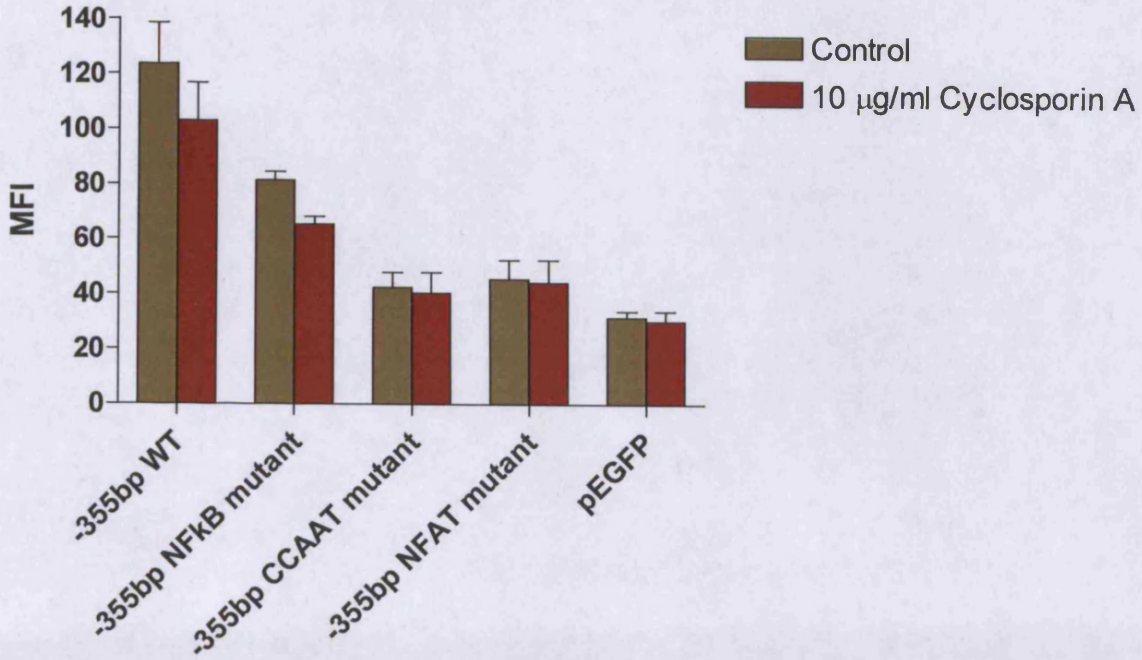


Figure 3.3.31 Effects of cyclosporin A on EGFP fluorescence in 355bp + pEGFP reporter construct. Following incubation of -355bp constructs with 10 μg/ml cyclosporin A for 2 days EGFP fluorescence was monitored by flow cytometry. Two-way ANOVA followed by Bonferroni post test showed that the construct used to transfect into the $U937_{EC}$ cell line significantly effected EGFP fluorescence ($p < 0.0001$), however, incubation with cyclosporin had no significant effect on EGFP fluorescence.

3.3.5 Site directed mutagenesis of 3'UTR AU rich elements and transfection of 3'UTR EGFP reporter constructs into the U937 cell line

To determine if the C5aR expression is regulated at the mRNA level, the 3'UTR was cloned down stream of the EGFP gene in the EF1α + pEGFP vector. The two AU-rich elements, one of which has been identified by ARED database, were mutated using site directed mutagenesis (figures 3.3.32 and 3.3.33). Again site directed mutagenesis introduced new restriction enzyme sites, which allowed rapid screening for mutants (table 3.2.4). Shown in figure 3.3.34 is a schematic diagram of the C5aR 3'UTR EGFP constructs which were transfected into the $U937_{EC}$ cell line. Once growing in log phase changes in EGFP fluorescence were detected by flow cytometry (figure 3.3.35). As shown in figure 3.3.35 the C5aR WT 3'UTR had lower EGFP fluorescence as compared to the SV40 3'UTR, which is what would be predicted as the SV40 3'UTR is very stable. Mutating either the 1st or the 2nd ARE appeared to have no significant effect on EGFP fluorescence compared to the WT, however, it was not investigated whether mutating both sites at the same time had an effect on mRNA stability (figure 3.3.35).

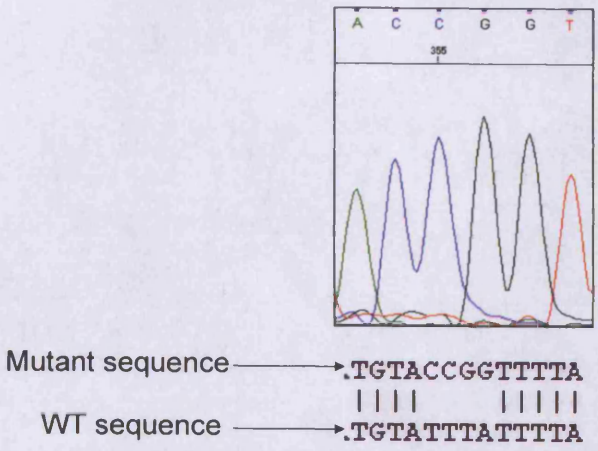


Figure 3.3.32 Sequencing results for 3'UTR 1st ARE mutant. Site directed mutagenesis introduced a new *Age I* site which was used to screen mutants. Shown here is the sequencing results aligned with the WT sequence.

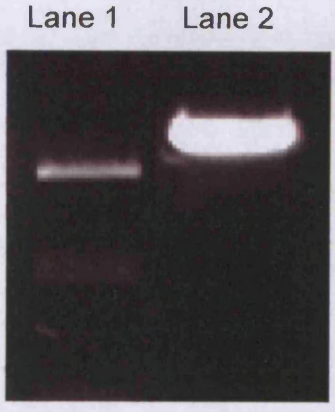


Figure 3.3.33 Restriction enzyme digest screening for 3'UTR 2nd ARE mutant. Site directed mutagenesis introduced a *Kpn I* site which was used to confirm the 3'UTR 2nd ARE had been mutated.

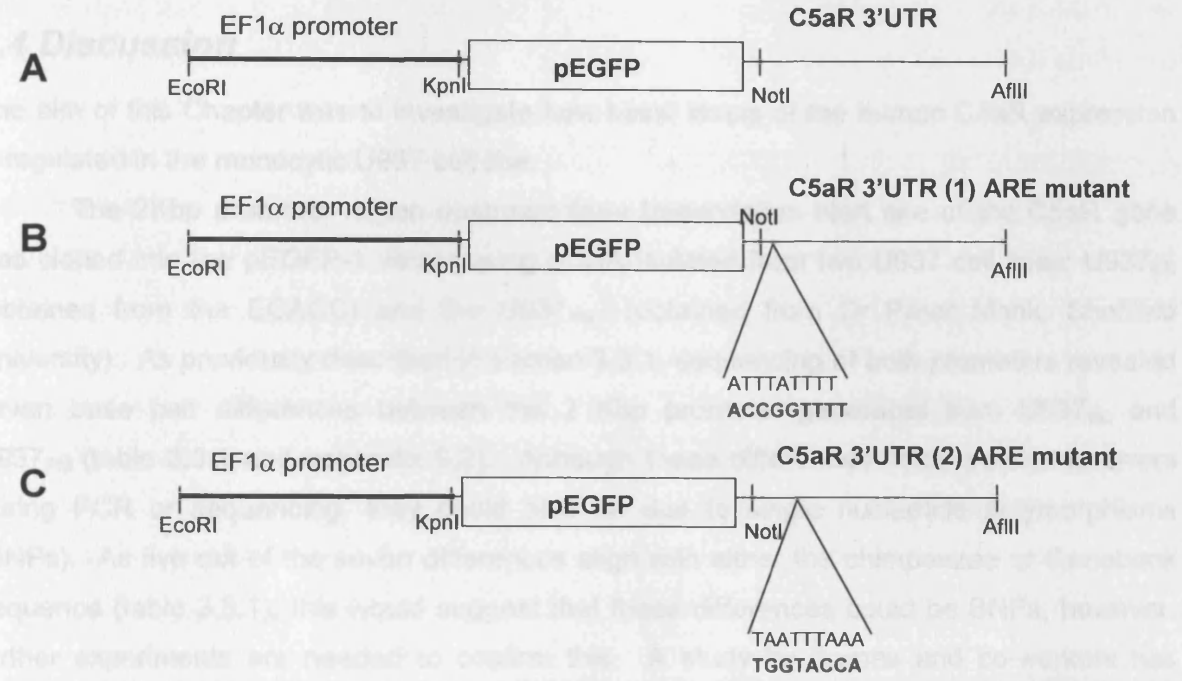


Figure 3.3.34 Schematic diagram of C5aR 3'UTR EGFP reporter constructs. The C5aR 3'UTR was cloned down stream of the EGFP gene in the EF1α+pEGFP vector using specific restriction enzyme sites as indicated in the figures. (A) wild type C5aR 3'UTR; (B) C5aR 1st ARE mutant; (C) C5aR 2nd ARE mutant. Blue indicates sequences mutated by site directed mutagenesis.

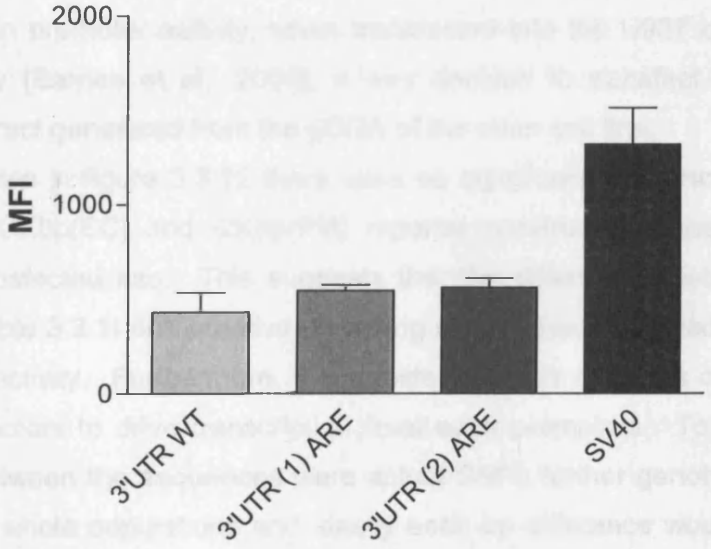


Figure 3.3.35 Effects of 3'UTR ARE mutations on EGFP fluorescence. Following transfection of $U937_{EC}$ cell line with the EGFP reporter constructs changes in EGFP fluorescence was monitored by flow cytometry. One-way ANOVA performed comparing the C5aR 3'UTR WT, 3'UTR(1) ARE mutant and 3'UTR(2) ARE mutant showed no significant difference.

3.4 Discussion

The aim of this Chapter was to investigate how basal levels of the human C5aR expression is regulated in the monocytic U937 cell line.

The 2Kbp promoter region upstream from transcription start site of the C5aR gene was cloned into the pEGFP-1 vector using gDNA isolated from two U937 cell lines: $U937_{EC}$ (obtained from the ECACC) and the $U937_{PM}$ (obtained from Dr Peter Monk, Sheffield University). As previously described in section 3.3.1, sequencing of both promoters revealed seven base pair differences between the 2 Kbp promoter generated from $U937_{EC}$ and $U937_{PM}$ (table 3.3.1 and appendix 9.2). Although these differences could be due to errors during PCR or sequencing, they could also be due to single nucleotide polymorphisms (SNPs). As five out of the seven differences align with either the chimpanzee or Genebank sequence (table 3.3.1), this would suggest that these differences could be SNPs, however, further experiments are needed to confirm this. A study by Barnes and co-workers has previously identified a novel T/C polymorphism within the promoter region of the C5aR (Barnes et al., 2004). This published SNP corresponds with the -253bp T/C base pair substitution observed between the $U937_{EC}$ and $U937_{PM}$ sequences (table 3.3.1). Although Barnes and colleagues had previously shown that this polymorphism was not associated with any changes in promoter activity, when transfected into the U937 cell line using luciferase reporter assay (Barnes et al., 2004), it was decided to transfect each cell line with the reporter construct generated from the gDNA of the other cell line.

As shown in figure 3.3.12 there were no significant differences in EGFP expression between the -2Kbp(**EC**) and -2Kbp(**PM**) reporter constructs irrespective of which cell line they were transfected into. This suggests that the differences between the two promoter sequences (table 3.3.1) and putative *cis*-acting sites (table 3.3.2) had no significant effect on the promoter activity. Furthermore, it suggests that both cell lines contained the necessary *trans*-acting factors to drive transcription from each promoters. To determine whether the differences between the sequences were actual SNPs further genotyping would need to be performed on whole populations and ideally each bp difference would then be investigated individually for differences in promoter activity.

Serial 5'deletion analysis of -2Kbp (**EC**) reporter construct revealed that the majority of the promoter was dispensable when transfected into both $U937_{EC}$ (figure 3.3.13). A significant reduction in EGFP fluorescence was observed in the -100bp reporter construct, although some promoter activity still remained within this region. Hunt and colleagues have previously shown similar results with the mouse C5aR promoter region. They attributed this reduction in promoter activity to a CCAAT motif present within the deleted region (Hunt et al., 2005) (as illustrated in figure 3.4.1). This study also suggested that the human C5aR may

also be regulated in a similar manner due to high sequence homology within this region of both the mouse and human promoter. Transfection of the $U937_{PM}$ cell line with the serial 5'promoter deletions produced a similar pattern as the $U937_{EC}$ cell line (figure 3.3.14). However they appeared to be more sensitive with deletion from -2Kbp to -500bp suggesting the presence of suppressor elements within this region and deletion from -500bp to -200bp suggesting promoter activity within this region (figure 3.3.14). It is important to note here that as the cells were stably transfected with the EGFP reporter constructs integration of the constructs into a position in the genome close to strong promoter or suppressor activity may have an effect on the rate at which the EGFP gene is transcribed. However, the position at which the reporter constructs are integrated into the genome is more favourable at loosely pack euchromatin rather than heterochromatin. As each transfection was repeated 4 times this would be a sufficient number of repeats to highlight whether integration near a strong promoter or suppressor activity may interfere with the transcription of the EGFP gene. Further still, the EGFP fluorescence was measured in mixed cell populations therefore the natural spread/range was recorded for each reporter construct transfected.

To test whether the putative CCAAT motif (-123bp to -119bp) within the human C5aR promoter was responsible for transcriptional activity of the human C5aR gene this site, along with putative NFκB (-238bp to -232bp) and NFAT (-93bp to -87bp) sites, were mutated by site directed mutagenesis. As shown in figure 3.3.19 site directed mutagenesis of the putative CCAAT resulted in a significant reduction in EGFP fluorescence compared with the wt -355bp promoter region. This correlates with results previously observed in mouse C5aR promoter region when transfected into mouse macrophage, RAW 264.7, and endothelial, b.End3 and mHEVc, cell lines (Hunt et al., 2005). The CCAAT motif is one of the most common promoter elements in eukaryotic promoters (Maity and de Crombrugghe, 1998). The motif can be found in either the forward or reverse orientation and is preferentially located -80/-100 bp to start site (Mantovani, 1998). As shown in table 3.4.1, several binding proteins have been found to recognise the CCAAT motif. MatInspector analysis of the human C5aR promoter sequence predicted that the CCAAT motif present at position -123bp to -119bp would likely bind NF-Y binding protein, which would correlate with the study by Hunt and co-workers who showed, using EMSA and super shift EMSA, that NF-Y, but not c-Fos or ETS1/2, bound to the homologous CCAAT motif present in the mouse C5aR promoter (Hunt et al., 2005).

Table 3.4.1 CCAAT binding proteins. Taken from (Mantovani, 1998).

Binding protein	Recognising sequence
CCAAT/enhance binding protein (c/EBP)	Palindromic repeats occasionally containing CCAAT pentanucleotide in the intervening sequences
CCAAT transcription factor (CTF/NF1)	Recognises TGG(N)6GCCAA sequence, with a T after CCAA occasionally but not strictly required
CCAAT displacement protein (CDP)	Recognises repeated CCAAT motifs
NF-Y (also called CCAAT binding factor CBF)	Recognises only exact CCAAT motifs

Although it was anticipated that mutating the CCAAT motif would reduce transcriptional activity based on previous published findings on the mouse C5aR promoter, the decision to mutate the NFAT site was one based on induced expression of the C5aR. NFAT (nuclear factor of activated T-cells) is a family of transcription factors that regulate inducible expression of many cytokine and cell surface receptors critical for the immune response (Lee and Park, 2006). Shown in table 3.4.2 is a list of stimuli which have been shown to elicit NFAT activation, and target proteins whose expression changes as a result of this activation. Of particular interest was that PMA can stimulate CCL23 expression in the U937 cell line via activation of NFAT (Shin et al., 2007). As PMA has been well documented in the literature to increase C5aR expression in the U937 cell line, this led to the putative NFAT site within the C5aR promoter region being investigated (Rubin et al., 1991a, Burg et al., 1996). Site directed mutagenesis of the putative NFAT site revealed a significant reduction in transcriptional activity in mutant promoter compared with the wild type -355bp fragment (figure 3.3.19). As described above this site was mutated on the presumption it may be involved in regulation of induced expression of the C5aR, which makes this finding more interesting.

Table 3.4.2 Stimuli that elicits NFAT activation. Adapted from (Rao et al., 1997).

Receptor/Function	Stimulus/Drug	Cell type tested	Reference
Immunoreceptors/ chemokines			
T cell receptor	Ag/MHC	T cells	(Rao, 1994)
CD40	CD40L plus IL-4	B cells	(Choi et al., 1994)
FcγR	Ag/IgG, αCD16	NK cells, macrophages	(Aramburu et al., 1995)
CCR2	Ionomycin	Neuron cell line F11	(Jung and Miller, 2008)
CCL23	PMA	U937 cell line	(Shin et al., 2007)
G protein coupled receptors			
H1 histamine receptor	Histamine	Endothelial cells (Human umbilical vein)	(Rao et al., 1997)
Thrombin receptor	Thrombin		(Rao et al., 1997)

Inactive NFAT normally resides in the cytoplasm in a heavily phosphorylated form (Lee and Park, 2006). As illustrated in figure 3.4.1, the stimulation of cell surface receptors that are linked to phospholipase C signalling pathway results in an increase in intracellular Ca^{2+}, which activates calmodulin. Activated calmodulin in turn activates calcineurin, which dephosphorylates NFAT allowing its translocation into the nucleus. As the site directed mutagenesis results showed a decrease in transcriptional activity following mutation of the putative NFAT site, this would suggest that in the 'resting' U937 cells NFAT is present in its active form within the nucleus. Although the transfection process itself could result in the stimulation and activation of the cells, here stable transfectants were generated in order to allow the cells time to recover from the shock of electroporation. Alternatively, this result may be observed if site directed mutagenesis introduced a new suppressor element or even if another promoter element was also disrupted by the mutagenesis. Although both these scenarios were checked by MatInspector prior to mutagenesis and no obvious new motif was introduced or deleted, this software can only predict known binding sites.

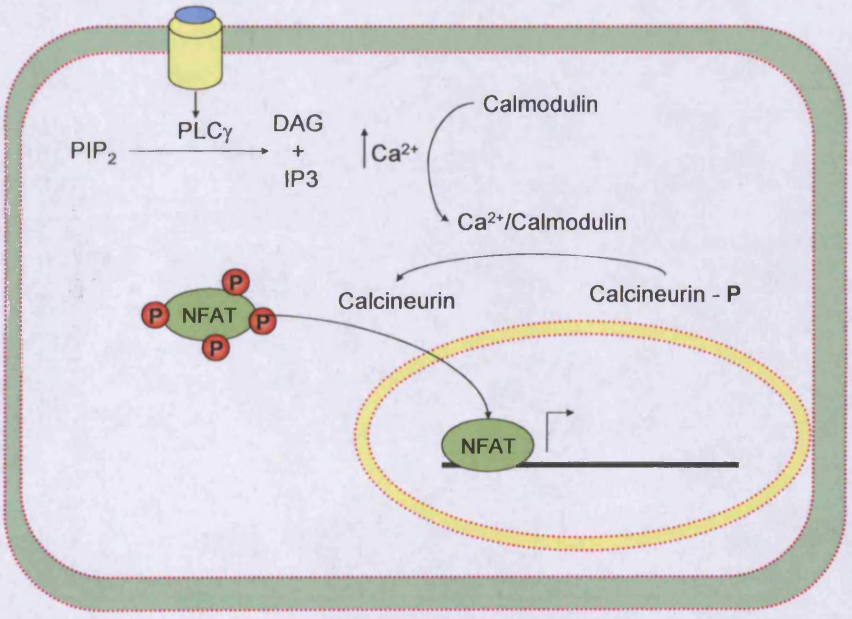


Figure 3.4.1 Schematic diagram illustrating NFAT activation

As shown in figure 3.3.19, mutation of the putative NFκB site at -238bp to -232bp also resulted in significant reduction in EGFP fluorescence compared to the WT fragment, albeit less than the CCAAT and NFAT mutants. NFκB is another family of transcription factors which is associated with the transcriptional regulation of genes whose proteins are involved in inflammation and the immune response (Hoffmann et al., 2006). NFκB transcription factors are dimers bound by inhibitory IκB proteins, which controls the DNA

binding activity and nuclear localisation of NFκB (Hoffmann et al., 2006). Upon stimulation by stimuli such as cytokines, reactive oxygen species, bacterial wall products, viral infection or DNA damage, IκB dissociates from NFκB, allowing its translocation into the nucleus where it can then regulate the transcription of target genes (Brasier, 2006). Although the results presented here suggest that the putative NFκB site in the human C5aR promoter contains a small amount of transcriptional activity, it has previously been shown that the homologous NFκB site within the mouse promoter contains little promoter activity when transfected into mouse macrophage, RAW 264.7, and endothelial, b.End3 and mHEVc, cell lines (Hunt et al., 2005).

Taken together the results from the site directed mutagenesis combined with 5'deletion analysis suggests that deletion of the putative CCAAT site at position -123bp to -119bp may explain why a reduction in EGFP fluorescence was observed at the -100bp deletion fragment and that the remaining promoter activity within the -100bp fragment may be attributed to the putative NFAT site (-93bp to -87bp), as illustrated in figure 3.4.2. However it is important to note that the -100bp fragment contains the core promoter TATA box which may be contributing towards the remaining transcriptional activity.

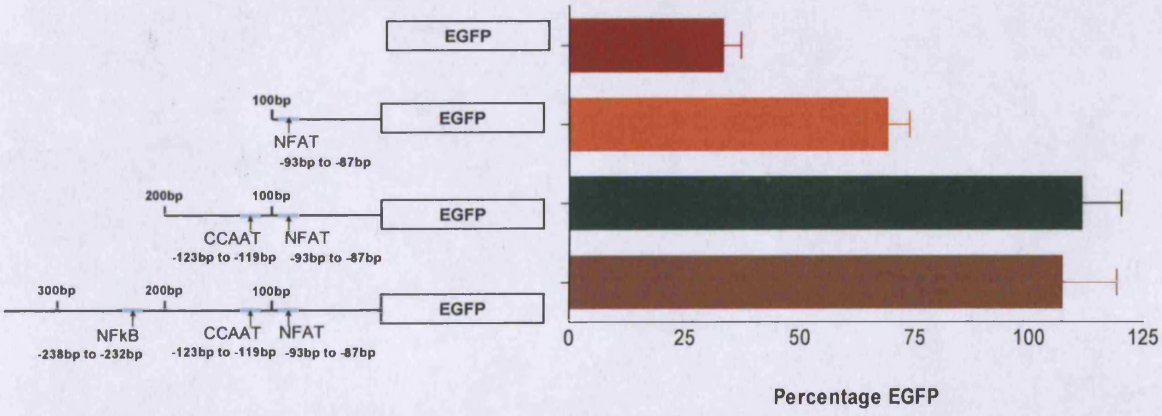


Figure 3.4.2 Schematic diagram illustrating how the putative NFκB, CCAAT and NFAT affect EGFP fluorescence during 5'promoter deletion analysis.

In an effort to determine which *trans*-acting factors may be binding to the putative CCAAT and NFAT sites, EMSAs were performed using several competitors including the CCAAT/NF-Y and NFAT consensus sequences. Unfortunately in both cases there appeared several proteins that recognised and bound to both CCAAT and NFAT labelled probes, which made interpreting the results more difficult (figures 3.3.20 and 3.3.22 respectively). However, it appeared that some of the proteins binding to the different probes were similar (figure 3.3.25 and 3.3.26). Although this could mean that the same non-specific protein has

bound to both probes, this did not appear to be the case as some specific displacement by the self and mutant competitors but not the NFAT consensus was observed. Alternatively this may be explained by the close proximity of the two sites, with only 9bp separating the two full sites as identified by MatInspector. Possibly there is some cooperation between the two sites and the transcription factors which bind to them (figure 3.3.1). For instance, NFAT1 which bind to the NFAT sequence can cooperate with adjacent, no more than a single bp separation, AP-1 dimers in murine IL-2 promoter (Rao et al., 1997). This cooperation results in the NFAT1:AP-1:DNA complex being significantly more stable, and higher affinity than the NFAT1 alone (Rao et al., 1997). Similar several studies have also document NFAT cooperating with the CCAAT binding protein C/EBP (Yang and Chow, 2003, Oh et al.). However no published data has reported any cooperation with NF-Y which was the CCAAT binding protein that was previously shown to interact with the mouse C5aR promoter (Hunt et al., 2005).

Although the addition of antibodies to the EMSA incubation mixture can be used to identify the proteins which bind to the labelled probes, it was decided to incubate the cells with cyclosporin A, an inhibitor of NFAT activation. It was anticipated that cyclosporin A should reduced C5aR expression if NFAT plays a key regulatory role in regulting expression of the receptor *in vivo*. However, C5aR expression was only significantly decreased at the higher concentration of cyclosporin A (10 μg/ml), and this decrease was not as significant as was expected based on the mutagenesis results of the NFAT site which abolished the promoter activity (figure 3.3.30). This result suggests that although it appears that the putative NFAT site is important for the transcriptional activity in the -355bp reporter fragment, *in vivo* the C5aR expression is not regulated by NFAT. To confirm NFATs involvement in the transcriptional activity of -355bp reporter fragment, this reporter construct along with the mutant reporter constructs were also incubated with cyclosporin A. Cyclosporin A had no effect on EGFP fluorescence in these reporter constructs, which provides further evidence that result observed by mutating the putative NFAT site is due to either the disruption of another important promoter element or the introduction of a new suppressor element. To confirm which is the likely scenario, this site should be mutated again using different residues.

As well as looking at transcriptional regulation of the C5aR, this chapter has also investigated whether its mRNA can be affected by AU-rich elements present within the 3'UTR. As shown in figure 3.3.35, site directed mutagenesis of either the 1st ARE or the 2nd ARE had no effect on EGFP fluorescence. Therefore, unlike other ARE belonging in class I (see table 3.1.2 for details), the AREs in the C5aR 3'UTR do not contribute towards mRNA stability. However this study only mutated the sites individually, it was not investigated whether mutating these sites together would have any effect. Although these sites are

approximately 100 bp apart, they may still be within close spatial proximity during folding of the mRNA and therefore allowing cooperation between the two AREs and their binding proteins.

3.4.1 Summary

This chapter has found that the majority of the -2Kbp promoter region was dispensable for expression, although some transcriptional activity was deleted between -200bp to -100bp in the $U937_{EC}$ cell line. Site directed mutagenesis of a putative CCAAT motif at position -123bp to -119bp resulted in a significant decrease in promoter activity compared to the WT -355bp fragment, suggesting that this site may be important for the transcriptional activity lost between -200bp to -100bp. Site directed mutagenesis of a putative NFAT site at position -93bp to -87bp, also suggested that this site may be responsible for some transcriptional activity. However, further analysis indicate that another promoter element may have been disrupted or a suppressor element introduce during mutagenesis as cyclosporin A, an inhibitor of NFAT, was unable to significantly reduced transcriptional activity. Site directed mutagenesis of the putative NFκB site at position -238bp to -232bp also found that this site contains some promoter activity, although not as significant as the CCAAT or NFAT motifs. This chapter has also shown that mutating either of the ARE within the C5aR mRNA had no effect on mRNA stability.

Chapter 4

Characterisation of induced C5aR expression and function in the U937 cell line

Chapter 4

Characterisation of induced C5aR expression and function in the U937 cell line

4.1 Introduction

The U937 cell line is frequently used to study the expression and function of the C5aR, but because C5aR is low on these cells, it is usually differentiated with a range of stimuli in order to induce a more macrophage like phenotype and increase expression of the C5aR. The aim of this chapter is to characterise and investigate which stimuli are the best inducers of C5aR expression in the U937 cell line in order to set up a model system to investigate effects of lipid lowering drugs on the expression and function of the C5aR.

4.1.1 Stimuli that regulate C5aR expression

Early studies involving differentiation in the U937 cell line mainly focused on artificial stimuli such as Bt_2cAMP, 1,25-dihydroxy vitamin D3, trans retinoic acid and PMA (Chenoweth et al., 1984, Rubin et al., 1986, Gavison et al., 1988, Nilsson et al., 1980). Although all these stimuli have been shown to differentiate the U937 cell line, they generate dissimilar maturation patterns such as PMA increases adherence whereas Bt_2cAMP causes cells to remain in suspension. Table 1.2 (Chapter 1) summarises which stimuli has been shown to regulate C5aR expression and the methods which have been used to detect changes in expression.

Bt_2cAMP, which is a membrane permeable cAMP analogue, was first shown to increase functional C5aR expression in the U937 cell by Chenoweth and co-workers in 1984. Although its main cellular target is believed to be protein kinase A, raised intracellular cAMP levels can also activate cyclic nucleotide-gated ion channels, as well as exchange proteins directly activated by cAMP (Epacs) (Sands and Palmer, 2008). Surprisingly other inducers of cAMP, such as prostaglandin E2, dimaprit and isoproterenol, have been unable to increase C5aR expression. It has been suggested by Shayo and co-workers that the histamine H_2 receptor agonist (dimaprit), which increases intracellular cAMP levels transiently, was unable to induce C5aR expression because of rapid desensitisation of the H2 receptor, whereas forskolin could induce differentiation and up-regulation of C5aR via a sustained elevated cAMP level within the cells (Shayo et al., 1997, Brodsky et al., 1998). Although prostaglandin E2 and isoproterenol, a β-adrenoreceptor agonist, are unable to induce C5aR expression on their own, when combined with 1,25-dihydroxy vitamin D3 they have been

shown to induced C5aR expression proportional to cAMP induction (Rubin et al., 1986). In these experiments, the induction of C5aR expression was not solely explained by PKA activation and it was suggested that protein kinase C (PKC) may also be activated (Rubin et al., 1991b). Further evidence that activation of PKC can contribute towards differentiation of the U937 cell line came from studies that have shown that PMA (PKC activator) is able to increase C5aR expression alone and it can be augmented with 1,25-dihydroxy vitamin D3 or Bt_2cAMP (Burg et al., 1996, Rubin et al., 1991b).

Other more physiological stimuli that have been reported to regulate C5aR expression are also shown in Chapter 1 table 1.2. Of particular interest to this project are the cytokines that have been implicated in atherosclerosis. Section 1.3.1 has previously described chemokine MCP-1 as important chemoattractant of monocytes into the intima during early stages of atherosclerosis (Libby, 2002). Although no published data has shown whether these stimuli are able to regulate C5aR expression, other pro-inflammatory and pro-atherogenic cytokines (TNFα, IL-1, IL-6 and IFNγ) are able to increase the receptor expression, see table 1.2. Shown in figure 4.1 are the principal signalling pathways used by these pro-inflammatory cytokines. The exact mechanisms by which these pro-inflammatory cytokines regulate C5aR expression is not known. However, as C5a has previously been shown to increase expression of TNFα, IL-1 and IL-6 cytokines, and these cytokines increase expression of the C5aR (Okusawa et al., 1987, Okusawa et al., 1988, Goodman et al., 1982, Scholz et al., 1990), it is likely that the mechanism is complex with 2-directional interaction.

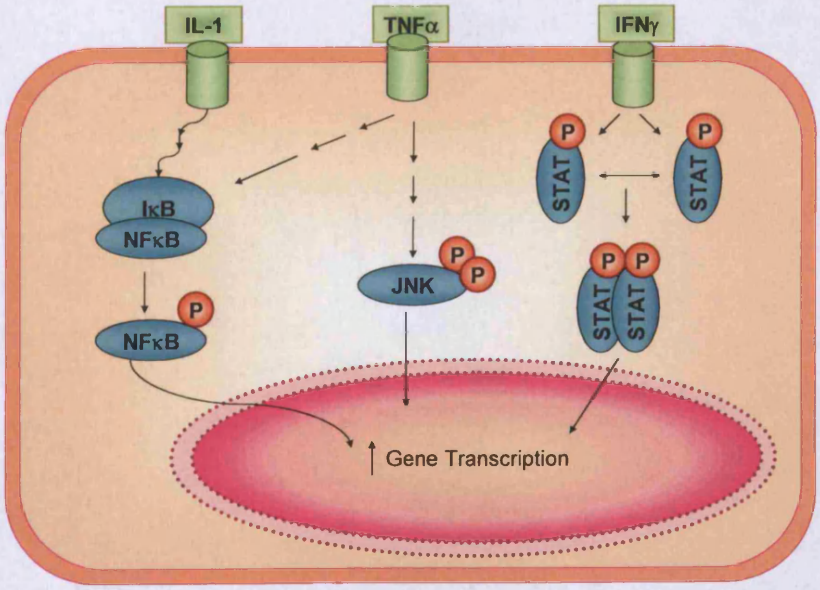


Figure 4.1 Schematic diagram illustrating the principal signalling pathways activated by the pro-inflammatory cytokines IL-1, TNFα and IFNγ. Adapted from (Tedgui and Mallat, 2006).

4.1.2 Methods for detecting changes in C5aR expression

As shown in table 1.2, several techniques have been employed to investigate changes in C5aR expression. Some investigators choose to monitor changes in mRNA, using techniques such as northern blotting, RT-PCR and Q-PCR, as an indication for changes in protein expression. Although the latter techniques are quite sensitive, changes in mRNA does not always equate to changes in protein expression. It is therefore important to also monitor changes in protein expression when these techniques are used.

Earlier studies used ligand binding studies using ^{125}I-C5a to monitor changes in the C5aR expression, however due to their radio-active nature this technique has become less popular. Furthermore results from ligand binding studies are complicated now by the discovery of the second C5a receptor C5L2, which also binds C5a with a similar affinity as the C5aR (Cain and Monk, 2002).

The wide spread availability of flow-cytometers has made flow cytometry a popular method for monitoring changes in surface receptor expression. Flow cytometry is advantageous over other techniques because it allows the user to monitor expression of several surface antigens at the same time on the same cells. To check whether changes in C5aR expression is reflected by changes in sensitivities towards C5a several studies also perform functional assays such as chemotaxis towards C5a using chemotactic chambers and C5a induced Ca^{2+} release as an indication for changes in functional C5aR expression.

In order to characterise induced C5aR expression in this chapter, techniques including western blotting and flow cytometry will be used to measure changes in receptor expression and C5a induced Ca^{2+} release will be monitored to determine whether changes in expression equate to changes in biological function of the receptor.

4.1.2.1 Flow cytometry

Flow cytometry allows the rapid and sensitive measurement of multiple parameters at any one time on a given cell. Using fluorescently labelled antibodies against the particular antigen of interest, flow cytometry provides the user with information regarding the fluorescence intensity of each cell which is proportional to the amount of fluorescent antibody bound to the antigen and therefore the expression levels of that particular antigen. It achieves this by passing a stream of single cells through the laser beam and capturing light emitted from each cell as depicted in figure 4.2. The use of different fluorophores attached to different antibodies allows multiple antigens to be analysed at the same time on one cell. Flow cytometry also provides information regarding the cell shape and granularity, which together allows the distinction between different cell populations. Due to this rapid and multi-

parametric nature of flow cytometry, this technique will be used to monitor changes in C5aR expression levels.

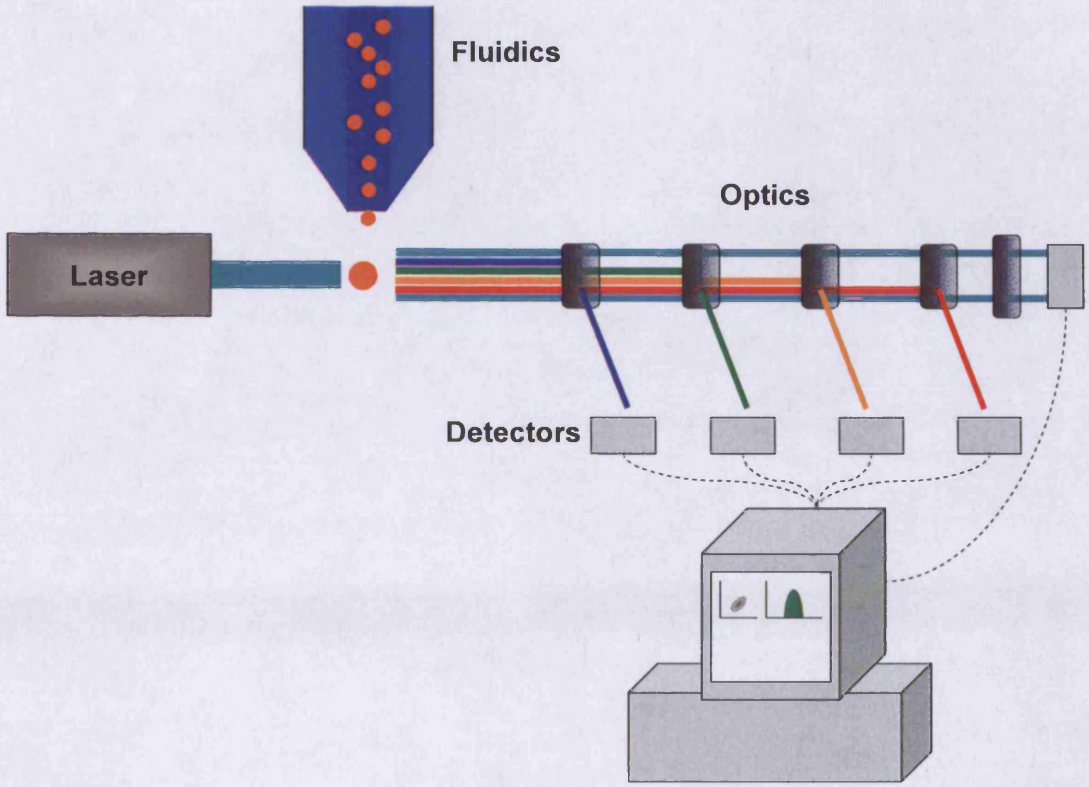


Figure 4.2 Schematic diagram of flow cytometry.

4.1.2.2 Western blotting

Western blotting will also be used to monitor changes in C5aR expression as it can detect changes in both extracellular and intracellular protein. The technique involves separating proteins from cell samples according to their molecular weight using SDS-PAGE. The separated proteins are then transferred from the SDS-PAGE onto a membrane, typically nitrocellulose or PVDF. Once on the membrane the proteins can be probed using antibodies against the specific antigen of interest. Antibody binding can be visualised using horse radish peroxidase conjugated secondary antibody, followed by either chemiluminescence or colourimetric analysis.

4.1.2.3 C5a induced intracellular Ca^{2+} release

Following activation of the C5aR one of the first responses observed within the cell is a rise in intracellular Ca^{2+} concentration which arises due to activation of PLCβ signalling pathway (figure 1.3) (Maurya and Subramaniam, 2007b, Maurya and Subramaniam, 2007a, Jiang et al., 1996, Camps et al., 1992). C5a induced Ca^{2+} release can therefore be used to monitor changes in functional C5aR expression. Intracellular Ca^{2+} acts as a diffusible secondary messenger which can bind and activate Ca^{2+} binding proteins. Although the exact Ca^{2+}

binding proteins activated through stimulation of C5aR are not well characterised, typically these proteins regulate cellular processes such as enzyme activation, motility, morphology, cell cycle progression and gene regulation.

As the U937 cell line is a cell suspension cell line, intracellular Ca^{2+} measurements will be recorded using the FLUOstar Optima plate reader. This method allows the average Ca^{2+} measurement of the total cell population as opposed to using techniques such as confocal microscopy which allow Ca^{2+} measurements of individual cells. The Ca^{2+} fluorescent indicator Fura-2-AM will be used to measure intracellular Ca^{2+} as its acetoxymethyl (AM) group allows it to diffuse into the cell, however once in the cell it is then cleaved by intracellular esterases causes it to become trapped within the cell. This form of dye is less invasive than dextran or salt forms which need to be microinjected or electroporated into the cells due to their cell impermeable nature. Another advantage of using the Fura-2-AM dye is that upon ion binding the dye exhibits a spectral shift in its excitation wavelength which allows ratiometric measurements of Ca^{2+} concentrations that are essentially independent of uneven dye loading, cell thickness, photobleaching and dye leakage (Grynkiewicz et al., 1985).

4.1.3 Hypothesis and aims

The hypothesis of this chapter is that stimulation of the U937 cell line with different stimuli, as listed in table 1.2, should cause up-regulation of C5aR expression and differentiation of the cells to a more macrophage like phenotype. The aim is to find the best inducer of C5aR expression in the U937 cell line and study their effects on functions of the C5aR.

4.2 Materials and methods

4.2.1 Chemicals and reagents

As previously described in section 2.1 all general reagents were purchased from Sigma. Lipopolysaccharide LPS was from Sigma (*E.coli* 0111:B4). Human recombinant tumour necrosis factor-alpha (TNFα) (TNFSF2), IL-6 (200-06-B) and interferon gamma (IFNγ) (H00003458) were from were from Tebu-bio.

4.2.2 Cell culture

Human monocytic cell lines $U937_{PM}$ and $U937_{EC}$ were routinely cultured in complete RPMI as already described in section 2.3.1. The cells were seeded at 1 $x10^5$ cell/ml ($U937_{EC}$) or 2.5 x 10^5 cell/ml ($U937_{PM}$) and stimulated for 48 hrs, unless otherwise stated, with various concentrations of PMA, Bt_2cAMP, IFNγ, TNFα, LPS or IL-6.

4.2.3 Flow cytometry

C5aR expression was analysed by flow cytometry as previously described in section 2.3.6. C5aR was detected using specific monoclonal anti-C5aR (S5/1, from SantaCruz) which according to the data sheet is an IgG1 isotype and therefore IgG1 isotype matched control anti-glycophorin (BGRL100, from IBGRL) was used to detect non-specific binding via the Fc receptors. However later investigation found that the monoclonal anti-C5aR S5/1 clone is an IgG2a isotype and therefore later experiments were incubated with aggregated IgG (56834, from Sigma) prior to staining for flow cytometry, where stated, to block non-specific binding via the Fc receptors as oppose to using an isotype matched control (Oppermann et al., 1993). Aggregated IgG was generated by heating IgG (1 mg/ml in PBS) at 63 °C until solution became cloudy, approximately 20 min. To monitor changes in Fc receptors expression flow cytometry was also performed using monoclonal anti-CD16 (3G8; IgG1; from ATCC) and CD32 (IV.3; IgG2b; from ATCC). The secondary antibody used for all experiments was a goat anti-mouse FITC conjugate, as previously described in section 2.3.6. Cells were stained in the absence of aggregated human IgG unless otherwise stated.

4.2.4 Western blotting

Western blotting was performed as previously described in section 2.3.7 using polyclonal anti-C5aR (H-100).

4.2.5 Ca^{2+} signalling

Intracellular Ca^{2+} was measured using Fura-2-AM as previously described in section 2.3.8.

4.2.6 Propidium iodide staining

Cell viability was assessed using propidium iodide exclusion as previously described in section 2.3.9.

4.3 Results

The U937 cell line is frequently used to study the C5aR expression and function. The cell line is differentiated using stimuli such as Bt_2cAMP, PMA and IFNγ, in order to obtain a more macrophage like phenotype and induce up regulation of the C5aR (see table 1.2). To investigate which stimulus is the best inducer of C5aR expression and function in our U937 cell lines, the cells were incubated with a variety of different stimuli and changes in C5aR expression and function were then analysed.

4.3.1 Comparison of two U937 cell lines in their responsiveness to PMA, Bt_2cAMP, IFNγ, IL-6, LPS and TNFα

Initial studies using the U937 cell line obtained from the ECACC ($U937_{EC}$) found that Bt_2cAMP, which has been well documented in the literature as a strong inducer of C5aR expression in the U937 cell line, failed to alter receptor expression in these cells (figure 4.3.1). Subsequently, a U937 cell line was obtained from Dr Peter Monk (Sheffield University; $U937_{PM}$). In this Chapter the two different cell lines were incubated with different stimuli for 48 hrs and changes in C5aR expression and function were compared.

Although both $U937_{EC}$ and $U937_{PM}$ had similar resting levels of C5aR expression, which was quite low, each cell line differed in their responses to the different stimuli. Figure 4.3.1 shows that incubating the $U937_{EC}$ cell line with PMA, IFNγ and IL-6 increased C5aR expression when detected by flow cytometry using the monoclonal antibody S5/1, however, only IFNγ and IL-6 induced effects were statistically significant. Although other stimuli, including Bt_2cAMP, TNFα and LPS, have been previously reported to increase C5aR expression in the U937 cell line (see table 1.2 for details), here they had no effect on its expression in the $U937_{EC}$ cell line (figure 4.3.1). The $U937_{PM}$ cells, however, responded with an increased C5aR expression when treated with Bt_2cAMP, PMA and IFNγ, while TNFα, LPS and IL-6 had no significant effects on its expression (figure 4.3.2). The results showed that these cells not only differ in which stimuli they respond to, they also differ in the magnitude of their responses. Figure 4.3.3 compares the relative change in C5aR expression in the $U937_{EC}$ with the $U937_{PM}$. Following treatment with the different stimuli, the most obvious difference is observed when comparing the effects of Bt_2cAMP treatment. In $U937_{PM}$ this stimuli resulted in average ~ 5 fold increase in C5aR expression compared with the control, however, in $U937_{EC}$, Bt_2cAMP had no effect on C5aR expression (figure 4.3.3). Although both cell lines responded to PMA, the $U937_{PM}$ cell line exhibited an average ~ 5 fold increase in C5aR expression compared with the ~ 2 fold increase shown in the $U937_{EC}$ cell line. Incubation with IFNγ resulted in the strongest responses with a ~ 13 and ~ 12 fold increase in

fluorescence in the $U937_{EC}$ and $U937_{PM}$ cell line respectively. $U937_{EC}$ increased C5aR expression, ~ 3 fold, following incubation with IL-6, whereas in the $U937_{PM}$ cell line the receptor expression remained unchanged (figure 4.3.3).

As well as differing in their C5aR expression following treatment with different stimuli, these cell lines also differ in their sensitivity to the cytotoxic effects of the stimuli. Using propidium iodide staining as a indicator of cell death, the $U937_{EC}$ was shown to be more sensitive to the cytotoxic effects of IFNγ compared with $U937_{PM}$ and that $U937_{PM}$ was more sensitive to PMA compared with $U937_{EC}$ (figure 4.3.4) The morphology of each cell line also differed slightly following incubation with either PMA or Bt_2cAMP (figure 4.3.5). PMA incubation caused both cell lines to become more adherent. The $U937_{PM}$ cells were completely adherent with only dead/dying cells present in the culture supernatant, whereas, $U937_{EC}$ cells had viable cells present in both the supernatant and adherent to the culture flask (figure 4.3.5). As PMA treatment resulted in cells becoming adherent, cells were harvested by cell scrapping which may contribute to the higher cell death seen in figure 4.3.4. Incubation of $U937_{PM}$ cells with Bt_2cAMP changed their cell morphology from a spherical like shape to an irregular shape with lots of protrusions. The $U937_{EC}$ cells however displayed little change in cell morphology following incubation with Bt_2cAMP (figure 4.3.5). The two cell lines also exhibited different morphologies when unstimulated with the $U937_{EC}$ being more spherical compared with the $U937_{PM}$ cells, furthermore the $U937_{EC}$ cells grew quicker in routine culture compared with the $U937_{PM}$ cells.

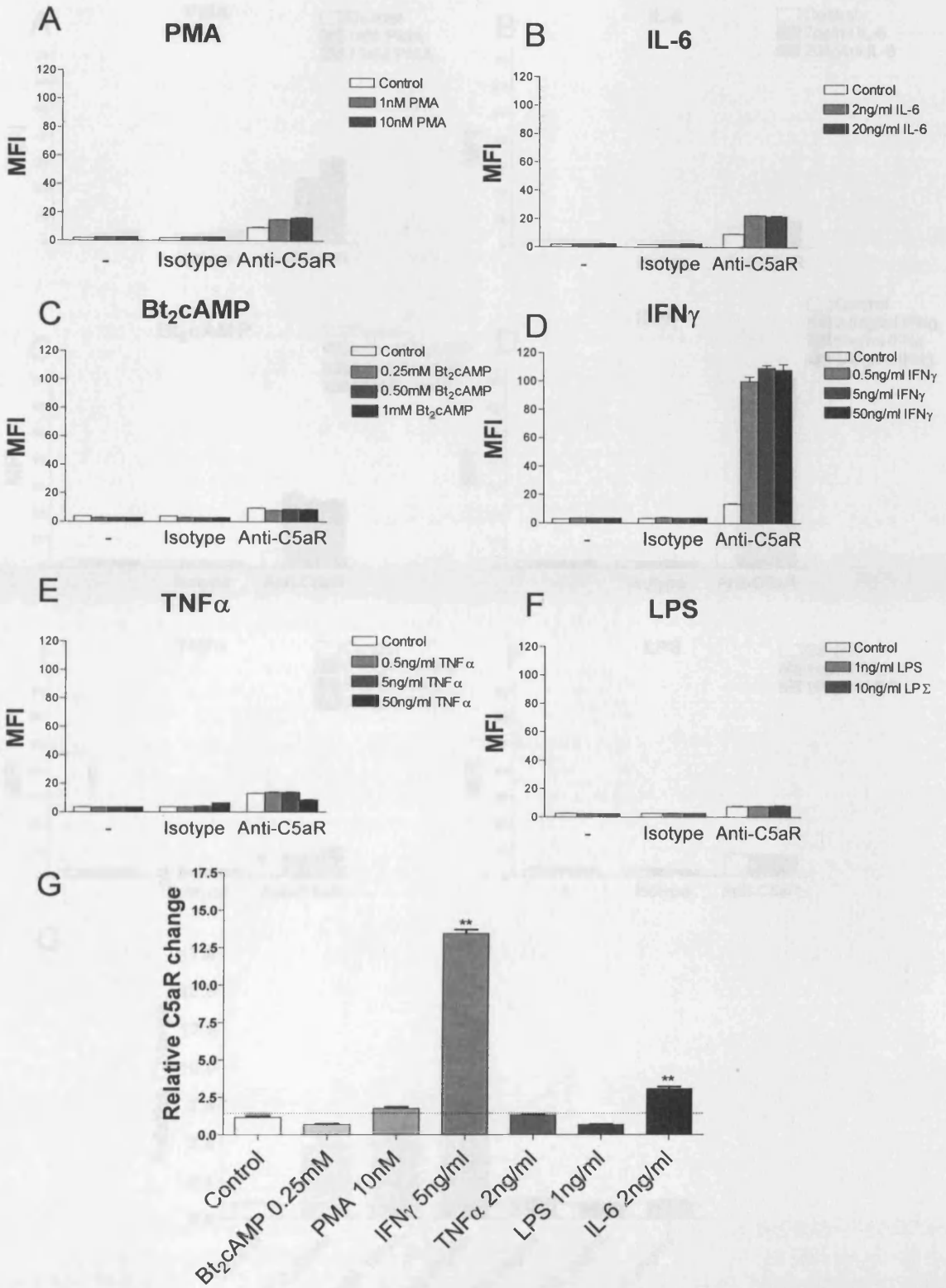


Figure 4.3.1 Effects of different stimuli on C5aR expression in the $U937_{EC}$ cell line. $U937_{EC}$ were incubated for 48 hr with various concentrations of PMA (A), IL-6 (B), Bt_2cAMP (C), IFNγ (D), TNFα (E) and LPS (F). C5aR expression was detected by flow cytometry as described in section 4.2.3. (G) Relative C5aR change was calculated as described in Chapter 2 section 2.3.6. One-way ANOVA followed by Dunnett's multiple comparison test was performed, **p<0.01 cf. control.

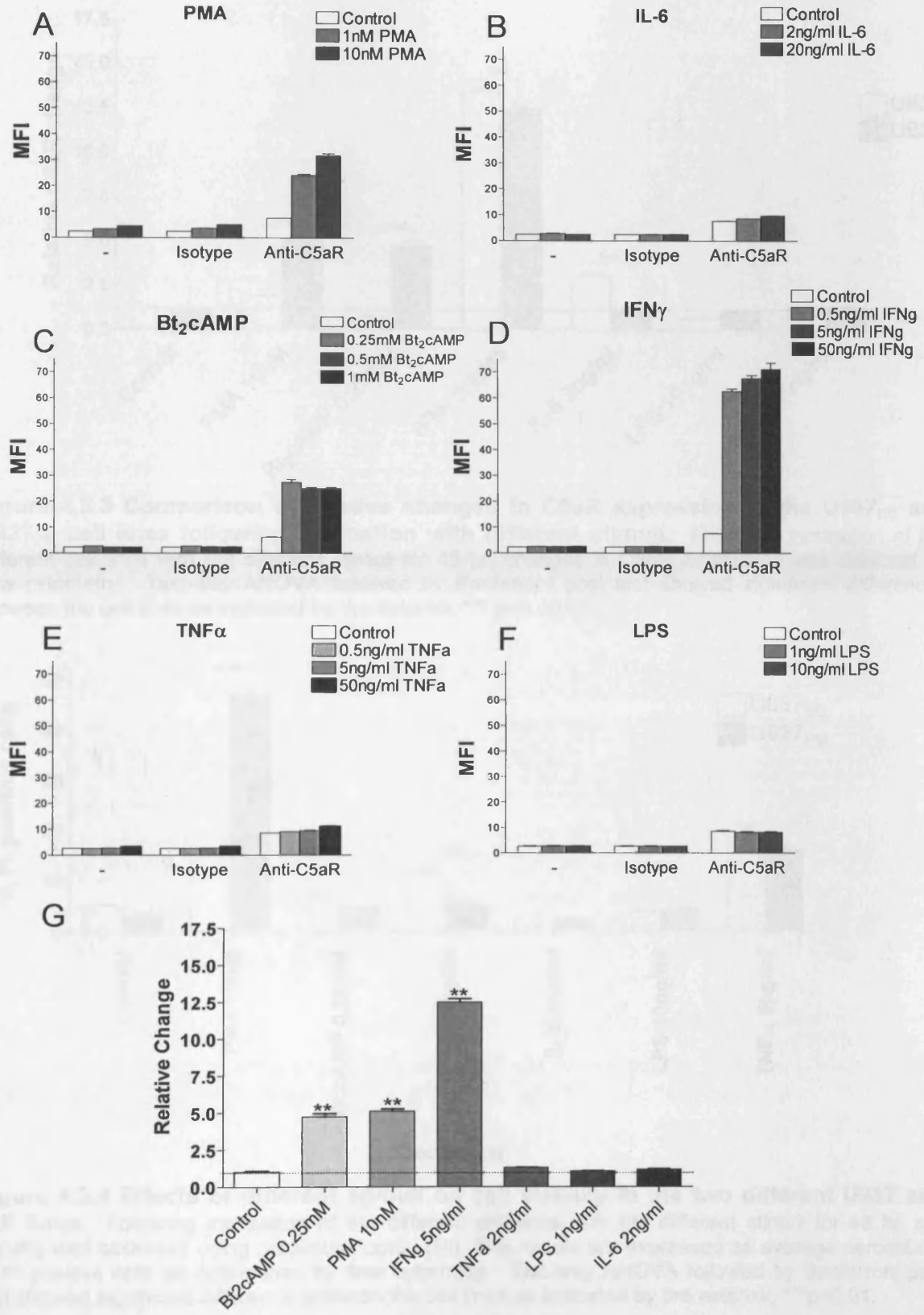


Figure 4.3.2 Effects of different stimuli on C5aR expression in the U937$_{PM}$ cell line. U937$_{PM}$ were incubated for 48 hr with various concentrations of PMA (A), IL-6 (B), Bt_2cAMP (C), IFNγ (D), TNFα (E) and LPS (F). C5aR expression was detected by flow cytometry as described in section 4.2.3. (G) Relative C5aR change was calculated as described in Chapter 2 section 2.3.6. One-way ANOVA followed by Dunnett's multiple comparison test was performed, **p<0.01 cf. control.

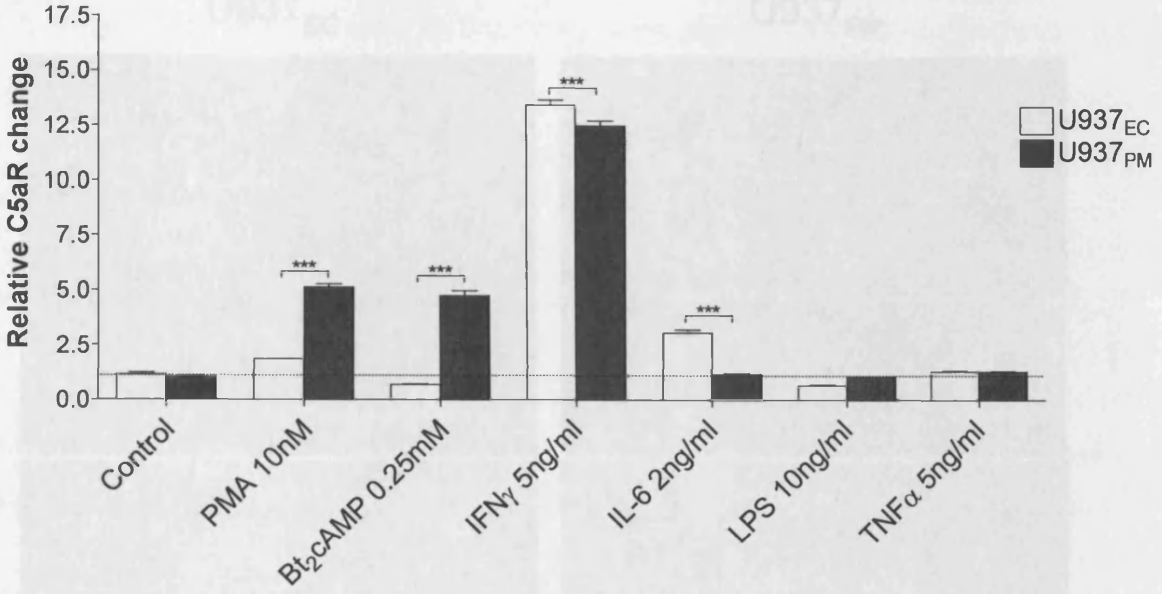


Figure 4.3.3 Comparison of relative changes in C5aR expression in the $U937_{EC}$ and $U937_{PM}$ cell lines following incubation with different stimuli. Following incubation of the different cell lines with the different stimuli for 48 hr, changes in C5aR expression was detected by flow cytometry. Two-way ANOVA followed by Bonferroni post test showed significant differences between the cell lines as indicated by the asterisk; *** $p<0.001$.

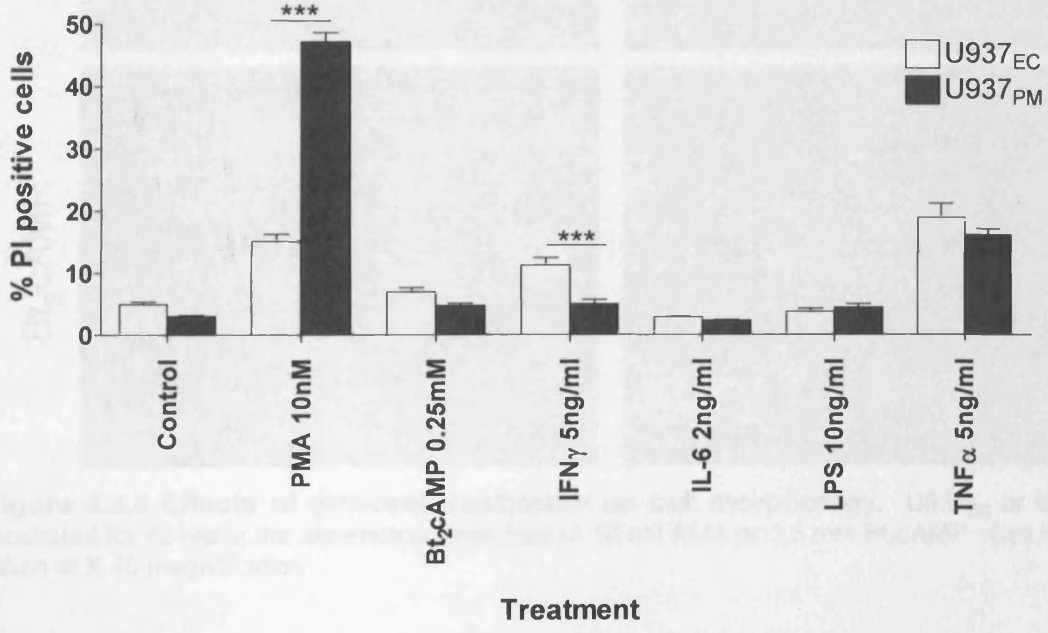


Figure 4.3.4 Effects of different stimuli on cell viability in the two different U937 sub cell lines. Following incubation of the different cell lines with the different stimuli for 48 hr, cell viability was assessed using propidium iodide (PI). The results are expressed as average percentage of PI positive cells as determined by flow cytometry. Two-way ANOVA followed by Bonferroni post test showed significant difference between the cell lines as indicated by the asterisk; ***$p<0.01$.

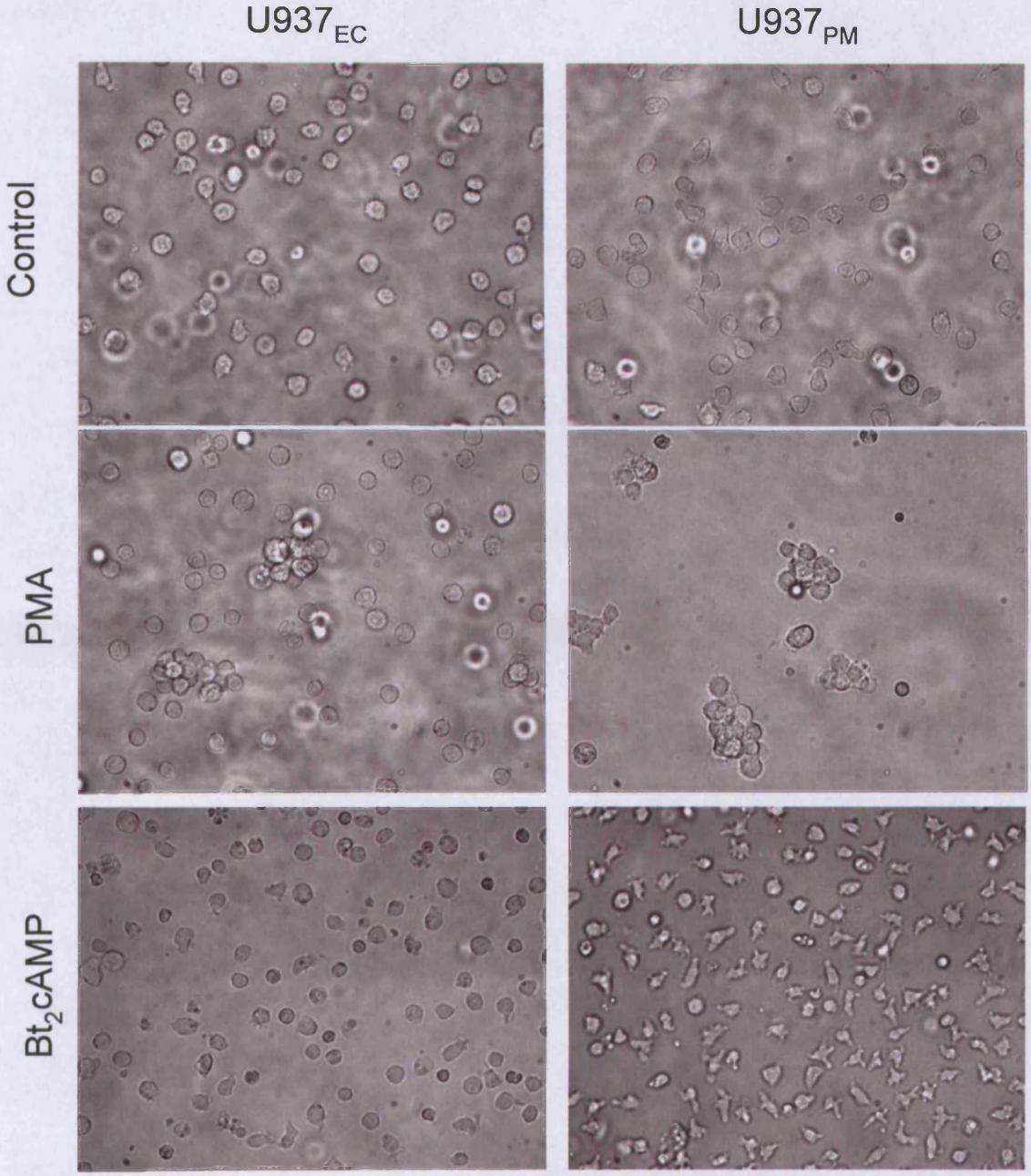


Figure 4.3.5 Effects of different treatments on cell morphology. $U937_{EC}$ or $U937_{PM}$ were incubated for 48 hrs in the absence or presence of 10 nM PMA or 0.5 mM Bt_2cAMP. Cell images were taken at X 40 magnification.

4.3.2 Comparison of changes in C5aR expression by western blotting

To confirm the results as observed by flow cytometry, both $U937_{EC}$ and $U937_{PM}$ cell lines were incubated with either PMA, Bt_2cAMP, IFNγ or IL-6 for 2 days and C5aR expression was detected by western blotting.

Shown in figure 4.3.6A is a representative western blot from the $U937_{EC}$ cells incubated with the different stimuli. The C5aR expression could not be detected in the control samples which was consistent with results from flow cytometry which had already shown that the basal levels of expression in these cells is quite low (figure 4.3.1). As flow cytometry had already shown that incubation with IFNγ and IL-6 increased C5aR expression in the $U937_{EC}$ cell line (figure 4.3.3) it was anticipated that following incubation with these stimuli we would be able to detect a specific molecular band at approximately 42 kDa corresponding to increased C5aR expression. However, western blotting of $U937_{EC}$ cells which had been incubated with IFNγ or IL-6 as well as PMA and Bt_2cAMP failed to detect C5aR expression (figure 4.3.6A). One explanation for this outcome could be that FACS only measures surface protein expression whereas western blotting measures total protein and if for instance IFNγ or IL-6 do not actually change total protein expression rather they act by redistributing proteins to the cell surface this would appear to increase levels by FACS but not by western blotting. The western blot of the $U937_{EC}$ cells treated with PMA and Bt_2cAMP displayed a high molecular weight band which appeared to remain in the stacking gel (figure 4.3.6). This band could be aggregated C5aR, however, as this film was exposed o/n this may explain why the same bands were not observed in the $U937_{PM}$ blot which was only exposed for 5 min (figure 4.3.6A and B). During the optimisation of western blotting it was observed that heat treating and both heat treatment plus a reducing reagent increased the amount of C5aR which aggregated in the stacking gel. Therefore it was decided not to heat treat or reduce the samples prior to western blotting to reduce the amount of aggregated C5aR.

Similar to the $U937_{EC}$ C5aR expression in the $U937_{PM}$ cells could not be detected in the control cells by western blotting which correspond to both cell lines having similar low levels of C5aR (figure 4.3.6B). Although IFNγ was previously shown to be the biggest inducer of C5aR expression in the $U937_{PM}$ cell line western blotting of the same samples failed to display any bands corresponding to the C5aR (figure 4.3.3 and 4.3.6B). Incubation with both PMA and Bt_2cAMP produced strong bands at the approximate molecular weight of the C5aR although flow cytometry had shown these stimuli were less able to induce C5aR expression compared with IFNγ (figures 4.3.6B and 4.3.3). Figure 4.3.6B also shows that the $U937_{PM}$ cells incubated with Bt_2cAMP produced a more intense C5aR band compared to

incubation with PMA (figures 4.3.6B). Western blot of $U937_{PM}$ cells which had been incubated with IL-6 also suggested that in these cells IL-6 had no effect on C5aR expression which correlates with flow cytometry results (figure 4.3.6B and 4.3.3).

Together these results suggest that although figure 4.3.3 shows IFNγ being the strongest inducer of C5aR expression in both $U937_{EC}$ and $U937_{PM}$ cell lines by flow cytometry, only Bt_2cAMP and PMA are able to increase C5aR expression in the $U937_{PM}$ cell line as detected by western blotting (figure 4.3.6).

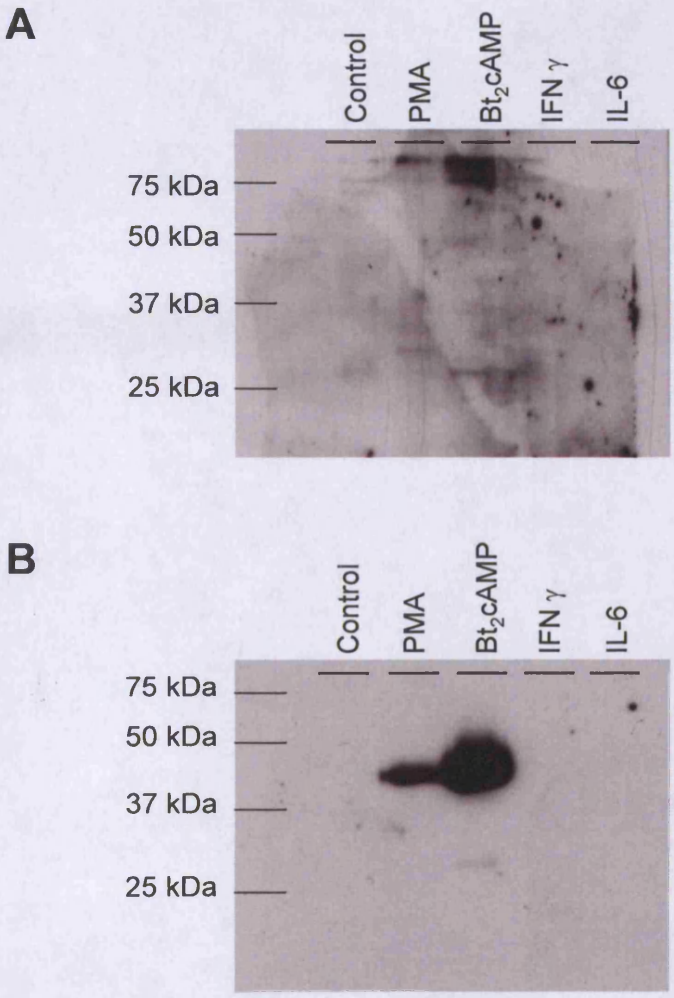


Figure 4.3.6 Western blot analysis of C5aR expression in $U937_{EC}$ (A) and $U937_{PM}$ (B) cells treated with different stimuli for 48 hr. $U937_{EC}$ (A) and $U937_{PM}$ (B) were incubated for 2 days in the absence or presence of 10 nM PMA, 0.5 mM Bt_2cAMP, 5 ng/ml IFNγ or 2 ng/ml IL-6. 1 X 10^6 cells were then loaded onto a 12.5% SDS-PAGE. C5aR expression was detected using polyclonal anti-C5aR (H-100, SantaCruz). Results presented are representative from multiple analysis. Panel (A) is an o/n exposure whereas panel (B) is 5 min exposure.

4.3.3 Effects of induction of C5aR on C5a induced Ca^{2+} release

The aim of this section was to investigate whether an increase in C5aR expression corresponded to an increased responsiveness to C5a. C5a induced Ca^{2+} release was measured using the ratiometric calcium indicator Fura-2AM.

Both the $U937_{EC}$ and $U937_{PM}$ control cells showed a similar increase in Ca^{2+} release following the addition of C5a, which correlates with their similar basal levels of C5aR expression (figure 4.3.7A and B). Pre-incubation with PMA failed to increase the responsiveness to C5a induced Ca^{2+} release in the $U937_{EC}$ cells, which corresponded to the observation that PMA did not induce significant changes in C5aR expression (figure 4.3.7A). Furthermore, incubation with either IFNγ or IL-6 also failed to increase the C5a induced Ca^{2+} response in these cells, despite their apparent higher C5aR cell surface expression (figure 4.3.7A). These results correlated with the results shown by western blot but not flow cytometry (sections 4.3.2 and 4.3.1 respectively). Treatment with Bt_2cAMP, however, showed a significant increase in Ca^{2+} release in response to either 5 nM or 50 nM C5a when compared to the control in the $U937_{EC}$ (figure 4.3.7A).

$U937_{PM}$ treated with Bt_2cAMP showed a strong and significant increase in Ca^{2+} release when stimulated with either 0.5 nM, 5 nM or 50 nM C5a, which correlates with results observed by flow cytometry and western blotting which showed an increased C5aR cell surface and protein expression induced by this agent (figure 4.3.7B). PMA treatment of $U937_{PM}$ also caused an increase in C5a induced Ca^{2+} release compared with the control, however, this increase was only statistically significant at 50nM C5a (figure 4.3.7B). This increased responsiveness to C5a after PMA incubation also correlates with the increased C5aR expression as detected by both FACS and western blotting. As with the $U937_{EC}$ cell line, incubation of $U937_{PM}$ cells with IFNγ failed to increase the cells responsiveness to C5a (figure 4.3.7B). This result correlates with the results from western blotting which showed that IFNγ had no effect on C5aR expression in the $U937_{PM}$ cell line but not flow cytometry (sections 4.3.2 and 4.3.1 respectively). Incubation of $U937_{PM}$ with IL-6 had no effect on the C5a induced Ca^{2+} release compared with the control which corresponds with both the flow cytometry and western blotting results that show that IL-6 had no effect on C5aR expression (figure 4.3.7B and 4.3.3).

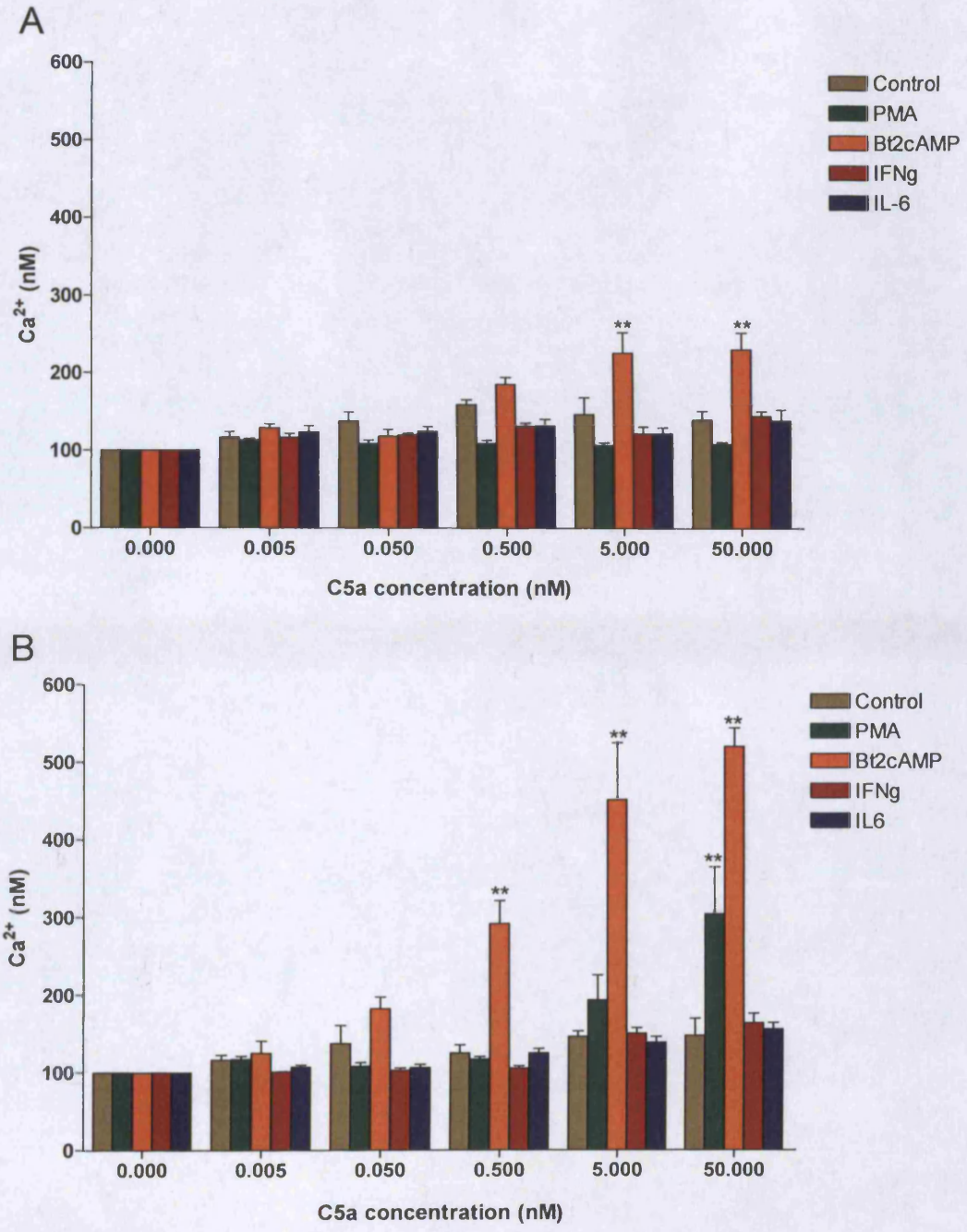


Figure 4.3.7 Effects of treatment on C5a induced Ca2+ release in the $U937_{EC}$ (A) or $U937_{PM}$ (B) cell lines. $U937_{EC}$ (A) or $U937_{PM}$ (B) cells were incubated for 48 hrs with either control, 10 nM PMA, 0.5 mM Bt_2cAMP, 5 ng/ml IFNγ or 2 ng/ml IL-6. Following incubation the cells were loaded with 10 μM Fura-2-AM. Average maximum Ca^{2+} release following the addition of C5a was calculated as described in section 2.3.8. Two-way ANOVA followed by Bonferroni post test showed significant difference compared with the control as marked by the asterisk, ** $p < 0.001$.

4.3.4 Apparent increase in cell surface C5aR expression induced by IFNγ and IL6, but not Bt_2cAMP and PMA, is caused by aspecific binding of the anti-C5aR antibody

Due to the nature of using monocytic cells it is crucial that the necessary controls are performed in order to identify non-specific binding via the Fc receptors. Typically using non-related isotype matched control is sufficient to detect any non-specific binding of antibodies via the Fc region, however, it is sometimes necessary to include human immunoglobulins in the buffer to block Fc receptors.

As results observed by C5a-induced calcium release and western blotting did not correlate with results observed by flow-cytometry, it was decided to repeat flow cytometry in the absence or presence of human aggregated IgG to block non-specific binding via Fc receptors. Although both cell types appeared to express some basal C5aR levels when stained in the absence of aggregated IgG, incubation with aggregated IgG prior to staining for flow cytometry resulted in the C5aR expression levels for the control cells to be barely above the background isotype control fluorescence (figure 4.3.8). The $U937_{EC}$, when stained in the absence of aggregated human IgG, both IFNγ and IL-6 appeared to cause significant upregulation of C5aR, whereas incubation with PMA and Bt_2cAMP had no effect. However, when the same cells were also stained in the presence of aggregated IgG the results showed that none of these stimuli, PMA, Bt_2cAMP, IFNγ or IL-6, actually upregulated cell surface C5aR expression (figure 4.3.8). Similarly, when $U937_{PM}$ were incubated with IFNγ the cells stained in the absence of aggregated IgG showed a significant increase in cell surface C5aR expression which was not observed when the cells were stained in the presence of aggregated IgG (figure 4.3.8). Although treatment of $U937_{PM}$ with PMA and Bt_2cAMP showed a significant increase in C5aR expression when stained in both the absence and presence of aggregated IgG (figure 4.3.8). These results taken together with results from the western blotting (section 4.3.1.2) and the functional studies (section 4.3.1.3) suggest that the increase in C5aR expression observed by IFNγ or IL-6 is due to an increase in non-specific binding of the antibody and not actually C5aR expression. However if this was the case it would be anticipated that the isotype control should also bind non-specifically and results shown in figure 4.3.8 show that binding of the isotype control did not alter when cells were incubated in the absence or presence of aggregated IgG. The isotype control used was anti-glycophorin (intracellular domain) IgG1 isotype. This antibody was chosen for the control as the data sheet for the monoclonal anti-C5aR S5/1 from SantaCruz stated that this antibody was an IgG1 isotype. Further investigation into the background of the anti-C5aR S5/1 antibody revealed that this antibody is in fact an IgG2a/k isotype which may explain why the

IgG1 isotype did not detect any non-specific binding via the Fcγ receptors (FcγR) (Oppermann et al., 1993).

Members of the FcγR family bind to the Fc portion of IgG class of antibodies. There are several isoforms of the FcγR, FcγR I (CD64), FcγR II (CD32), FcγR IIIa (CD16A) and FcγR IIIb (CD16B), which all differ in their antibody affinities (Indik et al., 1995). Flow cytometry was performed using antibodies against CD32 and CD16 to determine if these receptors were up regulated by any of treatments in the $U937_{PM}$ and $U937_{EC}$ cell lines. Both cell lines expressed high levels of CD32 but no CD16 (figure 4.3.9). Furthermore CD16 expression in $U937_{EC}$ and $U937_{PM}$ remained unchanged after incubation with PMA, Bt_2cAMP, IFNγ or IL-6, whereas, CD32 expression was significantly increased by Bt_2cAMP in both cell lines (figure 4.3.9). PMA, IFNγ and IL-6 had no effect on CD32 expression in $U937_{PM}$ cell line but slightly decreased CD32 expression in the $U937_{EC}$ cell line (figure 4.3.9). Although IFNγ or IL-6 were not shown here to up regulate CD16 or CD32 they may increase expression of CD64.

Together these results suggest that only Bt_2cAMP and PMA are able to induce C5aR expression in the $U937_{PM}$ cell line.

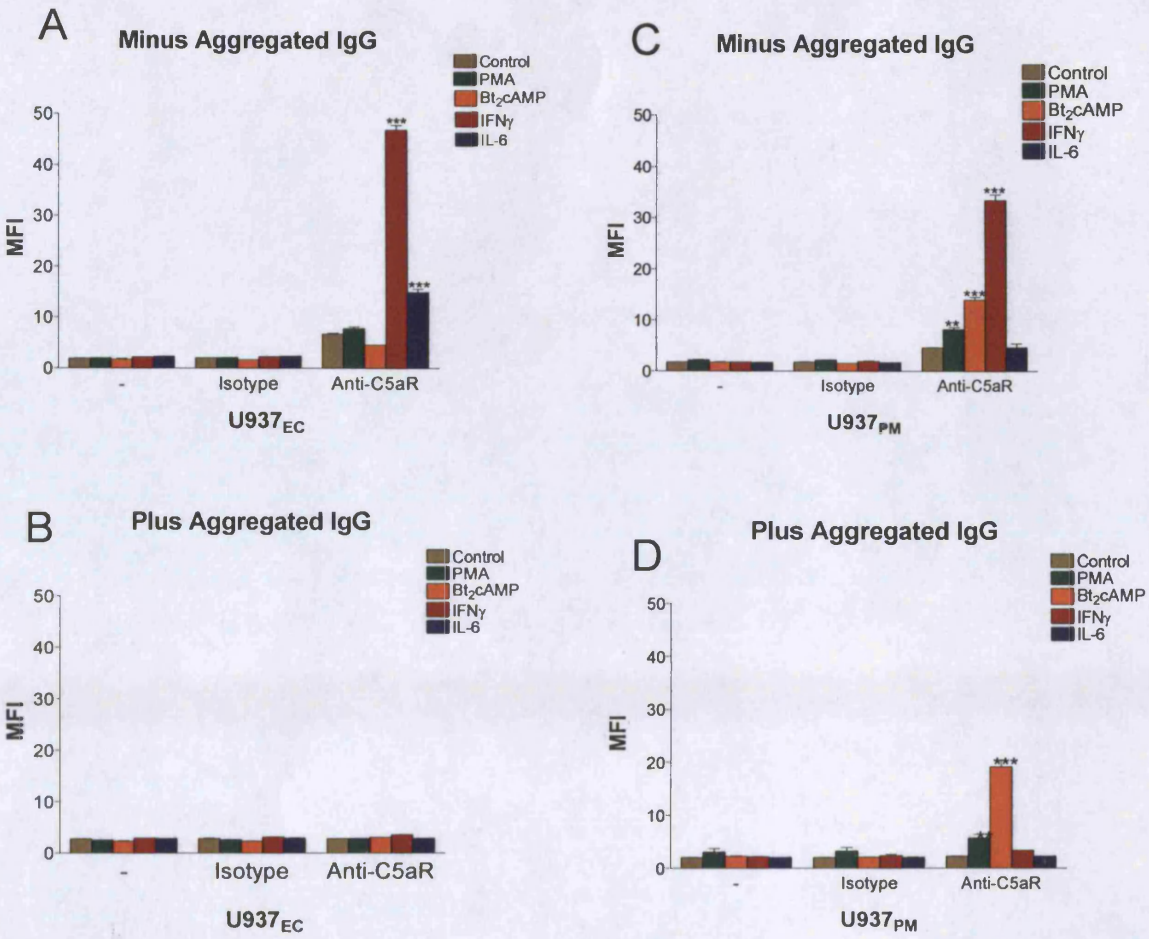


Figure 4.3.8 Effects of addition of aggregated human IgG on C5aR expression following treatment of different stimuli detected by FACS. Following treatment of $U937_{EC}$ (A and B) and $U937_{PM}$ (C and D) with different stimuli for 48 hrs, the cells were harvested and incubated in the absence (A and C) or presence (B and D) of aggregated human IgG (100ug/ml). Cells were stained for C5aR expression using anti-C5aR (S5/1) as described in section 4.2.3 and an IgG1 isotype control (anti-glycophorin C, intracellular epitope (BGRL100)) was also used to detect non-specific binding of the primary antibody. One-way ANOVA followed by Dunnett's test showed significant difference in C5aR expression compared with the control as marked by the asterisk, $^{**}p<0.01$, $^{***}p<0.001$.

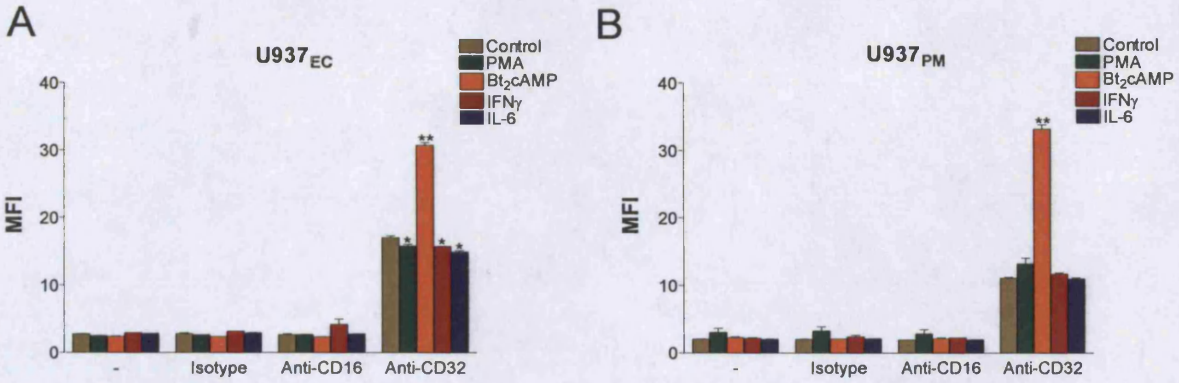


Figure 4.3.9 Effects of different treatment on FcγR III (CD16) and FcγR II expression (CD32). $U937_{EC}$ (A) and $U937_{PM}$ (B) were incubated for 48 hrs in the absence or presence of either 10 nM PMA, 0.5 mM Bt_2cAMP, 5 ng/ml IFNγ or 2 ng/ml IL-6. Cells were harvested and incubated with 100 μg/ml human aggregated IgG prior to staining for FACS analysis. CD16 expression was detected using anti-CD16 (3G8) and CD32 expression was detected using anti-CD32 (IV.3), as described section 4.2.3. One-way ANOVA followed by Dunnett's test shows significant difference from the control as marked by the asterisk, *$p<0.05$, **$p<0.01$.

4.3.5 Effects of Bt_2cAMP and PMA on C5aR promoter reporter expression

To investigate further whether the C5aR promoter contained any *cis*-acting elements which responded to either Bt_2cAMP or PMA, cloned from either $U937_{EC}$ (-2Kbp(**EC**)+pEGFP) or $U937_{PM}$ (-2Kbp(**PM**)+pEGFP) (see Chapter 3 for full details), both cell lines were transfected with the two reporter construct and changes in EGFP fluorescence were monitored by flow cytometry.

4.3.5.1 $U937_{EC}$ cell line transfected with C5aR promoter reporter constructs

When the $U937_{EC}$ cells transfected with the -2Kbp(**EC**)+pEGFP reporter construct were incubated with PMA, EGFP expression was shown to increase slightly although this was not statistically significant (figure 4.3.10). This result is in agreement with the finding that PMA had no significant effect on C5aR expression in the $U937_{EC}$ cell line (section 4.3.4). Similarly when the -2Kbp(**PM**)+pEGFP reporter construct was transfected into the $U937_{EC}$ cell line, incubation of these cells with PMA had no effect on EGFP fluorescence (figure 4.3.10).

Bt_2cAMP incubation of $U937_{EC}$ transfected with either -2Kbp(**EC**)+pEGFP or -2Kbp(**PM**)+pEGFP resulted in a decrease in EGFP fluorescence compared with the controls however, in the promoter-less pEGFP control vector fluorescence was also decreased, which suggest that this is a general effect on background fluorescence (figure 4.3.10).

Incubation of the $U937_{EC}$ cells, expressing the C5aR 3'UTR EGFP reporter construct, with PMA and Bt_2cAMP had no effect on EGFP fluorescence (figure 4.3.11). This result suggests that the ARE within the 3'UTR are not regulated by either of these stimuli which correlates with the finding that neither PMA nor Bt_2cAMP alter C5aR expression in the $U937_{EC}$ cell line.

4.3.5.2 $U937_{PM}$ cell line transfected with C5aR promoter reporter constructs

When the $U937_{PM}$ cell line transfected with -2Kbp(**EC**)+pEGFP and -2Kbp(**PM**)+pEGFP were incubated with PMA, EGFP fluorescence increased slightly compared with the control, although this was not classed as statistically significant (figure 4.3.12a). This result does not correlate with changes in C5aR expression which increased following incubation of the $U937_{PM}$ cell line with PMA (section 4.3.4).

Incubation of the $U937_{PM}$ cell line which had been transfected with the -2Kbp(**EC**)+pEGFP reporter construct with Bt_2cAMP showed a statistically significant increase in EGFP expression compared with the pEGFP control vector and -2Kbp(**PM**)+pEGFP transfected cells (figure 4.3.12b). However, incubation of the -2Kbp(**PM**)+pEGFP transfected cells with Bt_2cAMP showed no significant difference compared with the pEGFP control cells which is inconsistent with changes in C5aR expression which increased following incubation with Bt_2cAMP (figure 4.3.12b).

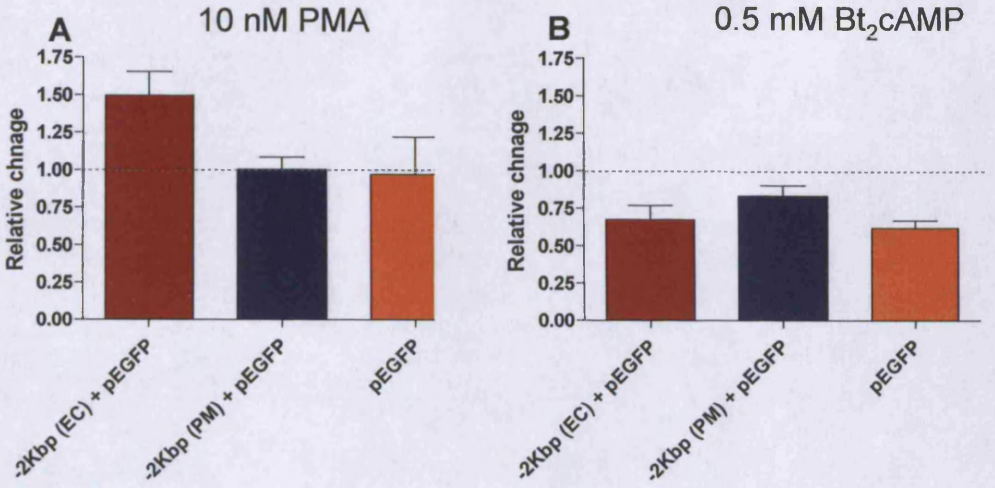


Figure 4.3.10 Effects of Bt_2cAMP and PMA incubation on EGFP expression in $U937_{EC}$ cell line transfected with the -2Kbp 5'promoter+pEGFP reporter constructs. $U937_{EC}$ cell line was transfected with either -2Kbp(**EC**)+pEGFP (red), -2Kbp(**PM**)+pEGFP (blue) or promoter-less pEGFP vector (orange). Once growing in log phase the cells were incubated for 2 days in the absence or presence of 10 nM PMA (A) or 0.5 mM Bt_2cAMP (B). Changes in EGFP fluorescence were then monitored by flow cytometry as described in section 4.2.3, dotted line represents the control value. One-way ANOVA followed by Tukey's multi comparison showed no significant difference between the different constructs when cells were treated with either PMA (A) or Bt_2cAMP (B).

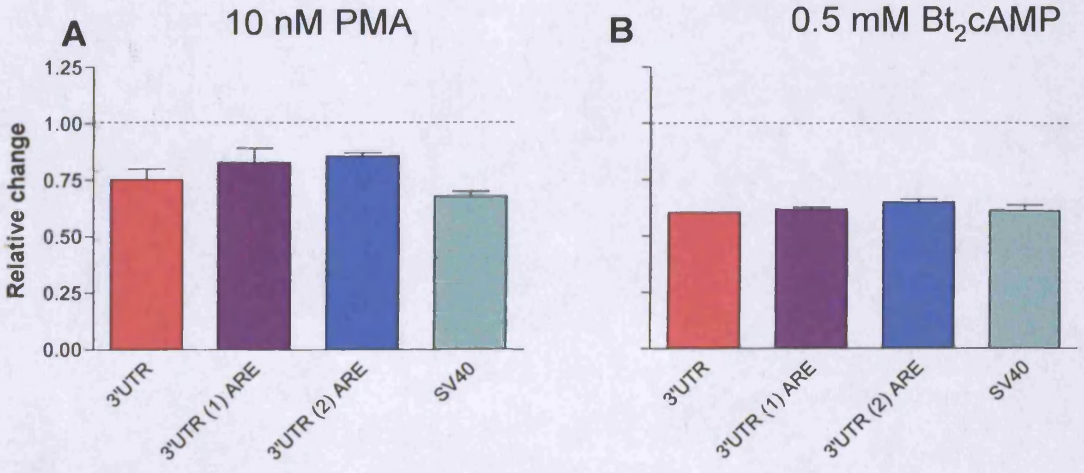


Figure 4.3.11 Incubation with PMA (A) or Bt_2cAMP (B) has no effect on the 3'UTR of C5aR. $U937_{EC}$ cell line which had been transfected with C5aR 3'UTR reporter constructs or SV40 control were incubated for 48 hr with either (A) 10 nM PMA or (B) 0.5 mM Bt_2cAMP. Changes in EGFP fluorescence were then monitored by flow cytometry as described in section 4.2.3, dotted line represents the control value. One-way ANOVA showed no significant difference between the different reporter constructs.

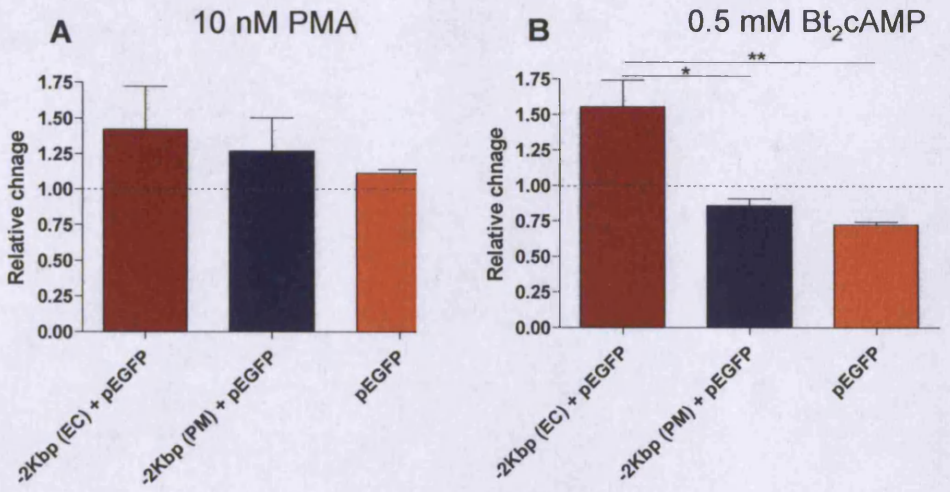


Figure 4.3.12 Effects of Bt_2cAMP and PMA incubation on EGFP expression in $U937_{PM}$ cell line transfected with the -2Kbp 5'promoter+pEGFP reporter constructs. $U937_{PM}$ cell line was transfected with either -2Kbp(**EC**)+pEGFP (red), -2Kbp(**PM**)+pEGFP (blue) or promoter-less pEGFP vector (orange). Once growing in log phase the cells were incubated for 2 days in the absence or presence of 10 nM PMA (A) or 0.5 mM Bt_2cAMP (B). Changes in EGFP fluorescence were then monitored by flow cytometry as described in section 4.2.3, dotted line represents the control value. One-way ANOVA followed by Tukey's multi comparison showed no significant difference between the different reporter constructs when the cells were treated with PMA (A), however a significant difference was observed when the cells were incubated with Bt_2cAMP, * $p< 0.05$, ** $p< 0.01$ (B).

4.4 Discussion

The aim of this chapter was to characterise and investigate which stimuli are the best inducers of C5aR expression in the U937 cell line in order to set up a model system to investigate the effects of statins on C5aR function and expression.

Results from this chapter have identified two U937 cell lines, $U937_{EC}$ and $U937_{PM}$, which differ in their responses to stimuli that have previously been documented in the literature to increase expression of the C5aR. These differences were most obvious when both cell lines were incubated with Bt_2cAMP and PMA for 48 hr and C5aR expression was detected by flow cytometry and western blotting (figures 4.3.3 and 4.3.4). Both these stimuli were able to increase C5aR expression in the $U937_{PM}$ cell line but had no significant effect on the receptor expression in the $U937_{EC}$ cell line. The cell lines also differed in their sensitivities to the cytotoxic effects following incubation with either Bt_2cAMP or PMA. As shown in figure 4.3.4, the $U937_{PM}$ cell line showed three times more cell death following incubation with PMA compared with the $U937_{EC}$ cell line, however, incubation with Bt_2cAMP produce very little cell death in both cell lines compared with the controls. The results observed by $U937_{PM}$ cell line correlate with previous published findings that have shown that 5 nM PMA is more cytotoxic than 1 mM Bt_2cAMP in the U937 cells when incubated for 2 days (Hetland, 1997). Incubation with Bt_2cAMP and PMA also induced different morphological changes in the cells, which also differed slightly between the two different cell lines. Incubation of both cell lines with PMA resulted in the cells becoming adherent with a macrophage morphology (figure 4.3.5). Although the $U937_{EC}$ cells still contained some cells in suspension, the $U937_{PM}$ were solely adherent with only dead looking cells in suspension (figure 4.3.5). Whereas, incubation with Bt_2cAMP caused both cell lines to remain in suspension however the $U937_{PM}$ became more macrophage like with lots of membrane protrusions (figure 4.3.5). The morphology of the $U937_{PM}$ cell line following stimulation with either PMA or Bt_2cAMP appears more similar to previous described morphological changes described in the literature such as becoming adherent following incubation with PMA and having a more ruffled membrane following stimulation with Bt_2cAMP (Sheth et al., 1988, Rubin et al., 1991a).

To coincide with increased C5aR expression following stimulation of the $U937_{PM}$ cell line with Bt_2cAMP and PMA, these cells release more intracellular Ca^{2+} following stimulation with C5a (figure 4.3.7B). However, the cells which were incubated with PMA required 50 nM C5a to show significant difference from the control, whereas cells which were incubated with Bt_2cAMP required 0.5 nM C5a to show significant difference from the control. These findings correspond with Bt_2cAMP being a stronger inducer of the C5aR compared with PMA in the $U937_{PM}$ cell line. Interestingly the $U937_{EC}$ cells which were incubated with Bt_2cAMP also

showed a significant increase in C5a induced Ca^{2+} release, at 5 nM and 50 nM C5a, although this stimulus did not increase C5aR expression in this cell line (figure 4.3.7A). This suggests that Bt_2cAMP may be able to increase signalling capacity of the C5aR on the cell surface. The mechanism by which this is achieved is not clear as signalling via the C5aR increases intracellular Ca^{2+} release following the stimulation of phospholipase C β2, whereas Bt_2cAMP is a cAMP analogue which activates PKA and exchange proteins directly activated by cAMP (Epacs) (Sands and Palmer, 2008).

Although both the $U937_{EC}$ and $U937_{PM}$ should technically be the same cell line, it is well known that when cells are over cultured or cultured in different laboratories this can lead to a selective pressure and genetic drift, to an extent in which the cells exhibit reduced or altered key functions, and no longer represent a reliable model of their original source material (Hughes et al., 2007). The U937 cell line was derived from a patient with histiocytic lymphoma and was characterised in 1976 by Sundstrom and Nilsson, since then this cell line has been widely used by many laboratories (Sundstrom and Nilsson, 1976). This is not the first time that different U937 cell lines have been identified. A study by Kaszubowska and colleagues found two U937 sublines which differed in their responses to TNFα, although this study did not look at changes in C5aR expression. Kaszubowska and co-workers found that the two sublines originated from a common origin, however cytogenetic studies revealed complex translocations and gene re-arrangements may be responsible for the different phenotypes observed between the two sub cell lines (Kaszubowska et al., 2001). Also in our laboratories two U937 sub-lines, one CD59 positive and the other CD59 negative have also been reported (van den Berg et al., 1994). Similarly to the $U937_{EC}$ cells, neither of these cells expressed the C5aR or responded to PMA with an increase in C5aR. Rubin and co-workers had previously found that Bt_2cAMP differentiation occurs only in a subset ~60-80 % of U937 cells (Rubin et al., 1986). It is therefore possible that over time in culture either the $U937_{EC}$ cell line has lost the subset of cells which respond to Bt_2cAMP and $U937_{PM}$ has lost the subset of cells which do not respond to Bt_2cAMP due to selective pressure. As initial experiments carried out using the $U937_{EC}$ cell line found these cells do not respond to PMA and Bt_2cAMP in the way in which the literature has reported, this resulted in the $U937_{PM}$ cell line being obtained by Dr Peter Monk from Sheffield University.

Incubation of both $U937_{PM}$ and $U937_{EC}$ cell lines with IFNγ and $U937_{EC}$ with IL-6 caused a significant apparent increase in C5aR cell surface expression when detected by flow cytometry (figure 4.3.3). However, these results were contradicted by the results from western blotting and C5a induced Ca^{2+} release, which suggested that neither IFNγ nor IL-6 altered C5aR expression when compared with the control (figures 4.3.6 and 4.3.7). As described in section 4.3.4, incubation of both $U937_{PM}$ and $U937_{EC}$ cells with aggregated

human IgG prior to staining for flow cytometry abolished the IFNγ and IL-6 induced C5aR expression in these cells, which suggested that the antibodies may be binding non-specifically via the Fc receptors (figure 4.3.8). This finding was unexpected as IgG1 isotype control did not detect any non-specific binding throughout the flow cytometry experiments. However, further investigation into the primary anti-C5aR (S5/1) from Santa Cruz found that although this monoclonal antibody was sold as an IgG1 isotype it is in fact an IgG2a isotype (Oppermann et al., 1993). This discovery had huge implications for our flow cytometry experiments as it meant that the isotype control was no longer relevant. As the U937 cell line is a monocytic cell line, they express cell surface FcγR which binds the Fc portion of IgG coated surfaces aiding their phagocytosis. Each of the class of FcγR have different affinities for the different isotypes of IgG class antibodies which makes using the correctly match isotype essential to detect non-specific binding of antibodies via the FcγR. To investigate whether increased expression of FcγRIII (CD16) or FcγRII (CD32) may be responsible for the apparent increase in C5aR expression following incubation with IFNγ or IL-6, flow cytometry was performed using anti-CD16 or anti-CD32 antibodies. Although staining for FcγRIII (CD16) and FcγRII (CD32) showed that neither of these receptors increased following treatment with IFNγ or IL-6, these stimuli may still up-regulate expression of the FcγRI (CD64) which was not investigated. Indeed, IFNγ (100U/ml) has previously been shown to up-regulate CD64 expression (three fold) in the U937 cell line which could explain the increased non-specific binding of the antibodies via their Fc portion (Karehed et al., 2007). Furthermore CD64 has 3 times more affinity for IgG2a isotype compared with IgG1 isotypes (van de Winkel et al., 1991). In contrast differentiation of the U937 cell line with Bt_2cAMP has been previously characterised to decrease CD64 expression in these cells, whilst increasing CD11b and CD14 (Sheth et al., 1988, Gavison et al., 1988, Brodsky et al., 1998).

Although results presented here suggest that the apparent increase in C5aR by IFNγ and IL-6 was due to non-specific binding of the antibody, these findings contradict several studies which have reported these stimuli to increase C5aR expression in the U937 cell line (Burg et al., 1996, Burg et al., 1995, Gasque et al., 1998). In order for Burg and co-workers to achieve an increase in C5aR expression in the U937 cell line following treatment with IFNγ, they carried out their experiments on a sub clonal population, which had considerable gene rearrangement (Burg et al., 1995). Furthermore, Burg and co-workers used ^{125}I-C5a ligand binding studies to monitor changes in C5aR expression following incubation with IFNγ (Burg et al., 1996, Burg et al., 1995). However, these experiments are now compromised by the discovery of a second C5a receptor, C5L2, which binds to C5a with similar affinities as C5aR (Cain and Monk, 2002). Although Gasque and colleagues used both flow cytometry and RT-PCR to monitor changes in C5aR in the U937 cell line following

incubation with IFNγ, their flow cytometry experiments used mouse anti-C5aR (P12/1) from Santa Cruz which is also an IgG2a isotype and they do not mention incubating the cells with aggregated IgG prior staining to eliminate non-specific binding via the FcγRs nor do they use an isotype control (Gasque et al., 1998). Furthermore their results from RT-PCR only produced a small change in mRNA levels following incubation with IFNγ while incubation with PMA saw a big increase by comparison in C5aR mRNA despite this stimulus being a weaker inducer for C5aR expression. Together these results suggest that his interpretation of the ability of IFNγ to increase C5aR expression is wrong (Gasque et al., 1998). As already discussed above, there is likely to be some heterogeneity between the U937 cell lines between different laboratories, due to selective pressure and over culturing of the cells. Taken together these results highlight the problem of using over cultured and over passaged cell lines.

To investigate whether differences between the cell lines is attributed to differences in their regulatory sequences or regulatory proteins within the cells, each -2Kbp EGFP reporter construct generated from each cell line was transfected into both U937 cell lines and were then incubated with the different stimuli. From these experiments it was shown that neither the -2Kbp(**EC**)+pEGFP or -2Kbp(**PM**)+pEGFP reporter constructs when transfected into either cell lines responded to PMA (figure 4.3.10A and figure 4.3.12A). Previously Gerard and colleagues found that a CAT reporter construct which contained the -355bp promoter region of the C5aR, and some of the intron, contained a regulatory element which was responsive to PMA when transfected into the RBL cells (Gerard et al., 1993). The difference between our results could be explained by several factors; firstly the C5aR promoter reporter construct studied by Gerard et al consisted of -355bp of the promoter region and ~500bp intronic sequence, therefore the element which was responsive to PMA might be present within the intron which was not cloned in this study; secondly there are 11bp differences between the sequences in the -355bp region published by Gerard and co-workers compared with the sequences used in this study (see appendix 9.2), although our results are most similar to other published results; and thirdly they transfect the rat basophilic leukaemia (RBL) cell line with their reporter constructs which may contain different transcription factors to the U937 cell line.

When the 2Kbp(**EC**)+pEGFP reporter construct was transfected into the $U937_{PM}$ cells a significant increase in EGFP fluorescence was observed when the cells were incubated with Bt_2cAMP compared with the other constructs (figure 4.3.12 b). However, this was not observed when the $U937_{EC}$ cells were transfected with this construct. This result suggests that the -2Kbp promoter region of the C5aR gene isolated from $U937_{EC}$ cells contains a *cis*-acting element which is responsive to Bt_2cAMP, however, only when it is transfected into the

$U937_{PM}$ cells does the activity become apparent (figure 4.3.10B and 4.3.12B). The extent of EGFP increase (~1.75 fold) is far less than the actual increase in C5aR expression (~6 fold). This could suggest that stronger regulatory activity is outside the region cloned. Interestingly however the -2Kbp promoter isolated from $U937_{PM}$ cell line showed no promoter activity towards PMA or Bt_2cAMP when transfected into the $U937_{PM}$ cell line, which was unexpected as this cell line responded to both PMA and Bt_2cAMP by increasing C5aR expression (figure 4.3.12). One explanation for these results could be that, as previously described in chapter 3, there are several bp differences between the $U937_{EC}$ and $U937_{PM}$ promoter region cloned, including a SNP at position -245bp (T/C) which has previously been identified by (Barnes et al., 2004). If for instance $U937_{PM}$ is heterozygous at a given SNP position, and the position of the SNP is critical for responsiveness to Bt_2cAMP, then the non-responder SNP may have been cloned in these studies. As the $U937_{EC}$ cell line was unresponsive to Bt_2cAMP, neither reporter construct containing the C or T SNP would respond, due to a lack of the necessary regulatory proteins. This theory could be tested by performing site directed mutagenesis on the promoter cloned from the $U937_{PM}$ cells to match the sequence obtained from the $U937_{EC}$ cell line and then transfecting it back into the $U937_{PM}$ cells. Future studies could further investigate whether the bp differences between the two cell lines are true SNPs and whether the $U937_{PM}$ cell line is heterozygous at any of these positions.

As previously described Bt_2cAMP is a cAMP analogue that has been suggested to regulate C5aR expression via activation of PKA. Following activation of PKA several transcription factors can be activated including cAMP regulatory element binding protein (CREB), cAMP response element modulator (CREM) and ATF-1 (Sands and Palmer, 2008). More recently raising intracellular cAMP levels has been shown to also activate cyclic nucleotide-gated ion channels, as well as exchange proteins directly activated by cAMP (Epacs) (Sands and Palmer, 2008). Epacs can also activate small GTPase binding proteins which can also regulate activity of several transcription factors, such as NFAT in cardiac myocytes (Morel et al., 2005). Taken together these results suggest that the $U937_{PM}$ cell line may contain one or more of these target transcription factors that are not present in the $U937_{EC}$ cell line. The results from this chapter suggest that there may be a regulatory protein or transcription factor, which is responsive to Bt_2cAMP, present in the $U937_{PM}$ but not $U937_{EC}$ cell line.

Although PMA and Bt_2cAMP can increase C5aR expression in the $U937_{PM}$ cell line, the promoter region cloned is only 2Kbp up-stream of the transcription start site of the C5aR gene and therefore the regulatory elements responsive to these stimuli could be out side the region cloned or even within the intron or 3'UTR. When the 3'UTR EGFP reporter constructs, generated from the $U937_{EC}$ cell line, were transfected into the $U937_{EC}$ cell line, incubation with PMA or Bt_2cAMP had no effect on EGFP fluorescence compared with the

SV40 control vector (figure 4.3.11). However, it was not investigated whether the 3'UTR isolated from the $U937_{PM}$ cell line could be regulated by either PMA or Bt_2cAMP.

4.4.1 Summary

This chapter has identified two U937 cell lines which differ in their responses to Bt_2cAMP and PMA. The differences between their C5aR expression patterns following stimulation by these stimuli appear to be due to both differences in the sequences of the -2Kbp promoter region cloned and the due to differences in expression of the regulatory proteins responsive to these stimuli. This chapter has also highlighted the need to use proper controls for non-specific binding when using cell lines which express Fc receptors. The results from this chapter have found that incubation of the $U937_{PM}$ cell line with Bt_2cAMP, results in the highest function and expression the C5aR, which provides a good model system to investigate the effects of lipid lowering drugs on the function and expression of the C5aR.

Chapter 5

The effects of statins on C5aR expression

Chapter 5

The effects of statins on C5aR expression

5.1 Introduction

Inhibition of HMG-CoA reductase by statins prevents biosynthesis of isoprenoid intermediates and cholesterol (figure 5.1.1). Isoprenoid intermediates are vital for the post-translational modification, isoprenylation, of small GTP binding proteins. These proteins are typically components in intracellular signalling cascades which can regulate the activity of several transcription factors. As cholesterol can also regulate the transcriptional activity of genes containing sterol-regulatory-elements present within their promoter regions, statins have the potential to regulate the transcriptional activity of several genes (figure 5.1.1). Furthermore statins can also affect gene expression by regulating mRNA stability, as well as altering membrane cholesterol content and therefore altering cell surface expression. The regulation of genes that encode pro-inflammatory, immunomodulating and pro-thrombotic proteins by statins has attracted a lot of interest over the last decades, and the regulation of expression of these genes has been attributed to some of the pleiotropic effects of statins.

Evidence suggests that C activation occurs within atherosclerotic plaques and that the C system contributes towards the development and maturation of these plaques; see section 1.3.2 for full details. As the C5aR has previously been shown to be present in atherosclerotic plaques (Oksjoki et al., 2007), this chapter aims to investigate whether statins regulate the expression of the receptor *in vitro*.

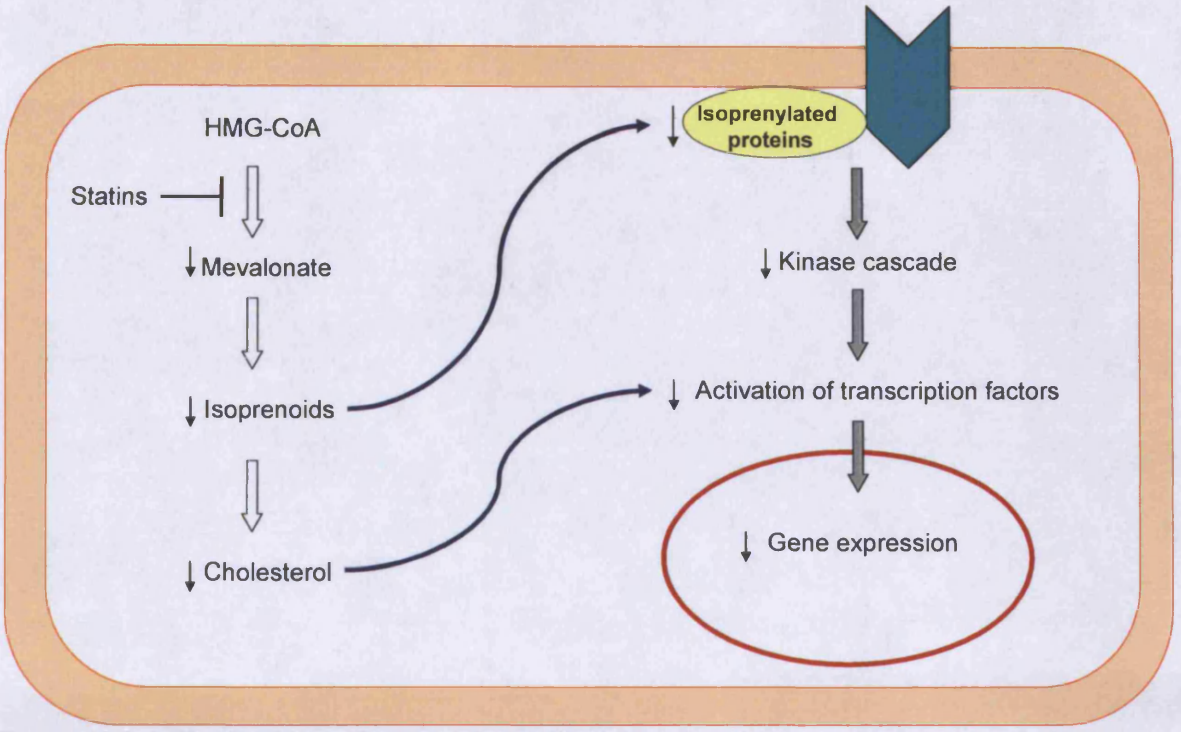


Figure 5.1.1 Mechanisms by which statins can regulate gene transcription. Adapted from (Massy and Guijarro, 2001).

5.1.1 Regulation of gene expression by statins independent of cholesterol lowering

Isoprenylated small GTP binding proteins are crucial components for several intracellular signalling pathways including extracellular signal-regulated kinase (ERK) and p38-MAPK, c-Jun N-terminal kinase (JNK) and phosphatidylinositol-3-kinase (PI3K) (Paumelle and Staels 2005). Inhibition of these signalling pathways by reducing isoprenylation is an important mechanism by which statins can regulate the expression of target genes and elicits a broad range of pleiotropic effects, table 5.1.1. The main transcription factors affected by statins are NFκB and PPARs but evidence has shown STAT-family members are similarly regulated. As well as regulating gene transcription, statins have also been shown to regulate mRNA stability via Rho family members. The mechanisms which statins affects these trans-acting factors will be discussed below in more detail.

Table 5.1 Effects of statins on gene regulation of target genes.

Pleiotropic effect	Target genes and mechanism for regulation
Improves endothelial function	• Increase eNOS expression by increasing mRNA stability via Rho/ROCK signalling pathway (Laufs et al., 1998, Laufs and Liao, 1998). • Lower adhesion molecules expression, such as ICAM-1 by reducing STAT-1α expression (Chung et al., 2002). • Increase DAF expression by via activation of protein kinase C and inhibition of RhoA signalling (Mason et al., 2002).
Decreases inflammation and cell infiltration	• Reduce LDL and oxidised LDL induced IL-6 expression by inhibition of NFκB activation (Massy et al., 2000, Guijarro et al., 1996). • Reduce MCP-1 expression by inhibition of RhoA signalling and therefore reduced NFκB activation (Veillard et al., 2006, Ortego et al., 1999). • Reduce chemokine receptors CCR2 and CCR5 expression on monocytes by depletion of isoprenoid GGPP and cellular cholesterol which together leads to increased PPARγ activation (Han et al., 2005, Chen et al., 2005, Yin et al., 2007). • Reduce IL-1β expression by increasing PPARα expression (Inoue et al., 2000).
Reduce T-cell activation: immunomodulation	• Reduce IFNγ induced MHC II expression therefore reducing T cell activation (Sadeghi et al., 2001, Kwak et al., 2000). • Reduce expression of co-stimulatory molecules such as IFNγ induced CD40 expression by reducing STAT-1α expression (Townsend et al., 2004, Lee et al., 2007). • Regulate Th1/Th2 balance by suppressing Th1 pro-inflammatory cytokines by inhibiting NFκB and promoting Th2 development (Jasinska et al., 2007). • Regulate Th1/Th2 lineage commitment by decreasing STAT-4 phosphorylation and increasing STAT-6 phosphorylation (Youssef et al., 2002).
Enhances stability of atherosclerotic plaques	• Reduce expression of MMPs such as MMP-2 and -9 via Rho/ROCK signalling pathways (Li et al., 2008, Turner et al., 2005).
Decreased thrombosis	• Inhibition of tissue factor expression by decreased NFκB activation (Colli et al., 1997).

5.1.2.1 Inhibition of NFκB by statins

NFκB exists as either a homodimer or heterodimer made up from the different members of the NFκB family. It is present in an inactive form in the cytoplasm bound by an inhibitory subunit IκB. Upon stimulation IκB is released and degraded allowing NFκB to translocate to the nucleus (figure 5.1.2). Once inside the nucleus, NFκB promotes the transcriptional activity of target genes which contain a NFκB promoter element. These genes are typically involved in the expression of pro-inflammatory molecules such as cytokines and adhesion molecules. The signal is eventually terminated by the new synthesis of IκB (Hoffmann et al., 2006).

NFκB is a crucial transcription factor in atherogenesis initiation and progression by regulating expression of adhesion molecules and pro-inflammatory cytokines (Thurberg and Collins, 1998). Although NFκB can be regulated by numerous MAPK signalling pathways, several studies have suggested that the reduced NFκB activation caused by statins is mediated by inhibition of the Rho/Rho Kinase (ROCK) signalling pathway however the exact mechanism still needs to be confirmed (Colli et al., 1997, Turner et al., 2005, Veillard et al., 2006, Guijarro et al., 1996).

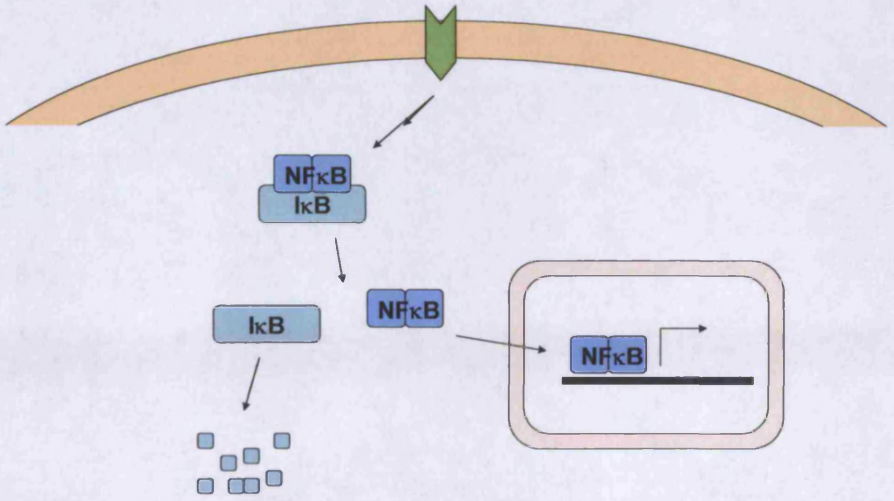


Figure 5.1.2 Schematic diagram of NFκB signalling pathway.

5.1.2.2 Activation of PPARs by statins

Peroxisome proliferator-activated receptors (PPAR) are transcription factors which become activated upon stimulation with their ligands (fatty acid derivatives and eicosanoids) (Paumelle and Staels, 2008). There are three different isotypes identified so far (PPARα, PPARγ and PPAR δ/β). Statins have been shown to activate both PPARα and PPARγ in endothelial cells, hepatocytes and macrophages (Yano et al., 2007, Inoue et al., 2000, Paumelle et al., 2006). Several mechanisms of how statins increase PPARα and PPARγ activation have been suggested (figure 5.1.3). One mechanism is that inhibition of RhoA by statins leads to a reduction in PKC phosphorylation of PPARα which would normally repress PPARα activity (Martin et al., 2001, Paumelle et al., 2006, Paumelle and Staels, 2008). Another study has also suggested that inhibition of RhoA/Cdc42 by statins leads to activation of p38 MAPK, which in turn increases and activates COX-2 expression (Yano et al., 2007). This statin induced COX-2 expression leads to an increase in the PPARα and PPARγ ligand 15-deoxy-delta (12,14)-prostaglandin J(2) (15d-PGJ(2). However, results from an earlier study found that incubation with statins reduced COX-2 expression, therefore this mechanism is still not clear (Inoue et al., 2000). It has also been suggested that statins may

regulate gene transcription of PPARα however the exact mechanism has yet to be established (Landrier et al., 2004, Paumelle and Staels, 2008). PPARα and PPARγ are also regulated by other drugs including fibrates, activators of PPARα, and their structural analogue anti-diabetic agents thiazolidinediones, which activate PPARγ (Jasinska et al., 2007).

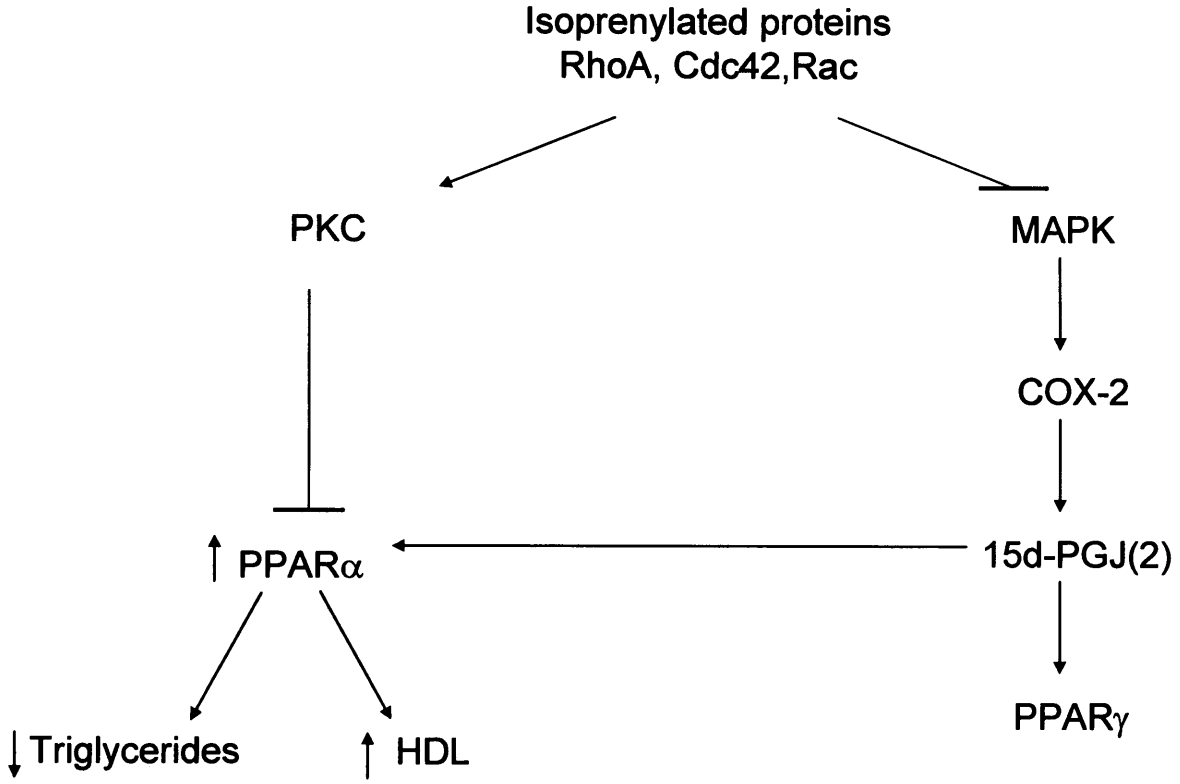


Figure 5.1.3 Potential mechanisms which statins activate PPARα and PPARγ. Adapted from (Jasinska et al., 2007).

5.1.2.3 Regulation of other transcription factors by statins

Statins have also been shown to inhibit IFNγ induced MHC II expression on endothelial cells, macrophages and microglia; however the exact mechanism in which they modulate this is controversial (Katznelson, 1999, Kwak et al., 2000, Sadeghi et al., 2001). Kwak and colleagues showed that IFNγ induced MHC II expression on endothelial cells and macrophages was inhibited via the down regulation of CIITA-PVI transcription factor, which normally mediates MHC II expression (Kwak et al., 2000, Kwak et al., 2001). However other studies have shown that CIITA-PVI mRNA levels remain unchanged following treatment with simvastatin (Kuipers and van den Elsen, 2007).

Statins have been shown to regulate members of the STAT-family. STAT activation is achieved by signalling of IFNγ via its receptor which leads to the activation of Janus kinases (JAK) 1 and 2 that can in turn activate STATs (Kuipers and van den Elsen, 2007). Decreased phosphorylation of STAT-4 and increased phosphorylation of STAT-6 by

atorvastatin *in vivo* is believed to reduce Th1 and promote Th2 lineage commitment (Youssef et al., 2002). Inhibition of STAT-1 by lovastatin has also been suggested to suppress IFNγ induced CD40 expression in microglia (Townsend et al., 2004, Lee et al., 2007).

5.1.2.4 Regulation of mRNA stability by statins

As previously described in section 3.1.3 regulating mRNA stability is another mechanism which gene expression can be effected. Previously statins have been shown to alter expression of eNOS by regulating mRNA stability. Laufs and colleagues found that ox-LDL was able to reduce mRNA expression of eNOS in human endothelial cells and that this reduction was prevented by incubation with simvastatin, lovastatin and mevastatin (Laufs et al., 1998). They identified that the inhibition of geranylgeranylation of Rho by statins improved the half life of the mRNA transcripts suggesting that Rho is a negative regulator for eNOS mRNA (Laufs and Liao, 1998). Although they did not investigate how Rho affects eNOS mRNA stability they suggested that as Rho is important for re-organisation of the actin-cytoskeleton Rho may effect the eNOS mRNA translation and stability via effects on cytoskeleton localisation of the mRNA (Laufs and Liao, 1998).

5.1.2 Regulation of gene expression by cholesterol

As cholesterol is a crucial component for maintaining membrane integrity, mammals have developed several feedback regulation mechanisms to sustain cholesterol homeostasis within the cell (see section 1.5) (Goldstein and Brown, 1990). Many genes that are involved in cholesterol metabolism are regulated by sterol-regulatory-element-binding protein (SREBP), which allows the cell to sense cholesterol levels and regulate the transcription of target genes accordingly. At high cholesterol levels, SREBP is trapped within the endoplasmic reticulum where it is bound to SREBP cleavage activator protein (SCAP) in its inactive form (Muller-Wieland et al., 1997, Maxfield and Tabas, 2005). Inactivation of SREBP leads to a reduction of cholesterol biosynthesis by reduction in gene transcription of HMG-CoA synthase and HMG-CoA reductase, as well as reducing uptake of LDL-cholesterol via down regulation of the LDL receptor gene. When cholesterol levels are low the SREBP-SCAP complex exits the endoplasmic reticulum and undergoes proteolytic cleavage to release SREBP. SREBP is translocated into the nucleus where it regulates the transcription of many genes such as HMG-CoA reductase, HMG-CoA synthase and LDL receptor gene (Muller-Wieland et al., 1997). Plasma LDL-cholesterol has also been shown to increase expression of the MCP-1 receptor, CCR2, in the THP-1 cell line; furthermore its expression was dramatically increased on monocytes from hypercholesterolemic patients (Han et al., 1998). Later investigations found that in a mouse hypercholesterolemia model the CCR2

gene in circulating monocytes is under the transcriptional control of SREBP and PPARγ within the promoter region (Chen et al., 2005).

As cholesterol is an integral part of cellular membranes it can also affect expression of genes which encode cell surface molecules by influencing membrane fluidity and therefore the proper processing and targeting to plasma membrane. For instance simvastatin has been suggested to reduce surface expression of MHC-II molecules by disrupting the cholesterol-containing microdomains which are important for the transport and concentration of MHC-II at the cell surface (Kuipers et al., 2005).

5.1.3 Regulation of C5aR expression by statins

C5aR expression has been shown to be altered in numerous inflammatory disease states (see section 1.2.8 for full details) including atherosclerotic plaques where C5aR expression was detected on macrophages, T-cells, mast cell, EC and SMC (Oksjoki et al., 2007). Few studies have investigated the mechanisms of regulation of C5aR expression and to date no published data has investigated whether statins may alter its expression (see 1.2.3 for full details). One potential mechanism by which statins could regulate C5aR expression would be to regulate gene transcription as is the case for chemokine receptors CCR2 and CCR5 (Han et al., 2005, Chen et al., 2005, Yin et al., 2007). This could be achieved by regulation of NFκB, PPARs or STATs transcription factors by statins. Chapter 3 has previously identified a NFκB site at position -238bp to -232bp within the -2Kbp promoter region of the C5aR, which offers a potential mechanism that statins may alter receptor expression.

Statins could also regulate C5aR expression by affecting its mRNA stability as is the case for eNOS (see section 5.1.2.4 for details) (Laufs et al., 1998). Messenger RNA stability can be affected by ARE with the 3'UTR (see section 3.1.3). Although it was shown in Chapter 3 that the C5aR gene contained two ARE within the 3'UTR and they had no effect on basal mRNA stability, these elements may still offer a mechanism by which statins may regulate C5aR expression. Recently DAF mRNA has been shown to be destabilised by an ARE present within the 3'UTR region (Gray et al., 2010). Although statins have been shown to increase DAF expression it has not been investigated whether they can increase mRNA stability via the ARE present within the 3'UTR (Mason et al., 2002, Gray et al., 2010).

As well as regulating gene transcription and mRNA stability, statins may also regulate surface C5aR expression by altering membrane cholesterol and thus affecting receptor internalisation and intracellular trafficking. As discussed in Chapter 1 section 1.3, GPCRs whose internalisation is regulated by membrane cholesterol content are localised in lipid raft regions of the plasma membrane or move in or out of such regions following stimulation with their ligand. Although initially there were conflicting results as to whether C5aR

internalisation was mediated by clathrin dependent or independent mechanism, it is now believed that following stimulation of the C5aR with C5a the receptor clusters into clathrin coated pits where it is then internalised in a β-arrestin, dynamin, and clathrin-dependent pathway (Gilbert et al., 2001, Licht et al., 2003, Braun et al., 2003, Suvorova et al., 2005, Huttenrauch et al., 2005). As lipid raft dependent endocytosis is characterised by clathrin-independence, this would suggest that the C5aR is unlikely to be internalised in a lipid raft dependent manner; however, it has not been investigated whether C5aR internalisation is dependent on cholesterol (Lajoie and Nabi, 2010). Membrane localisation of the C5aR will be investigated in Chapter 6.

5.1.4 Hypothesis and aims

As statins have been shown to down regulate expression of numerous other pro-inflammatory molecules, such as chemokine receptors CCR2 and CCR5 (see table 5.1 for details), this chapter hypothesises that statins may also down regulate expression of the C5aR and thereby exerting an anti-inflammatory effect. The aim of this chapter is to investigate whether statins regulate resting or induced C5aR expression *in vitro* using U937 cell lines and PBMCs.

5.2 Materials and methods

5.2.1 Chemicals and reagents

All general reagents were purchased from Sigma. Alamar blue was purchased from AbD Serotec. See section 2.1.1 for all other reagents and suppliers.

5.2.2 Cell culture

Both $U937_{PM}$ and $U937_{C5aR}$ cell lines were maintained in complete RPMI media as previously described in section 2.3.1. Unless otherwise stated cells were seeded at 2.5 $x10^5$ cell/ml and incubated for 48 hrs with appropriate stimuli prior to analysis (section 2.3.5). PBMCs isolated as previously described in sections 2.3.2, were seeded at 1 $x10^6$ cell/ml for 24 hr with the appropriate stimuli (section 2.3.5).

5.2.3 Flow cytometry

C5aR expression and CD14 expression was analysed by flow cytometry using specific monoclonal antibodies outlined in chapter 2 and GAM/FITC secondary antibody according to the method described in Chapter 2.3.6. EGFP fluorescence in EGFP reporter constructs transfected cells was also measured by flow cytometry. Relative change was calculated by subtracting the background fluorescence from all values and then dividing the treatment fluorescence by the control fluorescence.

5.2.4 Western blotting

Western blotting was performed as previously described in section 2.3.7 using polyclonal anti-C5aR (H-100, Santa Cruz) and anti-GAPDH (mAb, Santa Cruz SC47724) or anti-tubulin (mAb, Oncogen CP66) for equal loading control.

5.2.5 Alamar blue cell proliferation assay

Cells growing in log phase were washed in fresh complete RPMI media and counted. Cells were resuspended at $2x10^5$ cell/ml in complete RPMI. 50 µl of cells was added to 100 µl complete RPMI media containing stimulus. Following 40 hr incubation, 50 µl Alamar blue, diluted 1:1 with complete medium was added. For a 100% reduced control, Alamar blue diluted 1:1 with complete RPMI was autoclaved for 15 min and mixed with 150 µl complete RPMI. Fluorescence was monitored every 1 hr using FLUOstar Optima plate reader (BMG Labtech), excitation wavelength 560 nm and emission wavelength 590 nm. Percentage

Alamar blue reduction was then calculated using equation 1. Rate constant was calculated by one phase exponential association equation using GraphPad Prism 4.

Equation 1 $$\text{Percentage reduction} = \frac{\text{Fluorescence intensity of sample}}{\text{Fluorescence intensity of 100\% reduced Alamar blue}} \times 100$$

5.3 Results

The aim of this chapter was to investigate if statins can regulate basal levels or induced C5aR expression in the U937 cell line and human monocytes. Changes in C5aR expression were monitored by flow cytometry and western blotting.

5.3.1 Simvastatin had no effect on basal C5aR expression levels in $U937_{PM}$ cells

As the $U937_{PM}$ cell line behaved most like the U937 cell line as reported in the literature it was decided to continue with this cell line rather than the $U937_{EC}$ cell line (see Chapter 4 for full details). The effects of simvastatin were first investigated on basal C5aR expression levels.

Incubation of undifferentiated $U937_{PM}$ with increasing concentration of simvastatin initially showed a slight reduction in basal C5aR expression (figure 5.3.1.A). However, incubation of these cells with aggregated human IgG prior to staining for flow cytometry showed that treatment with simvastatin had no effect on basal C5aR expression (figure 5.3.1.B). Furthermore this experiment also showed that there is no detectable basal C5aR expression in these cells.

When the $U937_{PM}$ cell line transfected with -2Kbp(**PM**)+pEGFP promoter reporter construct was incubated with simvastatin no change in EGFP fluorescence was detected by flow cytometry (figure 5.3.2). This suggest that either there are no *cis*-acting elements in the promoter region cloned which are responsive to simvastatin or there could be a repressor present which over rides any enhancer activity. Simvastatin incubation was also shown to have no effect on the C5aR 3'UTR mRNA stability as incubation of the C5aR 3'UTR EGFP reporter constructs with simvastatin had no effect on EGFP fluorescence (figure 5.3.3). Similarly simvastatin had no effect on EGFP fluorescence in the 3'UTR mutant constructs 3'UTR(1)ARE and 3'UTR(2)ARE (figure 5.3.3).

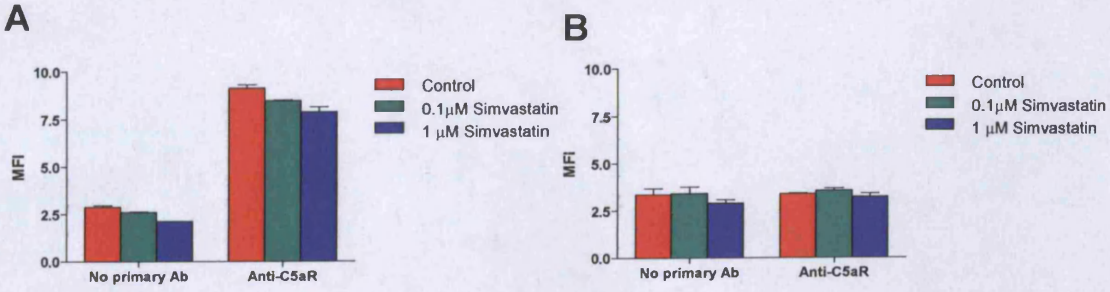


Figure 5.3.1 Simvastatin has no effect on basal levels of C5aR expression in $U937_{PM}$ cell line. $U937_{PM}$ cells were incubated with simvastatin for 48 hr. Cells were harvested and incubated in the absence (A) or presence (B) of human aggregated IgG prior to staining for flow cytometry, section 5.2.3 for details. One-way ANOVA showed no statistical difference with the control.

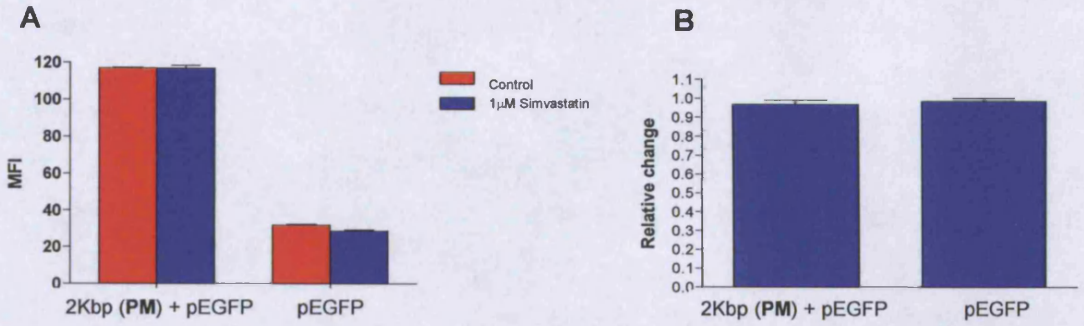


Figure 5.3.2 1uM simvastatin has no effect on EGFP expression in U937$_{PM}$ transfected with the 2Kbp (PM) + pEGFP reporter construct. U937$_{PM}$, transfected with the 2Kbp (**PM**) + pEGFP or pEGFP control vector were incubated with 1 μM simvastatin for 2 days. Following incubation changes EGFP fluorescence was monitored by flow cytometry, described section 5.2.3. (A) Result from representative experiment and (B) relative EGFP change comparing control with 1 μM simvastatin. Student t-test showed no statistical significance between the two groups.

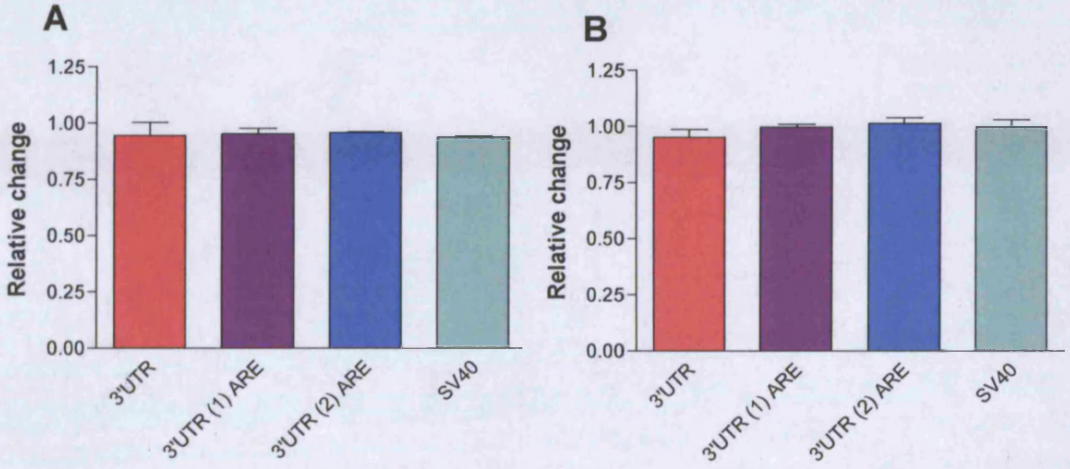


Figure 5.3.3 0.1 μM (A) and 1 μM (B) simvastatin have no effect on mRNA stability. U937$_{EC}$ transfected with the 3'UTR EGFP reporter constructs (see section 3.3.3 for more details) were incubated with 0.1 μM simvastatin (A) or 1 μM simvastatin (B) for 2 days. Following incubation changes in EGFP fluorescence was monitored by flow cytometry, described in section 5.2.3. One-way ANOVA showed no statistical significance.

5.3.2 Simvastatin reduces Bt$_2$cAMP induced C5aR expression in U937$_{PM}$ cells

As simvastatin had no effect on basal levels of C5aR expression in the U937$_{PM}$ cell line, the effects of simvastatin on induced expression were investigated. C5aR expression was previously shown to increase in the U937$_{PM}$ cell line by stimulation with PMA or Bt$_2$cAMP (section 4.3). As PMA was the weaker inducer of C5aR expression and it was shown to be cytotoxic to these cells (figure 4.3.4) it was decided to investigate the effects of simvastatin on Bt$_2$cAMP induced C5aR expression only.

Results from both flow cytometry, for cell surface expression, and western blots, total C5aR expression, showed that incubation with 1 μM simvastatin significantly reduced

Bt_2cAMP induced C5aR expression, whereas 0.1 μM simvastatin had no effect (figures 5.3.4 and 5.3.5).

To investigate if incubation for a longer duration with the lower concentration of simvastatin would have any effect on C5aR expression, $U937_{PM}$ were incubated with 0.1 μM simvastatin for 9 days, replacing the media every 2 days. After 7 days with 0.1 μM simvastatin the cells were incubated for a further 2 days with either 0.1 μM simvastatin or 0.5mM Bt_2cAMP plus 0.1 μM simvastatin, alongside cells incubated with Bt_2cAMP alone for 2 days. Results from these experiments showed that incubating $U937_{PM}$ cells with 0.1 μM simvastatin for 9 days increased C5aR expression slightly although this was not statistically significant and that treating these cells with Bt_2cAMP for the final 2 days with the simvastatin had no effect on its ability to up regulate C5aR expression (figure 5.3.6).

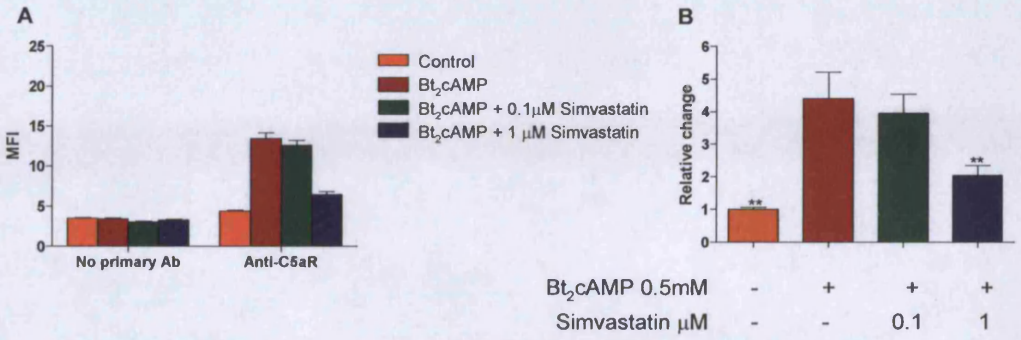


Figure 5.3.4 Incubation with 1 μM simvastatin reduces Bt_2cAMP induced C5aR expression detected by flow cytometry. $U937_{PM}$ cells were incubated with Bt_2cAMP and simvastatin for 48 hr. Cells were incubated with aggregated IgG prior to staining for flow cytometry, section 5.2.3 for details. Results expressed as mean fluorescence intensity from one representative experiment (A) and relative change from three separate experiments (B). One-way ANOVA followed by Dunnett's multiple comparison test was performed. Data marked with an asterisks represents significant difference from Bt_2cAMP treated cells, **p <0.01.

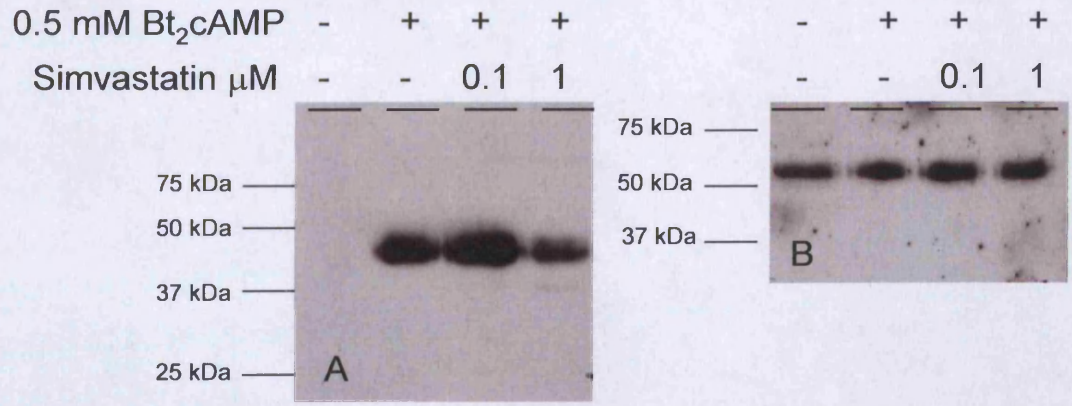
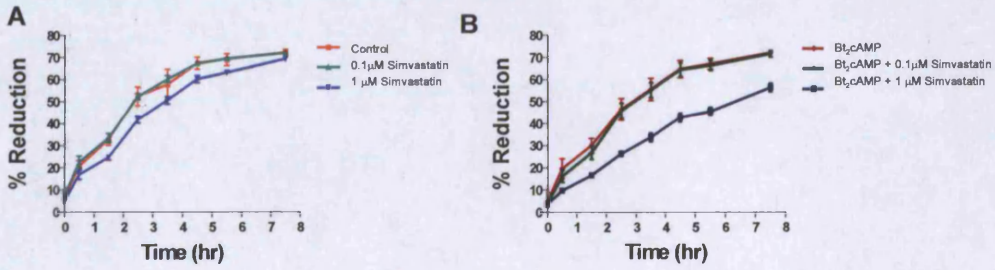


Figure 5.3.5 Incubation with 1 μM simvastatin reduces Bt_2cAMP induced C5aR expression in the $U937_{PM}$ cell line as detected by western blot. Following incubation of $U937_{PM}$ with Bt_2cAMP and simvastatin, expression of C5aR (A) and tubulin (B) were detected by western blotting as described in section 5.2.4. Results shown are from one experiment but are representative of multiple analysis (n=3).

Figure 5.3.6 Incubation of $U937_{PM}$ with 0.1 µM simvastatin for 9 days has no effect on C5aR expression. Following incubation for 7 days with 0.1 µM simvastatin $U937_{PM}$ cells were treated for a further 2 days with either 0.1 µM simvastatin or 0.1 µM simvastatin plus 0.5 mM Bt_2cAMP. C5aR expression was compared by FACS analysis with untreated control cells and cells incubated with 0.5mM Bt_2cAMP for 2 days. Cells were incubated with aggregated IgG prior staining for flow cytometry. C5aR expression was detected by flow cytometry as described in section 5.2.3. Results expressed as mean fluorescence intensity from one representative experiment (A) and relative C5aR change compared with untreated control $U937_{PM}$ (B). Paired student T-Test compared control against 0.1 µM simvastatin and 0.5 mM Bt_2cAMP against 0.1 µM simvastatin plus 0.5 mM Bt_2cAMP showed no significant difference.

5.3.3 Incubation of $U937_{PM}$ with Bt_2cAMP and 1uM simvastatin significantly reduced cell proliferation

To investigate whether simvastatin reduced cell proliferation following treatment of $U937_{PM}$ with simvastatin for 40 hrs, the rate of Alamar blue reduction was monitored. Figure 5.3.7 shows that incubation with both 0.5 mM Bt_2cAMP and 1 µM simvastatin significantly reduced cell proliferation rates, however alone these agents had no significant effect on proliferation.



	Rate constant (K) % reduction/hr
Control	0.4519 (+/- 0.06330)
0.1 µM Simvastatin	0.4736 (+/- 0.04763)
1 µM Simvastatin	0.3051 (+/- 0.03242)

	Rate constant (K) % reduction/hr
0.5 mM Bt_2cAMP	0.3796 (+/- 0.05639)
0.5 mM Bt_2cAMP+ 0.1 µM Simvastatin	0.3384 (+/- 0.04165)
0.5 mM Bt_2cAMP+ 1 µM Simvastatin	0.1649 (+/- 0.02621) *

Figure 5.3.7 Incubation of $U937_{PM}$ with 0.5 mM Bt_2cAMP plus 1 µM simvastatin significantly decreases cell proliferation. Following 40 hr incubation of $U937_{PM}$ with simvastatin, in the absence or presence of 0.5 mM Bt_2cAMP, Alamar blue was added to a final concentration 12 % and reduction in Alamar blue was then recorded every 1 hr for 7.5 hr. Rate constant was calculated using one phase exponential association equation and one-way ANOVA followed by Dunnett's multiple comparison test. Data marked with an asterisk is significantly different from Bt_2cAMP alone, *p <0.05.

5.3.4 Addition of mevalonate or GGPP prevented the effects of simvastatin

To further investigate how 1 μM simvastatin reduces Bt_2cAMP induced C5aR expression, $U937_{PM}$ were incubated with Bt_2cAMP plus 1 μM simvastatin and either 10 μM mevalonate or 10 μM GGPP. If simvastatin is having a direct effect on Bt_2cAMP induced C5aR expression by inhibition of cholesterol and/or isoprenoid synthesis, via HMG-CoA reductase, adding back mevalonate should reverse the effects of simvastatin (see Chapter 1, figure 1.8). Whereas adding back GGPP will reverse the effects of the simvastatin only if they are due to inhibition of isoprenoid synthesis (figure 1.8). Surprisingly, results from both flow cytometry and western blots showed that adding back GGPP but not mevalonate restored the ability of Bt_2cAMP to increase C5aR expression in the presence of 1 μM simvastatin (figures 5.3.8 and 5.3.9).

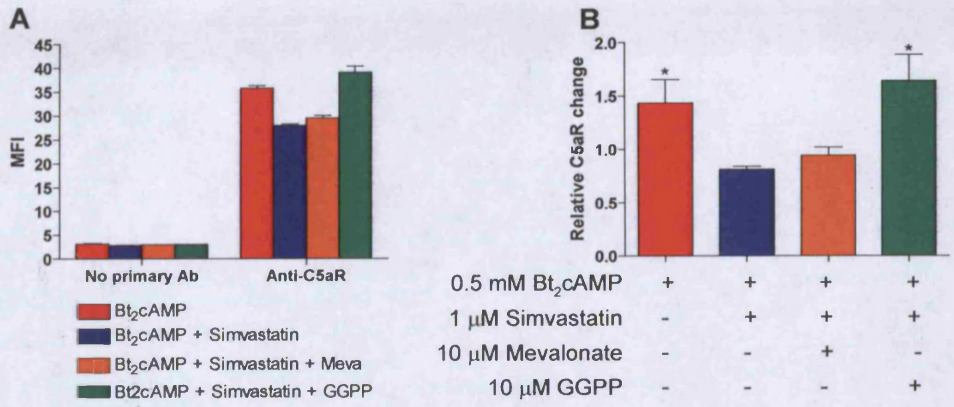


Figure 5.3.8 Adding back 10 μM GGPP, but not 10 μM mevalonate, restores Bt_2cAMP induced C5aR expression in the presence of 1 μM simvastatin as detected by flow cytometry. $U937_{PM}$ cells were incubated for 48 hrs with 0.5 mM Bt_2cAMP plus 1 μM simvastatin and either 10 μM mevalonate or 10 μM GGPP. Cells were incubated with human aggregated IgG and stained for C5aR expression as described in section 5.2.3. Results expressed as mean fluorescence intensity from one representative experiment (A) and relative C5aR change compared with Bt_2cAMP (B). One-way ANOVA followed by Dunnett's multiple comparison test shows significant difference from Bt_2cAMP + 1 μM simvastatin, * $p < 0.05$.

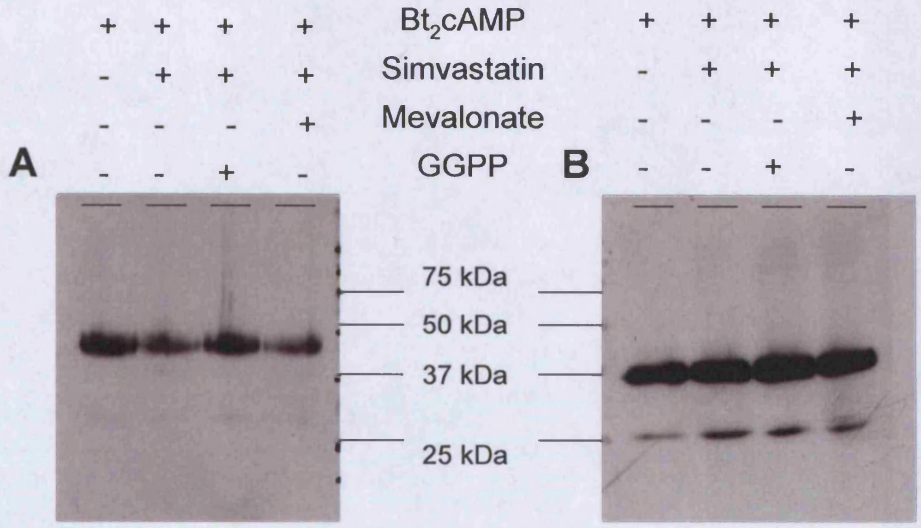


Figure 5.3.9 Adding back 10 μM GGPP, but not 10 μM mevalonate, restores Bt_2cAMP induced C5aR expression in the presence of 1 μM simvastatin as detected by western blot. $U937_{PM}$ cells were incubated for 48 hrs with 0.5 mM Bt_2cAMP plus 1 μM simvastatin and either 10 μM mevalonate or 10 μM GGPP. C5aR (A) and GAPDH (B) expression were detected by western blotting as described in section 5.2.4. Results shown are from one experiment but are representative of multiple analyses.

As previously described in section 4.3, incubation of $U937_{PM}$ with Bt_2cAMP causes the cells to develop an irregular cell shape morphology. Incubation of these cells with Bt_2cAMP and 1 μM simvastatin resulted in them obtaining a spherical cell morphology (figure 5.3.10). The addition of 10 μM GGPP, but not mevalonate, prevented the effects of simvastatin on the cell morphology and maintained the irregular shaped morphology of the Bt_2cAMP stimulated $U937_{PM}$ cell line (figure 5.3.10).

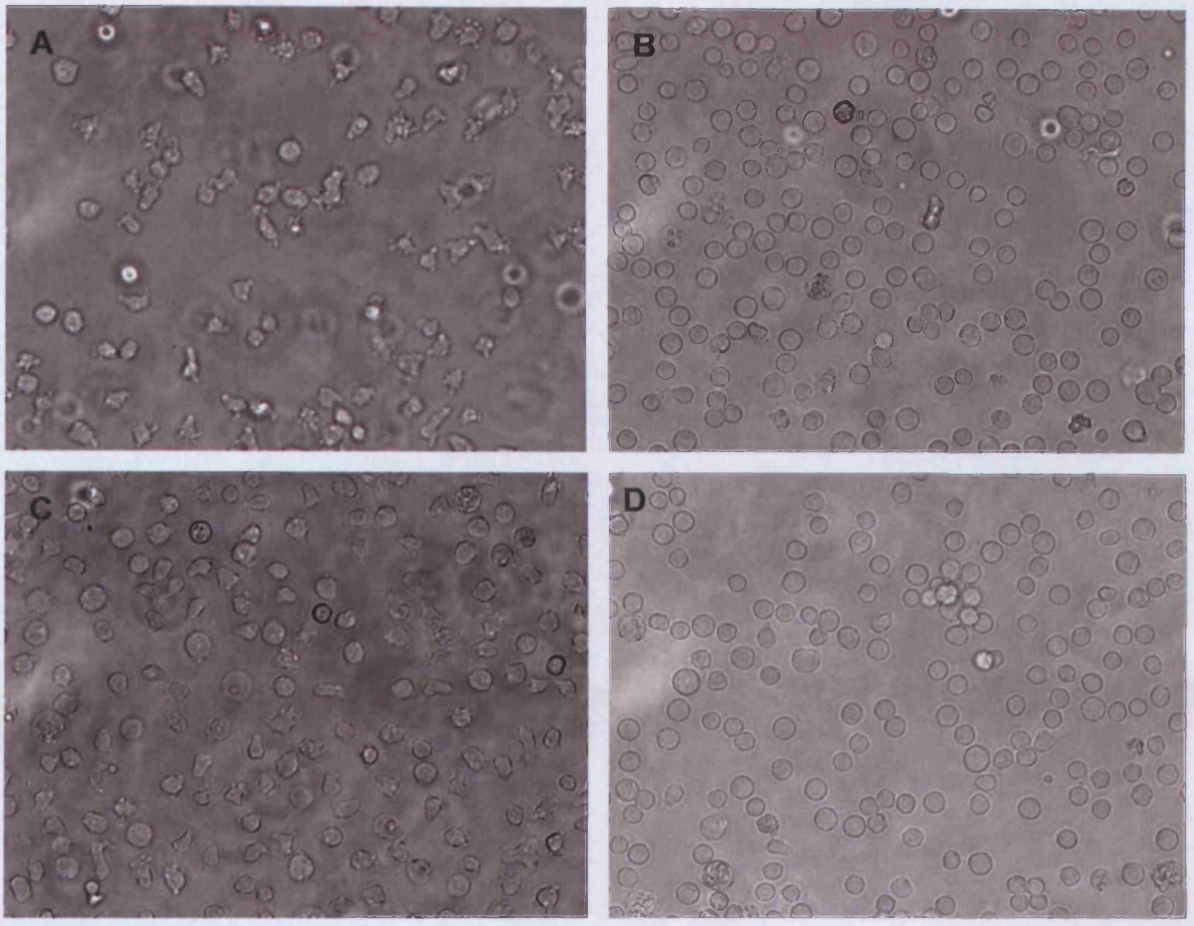


Figure 5.3.10 Adding back 10 μM GGPP, but not 10 μM mevalonate, restores Bt_2cAMP induced cell morphology. Images taken following 2 day incubation with (A) 0.5 mM Bt_2cAMP, (B) 0.5 mM Bt_2cAMP plus 1 μM simvastatin, (C) 0.5 mM Bt_2cAMP, 1 μM simvastatin and 10 μM GGPP and (D) 0.5 mM Bt_2cAMP, 1 μM simvastatin and 10 μM mevalonate.

As 10 μM GGPP but not 10 μM mevalonate appeared to prevent the effects of 1 μM simvastatin it was decided to repeat these experiments but using a higher concentration of mevalonate. Figures 5.3.11 and 5.3.12 show that the addition of 50 μM mevalonate was able to prevent the inhibitory effects of 1 μM simvastatin on Bt_2cAMP induced C5aR expression and retaining the $U937_{PM}$ cell morphology (figure 5.3.11 and 5.3.12).

To further investigate if adding back either 50 μM mevalonate or 10 μM GGPP prevented the anti-proliferative effect of 0.5 mM Bt_2cAMP plus 1 μM simvastatin an Alamar blue assay was performed. Figure 5.3.13 shows that adding back mevalonate or GGPP restored the proliferation rate of the $U937_{PM}$ cells when incubated with 0.5 mM Bt_2cAMP plus 1 μM simvastatin (figure 5.3.13). To investigate whether mevalonate or GGPP themselves had any affect on Bt_2cAMP induced C5aR expression, $U937_{PM}$ cells were incubated with Bt_2cAMP and either GGPP or mevalonate. Flow cytometry results from incubations with Bt_2cAMP plus mevalonate or GGPP showed that neither mevalonate nor GGPP had an effect on Bt_2cAMP induced C5aR expression (figure 5.3.14).

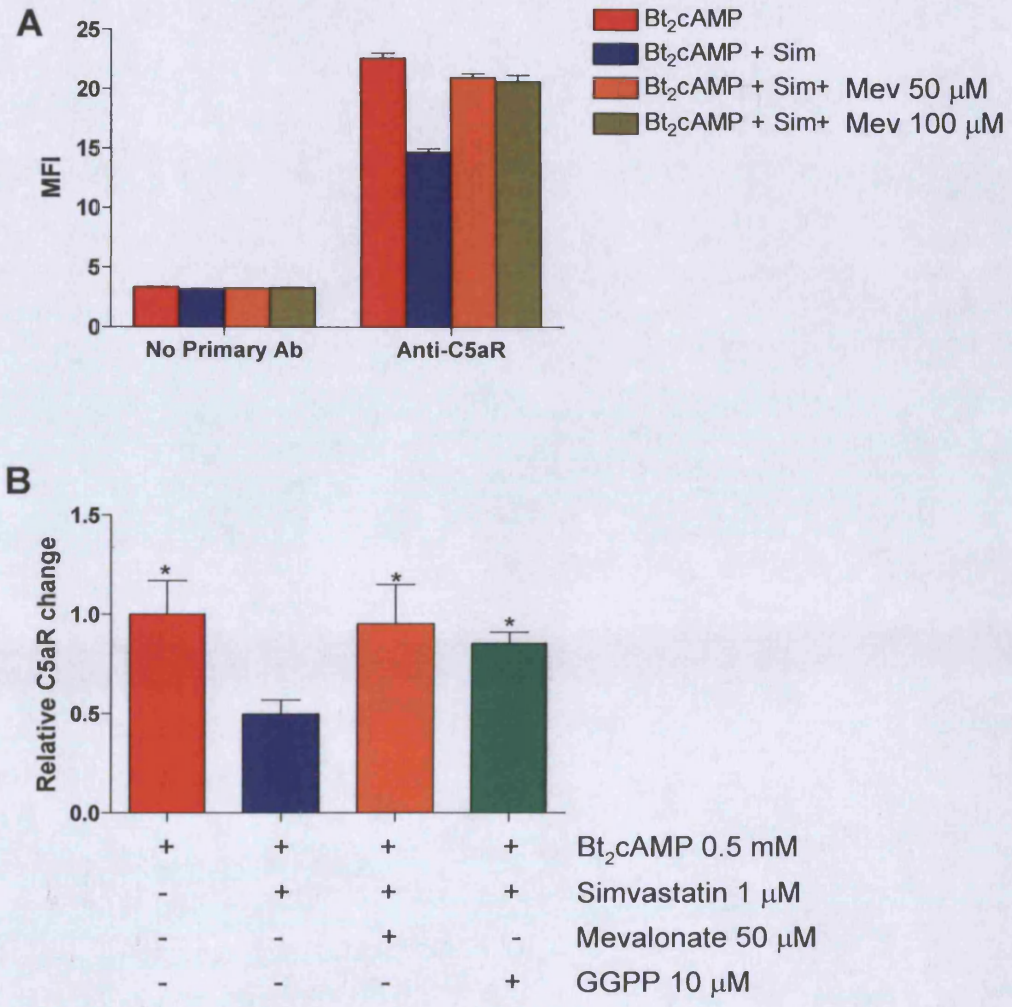


Figure 5.3.11 Adding back higher concentration of mevalonate restores the C5aR expression. (A) $U937_{PM}$ cells were incubated for 48 hrs with 0.5 mM Bt_2cAMP plus 1 μM simvastatin and either 50 μM or 100 μM mevalonate. Results expressed as mean fluorescence intensity from one representative experiment. (B) Relative C5aR change compared to 0.5mM Bt_2cAMP. One-way ANOVA followed by Dunnett's multiple comparison test shows significant difference from Bt_2cAMP plus simvastatin, *$p<0.05$.

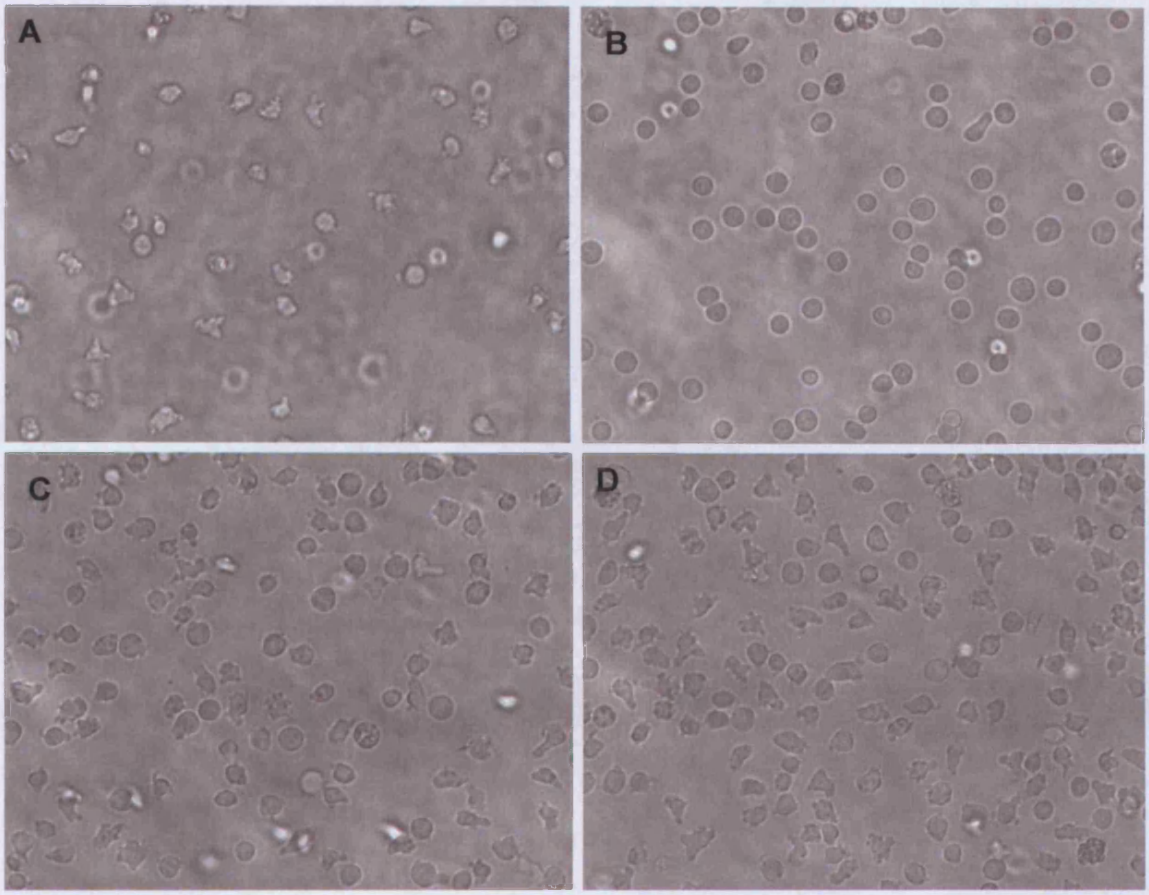
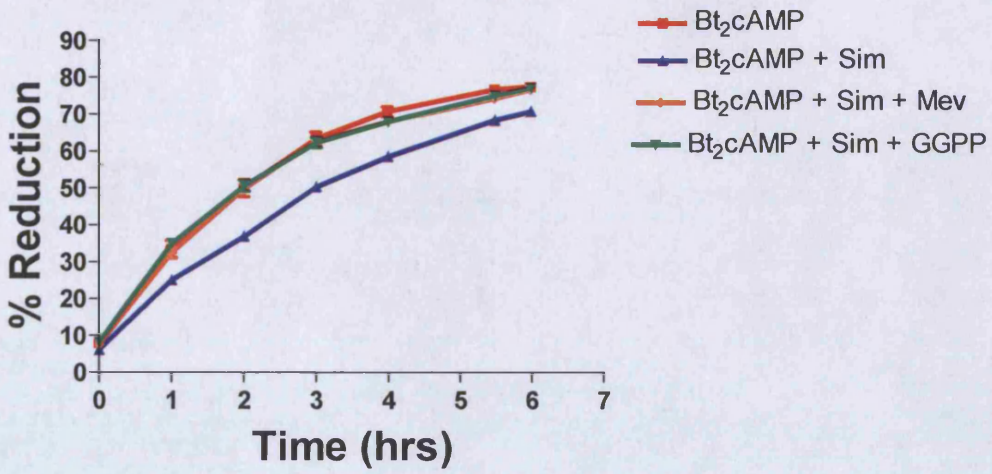


Figure 5.3.12 Adding back 50 μM mevalonate restores cell morphology. Images taken following 2 day incubation with (A) 0.5 mM Bt_2cAMP, (B) 0.5 mM Bt_2cAMP plus 1 μM simvastatin, (C) 0.5 mM Bt_2cAMP, 1 μM simvastatin and 10 μM GGPP and (D) 0.5 mM Bt_2cAMP, 1 μM simvastatin and 50 μM mevalonate, x40 magnification.



	Rate constant (K) % reduction/hr
0.5 mM Bt_2cAMP	0.4961 (+/- 0.04401) *
0.5 mM Bt_2cAMP + 1 μM Simvastatin	0.3094 (+/- 0.02862)
0.5 mM Bt_2cAMP + 1 μM Simvastatin + 50 μM Mevalonate	0.5020 (+/- 0.03705) *
0.5 mM Bt_2cAMP + 1 μM Simvastatin + 10 μM GGPP	0.5285 (+/- 0.04511) *

Figure 5.3.13 Mevalonate or GGPP prevents the inhibitory effects of Bt_2cAMP plus simvastatin on cell proliferation. Following 40 hr incubation of $U937_{PM}$ with 0.5 mM Bt_2cAMP, 1 μM simvastatin and 10 μM GGPP or 50 μM mevalonate Alamar blue was added to a final concentration 12 %. Reduction in Alamar blue was then recorded every 1hr for a further 7.5 hr using fluorescence. Rate constant was calculated using one phase exponential association equation and one-way ANOVA followed by Dunnett's multiple comparison test was performed. Data marked with an asterisk shows significant difference compared to the Bt_2cAMP + 1 μM simvastatin, * = $p < 0.05$.

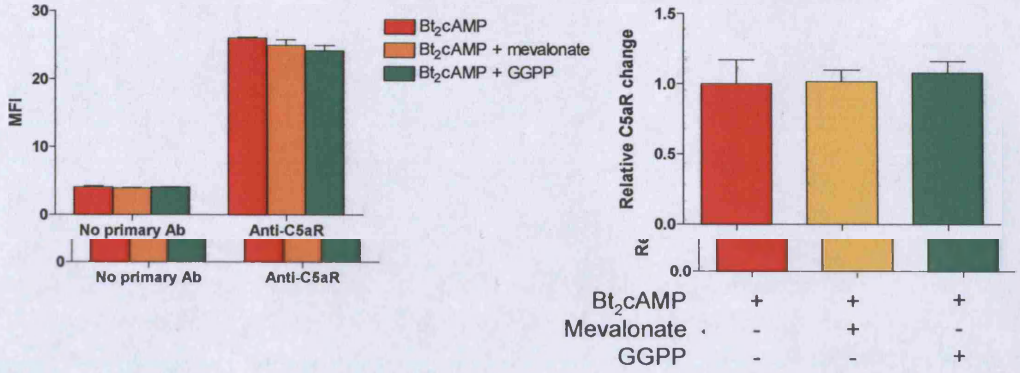


Figure 5.3.14 In the absence of simvastatin 50 μM mevalonate or 10 μM GGPP have no effect on Bt_2cAMP induced C5aR expression. $U937_{PM}$ cells were incubated for 48 hrs with 0.5 mM Bt_2cAMP plus 10 μM GGPP or 50 μM mevalonate. Cells were incubated with human aggregated IgG and stained for C5aR expression as described section 5.2.3. Results expressed as mean fluorescence intensity from one representative experiment (A) and relative C5aR change compared with Bt_2cAMP alone (B). One-way ANOVA shows no significant difference between the treatments.

5.3.5 Simvastatin did not inhibit Bt_2cAMP induced C5aR expression via *cis*-actin elements present within the -2Kbp promoter fragment

To investigate whether simvastatin was able to reduce the Bt_2cAMP induced C5aR expression at the gene transcription level the -2Kbp(**PM**)+pEGFP C5aR reporter construct (see Chapter 3 for full details) was transfected into the $U937_{PM}$ cell line. Once growing in log phase clonal cell populations were incubated for 2 days in the absence or presence of Bt_2cAMP, simvastatin, mevalonate or GGPP. Changes in EGFP expression were then monitored by flow cytometry.

Shown in figure 5.3.15 are the effects of the different treatments on EGFP fluorescence relative to the control. This figure shows that incubation with Bt_2cAMP decreased EGFP fluorescence compared with control which was unexpected as Bt_2cAMP increases C5aR expression on the cell surface (see section 4.3.5 for more details). This figure also showed that incubation with Bt_2cAMP and 1 μM simvastatin increased EGFP fluorescence slightly which was prevented by the addition of either mevalonate or GGPP (figure 5.3.15). However as the promoter-less pEGFP control vector also shows a similar pattern this suggests that the effect is not specifically due to regulation via *cis*-acting elements present within the -2Kbp(PM) promoter region cloned.

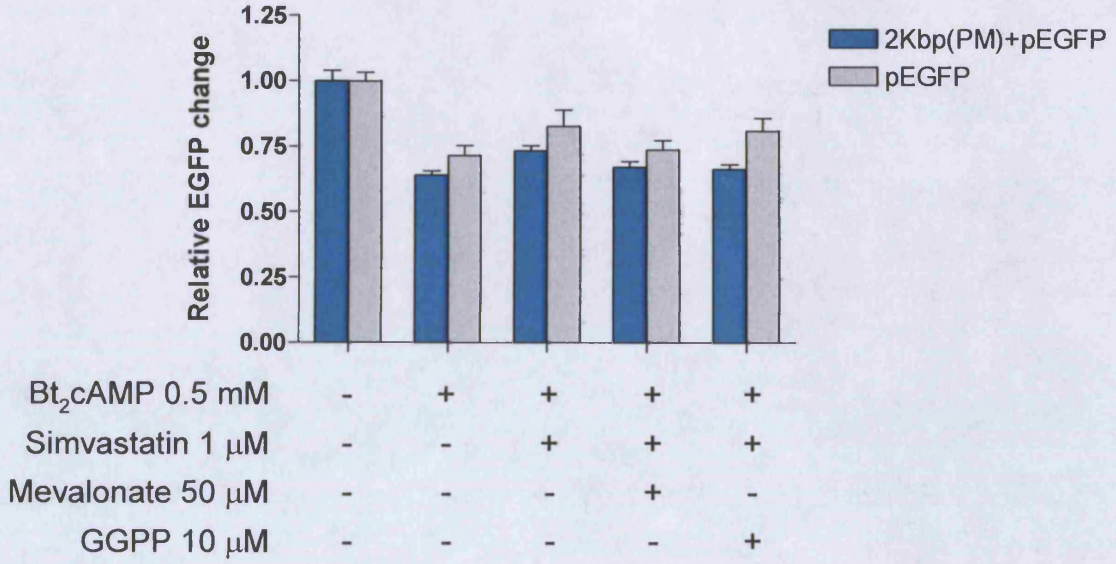


Figure 5.3.15 0.5mM Bt_2cAMP plus 1 μM simvastatin does not down regulate C5aR expression via any cis-actin elements present within the 2 Kbp promoter fragment. $U937_{PM}$ which have been transfected with the 2Kbp (PM) + pEGFP or pEGFP control vector were incubated with 0.5 mM Bt_2cAMP, 1 μM simvastatin, 10 μM GGPP or 50 μM mevalonate for 2 days. Following incubation changes EGFP fluorescence will be monitored by flow cytometry. Two-way ANOVA showed that the treatment had significant effect on EGFP fluorescence (p=<0.0001), however, transfection with the -2Kbp(**PM**)+pEGFP reporter construct showed no significant difference from the pEGFP control vector (p=0.0658). Bonferroni post-test showed no significant difference between the -2Kbp(**PM**)+pEGFP and pEGFP vector.

5.3.6 Zaragozic acid had no effects on Bt_2cAMP induced C5aR expression in $U937_{PM}$ cell line

To further investigate the effects of inhibition of the cholesterol synthesis pathway on Bt_2cAMP induced C5aR expression, $U937_{PM}$ cells were incubated with Bt_2cAMP plus the squalene synthase inhibitor zaragozic acid, either 0.1 μM or 1 μM (see figure 1.8). As squalene synthase converts two FFPP molecules into squalene, an important precursor of cholesterol, its inhibition will result in reduction of cholesterol synthesis independently of the HMG-CoA reductase inhibitors.

Incubation with Bt_2cAMP plus zaragozic acid had no effect on Bt_2cAMP induced C5aR expression nor cell proliferation, which further suggests that the effects seen by simvastatin are due to inhibition of isoprenoid synthesis rather than inhibition of cholesterol synthesis (figure 5.3.16 and 5.3.17).

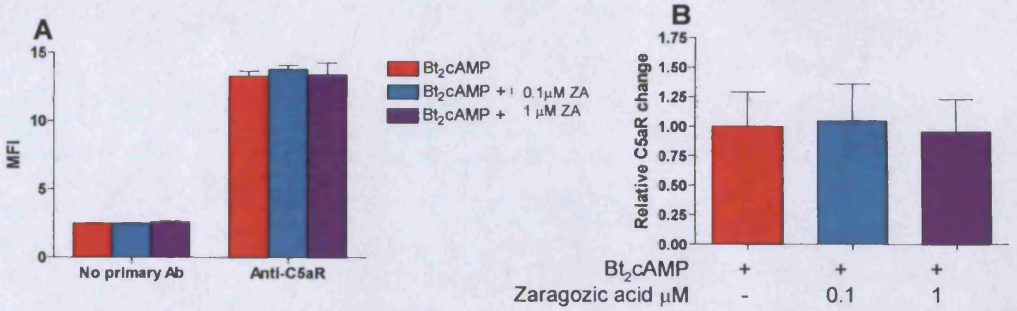
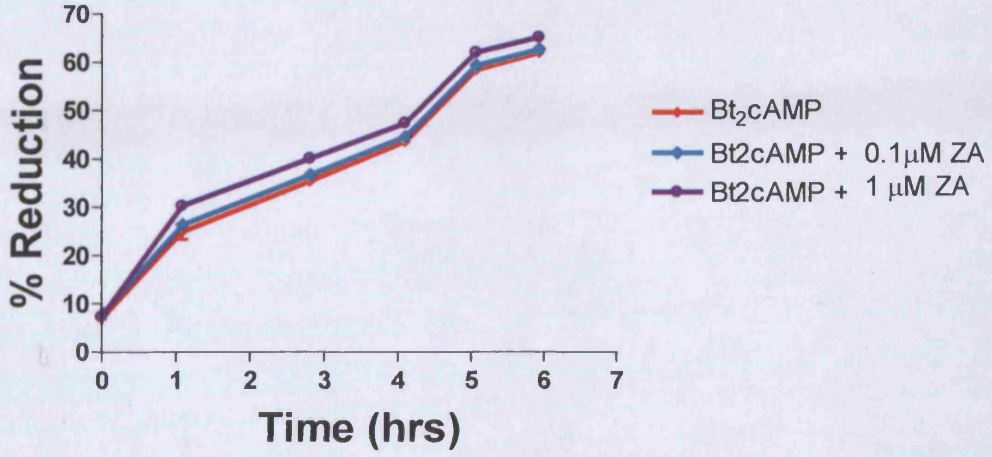


Figure 5.3.16 Zaragozic acid has no effect on Bt_2cAMP induced C5aR expression. $U937_{PM}$ cells were incubated for 48 hrs with 0.5mM Bt_2cAMP and 0.1 μM or 1 μM zaragozic acid (ZA). Cells were incubated with human aggregated IgG and stained for C5aR expression as described in section 5.2.3. Results expressed as mean fluorescence intensity from one representative experiment (A) and relative change compared with Bt_2cAMP alone (B). One-way ANOVA showed no statistical significance between the treatments.



	Rate constant (K) % reduction/hr
0.5 mM Bt_2cAMP	0.2149 (+/- 0.06906)
0.5 mM Bt_2cAMP + 0.1 μM Zaragozic acid	0.2461 (+/- 0.07438)
0.5 mM Bt_2cAMP + 1 μM Zaragozic acid	0.3309 (+/- 0.08372)

Figure 5.3.17 Zaragozic acid has no effect on cell proliferation. Following 40 hr incubation of $U937_{PM}$ with 0.5 mM Bt_2cAMP and 0.1 μM or 1 μM ZA, Alamar blue was added to a final concentration 12 %. Reduction in Alamar blue was then recorded every 2 hrs for a further 7.5 hr using fluorescence. Percentage reduction was calculated using 100 % reduced Alamar blue, achieved by autoclaving for 15 min. Rate constant was calculated using one phase exponential association equation and One-way ANOVA showed no significant difference between the treatments.

5.3.7 Increasing concentration of simvastatin reduced C5aR expression in the $U937_{C5aR}$ cell line

The $U937_{C5aR}$ cell line has previously been transfected with the human C5aR so that it expresses high levels of functioning receptor without the need to differentiate the cells (Kew et al., 1997). Although the C5aR expression on these cells is mainly exogenous expressed C5aR and not under the transcriptional control of the C5aR promoter it was still important to monitor the effects of simvastatin on the receptor expression in these cells as statins could still effect C5aR expression by altering membrane cholesterol content and therefore the surface expression (as described in section 5.1.2) and/or statins may regulate the activity of the promoter which drives transcription of the exogenous C5aR expression (although this is not known). It was anticipated that this cell line would be useful to monitor effects of membrane cholesterol composition and statins on C5aR function and expression.

Results from flow cytometry showed that following 2 day incubation with simvastatin the $U937_{C5aR}$ cell line reduces its C5aR expression in a concentration dependent manner (figure 5.3.18).

To determine if incubation with simvastatin reduced cell proliferation rates an Alamar blue assay was performed. Results in figure 5.3.19 show that although these cells treated with 1 μM simvastatin do have a slightly lower cell proliferation rate this reduction was not statistically significant (figure 5.3.19). Adding back either 50 μM mevalonate or 10 μM GGPP partially restored the change in C5aR expression induced by 1 μM simvastatin (figure 5.3.20).

Incubation of the $U937_{C5aR}$ cells with increasing concentration of zaragozic acid, a squalene synthesis inhibitor, had no effect on C5aR expression levels, which suggest that inhibition of cholesterol biosynthesis has no effect on C5aR expression in these cells (figure 5.3.21).

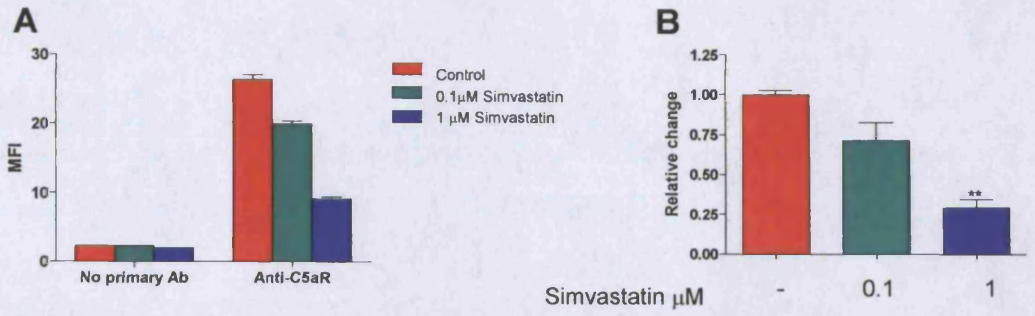
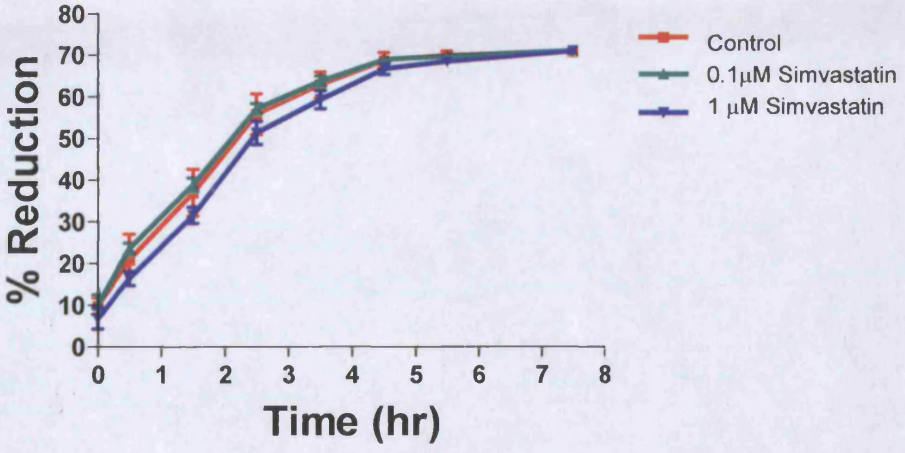


Figure 5.3.18 Incubation with simvastatin reduces C5aR expression in the U937$_{C5aR}$ cell line. U937$_{C5aR}$ cells were incubated with increasing concentration of simvastatin for 48 hr. Cells were harvested and incubated with human aggregated IgG prior to staining for flow cytometry. C5aR expression was detected by flow cytometry as described in section 5.3.2. (A) Results from one representative experiment expressed as mean fluorescence intensity and (B) relative C5aR change comapred with the control. One-way ANOVA followed by Dunnett's post-test shows significant difference to the control; ** $p<0.01$.



	Rate constant (K) % reduction^{-1} hr^{-1}
Control	0.5456 (+/- 0.07973)
0.1 μM Simvastatin	0.5985 (+/- 0.05984)
1 μM Simvastatin	0.4262 (+/- 0.04116)

Figure 5.3.19 Incubation of U937$_{C5aR}$ with simvastatin has no significant effect on cell proliferation. Following 40 hr incubation of U937C5aR with increasing concentration of simvastatin, Alamar blue was added to a final concentration 12 %. Reduction in Alamar blue was then recorded every 1 hr for 7.5 hrs using fluorescence. Percentage reduction was calculated using 100 % reduced Alamar blue, achieved by autoclaving for 15 min. Rate constant was calculated using one phase exponential association equation and One-way ANOVA showed no statistical difference between the treatments.

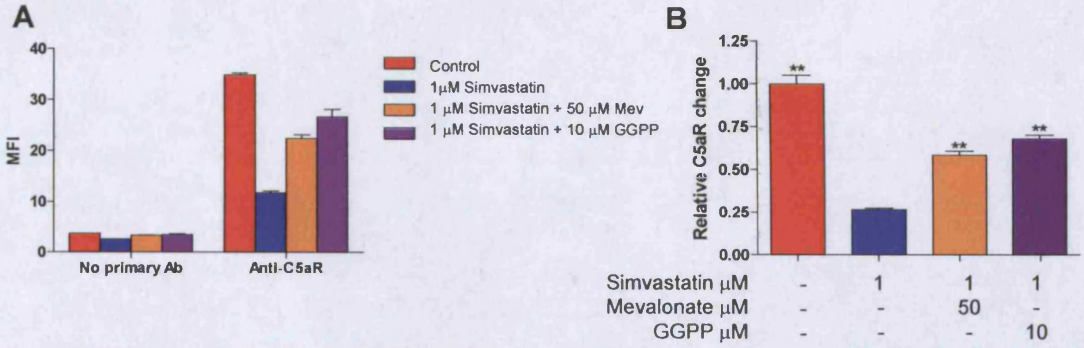


Figure 5.3.20 Adding back mevalonate or GGPP partially restores C5aR expression in U937$_{C5aR}$ cell line incubated with 1 μM simvastatin. U937$_{C5aR}$ were incubated for 48 hrs in the absence or presence of simvastatin plus 50 μM mevalonate or 10 μM GGPP. Cells were harvested and incubated with human aggregated IgG prior to staining for flow cytometry. C5aR expression was detected by flow cytometry as described in section 5.2.3. (A) Results from one representative experiment. (B) Relative C5aR change compared with control cells. One-way ANOVA followed by Dunnett's post-test shows significant difference to the 1 μM simvastatin treated cells; *** $p < 0.01$.

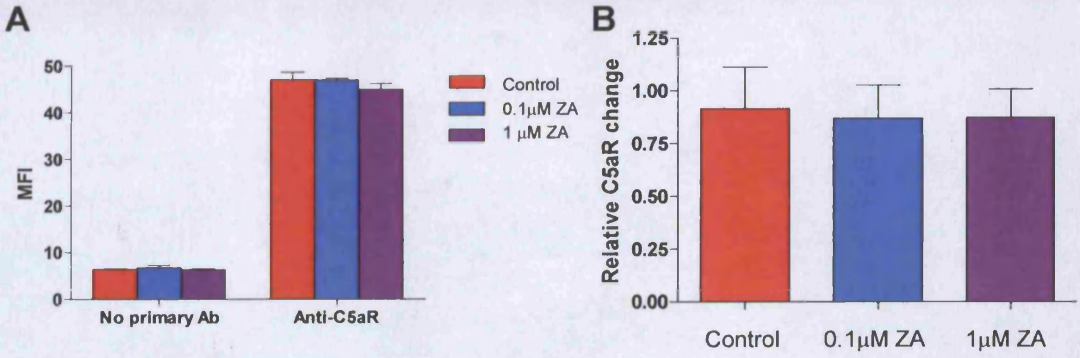


Figure 5.3.21 Effects of zaragozic acid incubation on C5aR expression in U937$_{C5aR}$ cell line. U937$_{C5aR}$ cells were incubated in the absence or presence of increasing concentration of zaragozic acid for 48 hrs. Cells were harvested and incubated with human aggregated IgG prior to staining for flow cytometry. C5aR expression was detected by flow cytometry as described in section 5.2.3. (A) Results from one representative experiment. (B) Relative C5aR change compared with the control. One-way ANOVA showed no significant difference between the treatments.

5.3.8 Incubation with fenofibrate but not simvastatin or lovastatin lowered C5aR expression in CD14 positive monocytes

As previously described in section 1.5.1, several naturally occurring and chemically synthesised statins are commercially available which have ranging inhibitory concentrations (Ki) for HMG CoA reductase. To further investigate whether different statins or other lipid lowering drugs had any effect on C5aR expression, human PBMCs were incubated with simvastatin, lovastatin or fenofibrate, a PPARα agonist (see section 1.3.6), for 24 hrs and changes in receptor expression were monitored by flow cytometry. By using the monocytic cell marker CD14 during FACS analysis allowed the C5aR expression to be monitored specifically on the CD14 positive monocytes.

Results shown in figure 5.3.22 show that incubation of human PBMC with simvastatin had no effect on C5aR or CD14 expression in CD14 positive monocytes (figure 5.3.22). Similarly, incubation of the PBMCs with lovastatin had no significant effect on C5aR or CD14 expression in CD14 positive cells (figure 5.3.23). Fenofibrate, however, significantly down regulated C5aR expression and it also lowered CD14 expression but this was not classed as statistically significant, p=0.0748 (figure 5.3.24).

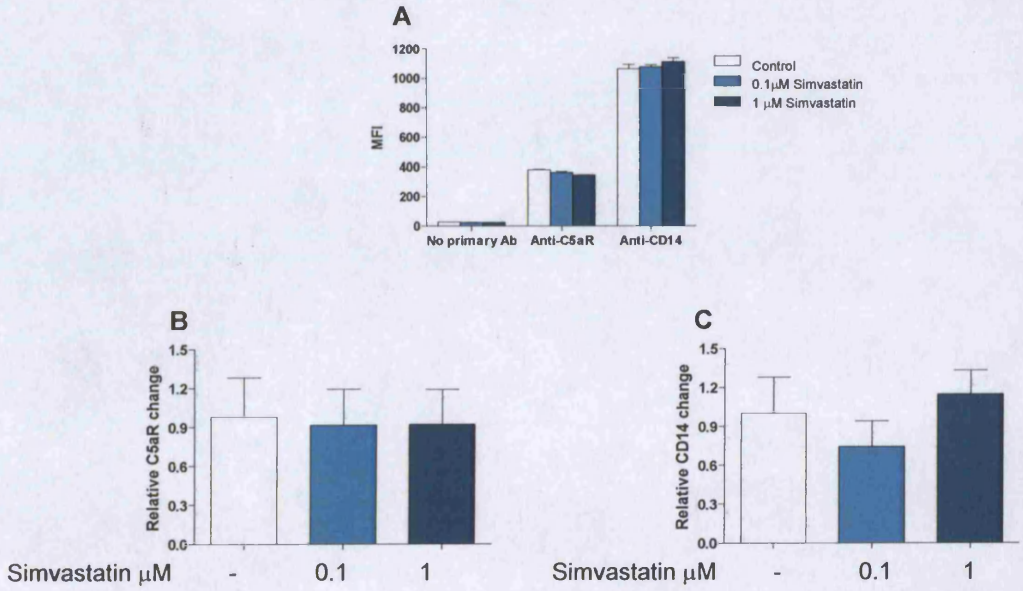
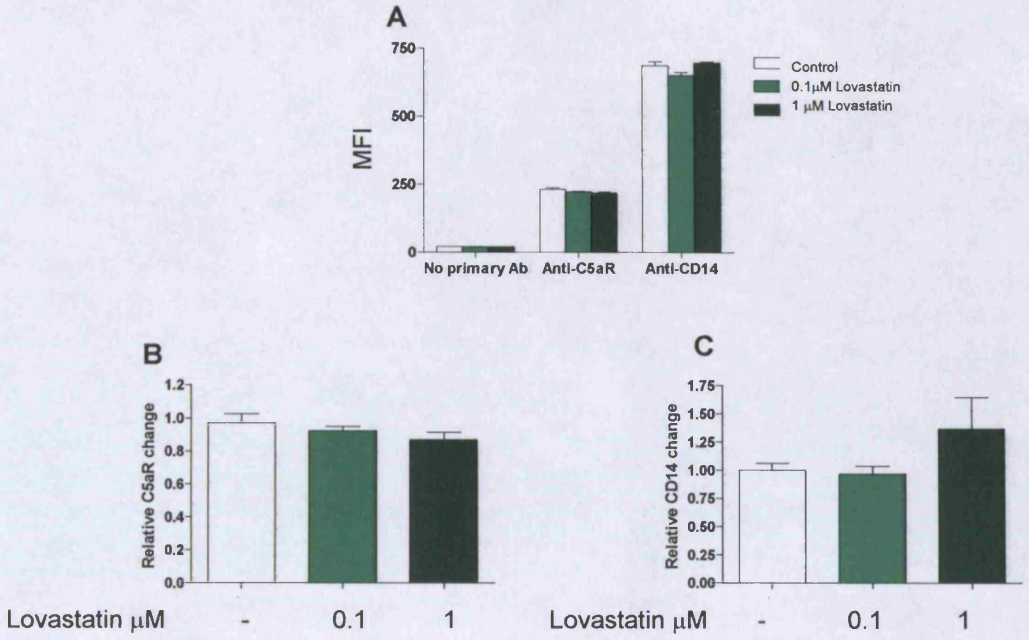
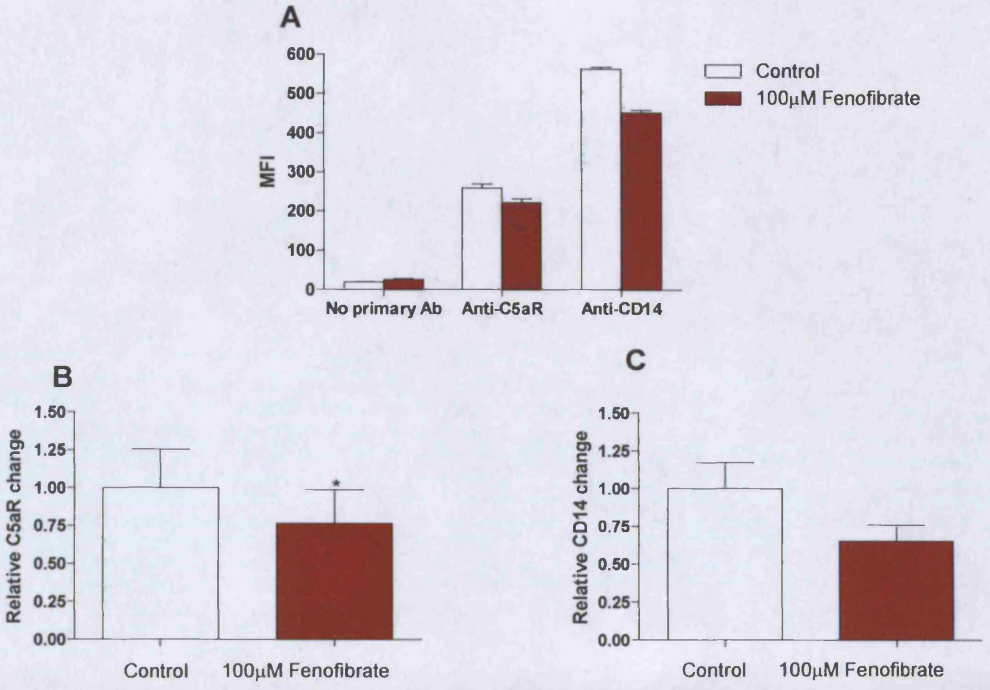


Figure 5.3.22 Simvastatin has no effect on C5aR or CD14 expression in CD14 positive monocytes. Human PBMCs isolated from buffy coat were incubated with increasing concentration of simvastatin for 24 hrs. Cells were harvested and stained for the monocyte cell surface marker CD14 or C5aR by flow cytometry as described in section 5.2.3. (A) Mean fluorescent intensity from CD14 positive population in one representative experiment. (B) Relative C5aR change compared with the control. (C) Relative CD14 change compared with the control, n=4. One-way ANOVA showed no statistical difference in C5aR or CD14 expression following incubation with simvastatin.



5.3.23 Lovastatin has no effect on C5aR or CD14 expression in CD14 positive monocytes. Human PBMCs isolated from buffy coat were incubated with increasing concentration of lovastatin for 24 hrs. Cells were harvested and stained for the monocyte cell surface marker CD14 or C5aR by flow cytometry as described in section 5.2.3. (A) Mean fluorescent intensity from CD14 positive population in one representative experiment. (B) Relative C5aR change compared with the control. (C) Relative CD14 change compared with the control, n=4. One-way ANOVA showed no statistical difference in C5aR or CD14 expression following incubation with lovastatin.



5.3.24 Fenofibrate significantly lowers C5aR expression in CD14 positive monocytes. Human PBMCs isolated from buffy coats were incubated with fenofibrate for 24 hrs. Cells were harvested and stained for the monocyte cell surface marker CD14 or C5aR by flow cytometry as described in section 5.2.3. (A) Mean fluorescent intensity from CD14 positive population in one representative experiment. (B) Relative C5aR change compared with the control. (C) Relative CD14 change compared with the control, n=4. Results marked with an asterisk represent significant difference from the control using paired Student T-test, ** $p<0.01$, n=4.

5.4 Discussion

The aim of this chapter was to investigate whether C5aR expression could be regulated by statins in the U937 cell line and in primary human monocytes isolated from buffy coats.

The effects of simvastatin on basal C5aR expression was investigated in the $U937_{PM}$ cell line. It was initially thought that simvastatin may down regulate basal levels of C5aR; however when C5aR staining was repeated in the presence of aggregated human IgG to prevent non-specific binding via the Fc receptors this effect disappeared, which suggests that simvastatin may have an effect on Fc receptor expression (figure 5.3.1). However a previous study by Hillyard and co-workers showed that fluvastatin had no effect on FcγRI or FcγRII expression in the U937 cell line (Hillyard et al., 2004). The incubation of the $U937_{PM}$ cell line with aggregated human IgG led to very little/no basal C5aR expression being detected in these cells which makes it difficult to determine whether statins have any effect on basal C5aR expression using this cell line (see Chapter 4 for more details). Incubation of the $U937_{PM,}$ which had been transfected with the -2 Kbp(**PM**)+pEGFP reporter construct or pEGFP vector control, with simvastatin suggested that there was no *cis*-acting element within the promoter region cloned that was regulated by simvastatin (figure 5.3.2). Similarly incubation of the C5aR 3'UTR EGFP reporter constructs with simvastatin had no effect on EGFP fluorescence, suggesting that simvastatin does not affect mRNA stability of the C5aR (figure 5.3.3).

To investigate the effects of simvastatin on induced C5aR expression, the $U937_{PM}$ cell line was differentiated with Bt_2cAMP in the absence or presence of simvastatin. As previously described in Chapter 4 it was originally proposed to differentiate the cells with a more physiologically relevant stimulus such as IFNγ, which has emerged as a significant factor in atherogenesis (McLaren and Ramji, 2009), however the C5aR expression in the $U937_{PM}$ cell line was unresponsive to such stimuli, see Chapter 4 for full details. Incubation of the $U937_{PM}$ with 1 μM simvastatin significantly reduced the Bt_2cAMP induced C5aR expression as observed by flow cytometry and western blotting (figures 5.3.4 and 5.3.5 respectively).

Although alone incubation with simvastatin or Bt_2cAMP had no effect on the rate of cell proliferation, when combined they significantly reduced the proliferation rate of the $U937_{PM}$ cells (figure 5.3.7). However the concentrations of statins used in this study are far greater, at least ten times, than the maximum pharmacological concentration of simvastatin (30-70 nM (Krishna et al., 2009)). Although 1-10 μM simvastatin is commonly used for most *in vitro* experiments published (reviewed by (Bjorkhem-Bergman et al., 2011), results obtained at these concentrations are therefore unlikely to have any therapeutic implications

due to *in vivo* toxicity. However these results yield information about the role of isoprenylation in the mechanism of C5aR expression.

Statins have previously been shown to inhibit cell growth by inhibiting isoprenylation of the growth-regulating $p21^{ras}$ proteins (Brown and Goldstein, 1980, Kaneko et al., 1978, Quesney-Huneeus et al., 1979). The effects of the 1 μM simvastatin and 0.5 mM Bt_2cAMP on cell proliferation rates were prevented by the addition of either mevalonate or GGPP, which also suggests that inhibition of isoprenylation rather than cholesterol biosynthesis were responsible for the effects on cell proliferation (figure 5.3.13). Interestingly the addition of mevalonate or GGPP were also able to prevent the inhibitory effects of 1 μM simvastatin on Bt_2cAMP induced C5aR expression, which also suggests that inhibition of isoprenylation was also responsible for this effect (figure 5.3.11). Incubation with Bt_2cAMP in the presence of zaragozic acid had no effect on C5aR expression or cell proliferation rates, therefore further suggesting that the effects of simvastatin are solely due to inhibition of isoprenylation (figures 5.3.16 and 5.3.17). Together these results could suggest that inhibition of Bt_2cAMP induced C5aR expression by simvastatin could be caused by the cells entering a quiescent state by inhibition of isoprenylation of cell growth regulating proteins, such as $p21^{ras}$, rather than specifically preventing the regulation of the C5aR gene by Bt_2cAMP. However, its has recently been shown in our lab that DAF expression increased in the $U937_{PM}$ cells following incubation with 0.5mM Bt_2cAMP and 1μM simvastatin which suggests that the findings presented in this thesis is due to specific regulation of the C5aR gene.

To circumvent the problems associated with using a higher concentration of simvastatin on cell proliferation, a lower concentration of simvastatin (0.1 μM) was also used for a longer duration however this had no effect on Bt_2cAMP induced C5aR expression (figure 5.3.6).

Incubation of $U937_{PM}$ cells transfected with -2Kbp(**PM**)+pEGFP reporter constructs with both Bt_2cAMP and simvastatin showed a slight increase in EGFP fluorescence compared with Bt_2cAMP alone, however this was also observed in the pEGFP promoter-less vector (figure 5.3.15). Although this result suggest that there is no *cis*-acting element within the -2Kbp promoter region cloned which is specifically regulated by Bt_2cAMP and simvastatin, these reagents could still regulate C5aR expression via regulatory elements outside the promoter region cloned. As previous results had shown that the mRNA stability was not to be regulated by incubation with simvastatin or Bt_2cAMP individually and as the 3'UTR EGFP reporter constructs were generated using 3'UTR isolated from $U937_{EC}$ it was not investigated whether dual incubation of these stimuli altered EGFP fluorescence (figure 4.3.12 and 5.3.3).

As described in Chapter 4 Bt_2cAMP is believed to differentiate the U937 cell line to a more macrophage like phenotype. As well as increasing C5aR expression, incubation with Bt_2cAMP has been documented to cause a reduction in cell size, inhibition in DNA synthesis, irregular morphology and often ruffled cell membranes (Sheth et al., 1988). The presence of 1 μM simvastatin during the incubation with 0.5 mM Bt_2cAMP prevented the irregular appearance of the $U937_{PM}$ cells caused by Bt_2cAMP treatment alone and instead caused cells to obtain a more spherical cell morphology (figure 5.3.12). Thus it appeared that the presence of 1 μM simvastatin prevented the 0.5 mM Bt_2cAMP induced differentiation of the $U937_{PM}$ cell line. This effect of simvastatin was reversed by the addition of either 50 μM mevalonate or 10 μM GGPP, which suggested that inhibition of isoprenylation or isoprenylation and cholesterol biosynthesis were responsible for these effects (figure 5.3.12). The cAMP analog, Bt_2cAMP, has previously been suggested to cause differentiation in the U937 cell line via activation of PKA (Shayo et al., 1997, Brodsky et al., 1998). The results presented here would therefore suggest that simvastatin effects Bt_2cAMP activation of PKA or its down stream targets. However more recently cAMP has also been found to activate two other targets, cAMP regulated ion channels and guanine nucleotide exchange factors activated by cAMP (known as Epac) (Bos, 2006). Activation of Epac can regulate small GTP binding proteins such as Rap which requires geranylgeranyl prenylation for correct functioning (Bos, 2006, Altschuler et al., 1995, Vasudevan et al., 1999). As Bt_2cAMP is not specific for either PKA or Epac activation, this therefore suggests that statins could potentially prevent the Bt_2cAMP induced C5aR expression via several mechanisms as outlined in figure 5.4.1. To confirm which mechanisms are involved, future experiments could involve the use of selective Epac activators such as 8-pCPT-2'OMe-cAMP (also known as 007), alternatively the same experiments could be repeated using FPP rather than GGPP as Rap is a gernylgeranylated protein.

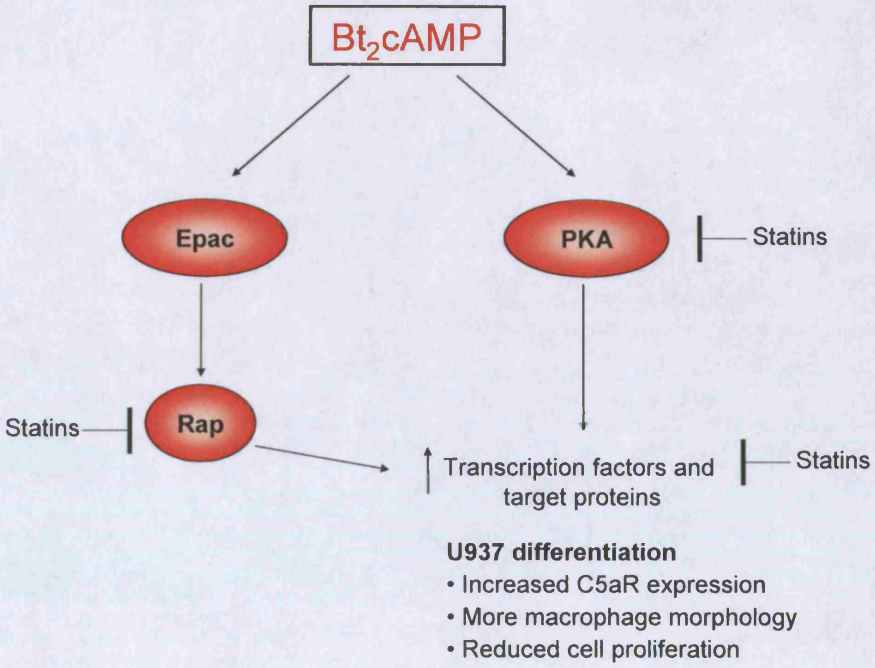


Figure 5.4.1 Potential mechanisms which simvastatin may inhibit Bt_2cAMP induced C5aR expression. Bt_2cAMP can activate guanine nucleotide exchange factor (Epac), which can in turn regulate small GTP binding proteins such as Rap and/or it can activate protein kinase A (PKA).

The incubation of the $U937_{C5aR}$ cell line with simvastatin found that in these cells the C5aR expression decreased in a concentration dependent manner (figure 5.3.18). As these cells had previously been transfected with the human C5aR, the majority of the receptor being expressed was due to exogenous expression (Kew et al., 1997). This makes it difficult to determine whether the decreased C5aR expression observed is due to specific regulation of simvastatin on a regulatory element with the promoter region or 3'UTR of the C5aR gene in these cells or whether the vector used to clone the C5aR into these cells is regulated by the statin. Unfortunately we were unable to identify which plasmid was used during the cloning process by Kew et al. (1997) and therefore unable to speculate whether its promoter or 3'UTR may be affected by incubation with statins. Alternatively C5aR surface expression may be reduced via a cholesterol dependent mechanism such as disrupting in the intracellular trafficking of the receptor; however as the results suggest the mechanism by which simvastatin down regulates receptor expression is via inhibition of isoprenoid synthesis rather than cholesterol biosynthesis, as both mevalonate and GGPP were able to prevent the effects of simvastatin, whereas zaragozic acid had no effect (figures 5.3.20 and 5.3.21), this makes this scenario unlikely.

Interestingly, in these cells 50 μM mevalonate and 10 μM GGPP did not completely prevent the effects of simvastatin on C5aR expression, whereas in the $U937_{PM}$ cell line these concentrations were able to completely prevent the effects. The concentration of mevalonate and GGPP used in other *in vitro* experiments involving statins is commonly 10 times the

concentration of statin, however this concentration may depend on how metabolically active the cells being studies are. For example the $U937_{C5aR}$ cells grow significantly quicker in routine culture compared with the $U937_{PM}$ cell line and therefore may need higher concentration of mevalonate or GGPP to prevent the effects of the statin. It would therefore be interesting to see if adding back a higher concentration of mevalonate or GGPP would completely prevent the effects of simvastatin on the C5aR expression in the $U937_{C5aR}$ cells. Interestingly the rate of cell proliferation was unaltered by incubation with 1 μM simvastatin in the $U937_{C5aR}$ cells unlike the $U937_{PM}$ cells which showed a slight reduction metabolic rate (figures 5.3.19 and 5.3.7).

As cell lines have their limitations, PBMCs were also incubated with simvastatin and other lipid lowering drugs and their effects on C5aR expression were monitored by flow cytometry. Results showed that incubation with simvastatin or lovastatin for 24 hrs had no effect on basal C5aR expression in CD14 positive cells (figures 5.3.22 and 5.3.23). These results were particularly disappointing as one of our hypothesis was that statins may exert anti-inflammatory effects via down regulation of the C5aR, as they have been previously shown to do for the chemokine receptors CCR2 and CCR5. However it was shown that incubation with fenofibrate reduced C5aR and CD14 expression in human primary monocyte (figure 5.3.25). This result is particularly interesting as fenofibrates can lower plasma triglycerides by increasing hepatic fatty acid uptake, reducing hepatic triglyceride biosynthesis and increasing clearance of VLDL. As previously described in sections 1.3.6 and 5.1.2.2, the mechanism in which fenofibrates achieve this is via activation of a family of nuclear hormone receptors called PPARs, who can in turn regulate the transcriptional activity of genes whose proteins encode enzymes in fatty acid metabolism such as fatty acid synthase and acetyl-CoA caroxylase (Staels et al., 1998). Although statins have also been demonstrated to activate PPARα and PPARγ which results in reduced IL-1β and TNFα expression (Inoue et al., 2000, Zhou and Liao, 2010), this result suggests that the C5aR expression may be regulated by fenofibrate via activation of PPARs through a mechanism that was not investigated in the $U937_{PM}$ and $U937_{C5aR}$ cell lines. Although these experiments use primary monocytes isolated from buffy coat from healthy individuals donating to the Welsh Blood Service they still have limitations as the plasma cholesterol levels are unknown in the donors. Ideally future experiments would involve measuring C5aR expression on monocytes isolated from patients with hypercholesterolemia and control volunteers, followed by lipid lowering drug therapy and then re-measuring C5aR expression once plasma cholesterol levels had been lowered.

As it was shown that simvastatin reduced the Bt_2cAMP induced C5aR expression in the $U937_{PM}$ cell line, it would be interesting to see if simvastatin would have any effect on induced expression in primary monocytes using a more physiologically relevant stimulus,

such as oxLDL, which is abundant within the atherosclerotic plaques. As previously discussed in Chapter 4 several physiological stimuli have been reported to increase C5aR expression, such as TNFα, IL-1 IL-6 and IFNγ, unfortunately none of these stimuli had any effect on C5aR expression in the $U937_{PM}$ cell line, however it was not investigated whether they can induce C5aR expression in primary monocytes. It would also be interesting to investigate effects of statins or fenofibrate on C5aR expression in animal models which have shown altered receptor expression, such as the increased C5aR on infiltrating neutrophils following I/R injury (Arumugam et al., 2003, Proctor et al., 2004).

5.4.1 Summary

This chapter has found that incubation with simvastatin prevented the Bt_2cAMP differentiation and up regulation of the C5aR in the $U937_{PM}$ cell line and this effect was mediated by inhibition of isoprenoid biosynthesis. However no effect could be detected on basal levels of C5aR expression. Investigations using the -2Kbp promoter reporter construct for the C5aR gene found that there was no *cis*-acting element within this region which was regulated by simvastatin. It was also shown that simvastatin and lovastatin had no effect on C5aR expression on primary monocytes *in vitro*, although fenofibrate significantly reduced it.

Chapter 6

The effects of statins on C5aR function

Chapter 6

The effects of statins on C5aR function

6.1 Introduction

As previously described in Chapter 1 section 1.3, cholesterol is a crucial component of biological membranes. As well as being essential for maintaining membrane fluidity, cholesterol also has an important role in the formation of distinct cholesterol enriched microdomains, termed lipid rafts (Burger et al., 2000, Maxfield and Tabas, 2005). As GPCRs are commonly found localised in these cholesterol enriched lipid rafts or move into such regions following stimulation with their ligand (see table 1.4, Chapter 1); their function and expression can often be regulated by membrane cholesterol.

Chapter 5 has already investigated whether statins can regulate expression of the C5aR in the human monocytic cell line, as well as primary monocytes isolated from human peripheral blood. Chapter 5 has shown that incubation with simvastatin inhibited the Bt_2cAMP induced expression of the C5aR in $U937_{PM}$ cells, and reduced C5aR expression in the stably transfected $U937_{C5aR}$ cell line. Further investigation of C5aR expression in primary monocytes found that *ex-vivo* statins did not alter C5aR expression in these cells. This chapter will investigate the effects of simvastatin on the biological functioning of the C5aR in PBMCs, as well as the monocytic cell lines $U937_{PM}$ and $U937_{C5aR}$.

6.1.1 Could statins have a cholesterol dependent anti-inflammatory effect on the function of the C5aR

As previously described in Chapter 1, section 1.3, membrane cholesterol can regulate several GPCRs functions by; altering sub-membrane compartmentalisation into lipid rapid rafts; altering the structural properties of GPCRs or their signalling components; and regulating internalisation and intracellular trafficking of the receptors and necessary components. Summarised below are the mechanisms by which statins could potentially regulate C5aR function by regulating membrane cholesterol content.

The sub-compartmentalisation of signalling components into lipid raft or non-lipid raft regions of the membrane can act as an important regulatory mechanism for the signalling cascade (Lingwood and Simons, 2010). While cholesterol plays an important role in this compartmentalisation, relatively few studies have actually investigated what effect statins may have on the enrichment of receptors or other signalling components within these regions upon stimulation. Recently atorvastatin has been shown to impair the selective recruitment of TLR4 and IL-2 receptor β chain into lipid raft regions following stimulation with their

respective ligands (Chansrichavala et al., 2010, Goebel et al., 2005). It has therefore been suggested that one of the anti-inflammatory properties of statins is their ability to alter raft enrichment of signalling components following stimulation with their ligands (Goebel et al., 2005).

To date no data have been published to indicate whether the C5aR is located in a lipid raft region and what effect C5a binding has on its membrane localisation. As a large number of other GPCR have been shown to be localised into lipid raft regions and their location can vary depending on whether they have been stimulated by their ligand (see Chapter 1, table 1.4), it is quite possible that the C5aR could also reside in a lipid raft region or is recruited in or out of such regions following stimulation with C5a. In this case statins could for example exert their anti-inflammatory effect by altering the C5aR membrane enrichment, as is the case for TLR4 and IL-2 receptor β chain (Chansrichavala et al., 2010, Goebel et al., 2005).

As already discussed in section 1.3.1 polarisation of cell surface receptors and associated proteins towards the leading edge is a crucial feature in chemoattractant stimulated cells (Manes et al., 2001). For example, interaction of the FPR with its ligand causes receptor clustering and redistribution towards the leading edge, followed by chemotaxis towards the source. However depletion of membrane cholesterol using methyl-β-cyclodextrin (MβCD) prevents this clustering and redistribution of the fMLP receptor towards the leading edge (Xue et al., 2004, Bath et al., 1991, Dunzendorfer et al., 1997, Wolach et al., 1992). Furthermore a study by Dunzendorfer and co-workers showed that treatment of neutrophils and monocytes with the statin pravastatin significantly decreased chemotaxis triggered by fMLP (Dunzendorfer et al., 1997). However, to contradict these findings another study by Sitrin et al. has suggested that the fMLP receptor is not located in lipid rafts neither does it require intact rafts for its signalling capacity (Sitrin et al., 2006).

As previously described (section 1.3.1) evidence suggests that the C5aR is unlikely to form clusters at the membrane edge towards C5a. A study by Servant et al. found that the C5aR appeared to cluster at the plasma membrane towards the C5a source, however this was instead due to an increase in relative abundance of plasma membrane rather than preferential accumulation of the receptor at the leading edge (Servant et al., 1999). Despite this a study by Nagao and co-workers found that cholesterol loading of mouse macrophages J774A.1 cells with cholesterol MβCD led to a significant decrease in transmigration towards C5a suggesting that the chemotactic functioning of the receptor is sensitive to membrane cholesterol (Nagao et al., 2007). Although this study did not investigate whether cholesterol loading had any effect on the clustering/membrane compartmentalisation of the C5aR, instead it found that cholesterol loading reduced activation of Rho A, a GTP binding protein involved in downstream signalling of the C5aR (Nagao et al., 2007).

As well as affecting sub-membrane compartmentalisation, membrane cholesterol can also affect the structural properties of the GPCRs or their signalling components (previously described in Chapter 1, section 1.3.2). GPCRs whos function can be regulated in such a manner are located within the lipid raft regions or move in or out of such regions following interaction with their ligand (Burger et al., 2000). To date little is known about the membrane localisation of the C5aR. This chapter will investigate whether the C5aR is localised in lipid raft regions of the plasma membrane or moves in or out of such regions following stimulation with C5a. From these findings we will be able to speculate whether the structural properties of the receptor will be affected either directly or indirectly by cholesterol.

Regulation of membrane cholesterol can also effect GPCR internalisation and intracellular trafficking (described in Chapter 1 section 1.3.4). Several studies have reported that C5a-C5aR interaction causes the receptor to cluster into clathrin coated pits where it is then internalised in a β-arrestin, dynamin, and clathrin-dependent pathway (Gilbert et al., 2001, Licht et al., 2003, Braun et al., 2003, Suvorova et al., 2005, Huttenrauch et al., 2005). To date no published studies have directly investigated whether the C5aR is located within the lipid raft region of the plasma membrane or whether it moves in or out of such regions following interaction with its ligand. Despite this lipid raft dependent endocytosis is characterised by clathrin-independence, which would therefore suggest that the C5aR is unlikely to be internalised in a lipid raft dependent manner; however it has not been investigated whether C5aR internalisation is dependent on cholesterol (Lajoie and Nabi, 2010).

6.1.2 Could statins have a cholesterol independent anti-inflammatory effects on the functioning of the C5aR

Inhibition of the mevalonate pathway by statins does not exclusively inhibit cholesterol biosynthesis, but also reduces the synthesis of isoprenoid intermediates (see figure 1.8). These isoprenoid intermediates, FPP and GGPP, serve as important lipid attachments in the post-translational modification isoprenylation (Zhang and Casey, 1996). Proteins which undergo this modification include the γ subunit of heterotrimeric G-proteins, heme-a, small GTP-binding proteins such as the Rho family members, and nuclear lamins (Liao and Laufs, 2005). As small GTP-binding proteins serve as important signalling intermediates in a variety of signalling cascades, their inhibition by preventing isoprenylation has been identified as an important cholesterol independent effect of statins.

6.1.2.1 Isoprenylation of small GTP-binding proteins

GTP-binding proteins are involved in a variety of signalling pathways which regulate cell growth, proliferation, shape and motility, membrane trafficking and transcriptional control

(Van Aelst and D'Souza-Schorey, 1997, Mackay and Hall, 1998, Jasinska et al., 2007). They can exist in an active GTP-bound form where they are found associated to the plasma membrane or an inactive GDP-bound form present in the cytoplasm. Isoprenylation of the GTP-binding proteins allows the translocation of the inactive form to the plasma membrane where it can then become active (Liao and Laufs, 2005). Statins can therefore lead to the accumulation of inactive GTP-binding proteins within the cytoplasm. Summarised in Table 6.2 is the pleiotropic effects of statins caused by inhibition of isoprenylation of GTP-binding.

Stimulation of the C5aR with C5a results in the activation of Cdc42, Rac and Rho G GTP-binding proteins (see section 1.2.6 for full details). It is believed that chemotaxis of leukocytes towards C5a is in part mediated by the activation Cdc42, which in turn activates the MAP-kinase signalling pathway (Li et al., 2003, Tardif et al., 2003). It has also been suggested that activation of Rac may have a role in mediating leukocyte functions such as chemotaxis, phagocytosis and respiratory burst upon stimulation of C5a (Welch et al., 2002a). Condliffe et al. have also shown that Rho G knockout mice have an impaired respiratory burst in response to C5a (Condliffe et al., 2006). Furthermore the HL-60 cell line has shown an impaired respiratory burst in response to fMLP in the presence of mevastatin which was attributed to the inhibition of isoprenylation of small GTP binding proteins (Bokoch and Prossnitz, 1992). As the C5aR signalling cascade involves the activation of numerous GTP-binding proteins which are important for the biological functioning of the C5aR it is possible that inhibition of isoprenylation by statins may have a direct effect on the signalling capacity of these intermediate proteins however to date no studies have investigated this.

Table 6.2 Pleiotropic effects of statins by inhibition of isoprenylation of small GTP-binding proteins

GTP-binding protein	Pleiotropic effects
Rho A	• Improve endothelial function by increasing the stability of eNOS mRNA (Laufs et al., 1998, Laufs and Liao, 1998). • Reduces vascular smooth muscle cell proliferation by inhibition of the Rho/Rho-kinase signalling pathway (Yang et al., 2000, Laufs et al., 1999). • Reduces monocyte adherence to endothelial cells by reducing integrin expression *in vitro* (Kawakami et al., 2002, Hiraoka et al., 2004). • Reduces cardiac hypertrophy by reducing myocardial expression of atrial natriuretic factor, fetal gene, and myosin light chain-2, contractile element (Laufs et al., 2002). • Improves plaque stability by inhibiting Rho dependent expression of MMPs (Turner et al., 2005, Massaro et al., 2010).
Rac 1	• Reduces cardiac hypertrophy by inhibiting myocardial oxidative stress caused by Rac NADPH oxidase activity (Takemoto et al., 2001, Zhou and Liao, 2010, Nakagami et al., 2003). • Reduces cardiac hypertrophy by reducing myocardial expression of atrial natriuretic factor, fetal gene, and myosin light chain-2, contractile element (Laufs et al., 2002).
Ras	• Reduces oxidised LDL induced macrophage proliferation (Senokuchi et al., 2005).

6.1.3 Monitoring the biological functions of the C5aR

Stimulation of the C5aR with C5a can elicit a broad range of biological functions depending on which cell type is being stimulated (see section 1.2.1 for full details). As this chapter is investigating the effects of statins on the functioning of the C5aR in primary monocytes and the monocytic U937 cell lines, below will discuss in detail the experimental techniques that will be used to monitor the biological functions in these cells. This chapter will also investigate the C5a induced Ca^{2+} release which has already been described in 4.1.2.3.

6.1.3.2 F-actin assembly

C5a is a potent chemoattractant for leukocytes. Following stimulation of the C5aR, GTP-binding proteins, Rac 1 and Cdc42, become activated by the exchange of GDP to GTP (Welch et al., 2002b, Tardif et al., 2003). The activation of both these GTP-binding proteins results in the stimulation of scaffold proteins, SCAR/WAVE and WASP respectively, which relay the signal from Rac/Cdc42 to the actin nucleation machinery, Arp2/3 (Affolter and Weijer, 2005, Charest and Firtel, 2007, Bi and Zigmond, 1999). The Arp2/3 complex is crucial for actin polymerisation, which is responsible for the formation of membrane protrusion during cell migration (Insall and Machesky, 2009). The Arp2/3 complex binds to existing actin filaments and allows the nucleation and elongation of new actin filaments,

filamentous actin (F-actin), by the addition of monomeric soluble actin, G-actin (Insall and Machesky, 2009).

Chemotaxis can be measured directly using techniques such as the Boyden chamber, which is composed of two medium-filled compartments separated by a microporous membrane. The cells can then migrate from the upper chamber through the membrane to the lower chamber which contains the chemoattractant (Chen, 2005). Chemotaxis can also be measured in agar plate assay where the cells are placed in one groove in the agarose and the chemoattractant is then placed in another and the cells then migrate through the agarose towards the chemoattractant source (Laevsky and Knecht, 2001).

An alternative to measuring chemotaxis directly are studies which measure the formation of F-actin as an indication of chemotactic activity. The monocytic cell line U937 has previously been shown to increase F-actin assembly following stimulation with C5a using phalloidin binding, a bicyclic peptide isolated from poisonous mushrooms (Banks et al., 1988). Phalloidin has been conjugated to numerous Alexa Fluor dyes which allows its rapid and sensitive detection by Flow Cytometry.

6.1.3.3 MMP secretion

The excess secretion of matrix metalloproteinases (MMPs) during late stages of atherosclerosis can degrade the fibrous cap surrounding the lesion, leading to plaque rupture and clinical events such as myocardial infraction or ischaemic stroke (Newby, 2007). Stimulation of neutrophils and eosinophils with C5a has previously been shown to induce MMP secretion, mainly MMP-1 and MMP-9 (DiScipio et al., 2006, Takafuji et al., 2003). More recently C5a has also been shown to induce MMP-1 and MMP-9 in human monocyte derived macrophages (Speidl et al., 2011). Secretion of MMPs from these cells is necessary for the transmigration of the cells towards the source of C5a (DiScipio et al., 2006). It has been shown that the secretion and activation of the proMMP to active MMP is dependent on the respiratory burst as inhibition of NADPH oxidase and nitric oxide synthase, by DPI and L-NIL respectively, abrogated C5a-mediated chemotaxis through basement membranes (DiScipio et al., 2006).

There are numerous ways to investigate MMP secretion, however the simplest and most traditional technique is zymography (Heussen and Dowdle, 1980). For MMPs belonging to the gelatinase family, MMP-2 and MMP-9, SDS-PAGE is carried out using gelatin impregnated gel. Samples are run in the same way as a standard SDS-PAGE under Laemlli non heat denaturing and non-reducing conditions, which allows the separation of proteins based on their molecular weight. Following electrophoresis the gel is washed to remove remaining SDS which allows the proteins to refold. The incubation of the gel

overnight at 37°C in a calcium containing zymography buffer allows the enzymatic activity of the MMPs to digest the gelatin within the gel. Staining of the gel with Coomassie Brilliant Blue R then allows the visualisation of the bands. This technique also allows the quantification and identification of the proforms as the process of denaturation and renaturation following electrophoresis causes the activation of the proforms, however these will have a higher molecular weight than the secreted active forms (Heussen and Dowdle, 1980). Although ELISAs have also been developed to quantify and discriminate between different MMPs they are much more expensive than zymography (Zucker et al., 1992).

6.1.3.5 Cytokine secretion

C5a stimulation of leukocytes can cause an increased secretion of a number of pro-inflammatory cytokines including IL-1, IL-6, IL-8, TNFα and MCP-1 (Goodman et al., 1982, Okusawa et al., 1988, Scholz et al., 1990, Vecchiarelli et al., 1998, Ember et al., 1994, Izumi et al., 1997). Of particular interest to this project is the chemotactic cytokines, chemokines IL-8 and MCP-1, as both have been implicated in atherosclerosis (see section 1.3.1 for full details) (Apostolakis et al., 2009, Weber et al., 2004, Barlic and Murphy, 2007). The most common method for measuring cytokine secretion is ELISAs, as they are sensitive and fairly easy to perform.

6.1.4 Methods for determining membrane localisation and cholesterol dependency

Several techniques are commonly used to investigate the sub-membrane compartmentalisation and cholesterol dependency of membrane proteins. Traditionally techniques involve indirect methods, such as cold detergent or mechanical extraction of lipid rafts or cholesterol depletion by cyclodextrins. These techniques, however, have an undesired disruptive nature, which can in turn induce artefacts (Lingwood and Simons, 2010). Despite this they are still commonly used to suggest lateral heterogeneity of membrane proteins and a dependency of cholesterol (Coskun and Simons, 2010). Recent advances in microscopy and spectroscopy techniques, such as single particle tracking and fluorescence resonance energy transfer, are now offering a more dynamic and physiological relevant approach to visualising membrane proteins and lipids (reviewed by (Lingwood and Simons, 2010). However as this study will use detergent extraction and cyclodextrins (CD) to assess membrane localisation and function of cholesterol on C5aR, only these techniques will be discussed in more detail below.

6.1.4.1 Detergent extraction of lipid rafts

Although it was known in the 1970s that different membrane proteins had different resistance towards detergents, it wasn't until Brown and Rose found that newly synthesised GPI-anchored proteins are sorted into these detergent insoluble fractions that the important application of these sphingolipid-cholesterol enriched fractions was realised (Yu et al., 1973, Brown and Rose, 1992). Following selective solubilisation of cellular membranes with Triton X-100 at 4°C, sphingolipid-cholesterol rafts remain insoluble and sucrose gradient centrifugation allows the separation of denser detergent soluble fractions from the lighter detergent insoluble fractions. Both the temperature and the type of detergent used during the extraction can greatly influence the lipid composition of the fractions collected (Brown and Rose, 1992). Once fractions are collected they can then be probed for proteins of interest by performing a standard SDS-PAGE followed by transfer to nitrocellulose membrane and western blotting using antibodies raised against the protein of interest.

6.1.4.2 Cholesterol depletion by cyclodextrins

CD are water soluble cyclic oligosaccharides which can encapsulate various hydrophobic molecules via their hydrophobic cavity (Zidovetzki and Levitan, 2007). β-CDs are efficient in extracting membrane cholesterol and the degree of cholesterol depletion is dependent on the concentration, incubation time, temperature and the type of cells (Irie et al., 1992). Although cholesterol is removed faster from lipid raft regions, it is also important to note that they can also remove cholesterol from non-lipid raft regions albeit a little more slowly (Zidovetzki and Levitan, 2007). It is therefore possible to selectively remove cholesterol from lipid raft regions alone under the correct conditions. However, experiments that completely remove cholesterol are non-physiological and can only hint towards a functional effect as removing cholesterol may have pleiotropic effects (Maxfield and Tabas, 2005). As well as removing cholesterol from the membrane β-CDs can also donate cholesterol to the membrane and increase cholesterol levels. Experiments involving β-CD, however, need to be approached with caution as they have been shown to interact with membrane phospholipids and hydrophobic amino acids of membrane proteins (Zidovetzki and Levitan, 2007).

6.1.5 Hypothesis and aims

As previous studies have shown a role of cholesterol on GPCR functioning by either influencing the membrane compartmentalisation into lipid rafts, or the structural properties of the receptor or its internalisation (see above), our hypothesis is that cell activation after interaction of the C5aR with its ligand C5a is dependent on association of the receptor within cholesterol rich lipid rafts. Furthermore, it is hypothesised that statins may negatively

regulate the functioning of the C5aR either by reducing membrane cholesterol levels or by inhibiting the isoprenylation of small GTP-binding proteins, which are important in downstream signalling cascade.

The aims for this chapter are:

- To investigate whether statins have cholesterol dependent or independent effects on the biological functioning of the C5aR by using techniques as discussed above.
- To determine whether the C5aR is localised within a lipid raft region of the plasma membrane or is recruited in or out of such regions following stimulation with C5a.

6.2 Materials and methods

6.2.1 Reagents

Alexa Fluor 488 phalloidin (A12379) was purchased from Invitrogen. Commercial IL-8 ELISA kit (Biosource International, CHC1303) was from purchased from Invitrogen. MCP-1 ELISA kit was purchased from R&D systems. All other chemicals and reagents were as described in section 2.1.

6.2.2 Cell culture

Both $U937_{PM}$ and $U937_{C5aR}$ cell lines were maintained in complete RPMI media as previously described in section 2.3.1. Unless otherwise stated cells were seeded at 2.5 $x10^5$ cell/ml and incubated for 48 hrs with appropriate stimuli prior to analysis (section 2.3.5). PBMCs isolated as previously described in sections 2.3.2, were seeded at 1 $x10^6$ cell/ml for 24 hr with the appropriate stimuli (section 2.3.5).

6.2.3 Antibodies

Primary antibodies used were monoclonal anti-CD59 (Bric229, obtained from International Blood Group Reference Laboratory IBGRL, Elstree, UK) and polyclonal rabbit anti-C5aR (H-100 from SantaCruz) for western blotting of sucrose gradient fractions. Secondary antibodies were as described in section 2.1.3.

6.2.4 Ca^{2+} signalling

Intracellular Ca^{2+} was measured using Fura-2-AM as previously described in section 2.3.8. For samples which were treated with either MβCD or ChMβCD, prior to adding Fura-2-AM MβCD (40 mM made up fresh everyday in RPMI) or ChMβCD were added to the cells (1 x 10^7 cell/ml) at final concentration of 10 mM. Fura-2-AM was added as normal and the cells were incubated for 30 min at RT. After washing, the cells were resuspended at 1 $x10^7$ cell/ml in Krebs buffer as described in section 2.3.8.

6.2.5 Measuring F-actin assembly

Cells were harvested and resuspended at 1 x 10^7 cell/ml in PBS unless otherwise stated. 200 μl of cells were stimulated with 10 μl C5a (final concentration 5 nM) for 5 min at RT. The F-actin content was determined by addition of 200 μl of cell lysis buffer and staining solution (containing 0.33 μM phallodin-Al488, 70 μg/ml lysophosphatidylcholine (LPC) and 0.5 % paraformaldehyde). After 20 minute incubation at RT, the cells were centrifuged for 5 min at

2,000 rpm. The cells were washed with 500 µl of PBS and centrifuged again. The cells were then resuspended in 200 µl FACS buffer and then cell fluorescence was measured immediately using flow cytometry.

6.2.6 Gelatin zymography

Prior to gelatin zymography cells were washed and incubated o/n in RPMI media (containing 100 U/ml penicillin, 100 µg/ml streptomycin, 290 µg/ml L-glutamine and 1 mM sodium pyruvate) in the absence of FCS. Cells (2 x 10^5 cell/ml) were then incubated in the absence or presence of C5a (5 nM), for 24 hrs. The s/n was harvested by centrifugation for 3 min at 1,200 rpm and stored at -20 °C until analysis. The s/n was mixed with an equal volume of protein sample buffer and 20 µl of sample was loaded onto a 12.5 % SDS-PAGE containing 1 mg/ml gelatine (from bovine skin, Sigma). Following electrophoresis (as described in section 2.3.7), the gel was washed twice with 0.1 % Triton-X100 for approximately 30 min to remove the SDS. The gel was incubated in zymography buffer (containing 50 mM Tris, 10 mM $CaCl_2$, and 0.05 % Brij35 (v/v) in dH_2O, pH8.2) o/n at 37°C.

The gel was washed twice in dH_2O to remove detergent and then stained using coomassie brilliant blue. Once excess SDS had been removed, the gel was incubated for approximately 3 hrs with gentle agitation (Red Rocker, Hoefer) with coomassie staining solution (40 % (v/v) methanol, 10 % (v/v) acetic acid, 0.1 % (w/v) coomassie brilliant blue (CBB) in dH_2O.). The gel was destained (40 % (v/v) methanol, 10 % (v/v) acetic acid in dH_2O) until the correct contrast had been achieved. Clear bands on a blue background are an indication of gelatinase activity. For presentation purposes, the gels were inverted so bands appear dark on a light background. Densitometry of bands was performed using Bio-Rad GS-710 scanner and software. Results were normalised by calculating OD/mm^2 for each treatment, minus or plus C5a, as a percentage compared to the control minus C5a.

6.2.7 IL-8 and MCP-1 ELISA

IL-8 secretion from culture fluid s/n (as described in 6.2.2) was quantified using a commercial kit (Invitrogen) according to the manufacturer's instructions. 96 well ELISA microtitre plates were coated o/n at 4 °C with 50 µl/well anti-IL-8 antibody (58.130.09, 1 µg/ml diluted in PBS). For MCP-1 quantification, the 96 well ELISA microtitre plates were coated o/n at 4 °C with 50 µl/well of capture polyclonal anti-MCP-1 (R&D systems; MAB679, 1 µg/ml diluted in PBS). Wells were washed four times with 200 µl PBS/Tween20 (0.1 %) and blocked with 200 µl/well 0.5 % BSA in PBS for 2 hr at 37°C. The wells were washed four times with PBS/Tween20 (0.1%) and 50 µl/well of sample and standard was added. For the IL-8 ELISA recombinant human IL-8 standards (58.130.10, diluted in 0.5 % BSA in PBS), ranging from

12.5 to 800 pg/ml was used. For the MCP-1 ELISA, standards ranged from 10 pg/ml to 1 ng/ml, were diluted in 0.5 % BSA in PBS. The microtitre plate was incubated at RT for 1hr and was washed again with PBS/Tween20. For IL-8 ELISA, 50 µl of biotinylated anti-IL-8 antibody (58.130.03, 0.4 µg/ml in 0.5 % BSA in PBS) was added and incubated at RT for 1 hr. For MCP-1 ELISA 50 µl of 50 ng/ml biotinylated anti-MCP-1 antibody was added to each well. After washing the plate, 50 µl of streptavidin-HRPO (41.000.03, diluted 1 in 2500 in 0.5 % BSA in PBS) was added to each well and incubated for 30 min at RT. Wells were again washed four times with PBS/Tween20 after which 50 µl of substrate was added (OPD kit Dako; 1 tablet plus dH_2O with 5 µl H_2O_2). Reaction was stopped after 2-4 min with 50 µl of 1 M H_2SO_4 and the absorbance of samples was read at 492 nm.

6.2.8 Sucrose gradient floatation

To determine the subcellular localisation of the C5aR, $U937_{C5aR}$ cells (7 x 10^6 cell/ml) were harvested and mixed with either $U937_{CD59\text{-}GPI}$ or $U937_{CD59\text{-}TM}$ (3 x 10^3 cell/ml). The $U937_{CD59\text{-}GPI}$ cells have been previously transfected with the GPI anchored CD59 which is present within lipid raft regions of the plasma membrane (Powell et al., 1997). Conversely the $U937_{CD59\text{-}TM}$ cells have been previously transfected with the CD59 chimera with the transmembrane region of MCP which is localised within non-lipid raft regions of the plasma membrane (Van Den Berg et al., 2002). Therefore these cells can allow the plasma membrane localisation of the C5aR to be determined by monitoring its co-localisation with either $U937_{CD59\text{-}GPI}$, a marker for lipid rafts, or $U937_{CD59\text{-}TM}$, a marker for non-lipid raft regions.

Sucrose gradient floatation was performed as previously described (Hiscox et al., 2002). Either $U937_{PM}$ differentiated with Bt_2cAMP or $U937_{C5aR}$ cell were cultured as described in section 6.2.2. Once harvested the cells were washed twice in PBS and resuspended in ice-cold sucrose gradient cell lysis buffer (20 mM Tris, 2 mM EDTA, 140 mM NaCl, pH 8.2) containing 40 % sucrose and 1 % Brij58. The lysate (1 ml) was loaded on the bottom of a 5 ml ultra-clear centrifuge tube. The lysis buffer (3.5 ml) containing 30 % sucrose and 1 % Brij58 was overlaid followed by 0.8 ml 1 % Brij58 in lysis buffer. Lysates were spun in a Beckmann SW55Ti swing-out rotor for 16 hr at 200,000g, 4°C. Fractions (0.5 ml) were collected starting from the top of the gradient. Samples were then separated on a 12.5 % SDS-PAGE gel under non-reducing conditions, as previously described in section 2.3.7. Western blotting of nitrocellulose membrane was performed using anti-CD59 (Bric229) and anti-C5aR (H-100), as previously described in section 2.3.7.2.

6.2.9 Propidium iodide exclusion assay

To monitor changes in cell viability propidium iodide cell exclusion assays were performed as previous described in section 2.3.9.

6.3 Results

The aims of this chapter were to investigate whether statins had any cholesterol dependent or independent effects on functioning of the C5aR and to determine whether C5aR is localised within a lipid raft region of the plasma membrane or is recruited in or out of such regions following stimulation with C5a. C5aR function was monitored in the $U937_{PM}$ cell line differentiated with Bt_2cAMP, the $U937_{C5aR}$ cell line, which has been previously transfected with the C5aR and therefore do not need stimulating to induce receptor expression, and in human PBMCs. The effects of lipid altering stimuli on the biological functions of the C5aR were investigated by detecting changes in C5a induced Ca^{2+} release, F-actin assembly, MMP production and cytokine production (IL-8 and MCP-1).

6.3.1. C5a induced Ca^{2+} release

Binding of C5a to the C5aR causes a rise in intracellular Ca^{2+} concentration via the activation of $PLC\beta_2$ (Jiang et al 1996). Intracellular Ca^{2+} concentrations following stimulation with C5a can easily be measured using the ratiometric Ca^{2+} indicator Fura-2-AM. Following incubation with simvastatin, cells were loaded with Fura-2-AM and the effects of simvastatin on C5a induced Ca^{2+} release was measured using the FluorStar plate reader.

6.3.1.1 Incubation of undifferentiated $U937_{PM}$ with 1 μM simvastatin reduces C5a induced Ca^{2+} release

The effects of simvastatin on C5a induced Ca^{2+} release was first investigated in undifferentiated $U937_{PM}$ cells. Although these cells had very little C5aR expression, as detected by FACS (Chapter 5, section 5.3.1), the cells still released some Ca^{2+} following stimulation with 5 nM C5a (figure 6.3.1). This concentration of C5a was chosen as it was shown in Chapter 4 to induce an intracellular Ca^{2+} release; furthermore this is the highest physiologically achievable C5a concentration (Guo and Ward, 2006). Figure 6.3.1 also shows that incubation with 1 μM simvastatin significantly reduced the C5a induced Ca^{2+} release when compared with the control. However treatment with MβCD, a cholesterol chelator, had no effect on C5a induced Ca^{2+} release which suggests that the effect of 1 μM simvastatin is unlikely to be due to its cholesterol lowering ability (figure 6.3.1). Loading the cells with cholesterol, using ChMβCD prior to Ca^{2+} signalling experiments, showed a significant decrease in C5a induced Ca^{2+} release when compared with the control. However, as shown in figure 6.3.2, incubation with ChMβCD caused a significant increase in cell death which suggests that the decrease in C5a induced Ca^{2+} could be due to its cytotoxicity rather than cholesterol loading (figures 6.3.1 and 6.3.2). Adding back both mevalonate and GGPP

restored the C5a induced Ca^{2+} release which was reduced by incubation with 1uM simvastatin, however, this was not statistically significant, p = 0.0551 (figure 6.3.3).

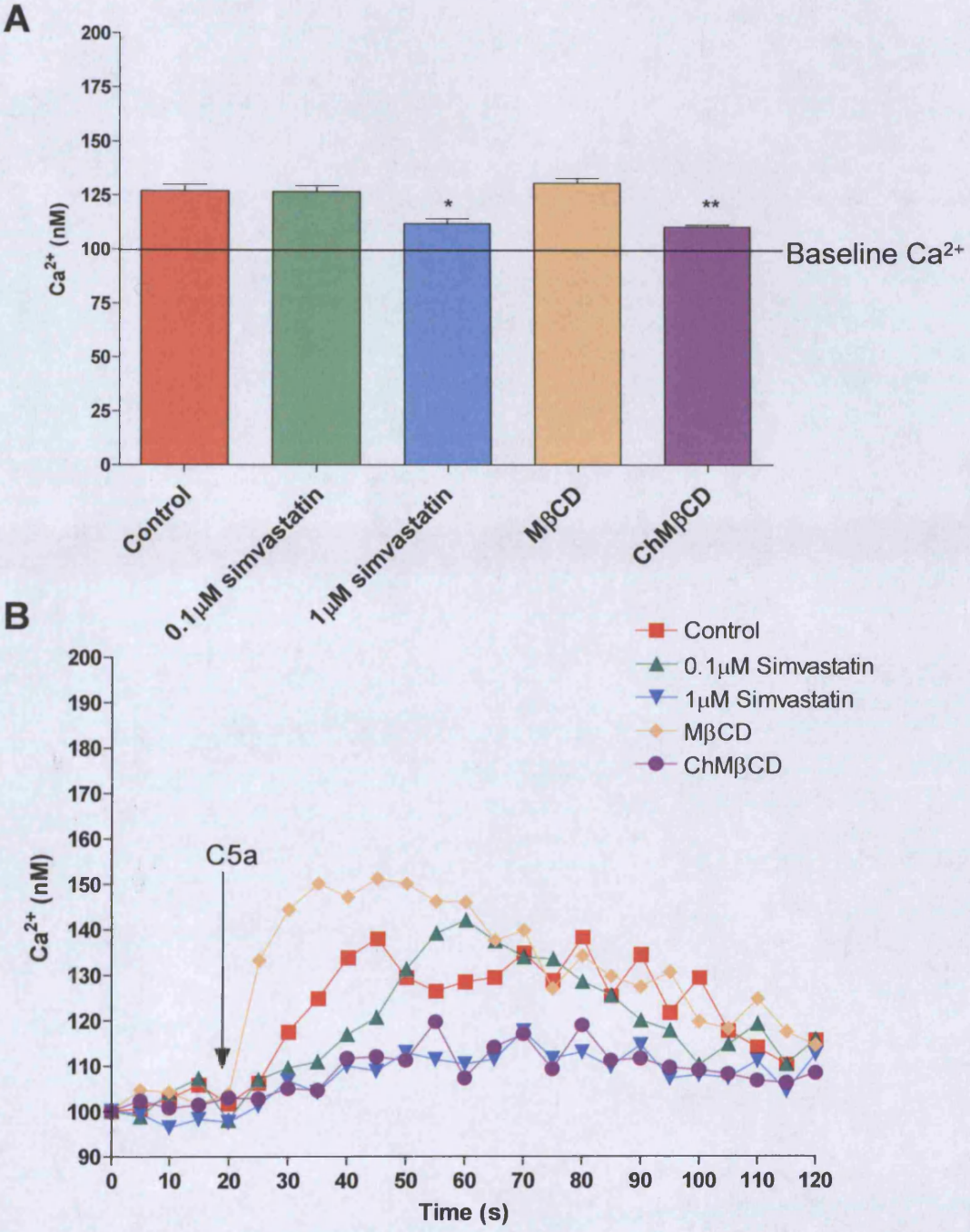


Figure 6.3.1 Incubation with 1 μM simvastatin reduces C5a induced Ca^{2+} release in the $U937_{PM}$ cell line. Cells were incubated for 2 days in the absence or presence of simvastatin. Cells were harvested and loaded with Fura-2-AM. As a control for cholesterol depletion cells were incubated with 10 mM MβCD, cholesterol chelator during Fura-2-AM loading or loaded with excess cholesterol using 10 mM ChMβCD. (A) Average maximum Ca^{2+} release following stimulation with 5 nM C5a from 3 separate experiments carried out in duplicate. One-way ANOVA followed by Dunnett's multiple comparison test compared significance to the control *p <0.05, **p <0.01. (B) Ca^{2+} release trace following the addition of 5 nM C5a, as indicated by arrow, from one representative experiment.

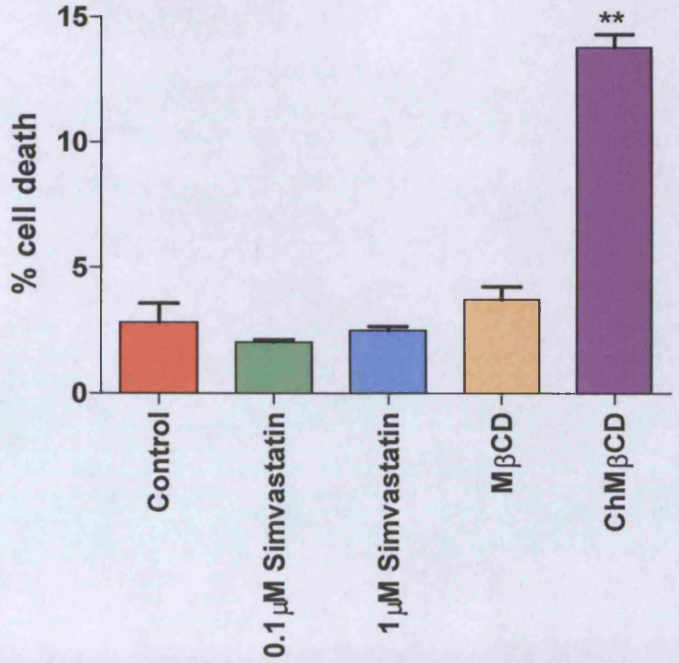


Figure 6.3.2 Cholesterol loading with ChMβCD significantly decreased cell viability in $U937_{PM}$ cell line. Cells were incubated either with simvastatin for 2 days or with 10 mM MβCD or 10 mM ChMβCD for 30 min at RT to staining with 0.5 μg/ml of propidium iodide. Percentage viability was then determined by flow cytometry. Shown is average of 3 experiments +/- SEM. One-way ANOVA followed by Dunnett's multiple comparison test compared significance to the control **= $p < 0.01$.

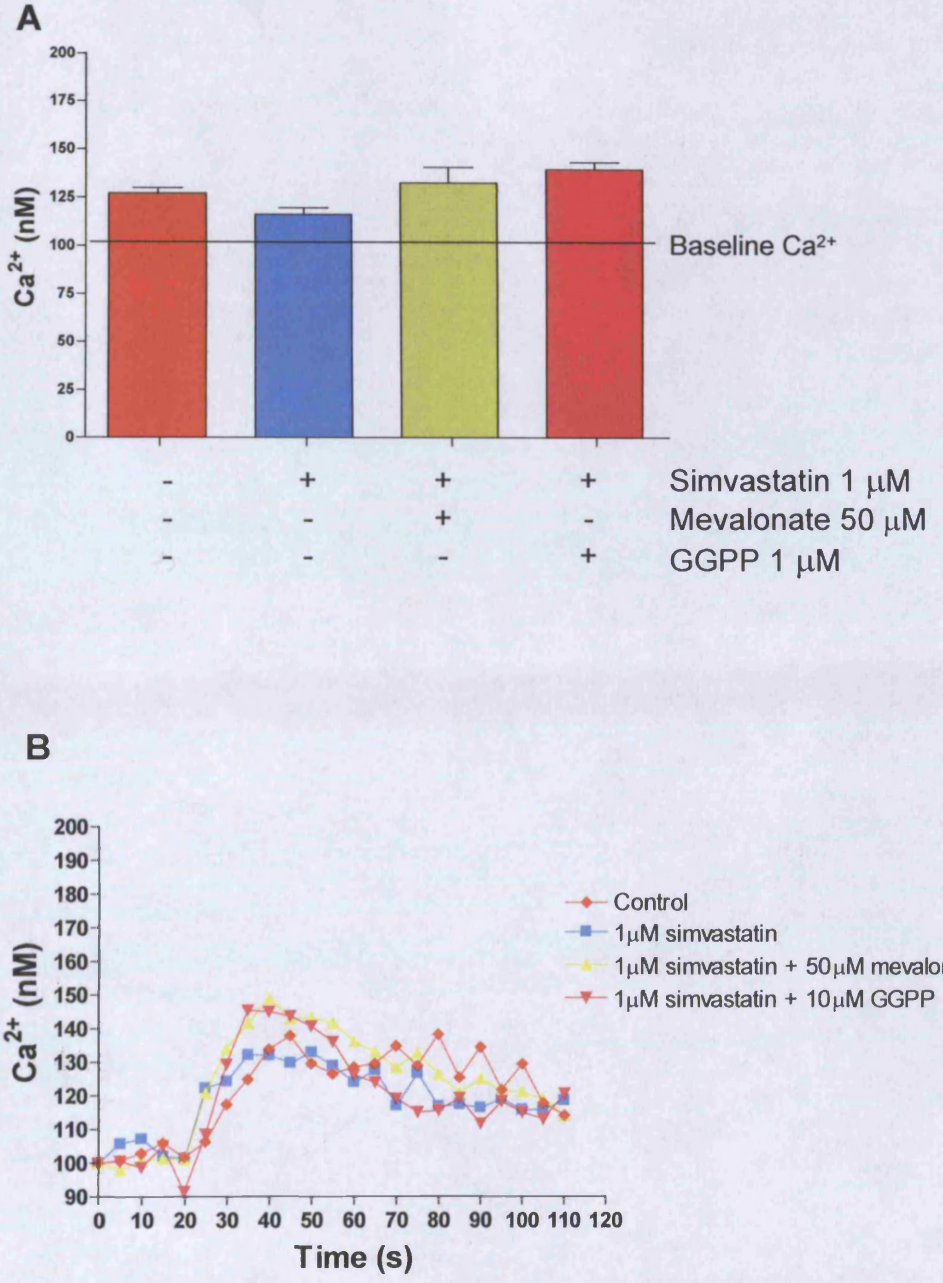


Figure 6.3.3 Effects of adding back mevalonate and GGPP on C5a induced Ca2+ release. $U937_{PM}$ cells were incubated for 2 days in the absence or presence of 1uM simvastatin plus 50uM mevalonate or 10uM GGPP. Cells were harvested and loaded with Fura-2-AM. (A) Average maximum Ca^{2+} release following stimulation with 5 nM C5a from 3 separate experiments. One-way ANOVA showed no significant difference ($p = 0.0551$). (B) Ca^{2+} release trace following the addition of 5 nM C5a, as indicated by arrow, from one representative experiment.

6.3.1.2 Incubation of Bt_2cAMP differentiated $U937_{PM}$ with 1 μM simvastatin reduces C5a induced Ca^{2+} release

As undifferentiated $U937_{PM}$ expressed very little C5aR and the C5a induced Ca^{2+} release was quite small (see Chapter 4 for details), the cells were differentiated with Bt_2cAMP to induce C5aR expression for 2 days in the absence or presence of simvastatin and the C5a induced Ca^{2+} release was assessed using Fura-2-AM.

As previously shown in section 4.3.1 incubation of $U937_{PM}$ with Bt_2cAMP significantly increased C5a induced Ca^{2+} release when compared with the control. Incubation of $U937_{PM}$ with 0.5 mM Bt_2cAMP and 0.1 μM simvastatin had no effect on C5a induced Ca^{2+} release (figure 6.3.4). However, incubation with 0.5 mM Bt_2cAMP and 1 μM simvastatin caused a significant decrease in C5a induced Ca^{2+} release in the $U937_{PM}$ cell line (figure 6.3.4). Cholesterol loading with ChMβCD also significantly reduced C5a induced Ca^{2+} release of Bt_2cAMP treated $U937_{PM}$ cells, however, this is likely due to its cytotoxicity (figures 6.3.4 and 6.3.2). Figure 6.3.4 also shows that incubation of Bt_2cAMP treated $U937_{PM}$ cells with MβCD during Fura-2-AM loading showed a slight decrease in C5a induced Ca^{2+} release although this was not significantly different from the Bt_2cAMP alone.

To further investigate the effect of 1 μM simvastatin on C5a induced Ca^{2+} release in Bt_2cAMP differentiated $U937_{PM}$, these experiments were performed in the presence of either 50 μM mevalonate or 10 μM GGPP. The addition of mevalonate, but not GGPP, prevented the reduction of 1 μM simvastatin on the C5a induced Ca^{2+} release (figure 6.3.5). This would suggest that the effects seen here by simvastatin are due to inhibition of cholesterol synthesis rather than isoprenoid synthesis, which is opposite to the results seen when looking at changes in Bt_2cAMP induced C5aR expression. To investigate if mevalonate or GGPP had an effect on C5a induced Ca^{2+} release, $U937_{PM}$ cells were incubated with Bt_2cAMP and either 50 μM mevalonate or 10 μM GGPP and changes in Ca^{2+} release were measured. Figure 6.3.6 shows that neither mevalonate nor GGPP had an effect on C5a induced Ca^{2+} release when co-incubated with Bt_2cAMP.

To confirm that the effects of 1 μM simvastatin were due to its inhibition of cholesterol synthesis rather than isoprenoid synthesis, $U937_{PM}$ cells were incubated with 0.5mM Bt_2cAMP and increasing concentrations of zaragozic acid, the squalene synthase inhibitor (see figure 1.8). Figure 6.3.7 shows that zaragozic acid had no effect on C5a induced Ca^{2+} release in Bt_2cAMP differentiated $U937_{PM}$ cells. This suggest that cholesterol synthesis plays no role in the C5a induced Ca^{2+} release, which contradicts the results observed by adding back mevalonate and GGPP as described above.

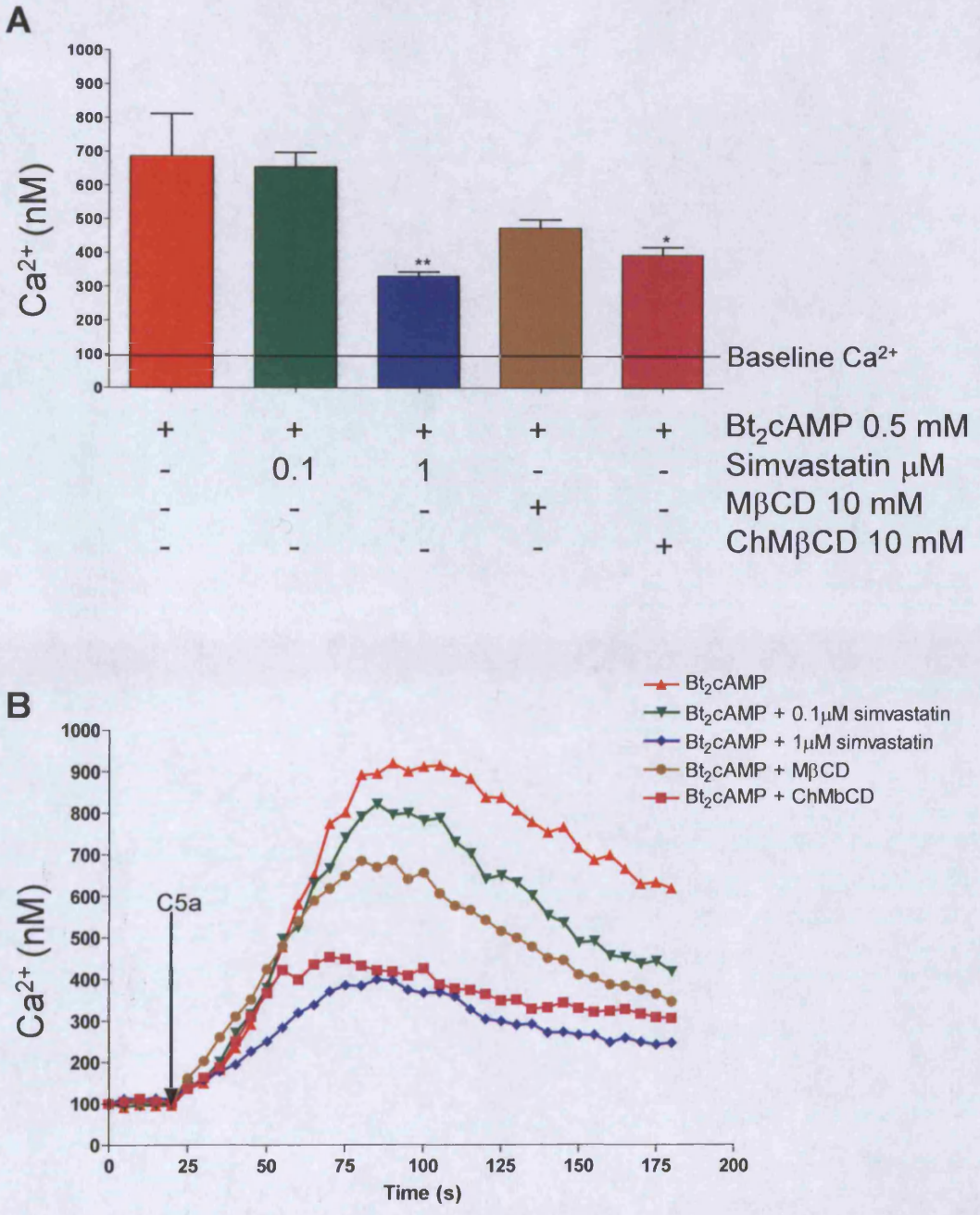


Figure 6.3.4 Incubation of $U937_{PM}$ with Bt_2cAMP plus 1 μM simvastatin significantly decreases C5a induced Ca^{2+} release in $U937_{PM}$ cell line. Cells were incubated for 2 days with 0.5 mM Bt_2cAMP in the absence or presence of simvastatin. Cells were harvested and loaded with Fura-2-AM. As a control for cholesterol depletion cells were incubated with MβCD, cholesterol chelator, during Fura-2-AM loading or loaded with excess cholesterol using ChMβCD. (A) Average maximum Ca^{2+} release following stimulation with 5 nM C5a from 3 separate experiments. One-way ANOVA followed by Dunnett's multiple comparison test compared significance to the Bt_2cAMP only treated cells *p <0.05, **p <0.01. (B) Ca^{2+} release trace following the addition of 5 nM C5a, as indicated by arrow, from one representative experiment.

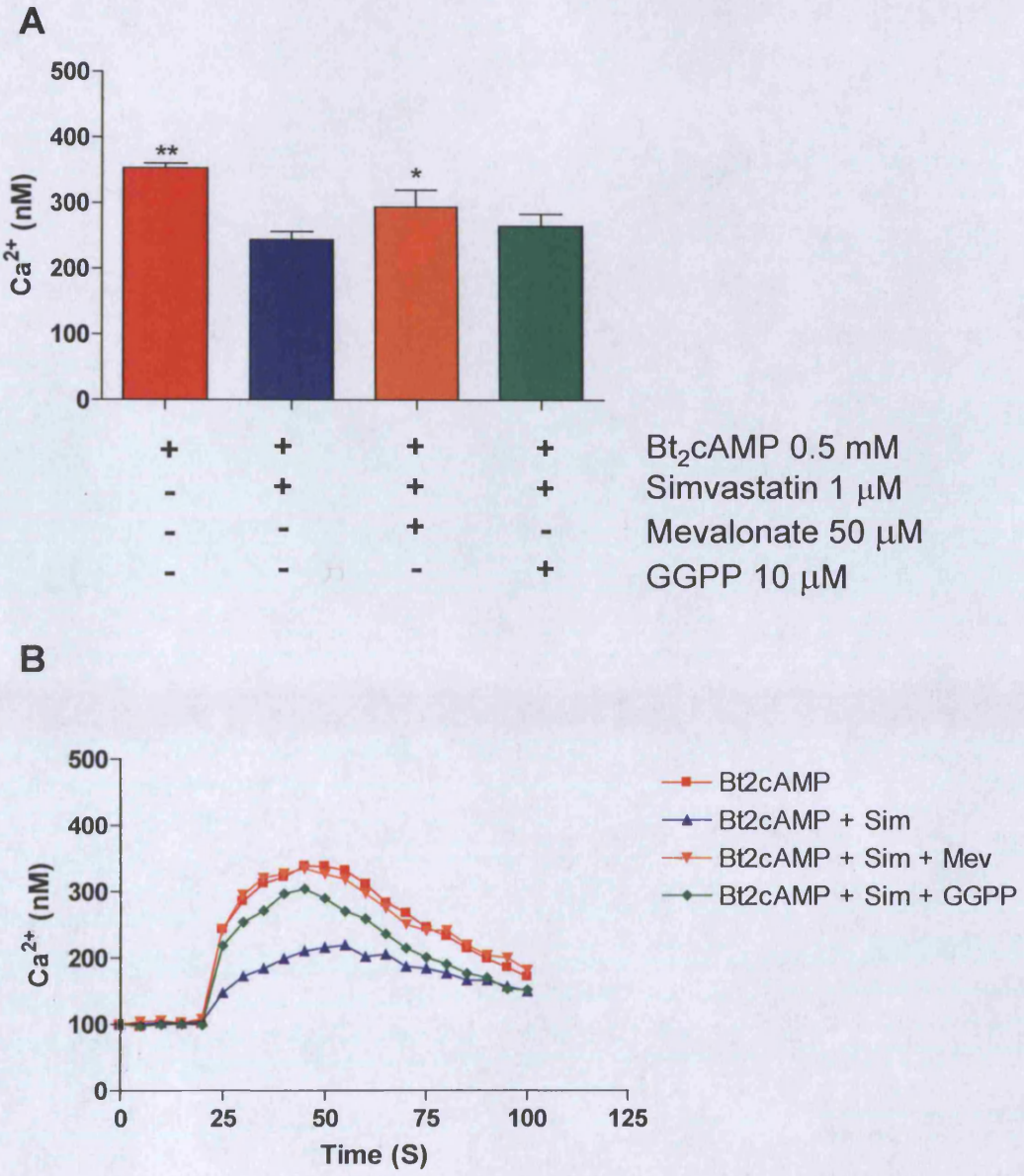


Figure 6.3.5 Adding back mevalonate but not GGPP restores C5a induced Ca^{2+} release in 0.5mM Bt_2cAMP plus 1 μM simvastatin differentiated $U937_{PM}$ cells. Cells were incubated for 2 days with 0.5 mM Bt_2cAMP plus 1 μM simvastatin in the absence or presence of either 50 μM mevalonate or 10 μM GGPP. Cells were harvested and loaded with Fura-2-AM. (A) Average maximum Ca^{2+} release following stimulation with 5 nM C5a from 3 separate experiments. One-way ANOVA followed by Dunnett's multiple comparison test compared significance to the Bt_2cAMP plus simvastatin treated cells *p <0.05, **p <0.01. (B) Ca^{2+} release trace following the addition of 5 nM C5a, as indicated by arrow, from one representative experiment.

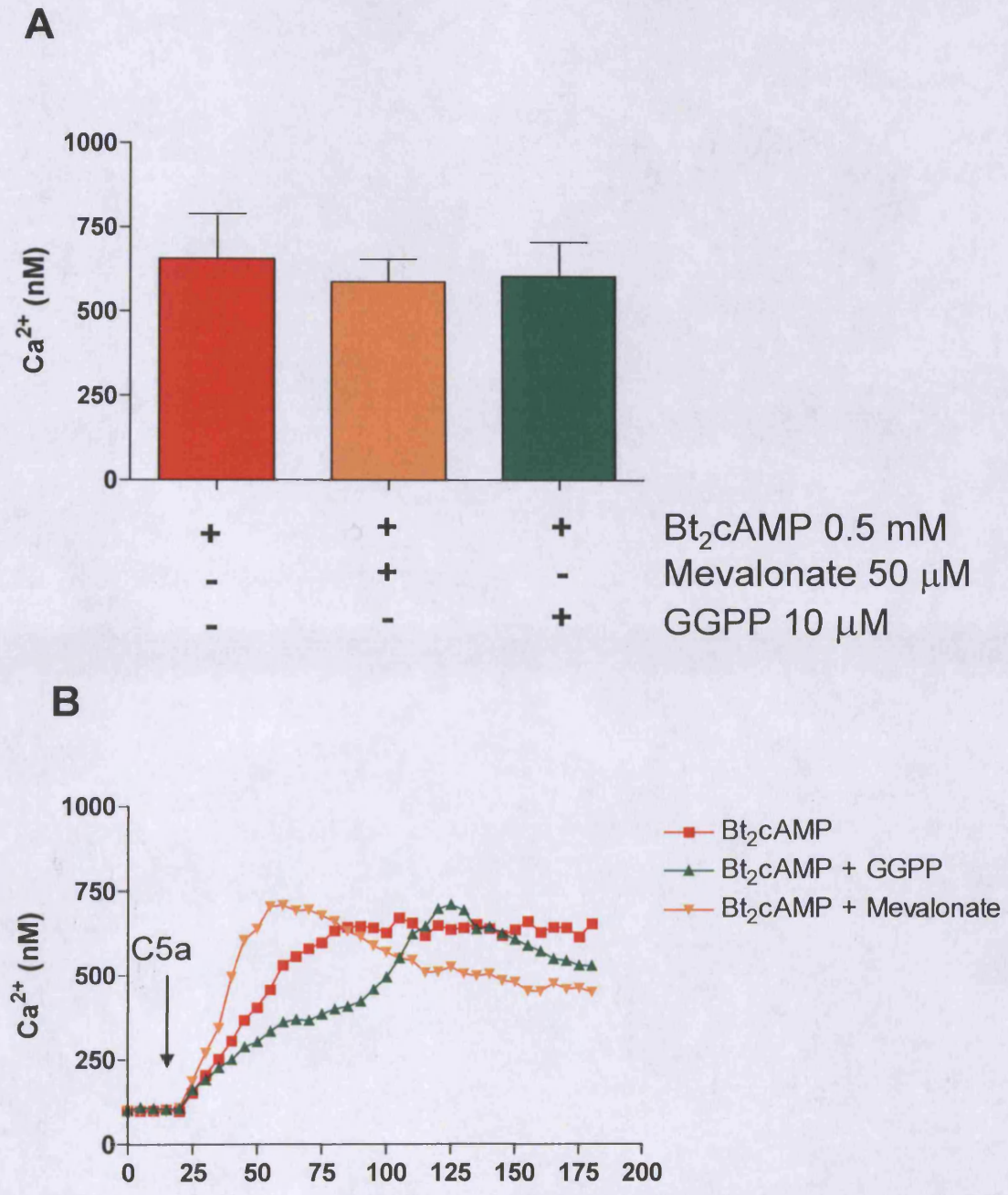
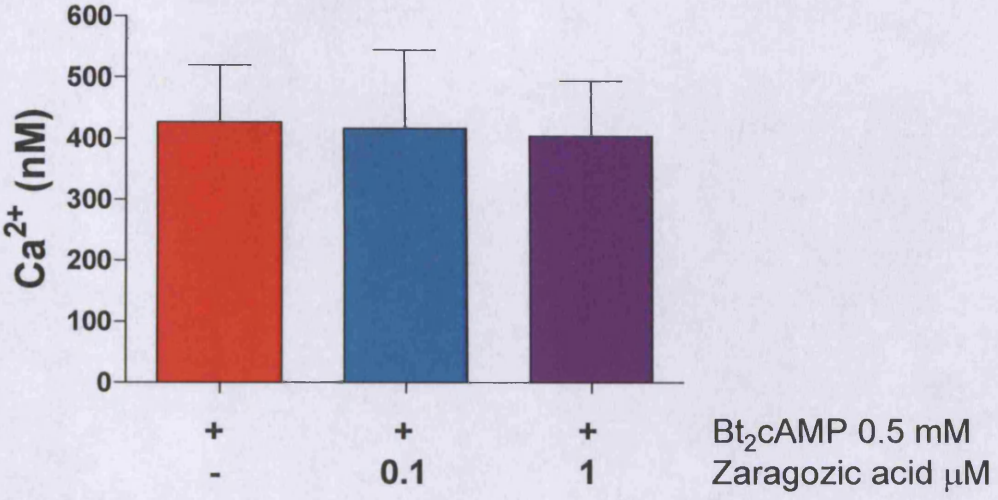


Figure 6.3.6 Incubation with mevalonate or GGPP in the absence of simvastatin had no effect on C5a induced Ca^{2+} release in 0.5mM Bt_2cAMP differentiated $U937_{PM}$ cells. Cells were incubated for 2 days with 0.5 mM Bt_2cAMP in the absence or presence of either 50 μM mevalonate or 10 μM GGPP. Cells were harvested and loaded with Fura-2-AM. (A) Average maximum Ca^{2+} release following stimulation with 5 nM C5a from 3 separate experiments. (B) Ca^{2+} release trace following the addition of 5 nM C5a, as indicated by arrow, from one representative experiment.



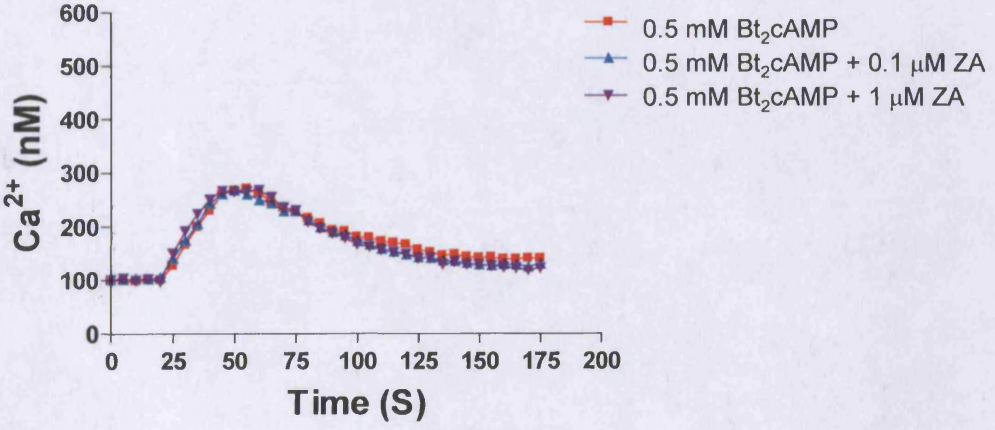


Figure 6.3.7 Zaragozic acid has no effect on C5a induced Ca^{2+} release in 0.5mM Bt_2cAMP differentiated $U937_{PM}$ cells. Cells were incubated for 2 days with 0.5 mM Bt_2cAMP in the absence or presence of zaragozic acid. Cells were harvested and loaded with Fura-2-AM. (A) Average maximum Ca^{2+} release following stimulation with 5 nM C5a from 3 separate experiments. (B) Ca^{2+} release trace following the addition of 5 nM C5a, as indicated by arrow, from one representative experiment.

6.3.1.3 Simvastatin has no effect on C5a induced Ca^{2+} release in $U937_{C5aR}$ cell line

To investigate whether simvastatin had any effect on C5a induced Ca^{2+} release in the $U937_{C5aR}$ once harvested, these cells were incubated with 1 μM Fura-2-AM and the C5a induced Ca^{2+} release was monitored. Results in figure 6.3.8 show that incubation with increasing concentration of simvastatin had no significant effect on the C5a induced Ca^{2+} release in this $U937_{C5aR}$ cell line (figure 6.3.8). At 50 nM C5a there was a significant difference between the control cells and cells incubated with the cholesterol chelator, MβCD (figure 6.3.8A).

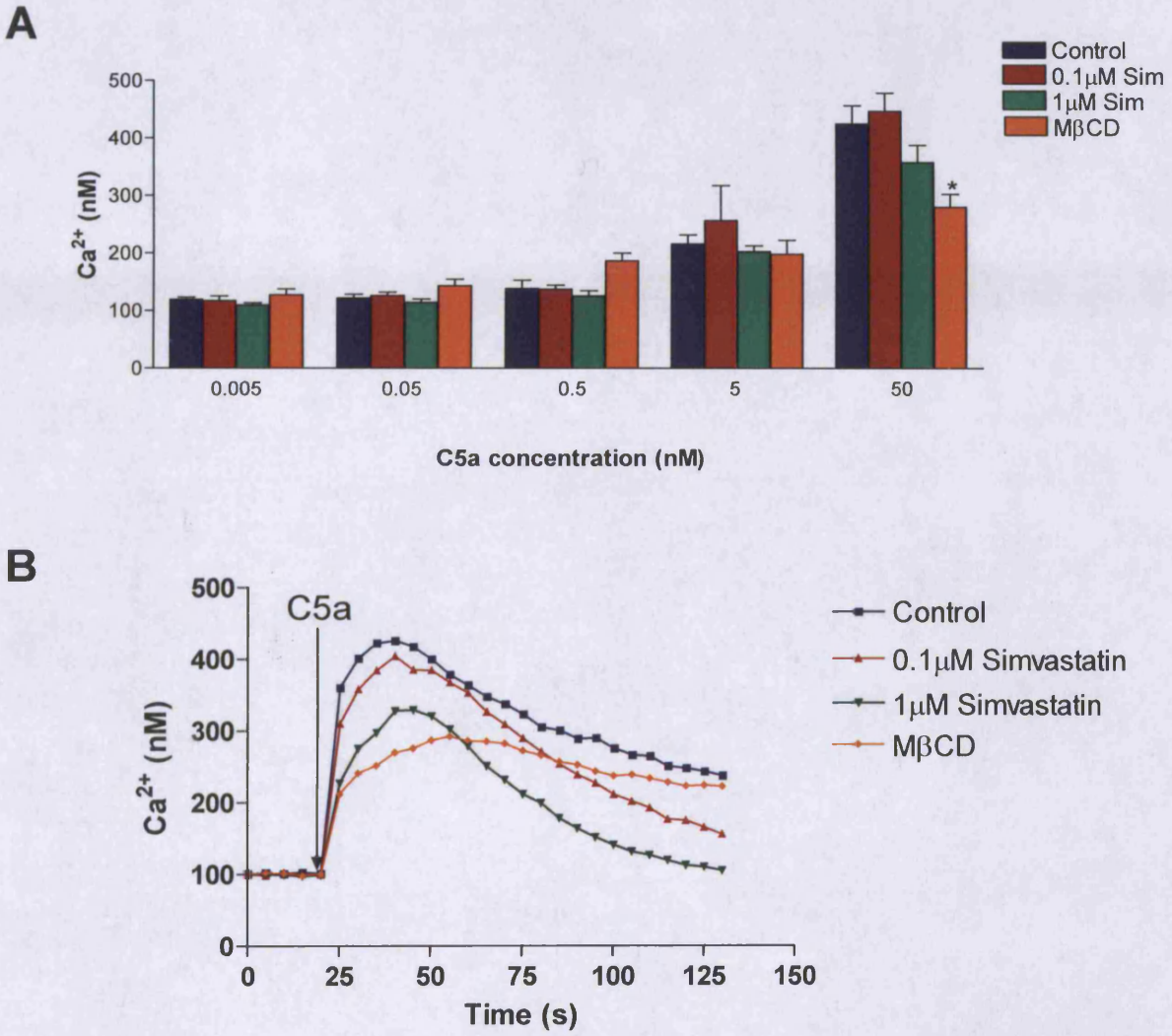


Figure 6.3.8 Simvastatin has no effect on C5a induced Ca^{2+} release in the $U937_{C5aR}$ cell line. Cells were incubated for 2 days in the absence or presence of simvastatin. Cells were harvested and loaded with 1 μM Fura-2-AM. As a control for cholesterol depletion cells were incubated with 10 mM MβCD during Fura-2-AM incubation. (A) Maximum Ca^{2+} following stimulation with various concentrations of C5a. Results expressed as mean Ca^{2+} release from 4 separate experiments. One-way ANOVA followed by Dunnett's multiple comparison test compared significance from the control at each separate C5a concentration; *p <0.05. (B) Ca^{2+} release trace following the addition of 50 nM C5a, as indicated by the arrow, from one representative experiment.

6.3.2 C5a induces F-actin assembly in human neutrophils but not in $U937_{PM}$ or $U937_{C5aR}$ cells

As previously described, C5a is a potent chemoattractant for leukocytes. Filamentous actin (F-actin) plays a central role in cell motility by forming the meshed framework for the protruding lamellipodia towards a chemotactic source (Small et al., 1999). Using Phalloidin-488, which binds tightly to actin filaments, allows changes in F-actin assembly to be easily detected by flow cytometry (see section 6.1.3.2).

Bt_2cAMP differentiated $U937_{PM}$ have previously been shown to increase F-actin following the addition of C5a, (Banks et al., 1988, Monk and Banks, 1991). In our experiments, however, the $U937_{PM}$ cells were unresponsive to C5a while the freshly isolated human neutrophils exhibited a dramatic increase in their F-actin staining (figure 6.3.9). Similarly when $U937_{C5aR}$ cells were stimulated with C5a no changes were observed in the F-actin assembly (figure 6.3.10).

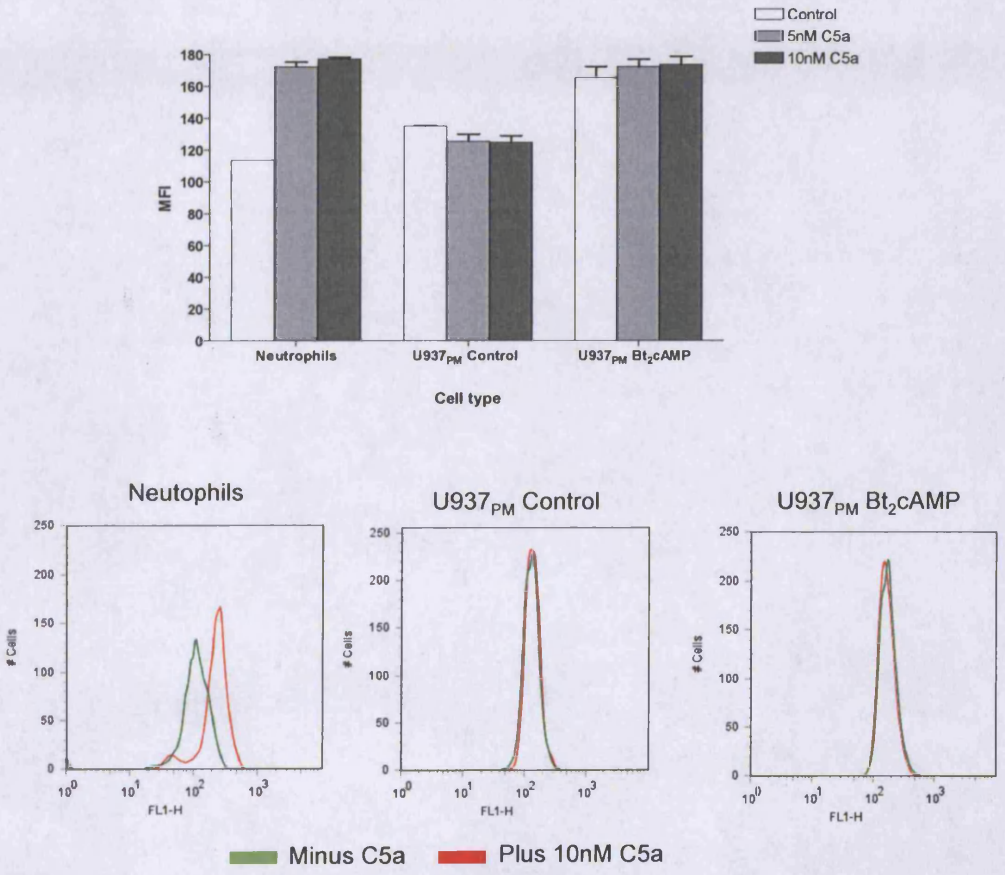


Figure 6.3.9 Flow cytometry staining of F-actin assembly following addition of C5a in presence of PBS. $U937_{PM}$ cells were incubated for 2 days in the absence or presence of 0.5 mM Bt_2cAMP. Cells were harvested and incubated in the absence or presence of C5a for 5 min. Following incubation with C5a the cells were incubated with F-actin staining solution, containing 0.33 μM phalloidin-Al488 and 70ug/ml LPC for 20 min. Cells were washed and F-actin assembly was detected by flow cytometry.

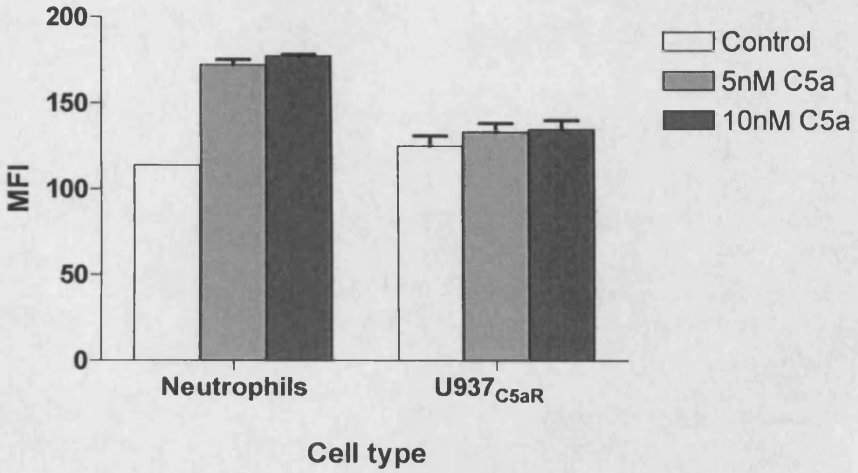


Figure 6.3.10 F-actin assembly in the U973$_{C5aR}$ cell line. U937$_{C5aR}$ cells and freshly isolated human neutrophils were incubated in the absence or presence or 5 nM C5a for 5 min. The cells were incubated with F-actin staining solution containing; 0.33 μM Phalloidin-Al488, 70 μg/ml LPC and 1 % paraformaldehyde, for 20 min. Cells were washed and F-actin assembly was detected by flow cytometry.

6.3.3 Effects of simvastatin on C5a induced MMP secretion

MMPs are capable of degrading components of the extracellular matrix, such as collagen, proteoglycans and elastin. C5a has been shown to cause release of MMP-1 and MMP-9 from neutrophils and eosinophils (Takafuji et al., 2003). The U937 cell line has been well documented in the literature for expressing different MMPs (Sundararaj et al., 2008, Yue et al., 2009, Liu and Chang, 2010), yet to our knowledge no one has investigated the effects of C5a on MMP secretion in these cells. Here the effect of simvastatin on C5a induced MMP secretion was investigated in both the $U937_{PM}$ and $U937_{C5aR}$ cell line using gelatin zymography.

6.3.3.1 Simvastatin increased C5a induced MMP-9 secretion in Bt_2cAMP differentiated $U937_{PM}$ cell line

Gelatin zymography of culture supernatant from $U937_{PM}$ cell line showed that these cells mainly secrete MMP-2 and that the addition of C5a did not change the amount being secreted (figure 6.3.11). It was also shown that incubation of the $U937_{PM}$ cells with increasing concentration of simvastatin had no effect on this MMP-2 secretion (figure 6.3.11).

However, stimulation of the $U937_{PM}$ cells with Bt_2cAMP caused the cells also to secrete MMP-9 (figure 6.3.12.A). Furthermore, the MMP-9 secretion was increased by the addition of C5a, whereas, MMP-2 secretion remained unchanged (figure 6.3.12 B and 6.3.12 C). Surprisingly, incubation with simvastatin significantly increased the C5a induced MMP-9 secretion, while it did not affect basal levels of MMPs (figure 6.3.12 B).

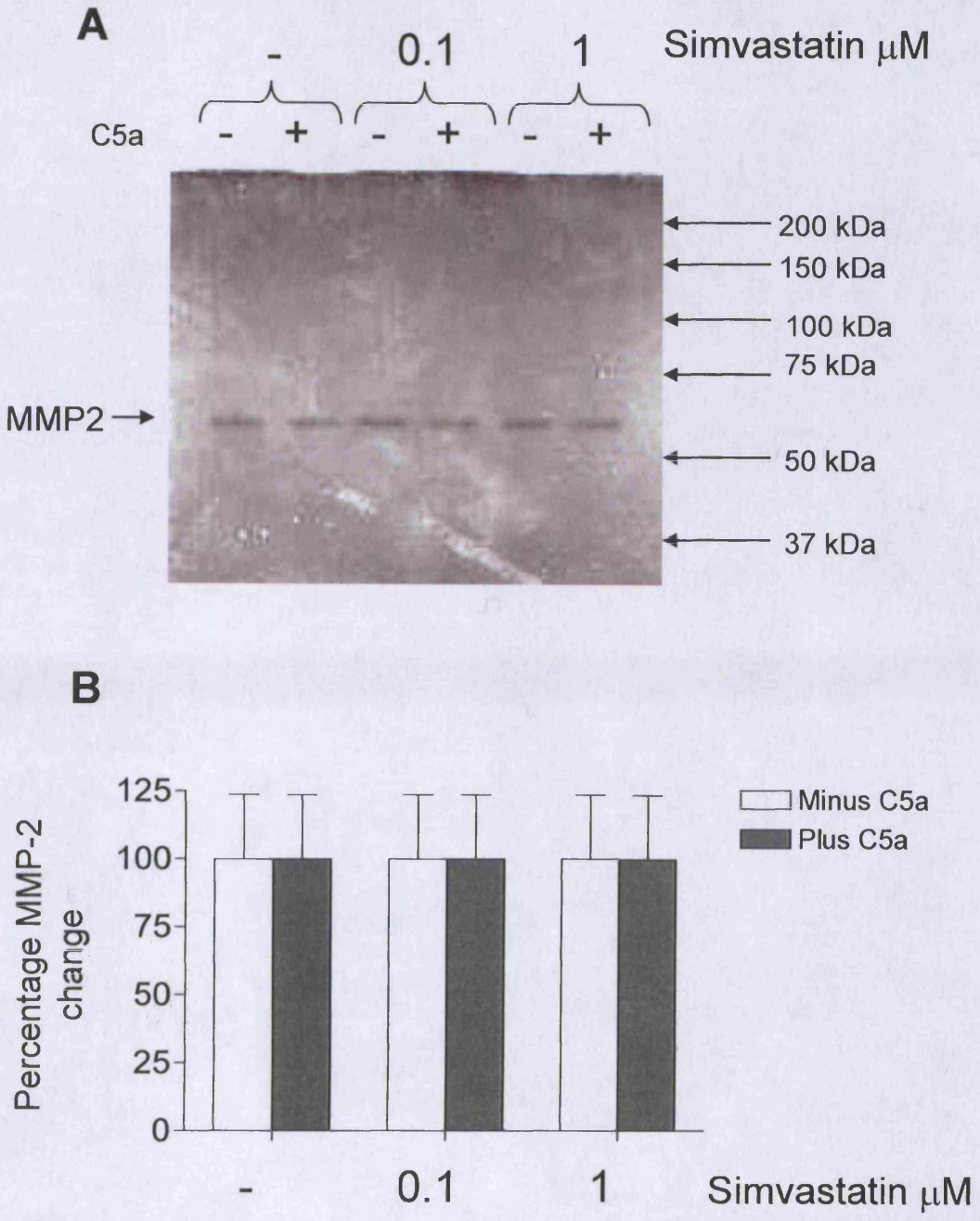


Figure 6.3.11 The $U937_{PM}$ cell line MMP-2 secretion remains unchanged by both the addition of C5a and/or incubation with simvastatin. $U937_{PM}$ cells were incubated for 2 days with simvastatin in serum free media. Following 24 hr, 5 nM C5a was added. The harvested s/ns were run on a 10 % SDS-gelatin polyacrylamide gel and bands were visualised after staining with Coomassie Brilliant Blue R. (A) Representative inverted gel image. (B) Percentage change in MMP-2 secretion from multiple experiments. Two-way ANOVA shows no significant effect of C5a or simvastatin.

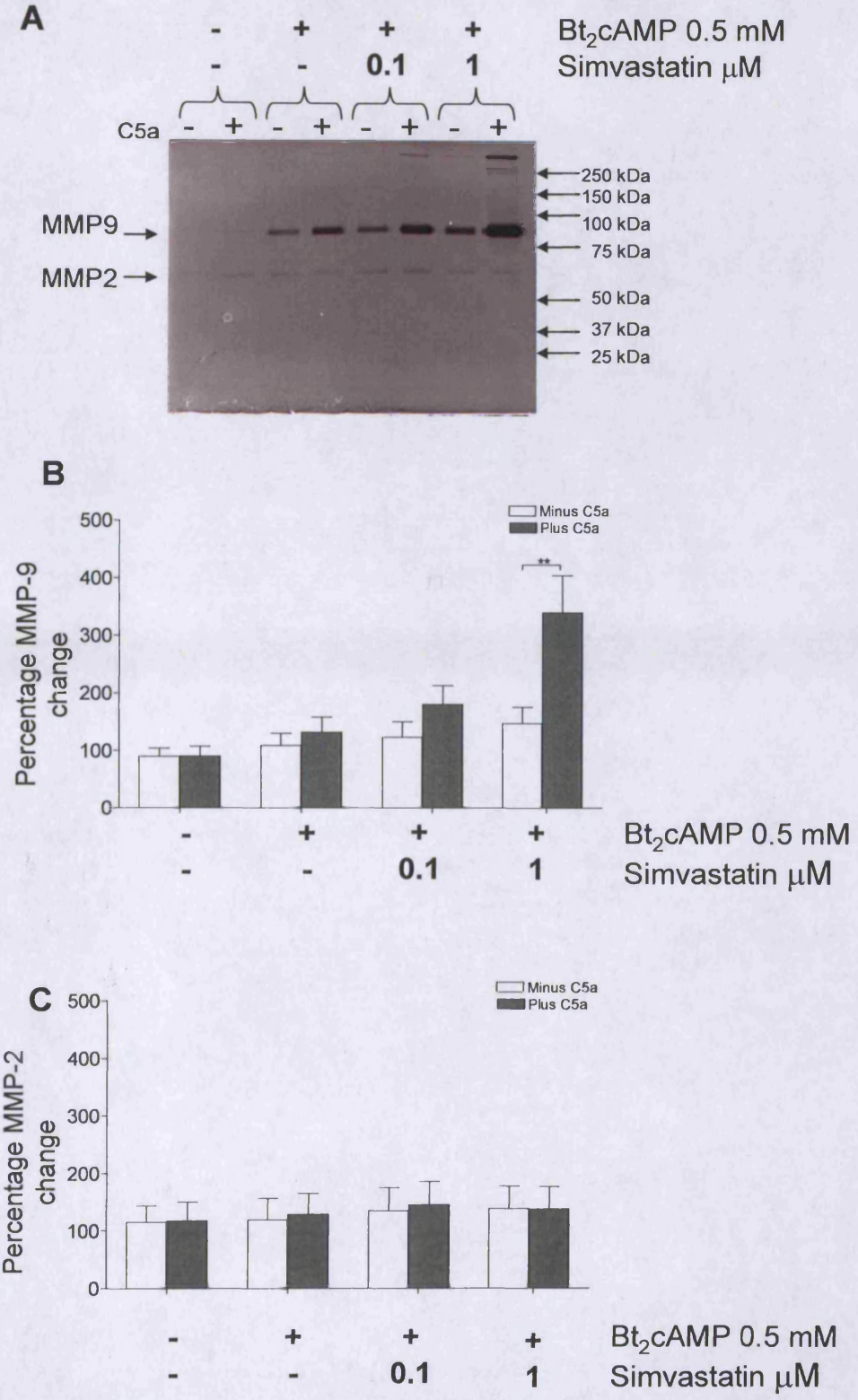


Figure 6.3.12 Effects of simvastatin on C5a induced MMP production in Bt_2cAMP differentiated $U937_{PM}$ cell line. $U937_{PM}$ cells were incubated for 2 days with simvastatin in serum free media. Following 24 hr, 5 nM C5a was added. The harvested s/ns were run on a 10 % SDS-gelatin polyacrylamide gel and bands were visualised after staining with Coomassie Brilliant Blue R. (A) Representative inverted gel image. (B) Percentage MMP-9 changed compared to Bt_2cAMP alone. Two-way ANOVA followed by Bonferroni post-test shows significant difference as marked by asterisks **p< 0.01. (C) MMP-2 secretion. Two-way ANOVA shows no significant effect of C5a or simvastatin on MMP-2 secretion.

6.3.3.2 Simvastatin had no effect on C5a induced MMP production in the U937$_{C5aR}$ cell line

The U937$_{C5aR}$ cell line was shown to secrete mainly MMP-2 and some MMP-9 (figure 6.3.13 A). MMP-2 and MMP-9 secretion remained unchanged by the addition of C5a (figure 6.3.13 B and C). Furthermore, incubation with simvastatin had no statistically significant effect on either MMP-2 or MMP-9 secretion (figure 6.3.13 B and C).

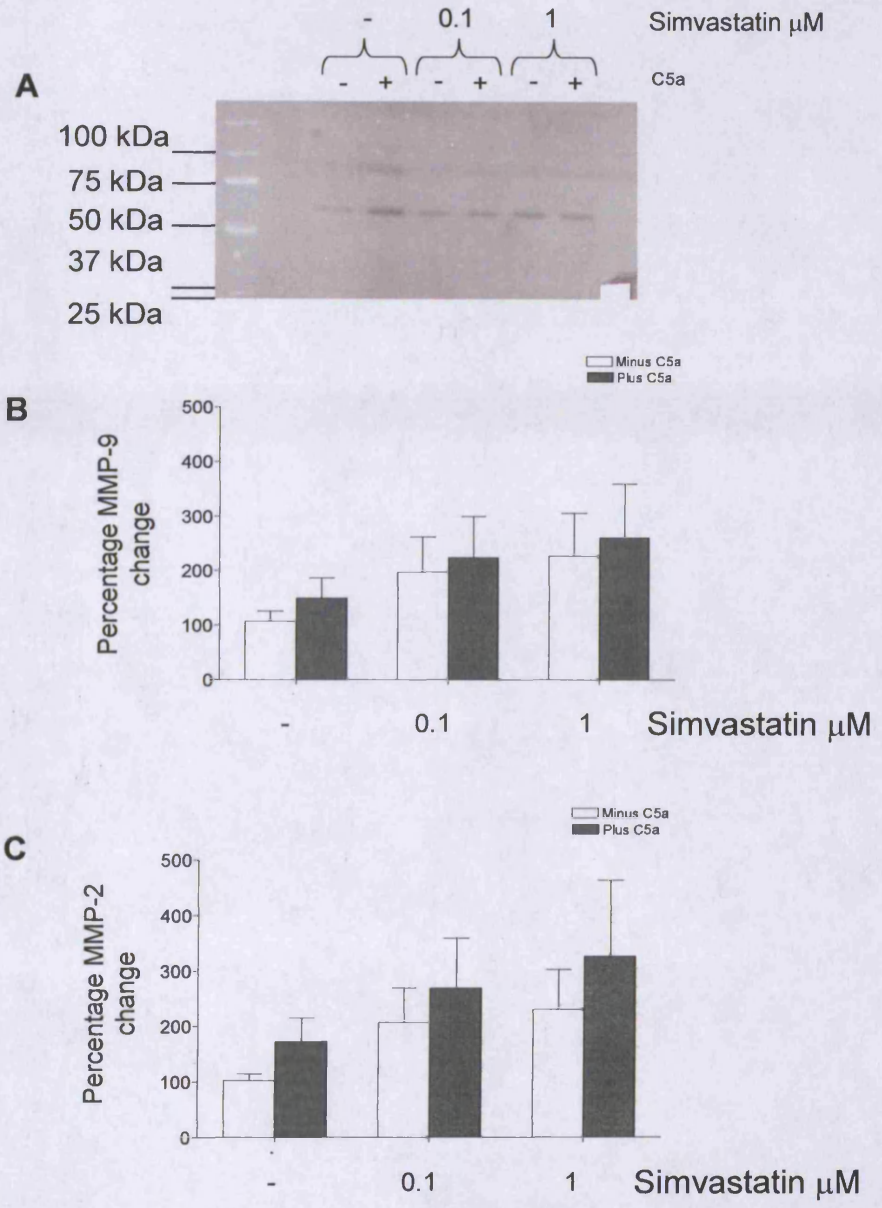


Figure 6.3.13 Effects of simvastatin on C5a induced MMP production in the U937$_{C5aR}$ cell line. U937$_{C5aR}$ cells were incubated for 2 days with simvastatin in serum free media. Following 24 hrs, 5 nM C5a was added. The harvested s/ns were run on a 10 % SDS-gelatin polyacrylamide gel and bands were visualised after staining with Coomassie Brilliant Blue R. (A) Representative inverted gel image; (B) Percentage MMP-9 change compared with the control. Two-way ANOVA showed no significant effect of simvastatin or C5a on MMP-9 secretion; (C) Percentage MMP-2 change compared with the control. Two-way ANOVA showed no significant effect of simvastatin or C5a on MMP2 secretion.

6.3.4 C5a induced cytokine production

Stimulation of leukocytes with C5a causes an increased production of a number of pro-inflammatory cytokines (Goodman et al., 1982, Okusawa et al., 1988, Scholz et al., 1990, Vecchiarelli et al., 1998, Ember et al., 1994, Izumi et al., 1997). Here the effects of C5a on IL-8 and MCP-1 production will be investigated in the undifferentiated and Bt_2cAMP differentiated $U937_{PM}$ cells and the $U937_{C5aR}$ line, as well as, human PBMCs. Using ELISA the effects of simvastatin and other lipid lowering agents on C5a induced IL-8 and MCP-1 will be investigated.

6.3.4.1 Simvastatin reduces C5a induced IL-8 production in Bt_2cAMP differentiated $U937_{PM}$ via inhibition of isoprenoid synthesis

Stimulation of $U937_{PM}$ cells with C5a caused a slight increase in IL-8 production, although this was not statistically significant ($p= 0.0519$) (figure 6.3.14). Incubation of these cells with simvastatin had no effect on resting or C5a induced IL-8 production (figure 6.3.14). Differentiation of $U937_{PM}$ cells with Bt_2cAMP, however, significantly increased both the resting and C5a induced IL-8 production (figure 6.3.15). Furthermore, the addition of simvastatin reduced the C5a induced IL-8 production (figure 6.3.15).

To determine if the effects of the simvastatin were caused by inhibition of cholesterol synthesis and/or isoprenoid synthesis, $U937_{PM}$ cells were incubated with Bt_2cAMP plus simvastatin and either 50 μM mevalonate or 10 μM GGPP. The addition of both mevalonate and GGPP restored the C5a induced IL-8 production, which was significantly reduced by the addition of 1 μM simvastatin (figure 6.3.16). This suggests that the effects of the simvastatin are due to inhibition of isoprenoid synthesis. To further investigate the role of the cholesterol synthesis pathway on C5a induced IL-8 production in Bt_2cAMP differentiated U937 cell line, these cells were incubated with the squalene synthesis inhibitor zaragozic acid. Figure 6.3.17 shows that incubation of $U937_{PM}$ cells with both Bt_2cAMP and zaragozic acid had no effect on the C5a induced production of IL-8. This result combined with results from above suggests that the effects seen by the simvastatin on IL-8 production are due to inhibition of isoprenoid synthesis alone.

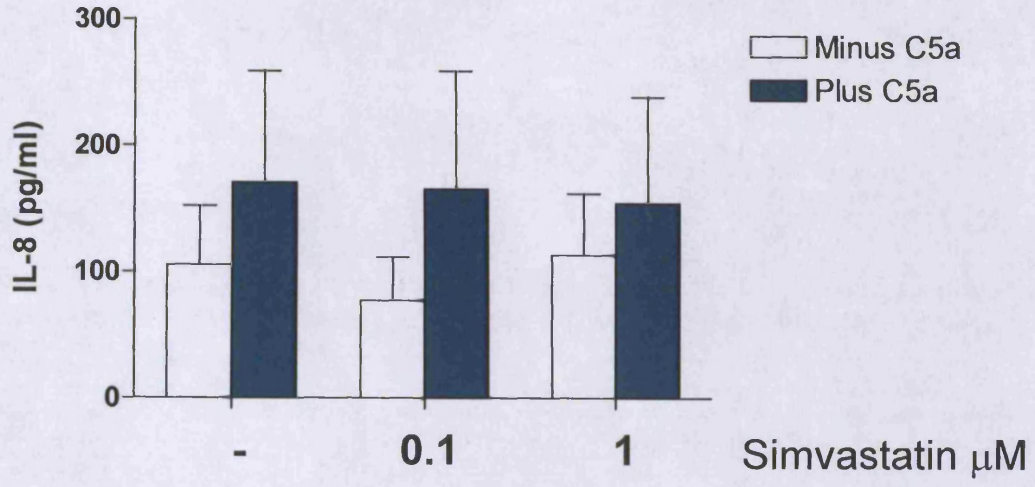


Figure 6.3.14 Incubation with simvastatin has no effect on C5a induced IL-8 production in the $U937_{PM}$ cell line. Cells were incubated for 48hr in the absence or presence of simvastatin. After 24hr 5nM C5a was added. Two-way ANOVA showed that neither C5a ($p = 0.0519$) nor simvastatin significantly affected IL-8 production ($p = 0.9830$).

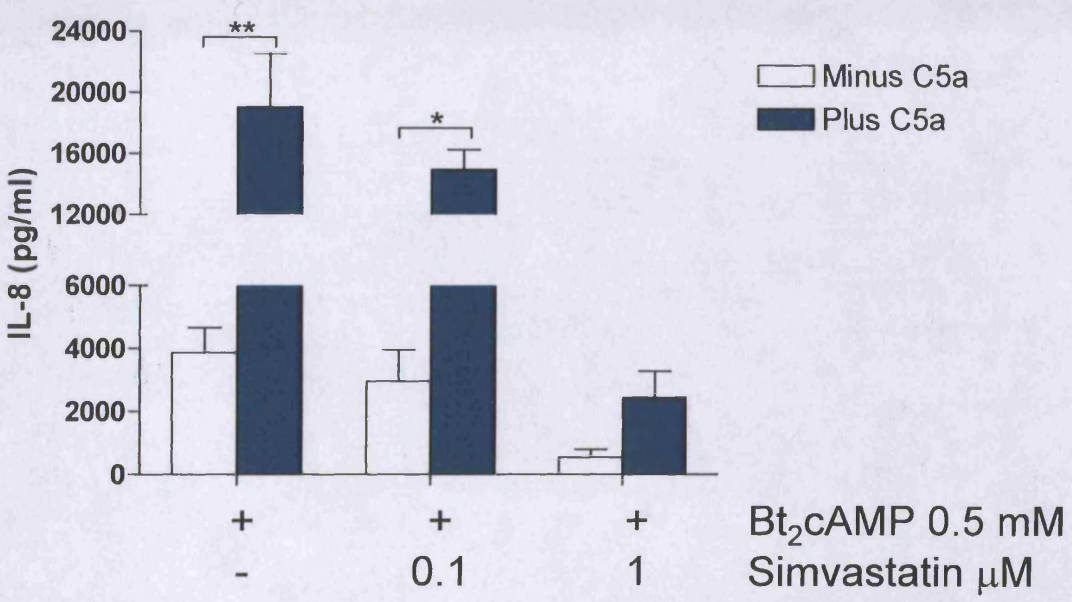
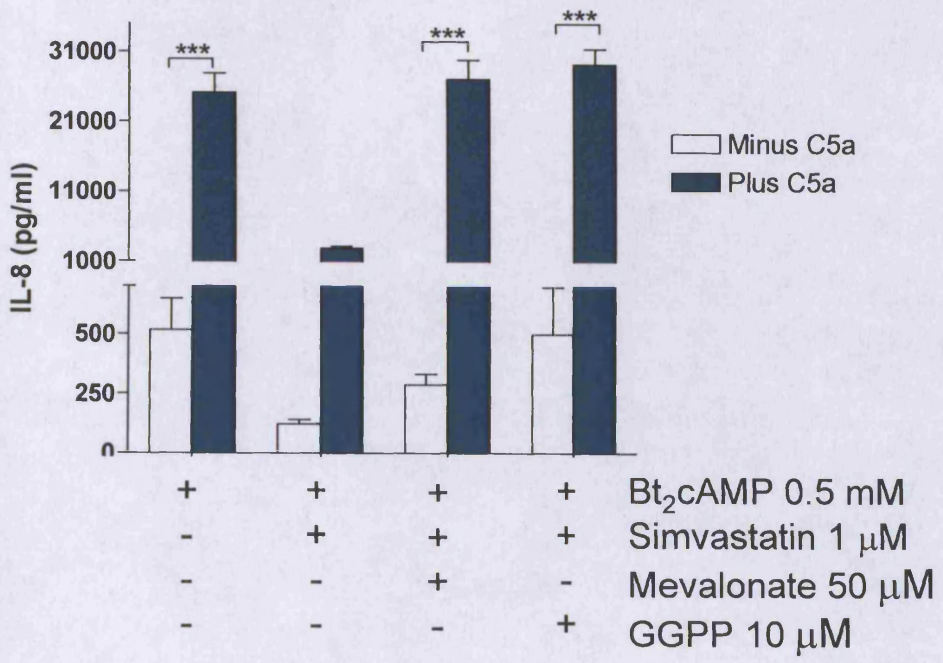
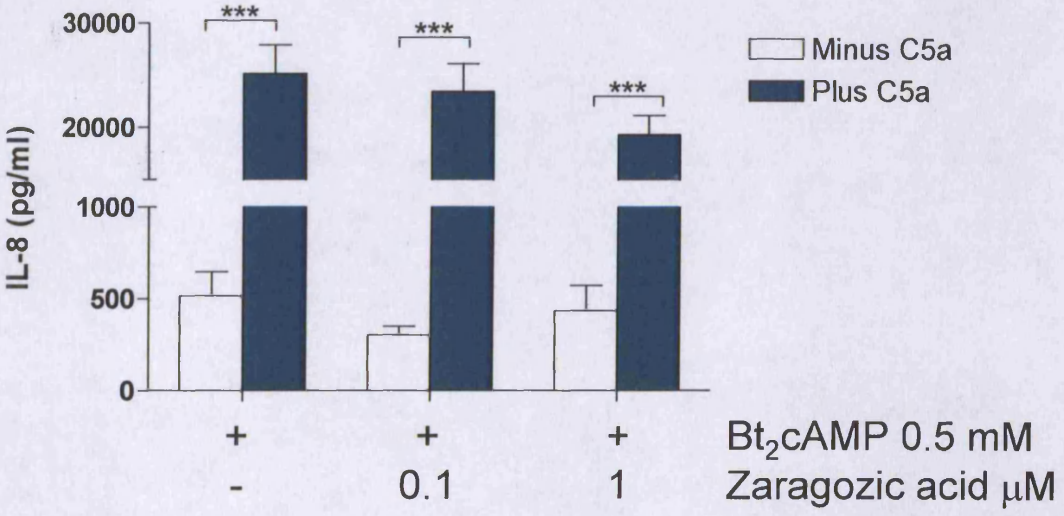


Figure 6.3.15 Incubation with simvastatin significantly decreases C5a induced IL-8 production in Bt_2cAMP differentiated $U937_{PM}$ cell line. $U937_{PM}$ cells were incubated with 0.5mM Bt_2cAMP in the absence or presence of increasing concentration of simvastatin for 48hr. Following 24hr 5nM C5a was added. Two-way ANOVA followed by Bonferroni post-test shows difference as marked by an asterisks *$p<0.05$ and **$p<0.01$.



6.3.16 Adding back mevalonate or GGPP restores C5a induced IL-8 production in Bt_2cAMP plus 1uM simvastatin treated $U937_{PM}$ cell line. $U937_{PM}$ cells were incubated with 0.5mM Bt_2cAMP plus 1 μM simvastatin and 50 μM mevalonate or 10 μM GGPP for 48hr. Following 24hr 5nM C5a was added. Two-way ANOVA followed by Bonferroni post-test shows difference as marked by an asterisks *** = p<0.001.



6.3.17 Zaragozic acid has no effect on IL-8 production in Bt_2cAMP stimulated $U937_{PM}$ cells. $U937_{PM}$ cells were incubated with 0.5mM Bt_2cAMP and zaragozic acid for 48hr. Following 24hr 5nM C5a was added. Two-way ANOVA followed by Bonferroni post-test shows difference as marked by an asterisks *** p<0.001.

6.3.4.2 MCP-1 secretion is not significantly altered by the addition of C5a or by incubation with simvastatin in the $U937_{PM}$ cell line

Although the $U937_{PM}$ cells secreted MCP-1, its levels were not significantly altered by the addition of C5a (figure 6.3.18). Incubation of these cells with simvastatin showed a slight reduction in resting and C5a induced MCP-1 secretion although this was not statistically significant (figure 6.3.18). $U937_{PM}$ cells, which had been differentiated with Bt_2cAMP, resulted in an increase in MCP-1 production following the addition of C5a although this was not statistically significant; however, combination of C5a and 0.1 μM simvastatin produced a statistically significant increase in MCP-1 secretion (figure 6.3.19).

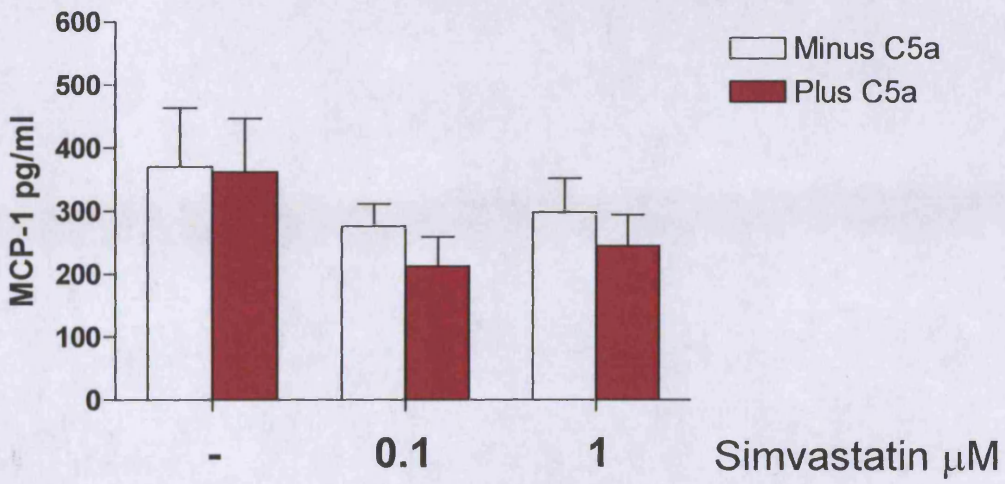


Figure 6.3.18 Simvastatin has no effect on C5a induced MCP-1 production in the $U937_{PM}$ cell line. $U937_{PM}$ cells were incubated with simvastatin for 48hr. Following 24hr, 5nM C5a was added. Two-way ANOVA followed by Bonferroni post-test showed no significant difference in MCP-1 secretion following addition of C5a or incubation with simvastatin.

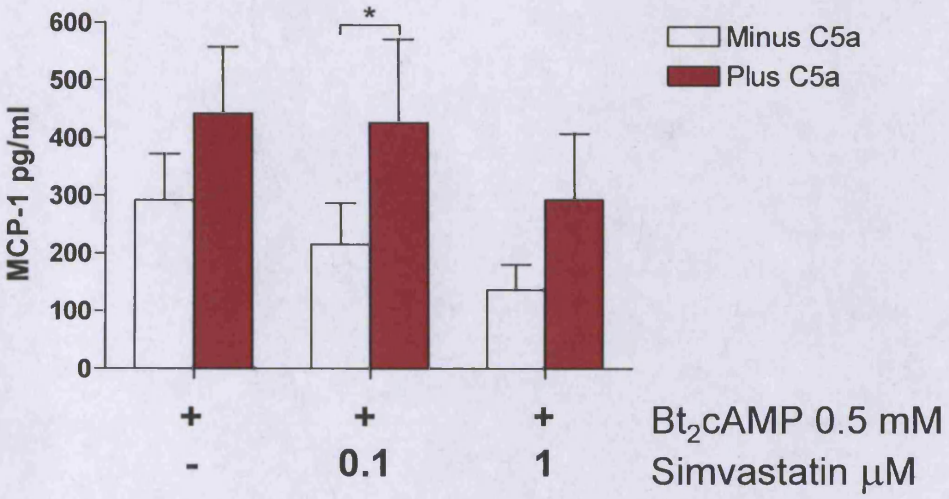


Figure 6.3.19 Effects of simvastatin on C5a induced MCP-1 production in the Bt_2cAMP differentiated $U937_{PM}$ cell line. $U937_{PM}$ cells were incubated with 0.5mM Bt_2cAMP and increasing concentration of simvastatin for 48hr. Following 24hr 5nM C5a was added. Two-way ANOVA followed by Bonferroni post-test shows difference as marked by an asterisks * = $p<0.05$.

6.3.4.3 Incubation of U937$_{C5aR}$ with simvastatin has no effect on C5a induced IL-8 or MCP-1 production

Results from the IL-8 ELISA showed that the U937$_{C5aR}$ cell line secreted IL-8 (figure 6.3.20). Following stimulation with C5a, IL-8 secretion increased, although this was only statistically significant at 1 μM simvastatin (figure 6.3.20) The U937$_{C5aR}$ cells were also found to secrete MCP-1 and in the control cells C5a appeared to increase MCP-1 secretion but this was not statistically significant (figure 6.3.21). However, incubation with simvastatin appears to reduce this C5a induced MCP-1 secretion, although this was not statistically significant (figure 6.3.21).

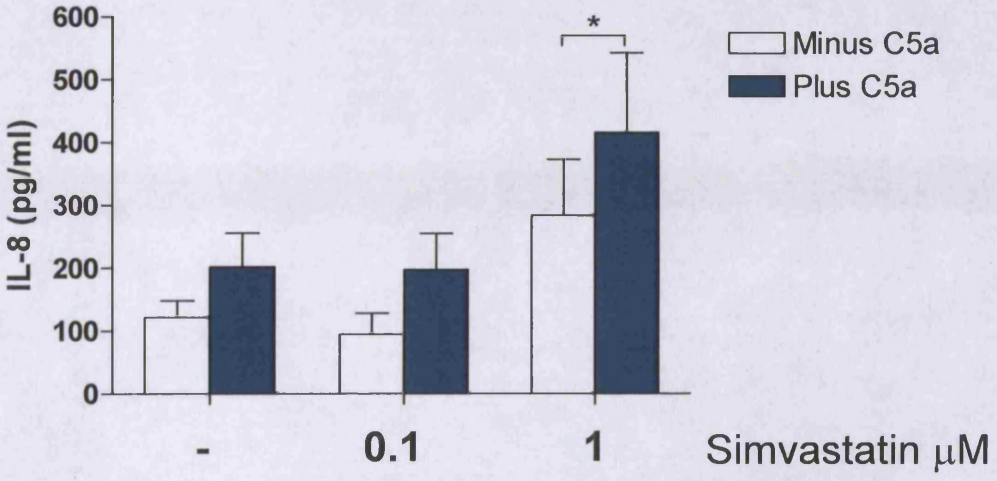


Figure 6.3.20 Incubation with simvastatin has no effect on C5a induced IL-8 production in the U937$_{C5aR}$ cell line. U937$_{C5aR}$ cells were incubated with simvastatin for 48 hr. Following 24 hr 5 nM C5a was added. Two-way ANOVA followed by Bonferroni post-test shows difference as marked by an asterisks * = $p<0.05$.

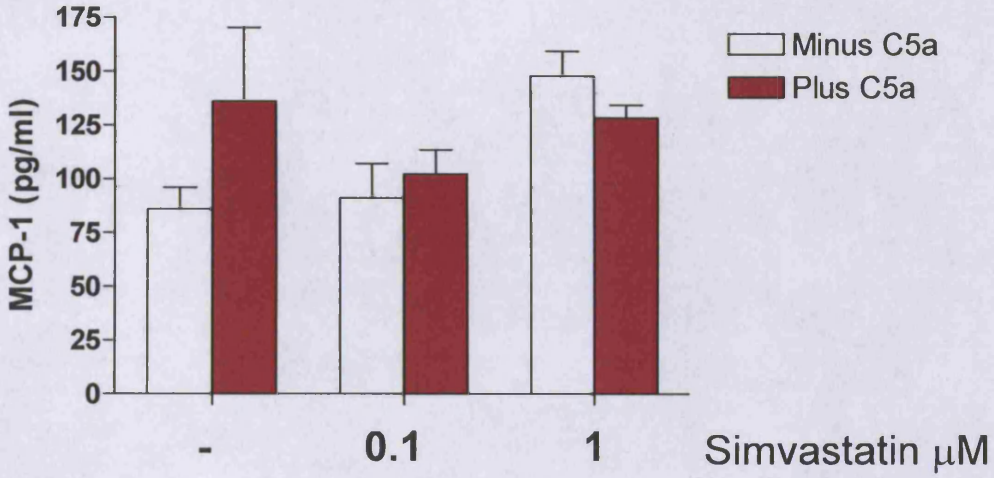


Figure 6.3.21 Incubation with simvastatin reduces C5a induced MCP-1 secretion in the $U937_{C5aR}$ cell line. $U937_{C5aR}$ cells were incubated with simvastatin for 48 hr. Following 24 hr C5a, 5 nM, was added. Two-way ANOVA followed by Bonferroni post-test showed no significant difference in MCP-1 secretion following addition of C5a or incubation with simvastatin.

6.3.4.4 Effects of lipid altering stimuli on C5a induced IL-8 and MCP-1 production in human PBMCs

To further investigate the effects of lipid lowering agents on C5aR function, human PBMCs were incubated with simvastatin, lovastatin or fenofibrate and their effects on C5aR expression and the C5a induced IL-8 production was monitored. By using mixed PBMCs population this will allow interactions between the different cell types and their different cytokine and chemokine secretions which will mimic a more *in vivo* situation.

Stimulation of the control human PBMCs with C5a had no significant effect on IL-8 secretion (figure 6.3.22). When the mononuclear cells were incubated with simvastatin there was no significant difference in resting IL-8 secretion compared with the controls (figure 6.3.22 A). Furthermore, incubation with simvastatin had no effect on C5a induced IL-8 secretion (figure 6.3.22 A). Similarly, incubation with lovastatin or fenofibrate had no significant effect on resting or C5a induced IL-8 secretion (figure 6.3.22 B and C).

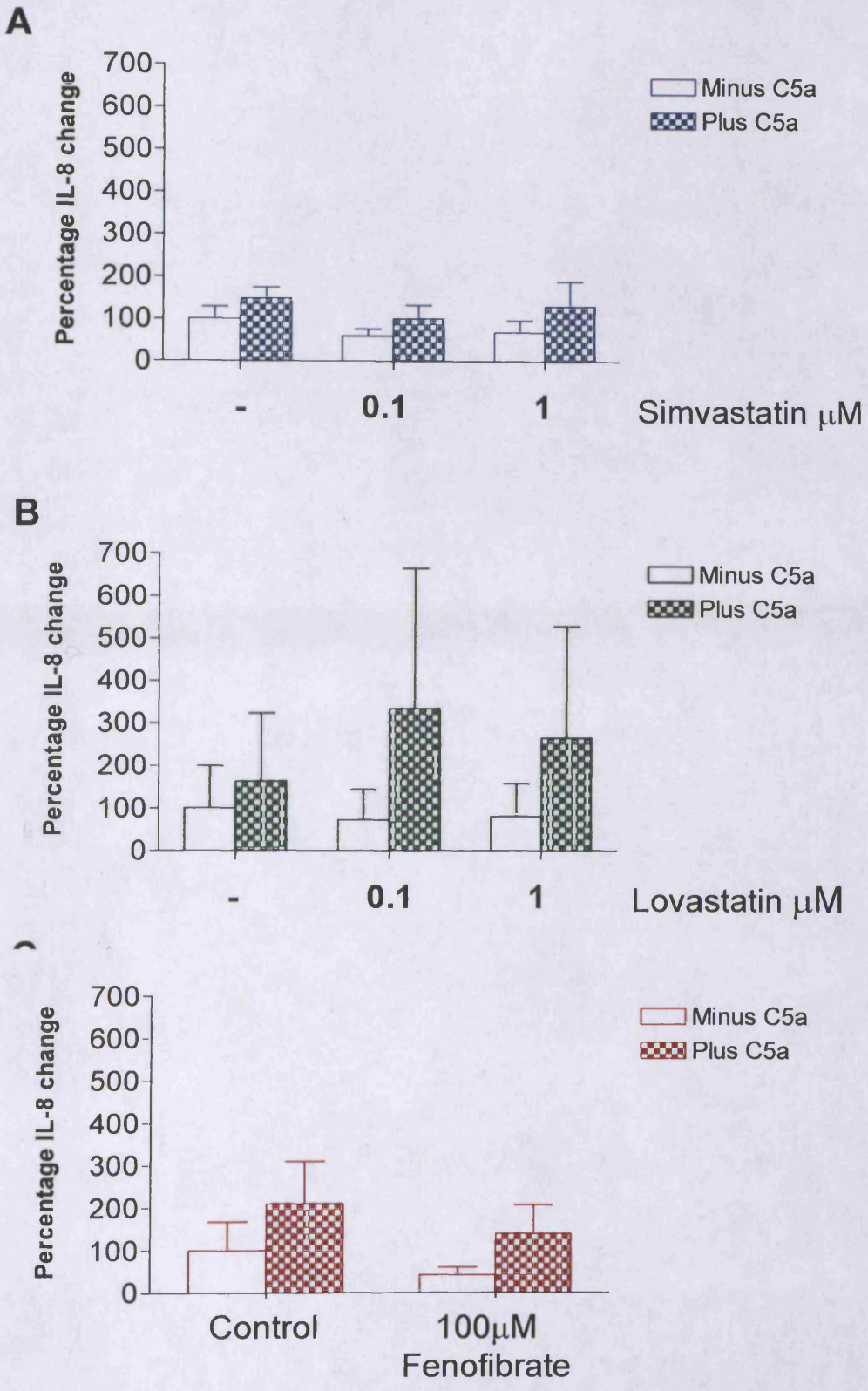


Figure 6.3.22 Effects of lipid lowering drugs on C5a induce IL-8 secretion in human PBMCs. Human PBMCs isolated from buffy coat were incubated with simvastatin (A), lovastatin (B) or fenofibrate (C) for 24 hrs in the absence or presence of 5 nM C5a. IL-8 ELISA was performed using harvested s/n. Data displayed as percentage IL-8 change compared with the control. Two-way ANOVA showed that C5a significantly increase IL-8 release whereas none of the treatments had any effect.

6.3.5 C5aR appears to be localised within the non-lipid raft region of the plasma membrane in both the $U937_{PM}$ and $U937_{C5aR}$ cell line

The membrane localisation of the C5aR was investigated in both the $U937_{PM}$ and the $U937_{C5aR}$ cell lines using a discontinuous sucrose gradient floatation in the presence of 1 % Brij58. The membrane localisation of GPI anchored CD59 ($U937_{CD59\text{-}GPI}$) and transmembrane anchored CD59 ($U937_{CD59\text{-}TM}$) were used as positive and negative controls respectively for lipid raft associated (detergent resistant) and non-lipid raft (detergent soluble) molecules; these constructs had been previously characterised using this method ((Hiscox et al., 2002) and van den Berg unpublished data). As described in section 6.2.8, the C5aR expressing cells were mixed with $U937_{CD59\text{-}TM}$ or $U937_{CD59\text{-}GPI}$ cells prior to sucrose gradient floatation. Due to the differences between the molecular weight of the C5aR (~42 kDa) and CD59 (between 18-20 kDa for GPI anchored and 20-25 kDa for TM), this allowed the same samples to be stained for both C5aR and CD59 by cutting the membrane in half at ~30kDa prior to incubation with the antibodies.

In order to detect the C5aR in the $U937_{PM}$ cell line using western blotting, these cells were differentiated with 0.5 mM Bt_2cAMP for 48 hrs prior to sucrose gradient floatation. As the C5aR was detected following sucrose gradient floatation solely in the lower fractions (fractions 7-9), alongside the detergent soluble transmembrane CD59, this suggests that the C5aR is localised in a non-lipid raft region of the plasma membrane (figure 6.3.23). If the C5aR was localised in a lipid raft region then it would be localised in the upper detergent resistant fractions alongside the GPI-anchored CD59 (fractions 1-3) (figure 6.3.23). Despite this however, both the GPI-anchored and the transmembrane CD59 were detected in other fractions, although very small amounts, which suggests that some of the CD59 from both cell lysates was partially detergent soluble and insoluble respectively.

To determine if the C5aR in the Bt_2cAMP differentiated $U937_{PM}$ cell line is clustered into the lipid rich microdomains following stimulation with C5a, these cells were incubated with 5 nM C5a prior to sucrose gradient floatation. As shown in figure 6.3.24 the C5aR remained localised in the detergent soluble fractions (fraction 6-8), alongside the transmembrane CD59 (fractions 7 and 8), even after C5a stimulation in the Bt_2cAMP differentiated $U937_{PM}$ cell line. Furthermore, C5aR expression following stimulation with C5a appears less in the $U937_{PM}$ cells differentiated with Bt_2cAMP (figure 6.3.24) compared with the western blot results in the absence of C5a (figure 6.3.23), suggesting that some C5aR may become degraded following stimulation with its ligand. Results from sucrose gradient floatation of the $U937_{C5aR}$ cell lysate also show that the C5aR localised in the lower detergent soluble fractions (fractions 6-8) both in the absence and presence of 5 nM C5a (figures 6.3.25 and 6.3.26).

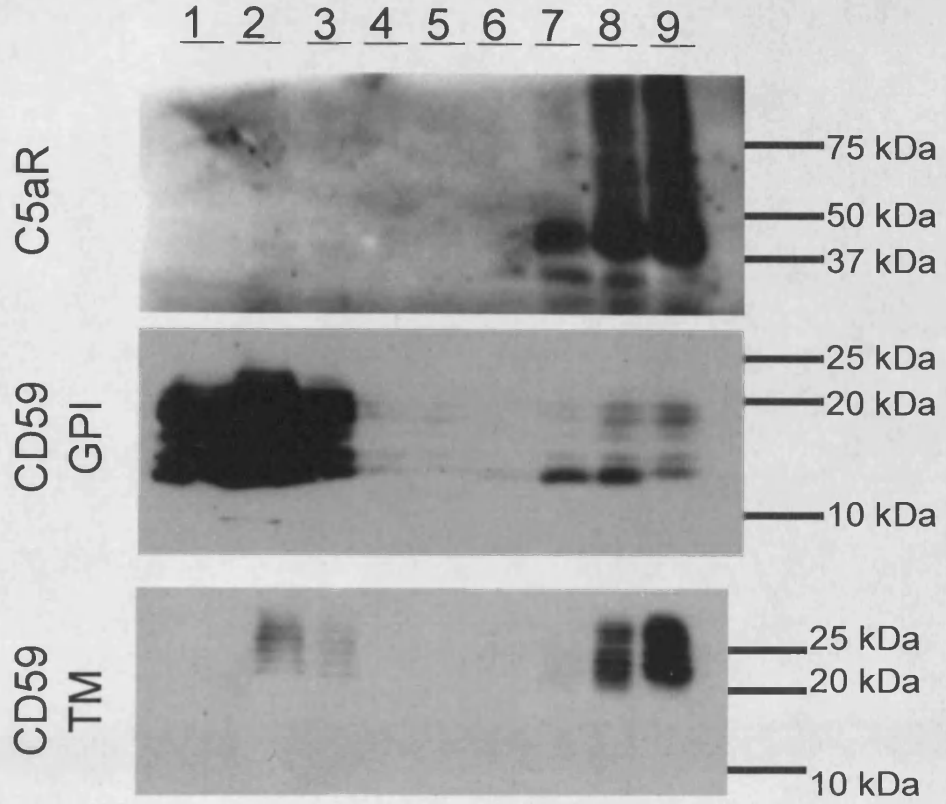


Figure 6.3.23 The C5aR in the Bt_2cAMP differentiated $U937_{PM}$ cell line is localised in the detergent soluble fractions of the sucrose gradient floatation. The $U937_{PM}$ cell line was differentiated with 0.5mM Bt_2cAMP for 48 hr to upregulate C5aR expression. 7 $x10^6$ Bt_2cAMP differentiated $U937_{PM}$ cells were mixed with 3 $x10^6$ U937 cells transfected with either the GPI-anchored CD59 or transmembrane CD59. The cell mixture was then solubilised in 40 % sucrose, 1 % Brij58 prior to gradient centrifugation at 45,000 rpm for 16 hr at 4 °C in order to isolate membrane microdomain associated proteins. Fractions (0.5 ml) were collected starting from the top of each sample and subjected to SDS-PAGE and western blotting with an anti-C5aR (H-100, Santa Cruz) or anti-CD59 (Bric229).

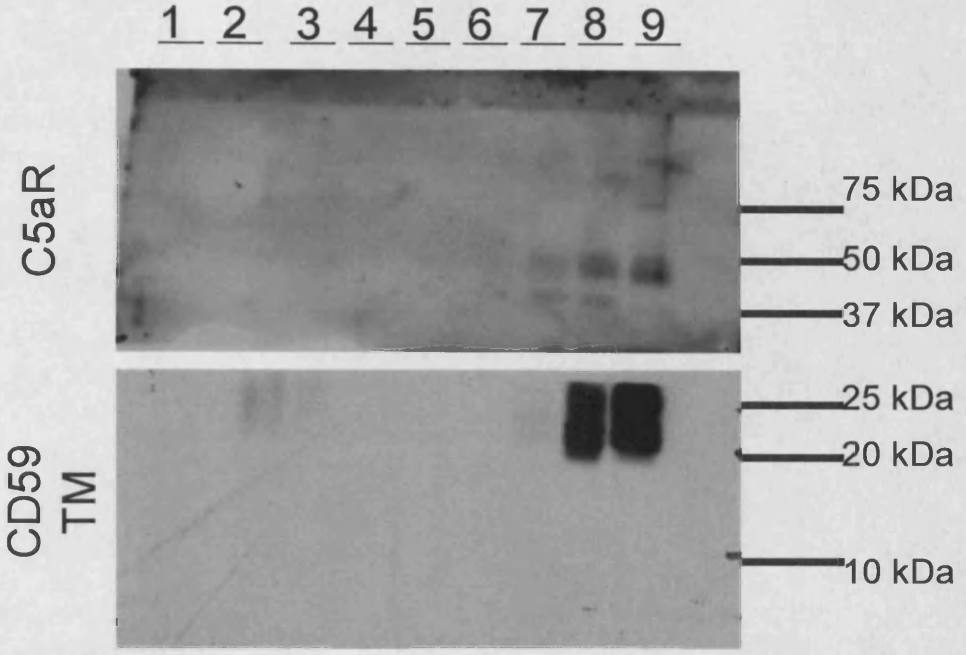


Figure 6.3.24 Stimulation of Bt_2cAMP differentiated $U937_{PM}$ cell line with C5a has no effect on the membrane localisation of the C5aR as observed by sucrose gradient floatation. The $U937_{PM}$ cell line was differentiated with 0.5mM Bt_2cAMP for 48 hr to upregulate C5aR expression. 7 $x10^6$ Bt_2cAMP differentiated $U937_{PM}$ cells were mixed with 3 $x10^6$ U937 cells transfected with transmembrane CD59. The cell mixture was then incubated with 5 nM C5a for 5 min prior to solubilising in 40 % sucrose, 1 % Brij58 prior to gradient centrifugation at 45,000 rpm for 16 hr at 4 ^{o}C in order to isolate membrane microdomain associated proteins. Fractions (0.5 ml) were collected starting from the top of each sample and subjected to SDS-PAGE and western blotting with anti-C5aR (H-100, Santa Cruz) or anti-CD59 (Bric229)

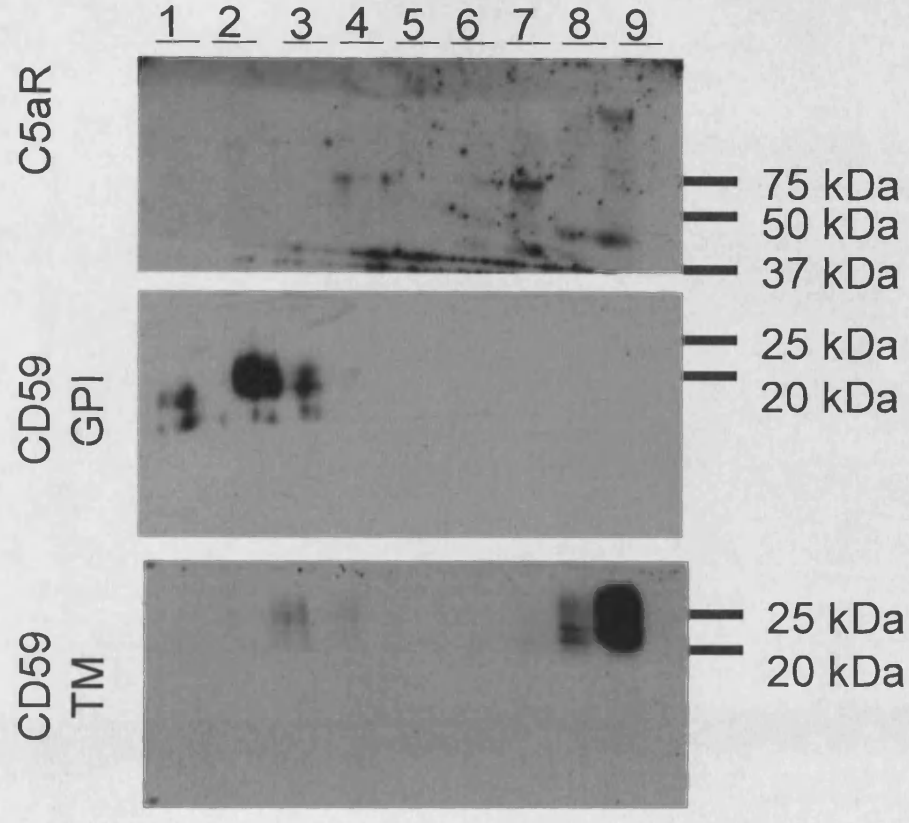


Figure 6.3.25 The C5aR in the U937$_{C5aR}$ cell line is localised in the detergent soluble fractions of the sucrose gradient floatation. 7 x10^6 U937$_{C5aR}$ cells were mixed with 3 x10^6 U937 cells transfected with either the GPI-anchored CD59 or transmembrane CD59 . The cell mixture was then incubated with 5 nM C5a for 5 min prior to solubilising in 40 % sucrose, 1 % Brij58 prior to gradient centrifugation at 45,000 rpm for 16 hr at 4 °C in order to isolate membrane microdomain associated proteins. Fractions (0.5 ml) were collected starting from the top of each sample and subjected to SDS-PAGE and western blotting with an anti-C5aR (H-100, Santa Cruz) or anti-CD59 (Bric229)

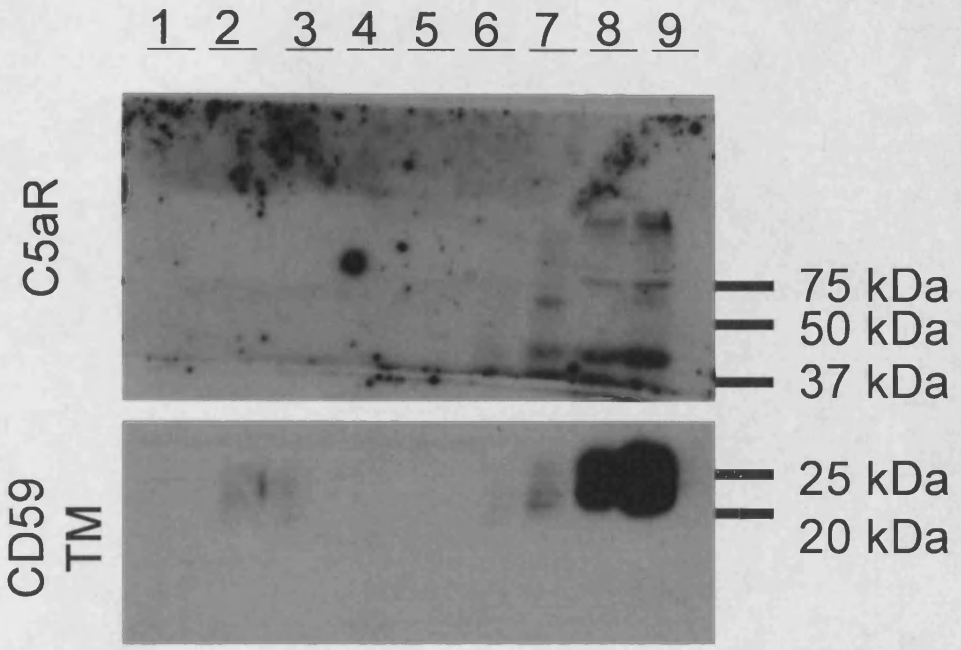


Figure 6.3.26 Stimulation of the $U937_{C5aR}$ cell line with C5a has no effect on the membrane localisation of the C5aR as observed by sucrose gradient floatation. 7 $x10^6$ $U937_{C5aR}$ cells were mixed with 3 $x10^6$ U937 cells transfected with transmembrane CD59. The cell mixture was then incubated with 5 nM C5a for 5 min prior to solubilising in 40 % sucrose, 1 % Brij58 prior to gradient centrifugation at 45,000 rpm for 16 hr at 4 ^{o}C in order to isolate membrane microdomain associated proteins. Fractions (0.5 ml) were collected starting from the top of each sample and subjected to SDS-PAGE and western blotting with an anti-C5aR (H-100, Santa Cruz) or anti-CD59 (Bric229)

6.4 Discussion

Evidence suggests that the cholesterol dependent and independent effects of statins have beneficial anti-inflammatory properties (Arnaud et al., 2005). As complement activation leads to the generation of C5a, which acts via the C5aR, this Chapter has investigated whether simvastatin can exert anti-inflammatory effects by modulating the function of the C5aR. Although the effects of simvastatin on the biological functioning of the C5aR will be discussed below, it is important to note that as Chapter 5 has previously shown that simvastatin can alter the receptor expression, this makes it difficult to determine whether the effects seen in this Chapter are due to lowered C5aR expression or both lowered expression and reduced biological function of the receptor.

6.4.1 Effects of simvastatin incubation on biological functioning of the C5aR

As previously described in section 6.1 statins can modulate GPCR functions by cholesterol dependent and independent mechanisms. The cholesterol dependent mechanisms are mediated by altering membrane cholesterol levels which can in turn alter the sub-membrane localisation, structural properties and internalisation of GPCRs and their signalling components. The cholesterol independent mechanisms are mediated by inhibition of isoprenoid biosynthesis, which are important for the post-translational modification isoprenylation of small GTP binding proteins. In this Chapter the biological functions of the C5aR were monitored in $U937_{PM}$ cells, which were either undifferentiated or differentiated with Bt_2cAMP to increase C5aR expression, $U937_{C5aR,}$ which had been stably transfected with the human C5aR, and PBMCs. Following incubation of these cells with statins, the C5a induced Ca^{2+} release, F-actin assembly, MMP-secretion and cytokine secretion were monitored, as previously described in sections 6.1 and 6.2.

6.4.1.1 C5a induced intracellular Ca^{2+} release

Stimulation of the C5aR with C5a leads to activation of PLCβ which results in a rise in intracellular Ca^{2+} (Maurya and Subramaniam, 2007a, Jiang et al., 1996, Camps et al., 1992). The effects of simvastatin on C5a induced Ca^{2+} release, in the $U937_{PM}$ and $U937_{C5aR}$ cell lines, was monitored using Fura-2-AM, as previously described in section 4.1.2.3.

As described in section 6.3.1.1 the undifferentiated $U937_{PM}$ cell line observed a decrease in C5a induced Ca^{2+} release following incubation with 1 μM simvastatin (figure 6.3.1). Although not statistically significant, this reduction was reversed by adding back either mevalonate or GGPP (figure 6.3.3). These results are particularly interesting as previously Chapter 5 (section 5.3.1) was unable to detect C5aR expression in these cells by

flow cytometry, yet here these cells were able to induce intracellular Ca^{2+} release following stimulation with C5a and furthermore this response decreased by incubation with simvastatin. Despite being unable to detect C5aR expression by flow cytometry or western blotting in these cells, we cannot rule out that simvastatin does not alter receptor expression levels. For this reason it is difficult to conclude whether the decrease in C5a induced Ca^{2+} release following incubation with simvastatin is due to functional changes in the receptor or its signalling components and/or changes in receptor expression. Figure 6.3.1 also shows that the C5a induced Ca^{2+} release was significantly reduced by cholesterol loading, which suggests that the receptor may have sensitivity towards cholesterol levels. This result correlates with other published reports that have found other GPCR, such as the fMLP receptor, to be sensitive to membrane cholesterol content (Bath et al., 1991, Dunzendorfer et al., 1997, Wolach et al., 1992, Xue et al., 2004). For the fMLP receptor it was shown that membrane depletion of cholesterol by using MβCD, reduced the ability of the neutrophils to undergo chemotaxis towards fMLP (Xue et al., 2004). However in our experiments only cholesterol loading had a significant effect on C5a induced Ca^{2+} release in the $U937_{PM}$ cell line whereas MβCD had no significant effect (figure 6.3.1). Our findings also correlate with a study by Nagao and co-workers, which found that cholesterol loading of mouse macrophages J774A.1 cells with ChMβCD led to a significant decrease in transmigration towards C5a (Nagao et al., 2007). Together these findings suggest that some of the C5aR biological functions, C5a induced Ca^{2+} release reported here and C5a induced migration reported by (Nagao et al., 2007), are sensitive to membrane cholesterol and that simvastatin exerts an anti-inflammatory effect by reducing the C5a induced Ca^{2+} in the $U937_{PM}$ cell line. It is also important to note that using CD to either deplete or load cell membranes of cholesterol have their limitations as their non-physiological actions can often cause non-specific artefacts (Maxfield and Tabas, 2005, Zidovetzki and Levitan, 2007). This was true for these experiments as incubation with ChMβCD significant decrease cell viability (figure 6.3.2).

Incubation of Bt_2cAMP differentiated $U937_{PM}$ cells with 1 μM simvastatin decreased C5a induced Ca^{2+} release (figure 6.3.4). As this effect was prevented by the addition of mevalonate but not GGPP, this suggests that the C5a induced Ca^{2+} release may be sensitive to lowered cholesterol levels (figure 6.3.5). However, to contradict this result incubation with MβCD, cholesterol chelator, (figure 6.3.4) or zaragozic acid, a squalene synthase inhibitor, (figure 6.3.7) had no effect on C5a induced Ca^{2+} release. One explanation for these results is differences in C5aR expression levels. As described in Chapter 5, incubation with simvastatin reduced the Bt_2cAMP induced C5aR expression, whereas zaragozic acid had no effect on receptor expression. Therefore it is difficult to determine whether these differences

are due to functional changes and/or changes in expression. If changes in expression were entirely to blame for the reduced C5a induced Ca^{2+} release then it would be expected that the addition of both mevalonate and GGPP reversed the effects of simvastatin on C5a induced Ca^{2+} release, as they did for Bt_2cAMP induced C5aR expression. However, this was not observed (figure 6.3.5), and one explanation could be that initial batches of Bt_2cAMP were more able to induce higher levels of C5a induced Ca^{2+} compared to later batches. This made it difficult to show statistical differences in later experiments as incubation with 1 μM simvastatin in initial experiments reduced the C5a induced Ca^{2+} release by half whereas later experiments it was reduced only by a quarter (figure 6.3.5 and 6.4.4 respectively). Another possibility could be that the intracellular signalling is more sensitive to inhibition of isoprenylation and therefore higher concentrations of mevalonate and GGPP are needed to completely reverse the effects of the simvastatin. In these experiments it was chosen to stimulate the cells with 5 nM C5a as this was shown in Chapter 4 to induce the maximum intracellular Ca^{2+} release; furthermore this is the highest physiologically achievable C5a concentration (Guo and Ward, 2006). However if future experiments could be performed using a suboptimal dose of C5a this might make any changes in the C5aR function more pronounced.

To circumvent problems associated with changes in expression, experiments were repeated using the $U937_{C5aR}$ cell line which had previously been stably transfected with the C5aR (Kew et al., 1997). However, these cells were also shown to be sensitive to simvastatin as incubation with the statin caused a significant reduction in C5aR expression, section 5.3.7. Despite this reduction in expression, simvastatin had no significant effect on C5a induced Ca^{2+} release (figure 6.3.8). This result was particularly interesting as it suggests that not all the C5aR on the cell surface is fully functional or that downstream signalling components are acting as a limiting factor in the functioning of the signalling pathway. This is further supported by the fact that although the $U937_{C5aR}$ cells express higher levels of C5aR on their cell surface compared with $U937_{PM}$ differentiated with Bt_2cAMP (general observations), they produced far less intracellular Ca^{2+} response following stimulation with 5 nM C5a compared with $U937_{PM}$ differentiated with Bt_2cAMP (figures 6.3.8 and 6.3.4 respectively).

6.4.1.2 C5a induced F-actin assembly

The Bt_2cAMP differentiated U937 cell line has frequently been shown to increase F-actin assembly following stimulation with C5a (Banks et al., 1988, Monk and Banks, 1991, Kew et al., 1997). Despite this our experiments failed to detect changes in F-actin assembly in either $U937_{PM}$ or $U937_{C5aR}$ cell lines following stimulation with C5a, although human neutrophils showed a significant increase in F-actin assembly after stimulation (figure 6.3.9

and 6.3.10). This result was particularly disappointing as it was hypothesised that statins would reduce F-actin assembly by preventing the isoprenylation of small GTP-binding proteins, Cdc42 and Rac, which have been implicated in chemotaxis of leukocytes towards C5a (Li et al., 2003, Tardif et al., 2003, Welch et al., 2002a). One explanation why we were unable to F-actin assembly in these cells might be explained by their cell morphology. Both cells exhibited an irregular cell morphology, which could mean that their F-actin assembly could already be maximum polymerised (figure 4.3.5). However, as incubation with simvastatin resulted in the cells becoming more spherical maybe these cells may have less F-actin staining using phalloidin compared with the Bt_2cAMP alone (figure 5.3.12). This would make an interesting future experiment. As F-actin assembly is not a direct measure for chemotaxis, future experiments could involve direct methods for measuring chemotaxis such as the Boyden chamber, however, as F-actin assembly is a prerequisite for chemotaxis it is quite possible these cells might not respond. It would also been interesting to see if the cells were responsive to other chemoattractants such as fMLP to determine whether these cells were just unresponsive to C5a or not able to re-arrange their F-actin.

Previously incubation with 1 mM Bt_2cAMP, for up to 48 hrs, has been used to differentiate the cells and cause an increase in F-actin assembly following stimulation with C5a (Banks et al., 1988). Although our initial experiments found 0.25 mM Bt_2cAMP had the same effect of increasing C5aR expression as 1 mM (figure 4.3.2); we later found that different batches of Bt_2cAMP were less able to up regulate C5aR expression and therefore used higher concentrations of Bt_2cAMP. Although the Bt_2cAMP concentration used was able to increase the C5aR expression, maybe a higher concentration is needed to induce F-actin assembly. As Bt_2cAMP differentiation of U937 cell line has been previously characterised by an increase in CD11b and CD14, a decrease in FcγRI/CD64 expression and increased non-specific phagocytosis, it may be useful to monitor these changes following Bt_2cAMP differentiation to confirm whether the concentration and duration of Bt_2cAMP incubation used is enough (Sheth et al., 1988, Gavison et al., 1988, Brodsky et al., 1998).

6.4.1.3 C5a induced MMP secretion

As C5a had previously been shown to increase MMP secretion in neutrophils and eosinophils it was decided to investigate whether C5a could induce MMP secretion in the U937 cell line. Furthermore, as one of the pleiotropic effects of statins is to improve plaque stability by reducing MMP secretion, it was investigated whether simvastatin incubation hadany impact on C5a induced MMP secretion in the U937 cell line.

Results shown in section 6.3.3 found that the $U937_{C5aR}$ cell line secreted both MMP-2 and MMP-9, however the addition of C5a had no significant effect on the secretion of either of these (figure 6.3.13). Furthermore, incubation with simvastatin had no significant effect on

MMPs secretion in these cells (figure 6.3.13). The $U937_{PM}$ cell line secreted MMP-2 and following differentiation with Bt_2cAMP, this cell line also secreted MMP-9 (figure 6.3.11 and 6.3.12). In both cases the MMP-2 secretion was unchanged by the addition of C5a; whereas in the Bt_2cAMP differentiated $U937_{PM}$ cells there was an increase in MMP-9 secretion following the addition of C5a (figure 6.3.12). These results are in line with a recent publication from Speidl et al. who found that differentiated monocytes are able to secrete MMP-9 and also MMP-1 following stimulation with C5a (Speidl et al., 2011). Although it was not investigated exactly how C5a may induce MMP secretion in this study one possible mechanism could be that MMP-9 expression is regulated by NFκB and C5a has previously been shown to activate NFκB (Hsu et al., 1999, Kastl et al., 2006). Surprisingly, incubation of the Bt_2cAMP differentiated $U937_{PM}$ cells with simvastatin significantly increased the C5a induced MMP-9 secretion. This result contradicts all published findings which show that statins prevent MMP secretion and therefore improve plaque stability (Aikawa et al., 2001, Fukumoto et al., 2001, Fujimoto et al., 2008, Sundararaj et al., 2008). Furthermore, this result does not correlate with changes in the C5aR expression or C5a induced Ca^{2+} release, which were both reduced when the cells were incubated with both Bt_2cAMP and simvastatin compared with Bt_2cAMP alone (figure 5.3.4 and 6.3.4). One possible reason for the opposite effects being observed could be that for MMP zymography the cells are incubated in the absence of FCS, to remove exogenous MMPs that will interfere with the assay, whereas, all other experiments were carried out in the presence of FCS. Possible future experiments could investigate whether C5aR expression changes in the absence of serum and the effect on statins. Alternatively the C5a induced MMP secretion may somehow involve the second C5a receptor C5L2, which was not investigated in these studies due to the lack of a specific antibody against the receptor. However to circumvent this problem, future experiments could be carried out in the presence of C5aR blocking antibodies or antagonist; in which case if these results were solely due to C5aR they will be prevented by such agents; whereas if C5L2 had an involvement then incubation with the C5aR blocking peptide or antagonist would have no effect on the overall outcome.

6.4.1.4 C5a induced cytokine secretion

C5a has previously been shown to increase pro-inflammatory cytokine secretion in leukocytes (section 6.1.3.5). As MCP-1 and IL-8 have been implicated in atherosclerosis, it was investigated whether the U937 cell lines expressed these cytokines and whether C5a induced their secretion in these cells. Furthermore, it was investigated whether simvastatin could exert anti-inflammatory properties by regulating the C5a induced secretion of these pro-inflammatory cytokines, IL-8 and MCP-1.

As described in section 6.3.4.1 the undifferentiated $U937_{PM}$ cells showed an increase in IL-8 secretion following stimulation with C5a, however this was not statistically significant p=0.0519 (figure 6.3.14). This result does not correlate with the Ca^{2+} signalling results which found that these cells were responsive to C5a by raising intracellular Ca^{2+} concentrations (figure 6.3.1). However, as Chapter 5 has previously shown that these cells express very little C5aR on their cell surface (figure 5.3.1), possibly there is not enough C5aR on the cell surface to produce an increase in IL-8 secretion following C5a stimulation. Although incubation of these cells with simvastatin reduced C5a induced Ca^{2+} release, figure 6.3.14 shows that simvastatin had no effect on IL-8 secretion or C5a induced IL-8 secretion.

Upon differentiation of the $U937_{PM}$ with Bt_2cAMP the basal IL-8 secretion increased dramatically which suggests that this cAMP analogue itself can regulate IL-8 secretion either by regulating gene expression of IL-8 directly or by a secondary effect such as regulating the expression of other cytokines (figure 6.3.15). This result confirms findings from a study by Kavelaars and co-workers demonstrating that Bt_2cAMP can increase basal IL-8 and LPS induced IL-8 production in the U937 cell line (Kavelaars et al., 1997). This study suggested that transcription factors such as NFκB, c-FOS and Jun-B, which are influenced by cAMP, may mediate this IL-8 production, although they cannot rule out secondary effects by other cytokines or regulatory proteins (Kavelaars et al., 1997). This result also correlates with MMP-9 secretion which increased following incubation with Bt_2cAMP in the absence of C5a (figure 6.3.12).

Additionally our results show that stimulation of Bt_2cAMP differentiated $U937_{PM}$ cells with C5a significantly increased IL-8 secretion (figure 6.3.15). This result correlates with these cells becoming more responsive to C5a by significantly increasing their intracellular Ca^{2+} release following its addition (figure 4.3.7). Furthermore, incubation with simvastatin significantly reduced the C5a induced IL-8 secretion (figure 6.3.15), which correlates with both a decrease in C5aR expression (figure 5.3.4) and C5a induced Ca^{2+} release (figure 6.3.4). Although it was not investigated here the exact signalling pathway through which C5a mediates IL-8 secretion in the $U937_{PM}$ cell line, it has been shown by Wang et al. that it is dependent on ERK 1/2 and p38 pathway but is independent of JNK 1/2 in human whole blood (Wang et al., 2010). Furthermore, a study by Hsu and co-workers found that NFκB activation following C5a stimulation in the RAW264.7 cell line was crucial for induced IL-8 gene expression (Hsu et al., 1999).

As Chapter 5 has previously shown that incubation with simvastatin reduces the Bt_2cAMP induced C5aR expression (figure 5.3.4), it is difficult to determine whether the reduced C5a induced IL-8 secretion is caused by reduced receptor expression or reduced function of the C5aR and its signalling components, or both. The addition of mevalonate or GGPP to the incubation was able to reverse the effects of simvastatin on C5a induced IL-8

secretion, which suggests isoprenoid biosynthesis is critical for the C5a induced IL-8 secretion in these cells (figure 6.3.16). However, the addition of these agents also reversed the effects of simvastatin on Bt_2cAMP induced C5aR expression, therefore we cannot say whether these effects are due to changes in expression alone or expression and function (figure 5.3.11).

Interestingly incubation with the squalene synthesis inhibitor zaragozic acid had no effect on basal Bt_2cAMP IL-8 secretion or C5a induced IL-8 secretion (figure 6.3.17). As zaragozic acid had no effect on C5aR expression, this suggests that the C5a induced IL-8 secretion in Bt_2cAMP differentiated $U937_{PM}$ cell was not affected by inhibition of cholesterol biosynthesis. However, it is important to note here that all these experiments were carried out in the presence of 10 % FCS (except for the gelatine zymography experiments), which means that although cholesterol biosynthesis was inhibited, the cells could still take up cholesterol-LDL via the LDL receptors. Although LDLR expression has not been characterised in this study, the U937 cell line has previously been shown to express mRNA for this receptor (Hammad et al., 2009). While some studies that investigate the effects of statins or cholesterol in cell culture in the absence of serum, to remove all exogenous cholesterol, serum starvation itself can cause non-specific effects independent of cholesterol such as cell cycle growth arrest (Lundberg and Weinberg, 1999, Pardee, 1989). Furthermore, to completely remove cholesterol by inhibiting cholesterol biosynthesis and preventing uptake from the environment by removing exogenous cholesterol is non-physiological and can only hint towards biological function (Maxfield and Tabas, 2005). Although FCS could provide an exogenous source of cholesterol, previous studies have demonstrated that supplementing RPMI media with 10 % FCS contains on average 80 mg/L free cholesterol of which 30 mg/L is LDL-cholesterol (Han et al., 2005, Han et al., 1998). When this value is compared with normal cholesterolemic LDL-cholesterol values in humans, which is ideally 1000 mg/L, the actual concentration of LDL-cholesterol in our experiments is far below physiologically normal concentrations (NCEP, 2002). Therefore the benefit of supplement the media with serum, to provide the cells with the necessary growth factors, far outweigh the negligible risk of LDL-cholesterol interference from the FCS. Further still, patients receiving statin therapy still consume cholesterol in their diet. For this reason it seemed more physiological to carry out experiments in the presence of 10% FCS for our experiments.

The $U937_{C5aR}$ cells also appeared to increase IL-8 secretion following stimulation with C5a; however, this was only statistically significant with 1 μM simvastatin due to large error bars (figure 6.3.20). The data from this experiment is particularly interesting as it shows that incubation with simvastatin increases the IL-8 secretion, whereas Chapter 5 has previously shown that statins down regulate C5aR expression in these cells (figure 5.3.18). These findings, together with the results from the C5a induced intracellular Ca^{2+} release, further

suggest that not all the C5aR on the cell surface may be functional; if it were all functional then you would expect to see a significant decrease in both C5a induced Ca^{2+} and IL-8 release.

In both the undifferentiated $U937_{PM}$ and $U937_{C5aR}$ cell lines, stimulation with C5a failed to increase MCP-1 secretion and incubation with simvastatin had no effect on the basal or C5a induced MCP-1 secretion (figure 6.3.18 and 6.3.21). However, differentiation of the $U937_{PM}$ cells with Bt_2cAMP, led to C5a stimulated increase in MCP-1 secretion but this was only significant at 0.1 μM simvastatin due to large error bars (figure 6.3.19). Although the exact mechanism by which C5a may induce MCP-1 secretion in these Bt_2cAMP stimulated $U937_{PM}$ cells has not been investigated in this study, one possible mechanism could be the increase in intracellular cAMP concentration has led to the activation of protein kinase A that can in turn phosphorylate target proteins such as CREB which can then regulate expression of target genes that regulate MCP-1 secretion. Alternatively other regulatory proteins such as cytokines could be stimulated by C5a which in turn regulate MCP-1 secretion by autocrine regulation.

As cell lines have their limitations it was decided to further investigate the effects of statins and fenofibrate on the C5a induced IL-8 secretion using human PBMCs. It was decided to use PBMCs rather than purified monocytes for these experiments as it would represent a more *in vivo* situation where you would have cytokine secretion and autocrine regulation between the different cell types. However, the disadvantage is that you are unable to monitor changes in one cell type alone. As described previously in section 6.1.3.5, several studies have demonstrated that stimulation of the human PBMCs with C5a increased their IL-8 secretion (Ember et al., 1994, Vecchiarelli et al., 1998). However, in our experiments C5a failed to significantly increase IL-8 secretion (figure 6.3.22). The results shown here may differ from that published in the literature as once the PBMCs were isolated from the buffy coat they were then frozen until they were needed. This freeze thaw process may have activated the cells; therefore, these experiments could be repeated using fresh PBMCs. Not surprisingly, incubation of the PBMCs with simvastatin, lovastatin or fenofibrate did not statistically alter either basal or C5a induced IL-8 secretion (figure 6.3.22). While these results for simvastatin and lovastatin correlate with no changes in C5aR expression being observed in Chapter 5; it was anticipated that fenofibrate may lower C5a induced IL-8 secretion, as this drug was previously been shown to down regulate expression of the receptor in CD14 monocytes (Chapter 5). The differences between these experiments may be explained by the fact that the ELISAs were performed using PBMCs whereas changes in C5aR expression were only monitored in CD14 positive monocytes. Therefore it would be interesting to monitor the effects of fenofibrate incubation on IL-8 secretion in CD14 positive monocytes.

6.4.2 Sub-membrane localisation of the C5aR

As previously described in section 6.1, cholesterol enriched micro domains, or lipid rafts, play an important role in the sub-membrane compartmentalisation of GPCR and their signalling components. As membrane cholesterol has been found to be crucial for clustering and redistribution of the FPR into lipid rafts following stimulation with fMLP (Xue et al., 2004, Bath et al., 1991, Dunzendorfer et al., 1997, Wolach et al., 1992), this study aimed to investigate the sub-membrane localisation of the C5aR, which is structurally homologous to the fMLP receptor.

Using detergent extraction and sucrose gradient floatation the results presented in section 6.3.6 suggest that in Bt_2cAMP differentiated $U937_{PM}$ cells the C5aR is localised in a non-lipid raft region of the plasma membrane (figure 6.3.23). Furthermore, stimulation of the receptor with C5a had no effect on the membrane distribution (figure 6.3.24). Similarly the C5aR was also found to be localised in the non-lipid raft region of the plasma membrane in the $U937_{C5aR}$ cells, and its membrane localisation was un-affected by the addition of C5a (figure 6.3.25 and 6.3.26). These findings could suggest that membrane cholesterol is unlikely to have a direct effect of the functioning on the C5aR. However, this study has only investigated the location of the receptor itself and has not investigated the location of other signalling components in the membrane. It is also important to note that detergent extraction has it limitations due to its disruptive nature, which can in turn induce artefacts (Lingwood and Simons, 2010). This is evident when different detergents are used during the extraction process, which can lead to different fraction composition being produced following sucrose floatation (Brown and Rose, 1992). Brij58 used in these studies, has a mild detergent action and was the only detergent that allowed the discrimination between GPI-anchored raft associated CD59 and non-raft associated molecules such as a transmembrane anchored form of CD59 under conditions used in this study (van den Berg et al., 1995, Hiscox et al., 2002) and unpublished data).

Although this is the first study to investigate whether the C5aR is present within a lipid raft region or is redistributed in or out of such regions following C5a stimulation, other research groups have found that the C5aR clusters following stimulation and is then internalised via β–arrestin, dynamin and clathrin-dependent pathway (Licht et al., 2003, Braun et al., 2003, Suvorova et al., 2005, Huttenrauch et al., 2005). As the results in this study suggest that the C5aR is located in a non-lipid raft region and is not distributed into such region following stimulation with C5a, this provides further evidence that C5aR is endocytosed via a clathrin-dependent pathway as this pathway is lipid raft independent (Lajoie and Nabi, 2010).

6.4.3 Summary

This chapter shows that incubation with simvastatin was able to reduce C5a induced Ca^{2+} release and IL-8 secretion, had no effect on the C5a induced MCP-1 secretion but increased C5a induced MMP-9 secretion in the Bt_2cAMP differentiated $U937_{PM}$ cell line. We were unable to detect C5a induced F-actin assembly, an indicator for chemotaxis in our cells. As Chapter 5 has previously shown that simvastatin was able to reduce C5aR expression in these cells, this makes it difficult to determine whether the effects observed by simvastatin in this chapter are due solely to reduced receptor expression or a combination of reduced expression and reduced function.

Although incubation of the $U937_{C5aR}$ cell line with simvastatin was previously shown to reduce C5aR expression (Chapter 5) here we found that incubation of these cells with simvastatin had no effect on C5a induced Ca^{2+}, MMP-2 or MMP-9 and IL-8 or MCP-1 secretion. This would suggest that not all the C5aR expressed on these cells is fully functional. Results from PBMCs show that incubation with simvastatin or lovastatin had no effect on the C5a induced IL-8 secretion in an *ex vivo* situation which correlated with unchanged C5aR expression in these cells, Chapter 5. Despite C5aR expression being reduced by fenofibrate in CD14 positive monocytes (Chapter 5), here it was shown that this drug had no effect on the C5a induced IL-8 release. However, these differences could be attributed to PBMCs being used for the IL-8 ELISAs as opposed to a pure monocytic population.

Results from detergent extraction and sucrose gradient floatation suggest that the C5aR is unlikely to be associated with lipid raft regions; neither does it move in or out of such areas following stimulation with its ligand, C5a.

Chapter 7

General conclusion

Chapter 7

General conclusion

7.1 Summary of findings and conclusions

Complement activation leads to the release of the potent pro-inflammatory anaphylatoxin C5a, which exerts its biological actions via the C5aR, a GPCR. Over expression or under regulation of C5a and the C5aR has been implicated in a variety of pathologies, including atherosclerosis and ischemic reperfusion injury. Cholesterol is an integral component of biological membranes and as well as regulating membrane fluidity, it can affect the function and expression of numerous GPCR. Statin therapy is widely used to reduce circulating serum cholesterol levels, a major risk factor in coronary heart disease.

The principal aim of this thesis was to determine if the C5aR expression and function can be modulated by cholesterol. It was hypothesised that statins can exert anti-inflammatory actions via the C5aR by:

- **Down regulating C5aR expression.** Statins may regulate gene transcription of the C5aR via transcription factors such as NFκB or PPARs, as is the case for MCP-1 and its receptor CCR2 (Veillard et al., 2006, Han et al., 2005, Chen et al., 2005, Yin et al., 2007). Alternatively statins may regulate its mRNA stability, as is the case for DAF and eNOS (Mason et al., 2002, Laufs et al., 1998, Laufs and Liao, 1998). Furthermore membrane cholesterol content may also affect cell surface C5aR expression by regulating receptor internalisation via lipid raft dependent endocytosis following interaction with its ligand.
- **Reducing functioning of the C5aR.** Statins may alter C5aR function by affecting the membrane sub-compartmentalisation of the receptor into lipid rafts, as is the case for the fMLP receptor whose membrane polarisation and chemotaxis towards its ligand is reliant on membrane cholesterol content (Bath et al., 1991, Dunzendorfer et al., 1997, Wolach et al., 1992). Alternatively the structural properties of the C5aR may be regulated by membrane cholesterol content, as is the case for the rhodopsin receptor whose activation is restricted in a high cholesterol environment due to the rigidification of the membrane (Albert and Boesze-Battaglia, 2005, Albert et al., 1996). Statins may further reduce the signalling capacity of the C5aR by cholesterol independent mechanisms affecting the isoprenylation of small GTP-binding proteins involved in down stream signalling cascades.

The findings of this thesis have provided data in several areas:

1. Regulation of basal C5aR expression in the U937 cell; the role of -2Kbp promoter region in transcriptional regulation of the gene and the effects of the 3'UTR on mRNA stability (Chapter 3).
2. Characterisation of reported inducers of C5aR expression in the U937 cell line; the identification and characterisation of two U937 cell lines (Chapter 4).
3. Characterisation of the effects of statins on C5aR expression; inhibition of Bt_2cAMP induced C5aR expression in the U937 cell line by simvastatin, although statins had no effect on basal expression in this cell line or human PBMCs (Chapter 5).
4. Effects of statins on the biological function of the C5aR; simvastatin significantly reduced C5a induced Ca^{2+} release and IL-8 secretion in Bt_2cAMP differentiated $U937_{PM}$ cell line, although statins had no effect on C5a induced IL-8 secretion in human PBMCs (Chapter 6).
5. The sub-membrane localisation of the C5aR; the receptor does not reside in lipid raft regions of the plasma membrane, neither does it move in or out of such regions following stimulation with C5a, as observed by sucrose gradient floatation (Chapter 6).

As very little was previously known about regulation of the human C5aR expression, it was decided to first investigate how basal C5aR expression was regulated. Using the U937 cell line, it was shown that the majority of the -2Kbp promoter region cloned was dispensable for transcriptional activity of the human C5aR (Chapter 3), which is similar to results already observed with the mouse C5aR promoter region (Hunt et al., 2005). Using site directed mutagenesis the role of three putative promoter elements, NFκB (-238bp to -232bp), CCAAT (-123bp to -119bp) and NFAT (-93bp to -87bp), were investigated. In line with results already published from the mouse C5aR promoter, it was shown that the CCAAT motif is important for the transcriptional control of the human C5aR promoter, as both its mutation and deletion resulted in a reduction in promoter activity (Hunt et al., 2005). It was also believed that the novel putative NFAT site was identified as critical for the transcriptional control of the human C5aR promoter; however these results are still inconclusive (Chapter 3). Furthermore, site directed mutagenesis of the putative NFκB site resulted in a reduction in promoter activity albeit to a lesser extent than the putative CCAAT or NFAT sites (Chapter 3). As statins have previously been shown to regulate expression of several genes, including MCP-1, by regulating activity of NFκB (Veillard et al., 2006, Ortego et al., 1999), it was anticipated that this putative site may facilitate a mechanism by which statins could alter C5aR expression. However, it was later shown that simvastatin had no effect on the basal C5aR expression in the U937 cell line (Chapter 5). Similarly, C5aR expression in human

PBMCs remained unchanged following incubation with lovastatin or simvastatin (Chapter 5). However, incubation with fenofibrate caused a significant reduction in C5aR expression in these cells (Chapter 5). Fenofibrate can activate transcription factors belonging to the PPARs family. Although statins have also been demonstrated to activate PPARα and PPARγ which results in reduced IL-1β, TNFα, CCR5 and CCR2 expression (Inoue et al., 2000, Zhou and Liao, 2010, Han et al., 2005, Chen et al., 2005, Yin et al., 2007), this result suggests that the C5aR expression may be regulated by fenofibrate via activation of PPARs through a mechanism that was not investigated in the U937 cell line.

As well as transcriptional regulation, this thesis also investigated regulation of mRNA stability. Site directed mutagenesis of the two ARE within the C5aR 3'UTR had no effect on mRNA stability, suggesting that unlike DAF (Gray et al., 2010), C5aR expression is not regulated by the AREs present within the 3'UTR (Chapter 3). Not surprisingly incubation of the 3'UTR reporter constructs with simvastatin had no significant effect on EGFP expression, suggesting that unlike eNOS (Laufs et al., 1998, Laufs and Liao, 1998), the C5aR mRNA stability is not affected by statins (Chapter 5).

To investigate whether statins would have any effect on induced receptor expression, the C5aR expression pattern in the U937 cell line following stimulation with various agents (including IFNγ, IL-6, LPS, TNFα, PMA and Bt_2cAMP) was characterised (Chapter 4). Although all of these stimuli had previously been reported in the literature to increase C5aR expression (Chapter 1, table 1.2 for full details), we found that only the artificial stimuli PMA and Bt_2cAMP were able to increase C5aR expression and this was only achieved in one, $U937_{PM}$, of the two U937 cell lines obtained for this research project (Chapter 4). Furthermore it was shown that although IL-6 and IFNγ appeared to increase C5aR expression via FACS analysis, this apparent increase was an artefact and due to non-specific binding of the antibody to Fcγ receptors (Chapter 4).

Although statins were unable to regulate basal C5aR expression, this thesis has found that simvastatin prevents the Bt_2cAMP differentiation and up-regulation of the C5aR in the $U937_{PM}$ cell line and this effect was mediated by the inhibition of isoprenoid biosynthesis (Chapter 5). However, the concentration of statin needed to obtain this effect was more than ten times the pharmacological concentration of simvastatin and at this concentration it was shown that simvastatin significantly reduced cell proliferation by inhibition of isoprenylation possibly involving cell growth regulating proteins (Chapter 5).

Other mechanisms by which simvastatin may regulate Bt_2cAMP differentiation and up-regulation of the C5aR possibly involve PKA and its down stream signalling components or via Epac, a guanine nucleotide exchange factor, which in turn regulates small GTP binding proteins. However the exact mechanism was not investigated here.

Using the -2Kbp promoter, isolated from $U937_{PM}$ cells, and the 3'UTR EGFP reporter constructs, it was shown that there were no *cis*-actin elements responsible for the Bt_2cAMP induced C5aR expression within these regions (Chapter 4). However the -2Kbp promoter region isolated from the $U937_{EC}$ cells contained a Bt_2cAMP responsive element when transfected into the $U937_{PM}$ cells (Chapter 4). A possible explanation for the differences between these promoters could be explained possibly by 4 SNPs identified between the sequences. However further studies are needed to confirm that one or more of these SNPs are responsible for these differences.

To investigate further whether statins had any effect on the functioning of the C5aR, this thesis monitored changes in the biological activities of the receptor. Although it was shown that incubation with simvastatin reduced the C5a induced Ca^{2+} release and IL-8 secretion in the Bt_2cAMP differentiated $U937_{PM}$ cell line, we were unable to determine whether this was due to changes in C5aR expression or both expression and function (Chapter 6). Similarly to changes in expression, it was shown that the reduced IL-8 secretion caused by simvastatin was due to inhibition of isoprenoid biosynthesis (Chapter 5 and 6).

This thesis has shown that C5a was able to induce MMP-9 secretion in Bt_2cAMP differentiated $U937_{PM}$ cell line (Chapter 6), which correlates with recent results published by Speidl et al. who found that differentiated human monocytes are able to secret MMP-9 and also MMP-1 following stimulation with C5a (Speidl et al., 2011). Interestingly, it was shown that the C5a induced MMP-9 secretion, in the Bt_2cAMP differentiated $U937_{PM}$ cells, increased following incubation with simvastatin (Chapter 6). This finding appears to be the first reported result that shows statins, which improve plaque stability by reducing MMP secretion (Aikawa et al., 2001, Fukumoto et al., 2001, Fujimoto et al., 2008, Sundararaj et al., 2008), increasing MMP secretion (Chapter 6).

As expression of the C5aR was altered by simvastatin in the Bt_2cAMP differentiated $U937_{PM}$ cell line, this thesis investigated the effects of simvastatin on function and expression of the C5aR in a U937 cell line previously transfected with the C5aR (Kew et al., 1997). Although the majority of the expressed C5aR is not under the transcriptional control of the C5aR promoter, incubation with simvastatin significantly reduced the receptor expression in these cells (Chapter 5). Furthermore, this reduction appeared to be due to inhibition of isoprenoid biosynthesis (Chapter 5). Interestingly, it was shown that simvastatin did not alter C5a induced Ca^{2+} release, MMP-2 or MMP-9 and IL-8 or MCP-1 secretion in these cells suggesting that not all expressed C5aR is fully functional (Chapter 6). This thesis also showed that incubation of PBMCs with simvastatin, lovastatin or fenofibrate had no effect on the biological functioning of the receptor, as the C5a induced IL-8 secretion remained unchanged (Chapter 6).

This thesis also investigated, for the first time, the membrane sub-localisation of the C5aR. Results from sucrose gradient floatation suggest that the receptor is unlikely to reside in a lipid raft region of the plasma membrane, neither does it move in or out of such regions following stimulation with C5a (Chapter 6). The finding that the C5aR does not reside within a cholesterol rich region of the plasma membrane makes it less likely that the receptor function would be regulated by membrane cholesterol content and thus affected by statins via regulation of cholesterol within the membrane.

7.2 Future experiments

The work carried out in the course of completing this thesis has led to a number of questions that could be answered by further experimental work: For instance what is the exact mechanism by which statins prevent the Bt_2cAMP differentiation and up-regulation of the C5aR? It would also be interesting to investigate whether statins could prevent the differentiation of human primary monocytes to macrophages using more physiologically relevant stimuli. It would also be interesting to monitor the effects of statin therapy on C5aR expression in an *in vivo* situation using PBMCs isolated from patients before and after statin therapy. Furthermore, it would be interesting to investigate how fenofibrate was able to reduce C5aR expression in the human monocytes; as well as investigating whether fenofibrate is able to regulate the receptor expression in the $U937_{PM}$ cell line.

As it was shown that incubation of simvastatin increased the C5a induced MMP-9 secretion in the Bt_2cAMP differentiated $U937_{PM}$ cell line; it would be extremely interesting to investigate the mechanism by which this is achieved.

Furthermore, it would be interesting to investigate what effects, in any, cholesterol and statins may have on the expression of the second C5a receptor, C5L2. This would be particularly interesting as it has been suggested that in humans C5L2 acts as a negative regulator of C5a by limiting its bioavailability to bind to the C5aR.

7.4 Concluding remarks

Unlike other GPCRs whose function and expression can be regulated by cholesterol and therefore statin therapy, this thesis has found that statins had little effect on basal C5aR expression in human monocytes; whereas incubation with simvastatin reduced the Bt_2cAMP induced differentiation and up-regulation of the C5aR in the $U937_{PM}$ cell line. Although a reduced C5a induced Ca^{2+} release and IL-8 secretion function was also observed in these cells we were unable to determine whether this reduction was solely due to reduced expression or reduced expression and function. Either way the effects of simvastatin appear to be due to inhibition of isoprenoid biosynthesis rather than inhibition of cholesterol biosynthesis. Furthermore sucrose gradient floatation suggests that the C5aR residues in a

non-lipid raft region of the plasma membrane therefore making it less likely to be susceptible to membrane cholesterol content.

Chapter 8

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Chapter 9

Appendix

9.1 Alignment of the U937$_{ECACC}$ C5aR 5' deletion promoter sequences.

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                    1                                                  50
  100bp EC  (-100) --------------------------------------------------
  200bp EC  (-200) --------------------------------------------------
  355bp EC  (-355) --------------------------------------------------
  500bp EC  (-506) --------------------------------------------------
   1kbp EC  (-994) --------------------------------------------------
 1.5kbp EC (-1529) --------------------------------------------------
   2kbp EC (-2037) CCCTTCGCTCATATTACCGCCTGATCTGTCAGATCAGGGGCGGAATTACA
 GeneBank  (-2035) CCCTTCGCTCATATTACCGCCTGATCTGTCAGATCAGGGGCGGAATTACA
                   51                                                 100
  100bp EC  (-100) --------------------------------------------------
  200bp EC  (-200) --------------------------------------------------
  355bp EC  (-355) --------------------------------------------------
  500bp EC  (-506) --------------------------------------------------
   1kbp EC  (-994) --------------------------------------------------
 1.5kbp EC (-1529) --------------------------------------------------
   2kbp EC (-1987) TTCTCATAAGAGCGTGAACCCAGGCCGGGCGCAGTGGCTCAAGCCTGTAA
 GeneBank  (-1985) TTCTCATAAGAGCGTGAACCCAGGCCGGGCGCAGTGGCTCAAGCCTGTAA
                   101                                                150
  100bp EC  (-100) --------------------------------------------------
  200bp EC  (-200) --------------------------------------------------
  355bp EC  (-355) --------------------------------------------------
  500bp EC  (-506) --------------------------------------------------
   1kbp EC  (-994) --------------------------------------------------
 1.5kbp EC (-1529) --------------------------------------------------
   2kbp EC (-1937) TCCCAGCATTTTGGGAGGCTGAGGCGGGTGGATCACGAGGTCAGGAGTTC
 GeneBank  (-1935) TCCCAGCATTTTGGGAGGCTGAGGCGGGTGGATCACGAGGTCAGGAGTTC
                   151                                                200
  100bp EC  (-100) --------------------------------------------------
  200bp EC  (-200) --------------------------------------------------
  355bp EC  (-355) --------------------------------------------------
  500bp EC  (-506) --------------------------------------------------
   1kbp EC  (-994) --------------------------------------------------
 1.5kbp EC (-1529) --------------------------------------------------
   2kbp EC (-1887) GAGATCAGCCTGGCCAAGATGGTGAAACCATGTCTCTACTAAAAA▌TACA
 GeneBank  (-1885) GAGATCAGCCTGGCCAAGATGGTGAAACCACGTCTCTACTAAAAA▌TACA
                   201                                                250
  100bp EC  (-100) --------------------------------------------------
  200bp EC  (-200) --------------------------------------------------
  355bp EC  (-355) --------------------------------------------------
  500bp EC  (-506) --------------------------------------------------
   1kbp EC  (-994) --------------------------------------------------
 1.5kbp EC (-1529) --------------------------------------------------
   2kbp EC (-1837) AAAATTAGCCGGGCGCAGTGGTAGGCACCTGTAGTCCCAGCTACTCGGGA
 GeneBank  (-1836) AAAATTAGCCGGGTGCAGTGGTAGGCACCTGTAGTCCCAGCTACTCGGGA
                   251                                                300
  100bp EC  (-100) --------------------------------------------------
  200bp EC  (-200) --------------------------------------------------
  355bp EC  (-355) --------------------------------------------------
  500bp EC  (-506) --------------------------------------------------
   1kbp EC  (-994) --------------------------------------------------
 1.5kbp EC (-1529) --------------------------------------------------
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  2kbp EC (-1787) GGCTGAGGCAGGAGAATCGCTTGAACCTGGGAGGCGGAGGTTGCAGTGTG
 GeneBank (-1786) GGCTGAGGCAGGAGAATCGCTTGAACCTGGGAGGCGGAGGTTGCAGTGTG
                  301                                                 350
 100bp EC  (-100) --------------------------------------------------
 200bp EC  (-200) --------------------------------------------------
 355bp EC  (-355) --------------------------------------------------
 500bp EC  (-506) --------------------------------------------------
  1kbp EC  (-994) --------------------------------------------------
1.5kbp EC (-1529) --------------------------------------------------
  2kbp EC (-1737) CCGAGATCGTGCCACTGCACTCTAGCCTGGGTGAGAGAGTGAGACTCCAT
 GeneBank (-1736) CCGAGATCGTGCCACTGCACTCTAGCCTGGGTGAGAGAGTGAGACTCCAT
                  351                                                 400
 100bp EC  (-100) --------------------------------------------------
 200bp EC  (-200) --------------------------------------------------
 355bp EC  (-355) --------------------------------------------------
 500bp EC  (-506) --------------------------------------------------
  1kbp EC  (-994) --------------------------------------------------
1.5kbp EC (-1529) --------------------------------------------------
  2kbp EC (-1687) CTCAAAAAAAAAAAAAAAAAAAAAAAGAGCATGAACCCTGTTGTGAACAG
 GeneBank (-1686) CTCAAAAAAAAAAAAAAAAAAAAAAAGAGCATGAACCCTGTTGTGAACAG
                  401                                                 450
 100bp EC  (-100) --------------------------------------------------
 200bp EC  (-200) --------------------------------------------------
 355bp EC  (-355) --------------------------------------------------
 500bp EC  (-506) --------------------------------------------------
  1kbp EC  (-994) --------------------------------------------------
1.5kbp EC (-1529) --------------------------------------------------
  2kbp EC (-1637) CGCATGTGAGGGATCTGGGTTGCAAGCTCCTTATGAGAATCTAATGCCTG
 GeneBank (-1636) CGCATGTGAGGGATCTGGGTTGCAAGCTCCTTATGAGAATCTAATGCCTG
                  451                                                 500
 100bp EC  (-100) --------------------------------------------------
 200bp EC  (-200) --------------------------------------------------
 355bp EC  (-355) --------------------------------------------------
 500bp EC  (-506) --------------------------------------------------
  1kbp EC  (-994) --------------------------------------------------
1.5kbp EC (-1529) --------------------------------------------------
  2kbp EC (-1587) ATGATCTGTCACTGTATCCCATCACCCCCAGATAGGACCATCTCGTTACA
 GeneBank (-1586) ATGATCTGTCACTGTATCCCATCACCCCCAGATAGGACCATCTCGTTACA
                  501                                                 550
 100bp EC  (-100) --------------------------------------------------
 200bp EC  (-200) --------------------------------------------------
 355bp EC  (-355) --------------------------------------------------
 500bp EC  (-506) --------------------------------------------------
  1kbp EC  (-994) --------------------------------------------------
1.5kbp EC (-1529) -----------TCAGGGATTCCACTGATTCTACATTATGTTGAATTGTAT
  2kbp EC (-1537) GGAAAACAAGCTCAGGGATTCCACTGATTCTACATTATGTTGAATTGTAT
 GeneBank (-1536) GGAAAACAAGCTCAGGGATTCCACTGATTCTACATTATGTTGAATTGTAT
                  551                                                 600
 100bp EC  (-100) --------------------------------------------------
 200bp EC  (-200) --------------------------------------------------
 355bp EC  (-355) --------------------------------------------------
 500bp EC  (-506) --------------------------------------------------
  1kbp EC  (-994) --------------------------------------------------
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  2kbp EC (-1487) AATTATTTCATTATATATTACAATGTAACAATAATAGATGGAGATGGTTG
 GeneBank (-1486) AATTATTTCATTATATATTACAATGTAACAATAATAGATGGAGATGGTTG
                  601                                                 650
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 GeneBank (-1436) TGGCTTGTGTAGTTGGGGGACAGGGTAGCTCCATTTTCTATTGCCAACTT
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 500bp EC  (-506) ----------------------------------------------------
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 GeneBank (-1386) TATAACCCTAGAAGGATGATTTTTTTCTTTTCTTTTCTTTTTCTTTTCTT
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 100bp EC  (-100) ----------------------------------------------------
 200bp EC  (-200) ----------------------------------------------------
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 500bp EC  (-506) ----------------------------------------------------
  1kbp EC  (-994) ----------------------------------------------------
1.5kbp EC (-1340) TTTTTTTTTTTTTGAGATTGAGTCTTGGTGTTGTCAGCCTGGGCTGGAGTG
  2kbp EC (-1337) TTTTTTTTTTTTTGAGATTGAGTCTTGGTGTTGTCAGCCTGGGCTGGAGTG
 GeneBank (-1336) TTTTTTTTTTTTTGAGATTGAGTCTTGGTGTTGTCAGCCTGGGCTGGAGTG
                  751                                             800
 100bp EC  (-100) ----------------------------------------------------
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 355bp EC  (-355) ----------------------------------------------------
 500bp EC  (-506) ----------------------------------------------------
  1kbp EC  (-994) ----------------------------------------------------
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  2kbp EC (-1287) CAATGGTGCAATCTCTGCTCACTGCAACCTCCGCCTCCTGGGTTCCAGCA
 GeneBank (-1286) CAATGGTGCAATCTCTGCTCACTGCAACCTCCGCCTCCTGGGTTCCAGCA
                  801                                             850
 100bp EC  (-100) ----------------------------------------------------
 200bp EC  (-200) ----------------------------------------------------
 355bp EC  (-355) ----------------------------------------------------
 500bp EC  (-506) ----------------------------------------------------
  1kbp EC  (-994) ----------------------------------------------------
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 GeneBank (-1236) ATTCTCCTGCCTCAGCCTTCTGAGTAGCTGAGATTACAGGCACCCACCAC
                  851                                             900
 100bp EC  (-100) ----------------------------------------------------
 200bp EC  (-200) ----------------------------------------------------
 355bp EC  (-355) ----------------------------------------------------
 500bp EC  (-506) ----------------------------------------------------
  1kbp EC  (-994) ----------------------------------------------------
1.5kbp EC (-1190) CACGCCTGGCTAATTTTTGTATTTTTAGTAGAGATGGGGTTTCACCATAT
  2kbp EC (-1187) CACACCTGGCTAATTTTTGTATTTTTAGTAGAGATGGGGTTTCACCATAT
 GeneBank (-1186) CACGCCTGGCTAATTTTTGTATTTTTAGTAGAGATGGGGTTTCACCATAT
                  901                                             950
 100bp EC  (-100) ----------------------------------------------------
 200bp EC  (-200) ----------------------------------------------------
 355bp EC  (-355) ----------------------------------------------------
 500bp EC  (-506) ----------------------------------------------------
  1kbp EC  (-994) ----------------------------------------------------
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  2kbp EC (-1137) TGGCCAGGCTGGTGACGATGGTTTTTTTTTTTT--GAGACAGAGTCTTGC
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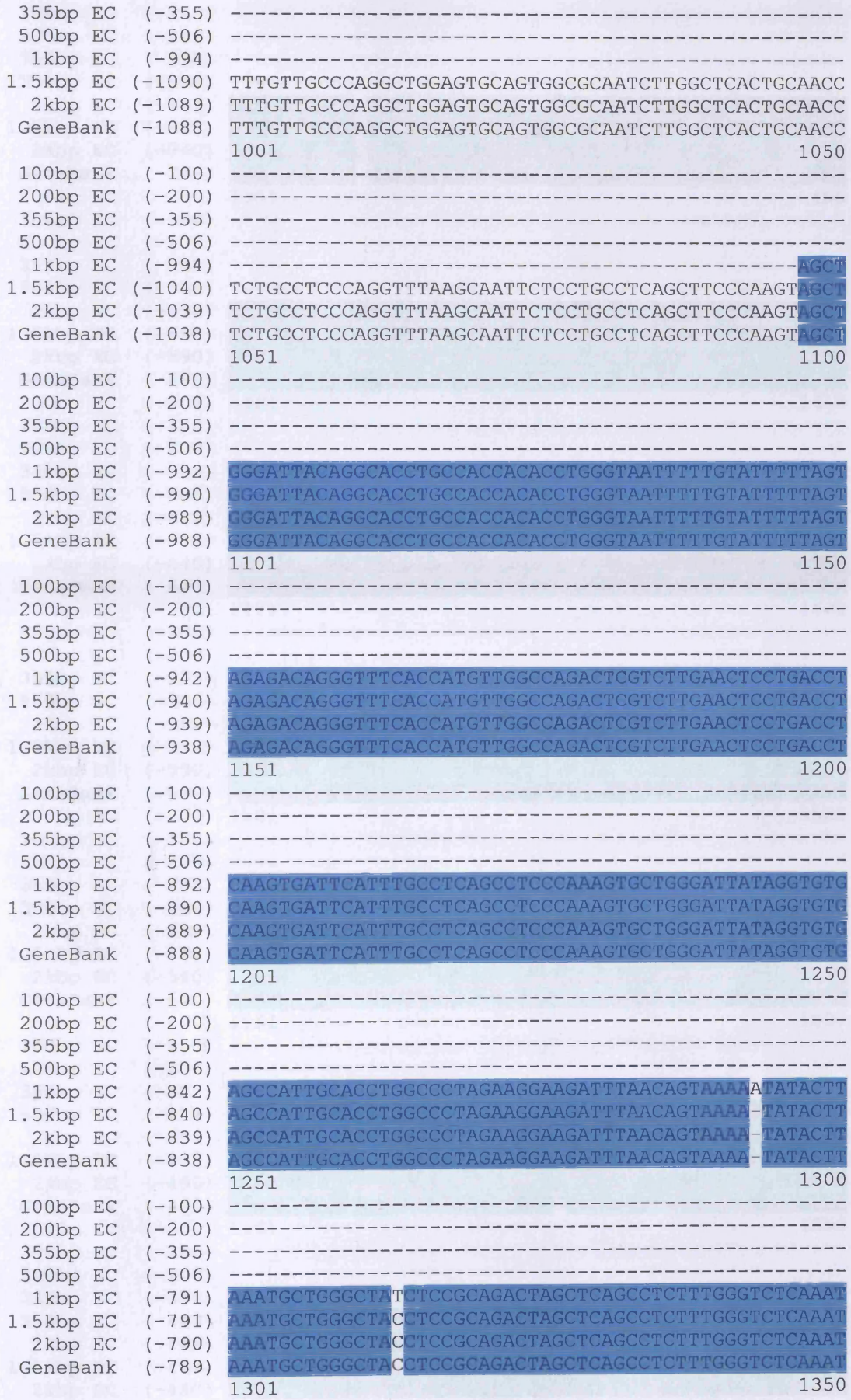
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GeneBank (-1088) TTTGTTGCCCAGGCTGGAGTGCAGTGGCGCAATCTTGGCTCACTGCAACC
1001
1050
100bp EC (-100)
200bp EC (-200)
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1.5kbp EC (-1040) TCTGCCTCCCAGGTTTAAGCAATTCTCCTGCCTCAGCTTCCCAAGTAGCT
2kbp EC (-1039) TCTGCCTCCCAGGTTTAAGCAATTCTCCTGCCTCAGCTTCCCAAGTAGCT
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1051
1100
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1101
1150
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500bp EC (-506)
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1151
1200
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355bp EC (-355)
500bp EC (-506)
1kbp EC (-892) CAAGTGATTCATTTGCCTCAGCCTCCCAAAGTGCTGGGATTATAGGTGTG
1.5kbp EC (-890) CAAGTGATTCATTTGCCTCAGCCTCCCAAAGTGCTGGGATTATAGGTGTG
2kbp EC (-889) CAAGTGATTCATTTGCCTCAGCCTCCCAAAGTGCTGGGATTATAGGTGTG
GeneBank (-888) CAAGTGATTCATTTGCCTCAGCCTCCCAAAGTGCTGGGATTATAGGTGTG
1201
1250
100bp EC (-100)
200bp EC (-200)
355bp EC (-355)
500bp EC (-506)
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1.5kbp EC (-840) AGCCATTGCACCTGGCCCTAGAAGGAAGATTTAACAGTAAAA-TATACTT
2kbp EC (-839) AGCCATTGCACCTGGCCCTAGAAGGAAGATTTAACAGTAAAA-TATACTT
GeneBank (-838) AGCCATTGCACCTGGCCCTAGAAGGAAGATTTAACAGTAAAA-TATACTT
1251
1300
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200bp EC (-200)
355bp EC (-355)
500bp EC (-506)
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1.5kbp EC (-791) AAATGCTGGGCTACCTCCGCAGACTAGCTCAGCCTCTTTGGGTCTCAAAT
2kbp EC (-790) AAATGCTGGGCTACCTCCGCAGACTAGCTCAGCCTCTTTGGGTCTCAAAT
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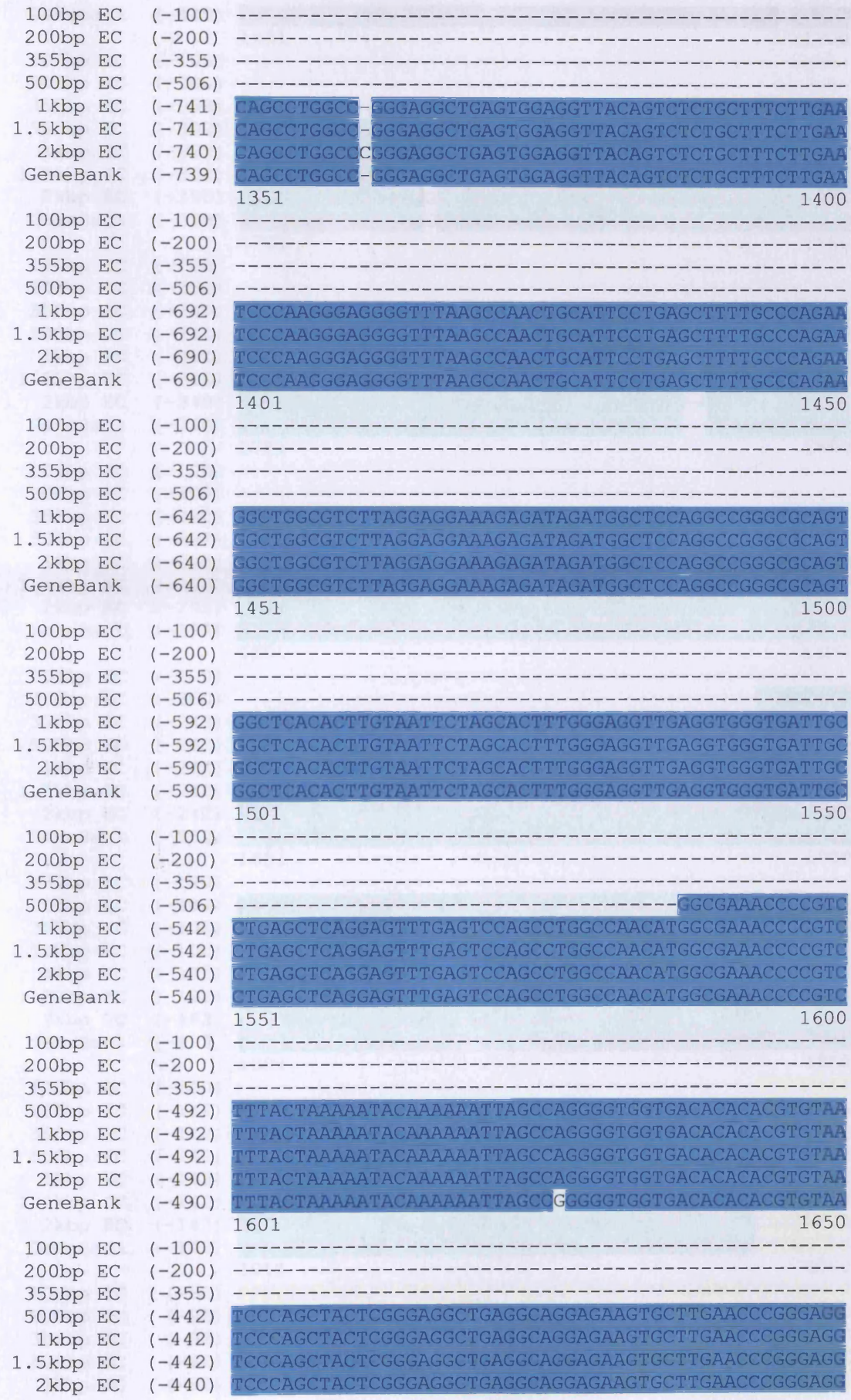
1301
1350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1.5kbp EC (-741) CAGCCTGGCC-GGGAGGCTGAGTGGAGGTTACAGTCTCTGCTTTCTTGAA
2kbp EC (-740) CAGCCTGGCCCGGGAGGCTGAGTGGAGGTTACAGTCTCTGCTTTCTTGAA
GeneBank (-739) CAGCCTGGCC-GGGAGGCTGAGTGGAGGTTACAGTCTCTGCTTTCTTGAA
1351 1400
100bp EC (-100)
200bp EC (-200)
355bp EC (-355)
500bp EC (-506)
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1.5kbp EC (-692) TCCCAAGGGAGGGGTTTAAGCCAACTGCATTCCTGAGCTTTTGCCCAGAA
2kbp EC (-690) TCCCAAGGGAGGGGTTTAAGCCAACTGCATTCCTGAGCTTTTGCCCAGAA
GeneBank (-690) TCCCAAGGGAGGGGTTTAAGCCAACTGCATTCCTGAGCTTTTGCCCAGAA
1401 1450
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200bp EC (-200)
355bp EC (-355)
500bp EC (-506)
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1.5kbp EC (-642) GGCTGGCGTCTTAGGAGGAAAGAGATAGATGGCTCCAGGCCGGGCGCAGT
2kbp EC (-640) GGCTGGCGTCTTAGGAGGAAAGAGATAGATGGCTCCAGGCCGGGCGCAGT
GeneBank (-640) GGCTGGCGTCTTAGGAGGAAAGAGATAGATGGCTCCAGGCCGGGCGCAGT
1451 1500
100bp EC (-100)
200bp EC (-200)
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500bp EC (-506)
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1.5kbp EC (-592) GGCTCACACTTGTAATTCTAGCACTTTGGGAGGTTGAGGTGGGTGATTGC
2kbp EC (-590) GGCTCACACTTGTAATTCTAGCACTTTGGGAGGTTGAGGTGGGTGATTGC
GeneBank (-590) GGCTCACACTTGTAATTCTAGCACTTTGGGAGGTTGAGGTGGGTGATTGC
1501 1550
100bp EC (-100)
200bp EC (-200)
355bp EC (-355)
500bp EC (-506) ----------GGCGAAACCCCGTC
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1.5kbp EC (-542) CTGAGCTCAGGAGTTTGAGTCCAGCCTGGCCAACATGGCGAAACCCCGTC
2kbp EC (-540) CTGAGCTCAGGAGTTTGAGTCCAGCCTGGCCAACATGGCGAAACCCCGTC
GeneBank (-540) CTGAGCTCAGGAGTTTGAGTCCAGCCTGGCCAACATGGCGAAACCCCGTC
1551 1600
100bp EC (-100)
200bp EC (-200)
355bp EC (-355)
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1.5kbp EC (-492) TTTACTAAAAATACAAAAAATTAGCCAGGGGTGGTGACACACACGTGTAA
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1601 1650
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1kbp EC (-442) TCCCAGCTACTCGGGAGGCTGAGGCAGGAGAAGTGCTTGAACCCGGGAGG
1.5kbp EC (-442) TCCCAGCTACTCGGGAGGCTGAGGCAGGAGAAGTGCTTGAACCCGGGAGG
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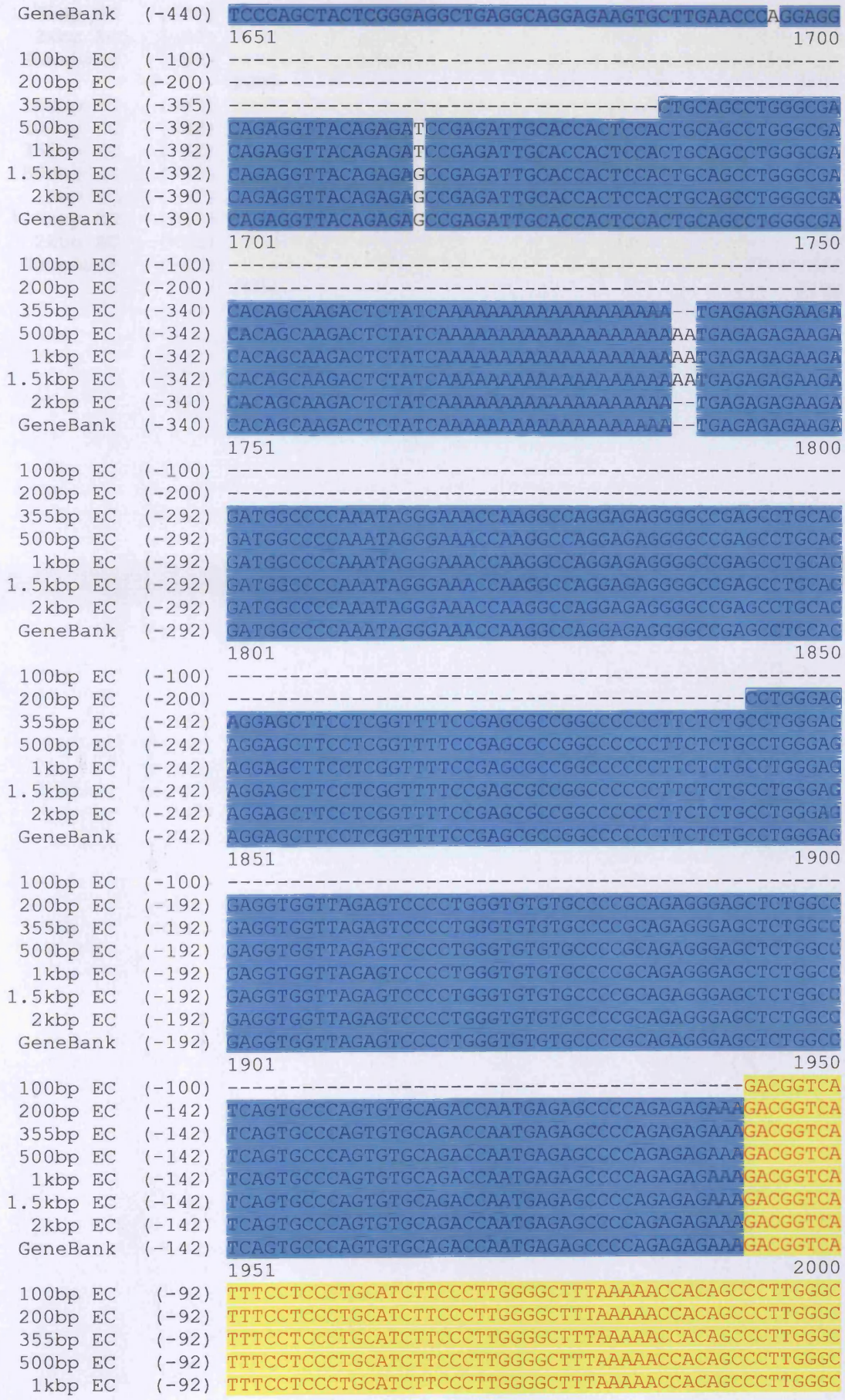
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1701 1750
100bp EC (-100)
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2kbp EC (-340) CACAGCAAGACTCTATCAAAAAAAAAAAAAAAAAAA--TGAGAGAGAAGA
GeneBank (-340) CACAGCAAGACTCTATCAAAAAAAAAAAAAAAAAAA--TGAGAGAGAAGA
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1801 1850
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500bp EC (-242) AGGAGCTTCCTCGGTTTTCCGAGCGCCGGCCCCCCTTCTCTGCCTGGGAG
1kbp EC (-242) AGGAGCTTCCTCGGTTTTCCGAGCGCCGGCCCCCCTTCTCTGCCTGGGAG
1.5kbp EC (-242) AGGAGCTTCCTCGGTTTTCCGAGCGCCGGCCCCCCTTCTCTGCCTGGGAG
2kbp EC (-242) AGGAGCTTCCTCGGTTTTCCGAGCGCCGGCCCCCCTTCTCTGCCTGGGAG
GeneBank (-242) AGGAGCTTCCTCGGTTTTCCGAGCGCCGGCCCCCCTTCTCTGCCTGGGAG
1851 1900
100bp EC (-100)
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500bp EC (-192) GAGGTGGTTAGAGTCCCCTGGGTGTGTGCCCCGCAGAGGGAGCTCTGGCC
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1901 1950
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1951 2000
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1kbp EC (-92) TTTCCTCCCTGCATCTTCCCTTGGGGCTTTAAAAACCACAGCCCTTGGGC

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 GeneBank    (-92) TTTCCTCCCTGCATCTTCCCTTGGGGCTTTAAAAACCACAGCCCTTGGGC
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   1kbp EC   (-42) AGGAGGGACCTTCGATCCTCGGGGAGCCCAGGAGACCAGAAC--------
 1.5kbp EC   (-42) AGGAGGGACCTTCGATCCTCGGGGAGCCCAGGAGACCAGAAC--------
   2kbp EC   (-42) AGGAGGGACCTTCGATCCTCGGGGAGCCCAGGAGACCAGAAC--------
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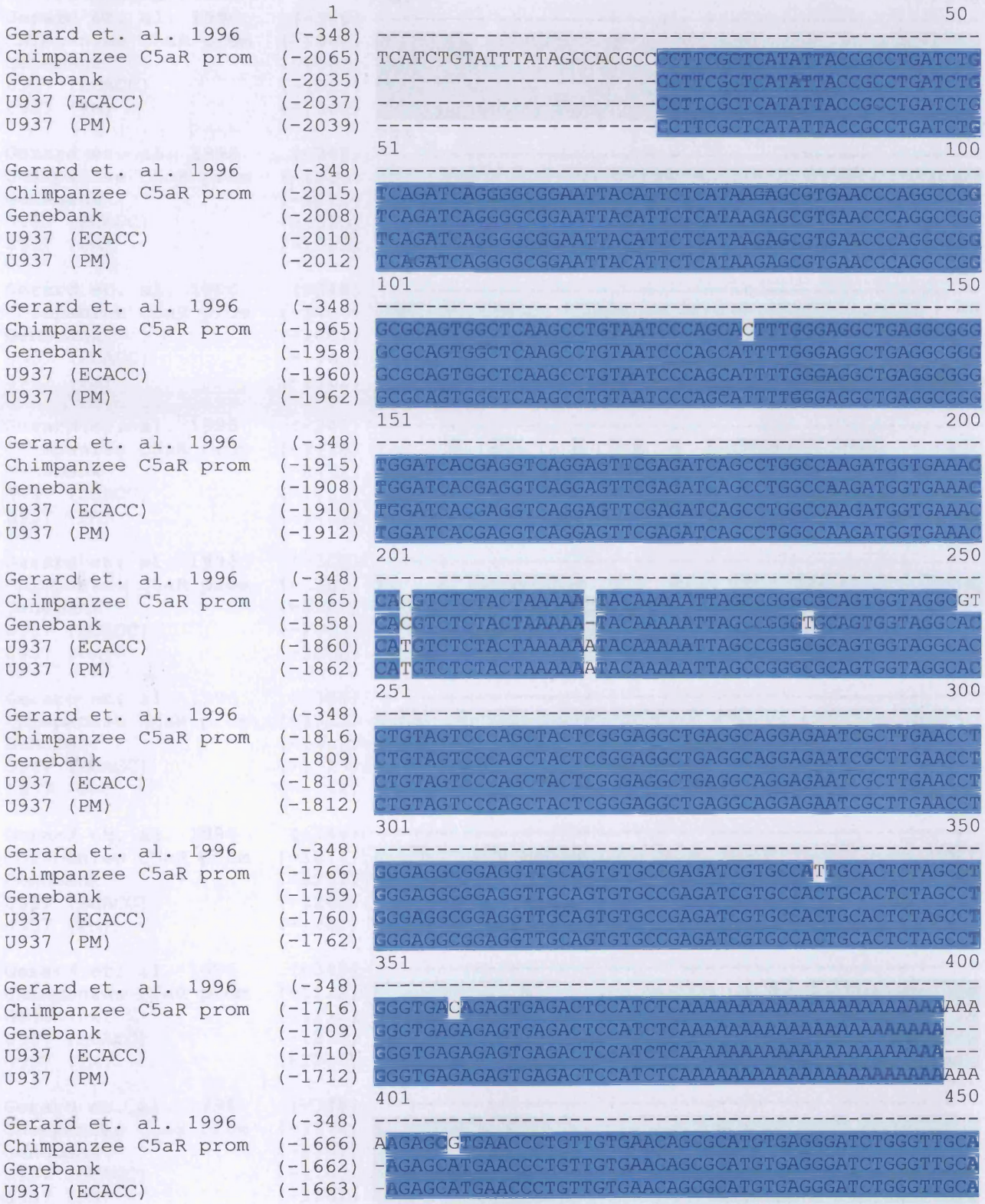
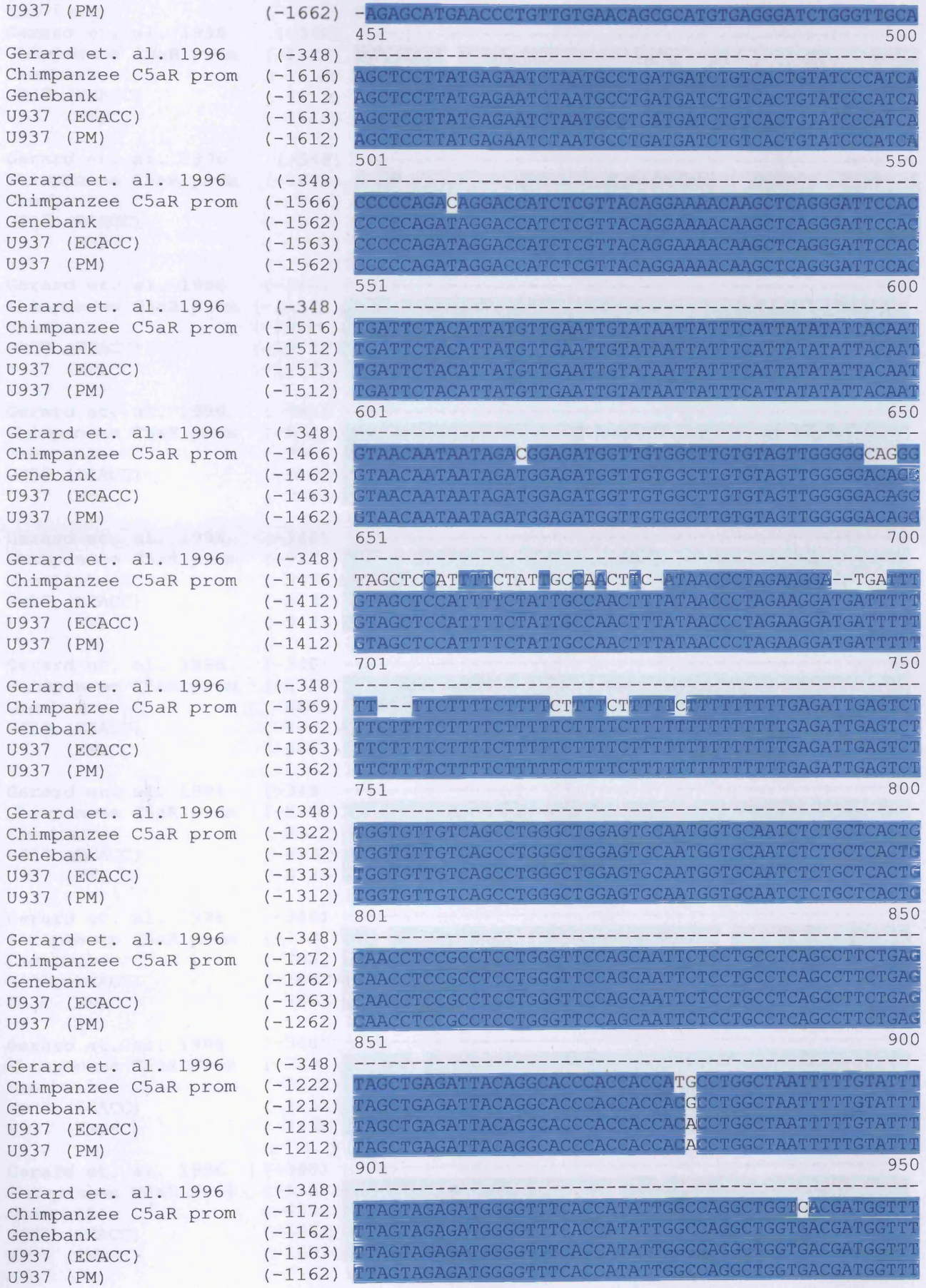
9.2 Alignment of C5aR 2 Kbp promoter sequence isolated from the U937 cell lines with human GENEBANK sequence and the chimpanzee sequence

GeneBank AC_099491.

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Genebank (-1612) AGCTCCTTATGAGAATCTAATGCCTGATGATCTGTCACTGTATCCCATCA
U937 (ECACC) (-1613) AGCTCCTTATGAGAATCTAATGCCTGATGATCTGTCACTGTATCCCATCA
U937 (PM) (-1612) AGCTCCTTATGAGAATCTAATGCCTGATGATCTGTCACTGTATCCCATCA
501 550
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U937 (ECACC) (-1563) CCCCCAGATAGGACCATCTCGTTACAGGAAAACAAGCTCAGGGATTCCAC
U937 (PM) (-1562) CCCCCAGATAGGACCATCTCGTTACAGGAAAACAAGCTCAGGGATTCCAC
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Gerard et. al. 1996 (-348) ----------
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Genebank (-1512) TGATTCTACATTATGTTGAATTGTATAATTATTTCATTATATATTACAAT
U937 (ECACC) (-1513) TGATTCTACATTATGTTGAATTGTATAATTATTTCATTATATATTACAAT
U937 (PM) (-1512) TGATTCTACATTATGTTGAATTGTATAATTATTTCATTATATATTACAAT
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Gerard et. al. 1996 (-348) ----------
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U937 (PM) (-1462) GTAACAATAATAGATGGAGATGGTTGTGGCTTGTGTAGTTGGGGGACAGG
651 700
Gerard et. al. 1996 (-348) ----------
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U937 (PM) (-1412) GTAGCTCCATTTTCTATTGCCAACTTTATAACCCTAGAAGGATGATTTTT
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U937 (PM) (-1362) TTCTTTTCTTTTCTTTTTCTTTTCTTTTTTTTTTTTTTTGAGATTGAGTCT
751 800
Gerard et. al. 1996 (-348) ----------
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U937 (PM) (-1312) TGGTGTTGTCAGCCTGGGCTGGAGTGCAATGGTGCAATCTCTGCTCACTG
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U937 (PM) (-1262) CAACCTCCGCCTCCTGGGTTCCAGCAATTCTCCTGCCTCAGCCTTCTGAG
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Gerard et. al. 1996 (-348) ----------
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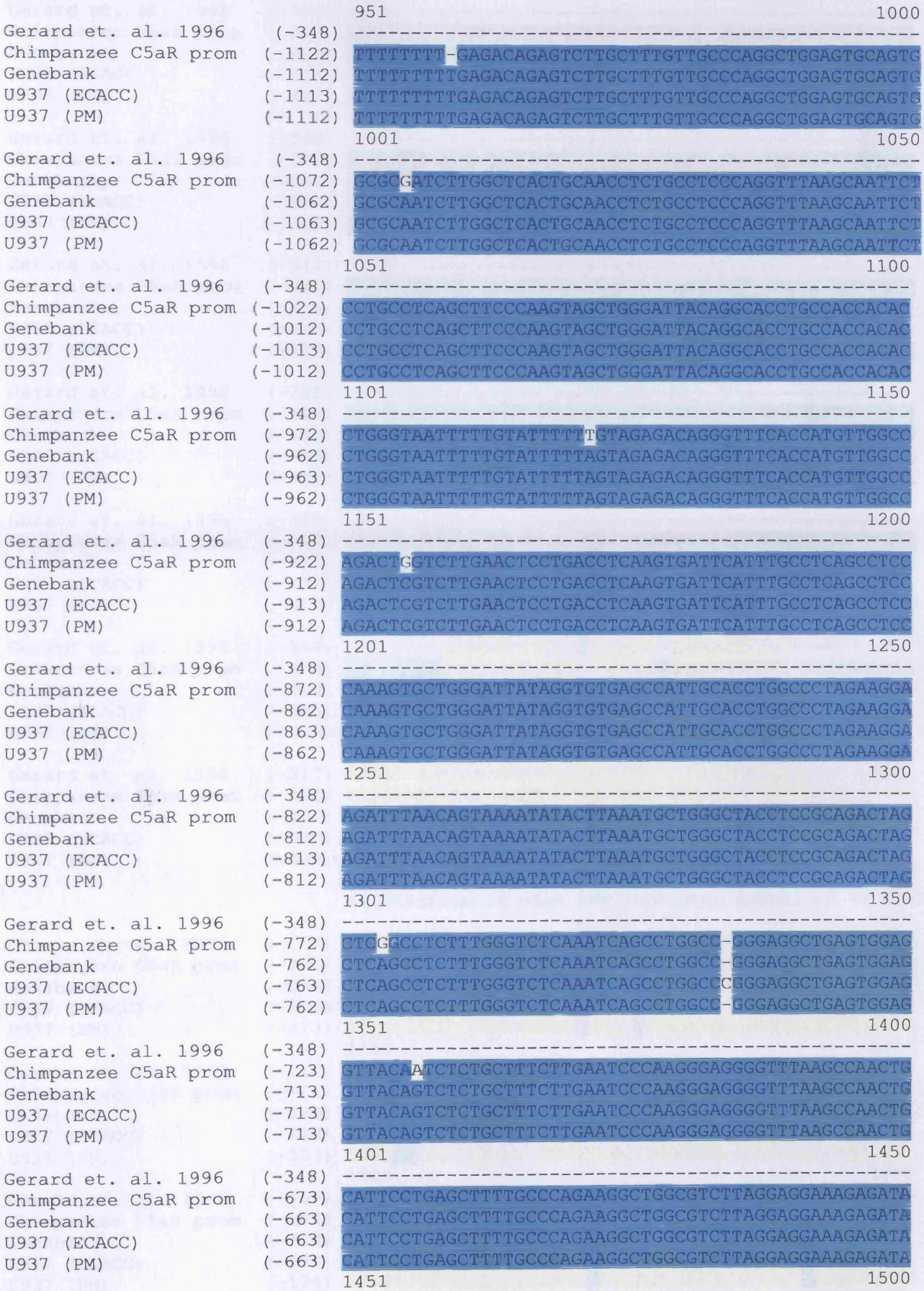
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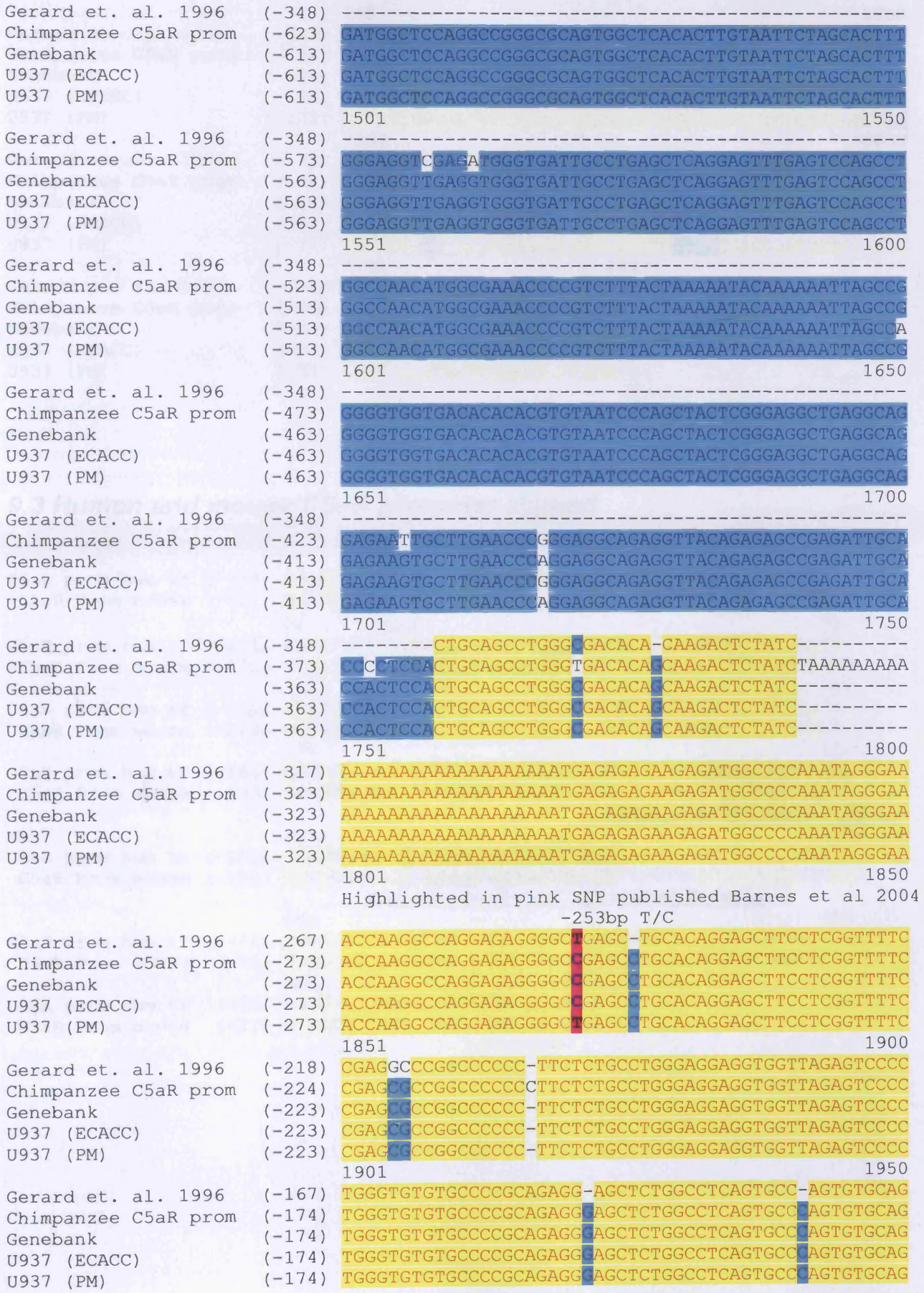
Genebank (-1112) TTTTTTTTTGAGACAGAGTCTTGCTTTGTTGCCCAGGCTGGAGTGCAGTG
U937 (ECACC) (-1113) TTTTTTTTTGAGACAGAGTCTTGCTTTGTTGCCCAGGCTGGAGTGCAGTG
U937 (PM) (-1112) TTTTTTTTTGAGACAGAGTCTTGCTTTGTTGCCCAGGCTGGAGTGCAGTG
1001 1050
Gerard et. al. 1996 (-348)
Chimpanzee C5aR prom (-1072) GCGCGATCTTGGCTCACTGCAACCTCTGCCTCCCAGGTTTAAGCAATTCT
Genebank (-1062) GCGCAATCTTGGCTCACTGCAACCTCTGCCTCCCAGGTTTAAGCAATTCT
U937 (ECACC) (-1063) GCGCAATCTTGGCTCACTGCAACCTCTGCCTCCCAGGTTTAAGCAATTCT
U937 (PM) (-1062) GCGCAATCTTGGCTCACTGCAACCTCTGCCTCCCAGGTTTAAGCAATTCT
1051 1100
Gerard et. al. 1996 (-348)
Chimpanzee C5aR prom (-1022) CCTGCCTCAGCTTCCCAAGTAGCTGGGATTACAGGCACCTGCCACCACAC
Genebank (-1012) CCTGCCTCAGCTTCCCAAGTAGCTGGGATTACAGGCACCTGCCACCACAC
U937 (ECACC) (-1013) CCTGCCTCAGCTTCCCAAGTAGCTGGGATTACAGGCACCTGCCACCACAC
U937 (PM) (-1012) CCTGCCTCAGCTTCCCAAGTAGCTGGGATTACAGGCACCTGCCACCACAC
1101 1150
Gerard et. al. 1996 (-348)
Chimpanzee C5aR prom (-972) CTGGGTAATTTTTGTATTTTTTGTAGAGACAGGGTTTCACCATGTTGGCC
Genebank (-962) CTGGGTAATTTTTGTATTTTTAGTAGAGACAGGGTTTCACCATGTTGGCC
U937 (ECACC) (-963) CTGGGTAATTTTTGTATTTTTAGTAGAGACAGGGTTTCACCATGTTGGCC
U937 (PM) (-962) CTGGGTAATTTTTGTATTTTTAGTAGAGACAGGGTTTCACCATGTTGGCC
1151 1200
Gerard et. al. 1996 (-348)
Chimpanzee C5aR prom (-922) AGACTGGTCTTGAACTCCTGACCTCAAGTGATTCATTTGCCTCAGCCTCC
Genebank (-912) AGACTCGTCTTGAACTCCTGACCTCAAGTGATTCATTTGCCTCAGCCTCC
U937 (ECACC) (-913) AGACTCGTCTTGAACTCCTGACCTCAAGTGATTCATTTGCCTCAGCCTCC
U937 (PM) (-912) AGACTCGTCTTGAACTCCTGACCTCAAGTGATTCATTTGCCTCAGCCTCC
1201 1250
Gerard et. al. 1996 (-348)
Chimpanzee C5aR prom (-872) CAAAGTGCTGGGATTATAGGTGTGAGCCATTGCACCTGGCCCTAGAAGGA
Genebank (-862) CAAAGTGCTGGGATTATAGGTGTGAGCCATTGCACCTGGCCCTAGAAGGA
U937 (ECACC) (-863) CAAAGTGCTGGGATTATAGGTGTGAGCCATTGCACCTGGCCCTAGAAGGA
U937 (PM) (-862) CAAAGTGCTGGGATTATAGGTGTGAGCCATTGCACCTGGCCCTAGAAGGA
1251 1300
Gerard et. al. 1996 (-348)
Chimpanzee C5aR prom (-822) AGATTTAACAGTAAAATATACTTAAATGCTGGGCTACCTCCGCAGACTAG
Genebank (-812) AGATTTAACAGTAAAATATACTTAAATGCTGGGCTACCTCCGCAGACTAG
U937 (ECACC) (-813) AGATTTAACAGTAAAATATACTTAAATGCTGGGCTACCTCCGCAGACTAG
U937 (PM) (-812) AGATTTAACAGTAAAATATACTTAAATGCTGGGCTACCTCCGCAGACTAG
1301 1350
Gerard et. al. 1996 (-348)
Chimpanzee C5aR prom (-772) CTCGGCCTCTTTGGGTCTCAAATCAGCCTGGCC-GGGAGGCTGAGTGGAG
Genebank (-762) CTCAGCCTCTTTGGGTCTCAAATCAGCCTGGCC-GGGAGGCTGAGTGGAG
U937 (ECACC) (-763) CTCAGCCTCTTTGGGTCTCAAATCAGCCTGGCCCGGGAGGCTGAGTGGAG
U937 (PM) (-762) CTCAGCCTCTTTGGGTCTCAAATCAGCCTGGCC-GGGAGGCTGAGTGGAG
1351 1400
Gerard et. al. 1996 (-348)
Chimpanzee C5aR prom (-723) GTTACAATCTCTGCTTTCTTGAATCCCAAGGGAGGGGTTTAAGCCAACTG
Genebank (-713) GTTACAGTCTCTGCTTTCTTGAATCCCAAGGGAGGGGTTTAAGCCAACTG
U937 (ECACC) (-713) GTTACAGTCTCTGCTTTCTTGAATCCCAAGGGAGGGGTTTAAGCCAACTG
U937 (PM) (-713) GTTACAGTCTCTGCTTTCTTGAATCCCAAGGGAGGGGTTTAAGCCAACTG
1401 1450
Gerard et. al. 1996 (-348)
Chimpanzee C5aR prom (-673) CATTCCTGAGCTTTTGCCCAGAAGGCTGGCGTCTTAGGAGGAAAGAGATA
Genebank (-663) CATTCCTGAGCTTTTGCCCAGAAGGCTGGCGTCTTAGGAGGAAAGAGATA
U937 (ECACC) (-663) CATTCCTGAGCTTTTGCCCAGAAGGCTGGCGTCTTAGGAGGAAAGAGATA
U937 (PM) (-663) CATTCCTGAGCTTTTGCCCAGAAGGCTGGCGTCTTAGGAGGAAAGAGATA
1451 1500


Gerard et. al. 1996 (-348)
Chimpanzee C5aR prom (-623)
Genebank (-613)
U937 (ECACC) (-613)
U937 (PM) (-613)
1501 1550
Gerard et. al. 1996 (-348)
Chimpanzee C5aR prom (-573)
Genebank (-563)
U937 (ECACC) (-563)
U937 (PM) (-563)
1551 1600
Gerard et. al. 1996 (-348)
Chimpanzee C5aR prom (-523)
Genebank (-513)
U937 (ECACC) (-513)
U937 (PM) (-513)
1601 1650
Gerard et. al. 1996 (-348)
Chimpanzee C5aR prom (-473)
Genebank (-463)
U937 (ECACC) (-463)
U937 (PM) (-463)
1651 1700
Gerard et. al. 1996 (-348)
Chimpanzee C5aR prom (-423)
Genebank (-413)
U937 (ECACC) (-413)
U937 (PM) (-413)
1701 1750
Gerard et. al. 1996 (-348)
Chimpanzee C5aR prom (-373)
Genebank (-363)
U937 (ECACC) (-363)
U937 (PM) (-363)
1751 1800
Gerard et. al. 1996 (-317)
Chimpanzee C5aR prom (-323)
Genebank (-323)
U937 (ECACC) (-323)
U937 (PM) (-323)
1801 1850
Highlighted in pink SNP published Barnes et al 2004
-253bp T/C
Gerard et. al. 1996 (-267)
Chimpanzee C5aR prom (-273)
Genebank (-273)
U937 (ECACC) (-273)
U937 (PM) (-273)
1851 1900
Gerard et. al. 1996 (-218)
Chimpanzee C5aR prom (-224)
Genebank (-223)
U937 (ECACC) (-223)
U937 (PM) (-223)
1901 1950
Gerard et. al. 1996 (-167)
Chimpanzee C5aR prom (-174)
Genebank (-174)
U937 (ECACC) (-174)
U937 (PM) (-174)

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                                1951                                        2000
Gerard et. al. 1996    (-119)   ACCAATGAGAGCCCCAGAGAGAAAGACGGTCATTTCCTCCCTGCATCT-C
Chimpanzee C5aR prom   (-124)   ACCAATGAGAGCCCCAGAGAGAAAGACGGTCATTTCCTCCCTGCATCTTT
Genebank               (-124)   ACCAATGAGAGCCCCAGAGAGAAAGACGGTCATTTCCTCCCTGCATCTTC
U937 (ECACC)           (-124)   ACCAATGAGAGCCCCAGAGAGAAAGACGGTCATTTCCTCCCTGCATCTTC
U937 (PM)              (-124)   ACCAATGAGAGCCCCAGAGAGAAAGACGGTCATTTCCTCCCTGCATCTTC
                                2001                                        2050
Gerard et. al. 1996     (-70)   CCTTGGG-CTTTAAAAACCACAGCCCTTGC---GGAGGGACCTTCGATCC
Chimpanzee C5aR prom    (-74)   CCTTGGGGCTTTAAAAACCACAGCCCTTGGGCAGGAGGGACCTTCGATCC
Genebank                (-74)   CCTTGGGGCTTTAAAAACCACAGCCCTTGGGCAGGAGGGACCTTCGATCC
U937 (ECACC)            (-74)   CCTTGGGGCTTTAAAAACCACAGCCCTTGGGCAGGAGGGACCTTCGATCC
U937 (PM)               (-74)   CCTTGGGGCTTTAAAAACCACAGCCCTTGGGCAGGAGGGACCTTCGATCC
                                2051                    2077
Gerard et. al. 1996     (-24)   TCGGGGAGCCCAGGAGACCAGAACATG
Chimpanzee C5aR prom    (-24)   TCGGGGAGCCCAGGAGACCAGAAC---
Genebank                (-24)   TCGGGGAGCCCAGGAGACCAGAAC---
U937 (ECACC)            (-24)   TCGGGGAGCCCAGGAGACCAGAAC---
U937 (PM)               (-24)   TCGGGGAGCCCAGGAGACCAGAAC---
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9.3 Human and mouse C5aR promoter aligned

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                                1                                           50
C5aR prom hum tr  (-399)   GAGAAG-AGATG-GCCCCAAATAGGGAAACCAAGGCCAGGAGAGGGGCCG
 C5aR Prom mouse  (-321)   GAAAAGCAGCTGCGTCCCCAAAAGTGAAG--AAGTCCTGGAAATTGCCTT
                                51       NFκB                               100
C5aR prom hum tr  (-251)   AGCCTGCACAGGAGC-------TTCCTCGGTTTTCCGAGC-GCCGGCCC-
 C5aR Prom mouse  (-273)   TTCCCCTTCTGGGGCCAGAGACTTCCTTCCTTTTCCAAGTTGACATCTCT
                                101                                         150
C5aR prom hum tr  (-210)   CCCTTCTCTGCCTGGGA---GGAGGTGGTTAGAGTCCCCTGGGTGTGTGC
 C5aR Prom mouse  (-223)   CCCCTGGCTGGTTGGTACTGGGTGGTGCTGAGGGTGTACTGGGTAAGCAC
                                151                                         200
C5aR prom hum tr  (-163)   CCCGCAGAGGGAGCTCTGGCCTCAGTGCCCAGTGTGCAGACCAATGAGAG
 C5aR Prom mouse  (-173)   CG-GTGGAGGGAGCCTCAGCTAGGATGGTCAGTGAGT-GACCAATGAGCA
                                                    CCAAT binding factor(NFY)
                                201                                         250
C5aR prom hum tr  (-113)   CCCCAGAGAGAA-AAGACGGTCATTTCCTC-------CCTGCATCTTCCCT
 C5aR Prom mouse  (-125)   CCTCCAGGAGACAAGACAGTCATTTCCTCTCAGTTGCCTGCATCTCTTCT
                                Nuclear factor of activated T-cells
                                251                                         300
C5aR prom hum tr   (-71)   TGGGGCTTTAAAAACCACAGCCCTTGGGCAGGAGGGACCTTCG---ATCC
 C5aR Prom mouse   (-75)   TGAGGGTTTAAAAGGCACAGCC--TGGGTGACAGGGACCTTCAGGCATCC
                                301                     327
C5aR prom hum tr   (-24)   -TCG--GGGAGCCCAGGAGACCAGAAC
 C5aR Prom mouse   (-27)   GTCGCTGGTTACCACAGAACCCAGGAG
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E2F-myc activator/cell cycle regulator	-2030	-2014	-	1.000
PAX-4/PAX-6 paired domain biding sites	-2027	-2009	+	1.000
TALE homedomain class recognizing TG motiffs	-2015	-2005	+	1.000
Zinc binding protein factors	-2011	-1989	-	1.000
GC-Box factors SP1/GC	-2005	-1991	+	1.000
Pancreatic and intestinal homeodomain trascription factor	-2002	-1982	-	1.000
Factors with moderate activity to homeo domain consensus sequence	-2000	-1984	-	1.000
PAR/bZIP family	-1997	-1981	-	1.000
Signal transducer and activtor of transcription	-1994	-1976	-	0.807
Octamer binding protein	-1993	-1979	-	1.000
PAX-4/PAX-6 paired domain biding sites	-1987	-1969	-	1.000
Camp-responsive element binding proteins	-1986	-1966	-	0.784
E2F-myc activator/cell cycle regulator	-1982	-1966	+	0.847
Metal induced transcription factor	-1967	-1953	-	0.949
Selenocysteine tRNA activating factor	-1942	-1920	-	1.000
v-ERB and RAR-related orphan receptor alpha	-1917	-1895	+	0.750
RXR heterodimer binding sites	-1916	-1892	+	1.000
E-box binding factors	-1909	-1897	+	1.000
v-ERB and RAR-related orphan receptor alpha	-1909	-1887	+	1.000
RXR heterodimer binding sites	-1902	-1878	+	0.888
AARE binding factors	-1869	-1861	-	1.000
Vertebrate caudal related homeodomain transcription factors	-1857	-1839	-	1.000
Metal induced transcription factor	-1833	-1819	-	0.949
myoblast determining factors	-1818	-1802	-	1.000
Two-handed zinc finger homeodomain	-1817	-1805	+	1.000
Pur-alpha binds both single-stranded and double stranded DNA in sequence-specific manner	-1759	-1747	+	1.000
C/EBP homologous protein (CHOP)	-1753	-1741	-	1.000
PAX-3 binding sites	-1752	-1734	-	0.780
X-box binding factors	-1752	-1734	-	1.000
Nuclear receptor subfamily 2 factors	-1729	-1705	-	0.777
Peroxisome proliferator-activated receptor	-1726	-1704	-	0.807
PAX-4/PAX-6 paired domain biding sites	-1725	-1707	+	1.000
PAX-9 binding sites	-1724	-1704	-	1.000
GC-Box factors SP1/GC	-1700	-1686	-	1.000

Fork head domain factors	-1648	-1632	+	1.000	0.785
Pancreas transcription factor 1, heterotrimeric transcription factor	-1646	-1626	-	0.761	0.800
AP4 and related proteins	-1627	-1611	-	1.000	0.931
Signal transducer and activtor of transcription	-1609	-1591	+	0.807	0.876
Octamer binding protein	-1606	-1592	+	1.000	0.826
Pancreatic and intestinal homeodomain trascription factor	-1603	-1583	+	1.000	0.879
PAX-4/PAX-6 paired domain biding sites	-1600	-1582	-	0.754	0.758
MAF and AP1 related factors	-1589	-1569	-	1.000	0.810
HOX - MEIS1	-1589	-1575	-	1.000	0.806
PAX-5 B-cell-specific activator protein	-1582	-1554	+	0.947	0.845
TALE homedomain class recognizing TG motiffs	-1582	-1572	+	1.000	0.971
GLI zinc finger family	-1568	-1554	+	1.000	0.900
Serum response element binding factor	-1563	-1545	-	0.757	0.751
GATA binding factors	-1559	-1547	+	1.000	0.968
bHLH transcription factor dimer of HAND2 and E12	-1555	-1541	+	0.758	0.764
Cellular and viral myb-like transcriptional regulators	-1548	-1536	-	1.000	0.891
Signal transducer and activtor of transcription	-1546	-1528	-	0.762	0.783
PAR/bZIP family	-1544	-1528	-	1.000	0.862
Signal transducer and activtor of transcription	-1544	-1526	+	1.000	0.953
X-box binding factors	-1544	-1526	-	1.000	0.799
Fork head domain factors	-1539	-1523	+	1.000	0.980
Brn POU domain	-1511	-1493	-	1.000	0.743
Factors with moderate activity to homeo domain consensus sequence	-1511	-1495	-	1.000	0.826
snRNA-activating protein complex	-1510	-1492	-	0.892	0.752
Factors with moderate activity to homeo domain consensus sequence	-1508	-1492	-	1.000	0.872
SOX/SRY-sex/testis determinig and related HMG box	-1499	-1483	-	1.000	0.872
CLOX and CLOX homology (CDP) factors	-1498	-1476	+	1.000	0.714
Cart-1 (cartilage homeoprotein 1)	-1496	-1480	+	0.750	0.835
PAR/bZIP family	-1496	-1480	+	0.800	0.845
Cart-1 (cartilage homeoprotein 1)	-1495	-1479	-	1.000	0.832
Homeodomain transcription factors	-1495	-1479	+	1.000	0.994
PAR/bZIP family	-1495	-1479	-	0.860	0.792
Brn POU domain	-1493	-1475	+	1.000	0.906
Brn POU domain	-1492	-1474	-	1.000	0.782
Pancreatic and intestinal homeodomain trascription factor	-1492	-1472	-	1.000	0.743
NKX homeodomain	-1491	-1477	+	0.773	0.845
Tata-binding protein factors	-1491	-1475	+	1.000	0.826
Factors with moderate activity to homeo domain consensus sequence	-1490	-1474	-	1.000	0.854
NKX homeodomain	-1490	-1476	-	0.773	0.868
Lim homeodomain factors	-1489	-1473	-	1.000	0.932

Cart-1 (cartilage homeoprotein 1)	-1488	-1472	+	1.000	0.940
Homeodomain transcription factors	-1488	-1472	-	1.000	0.992
Cart-1 (cartilage homeoprotein 1)	-1487	-1471	-	1.000	0.919
Cart-1 (cartilage homeoprotein 1)	-1485	-1469	+	0.797	0.888
PAR/bZIP family	-1484	-1468	-	0.884	0.847
Tata-binding protein factors	-1484	-1468	-	1.000	0.823
Tata-binding protein factors	-1482	-1466	-	0.891	0.909
Lim homeodomain factors	-1481	-1465	-	1.000	0.828
Special AT-rich sequence binding protein	-1478	-1464	-	1.000	0.957
OCT6 binding factors-astrocytes + glioblastoma cells	-1473	-1461	+	1.000	0.913
PAR/bZIP family	-1470	-1454	+	1.000	0.895
SOX/SRY-sex/testis determinig and related HMG box	-1463	-1447	+	1.000	0.993
Fork head domain factors	-1461	-1445	+	1.000	0.879
Brn POU domain	-1460	-1442	-	1.000	0.832
CLOX and CLOX homology (CDP) factors	-1460	-1438	-	0.960	0.731
CLOX and CLOX homology (CDP) factors	-1458	-1436	+	1.000	0.840
Factors with moderate activity to homeo domain consensus sequence	-1458	-1442	+	1.000	0.875
FAST-1 SMAD interacting proteins	-1442	-1426	+	1.000	0.826
human actute myelogenous leukemia factors	-1440	-1426	+	1.000	0.870
Basonuclein rDNA transcription factor (PolI)	-1422	-1404	-	1.000	0.895
CLOX and CLOX homology (CDP) factors	-1412	-1390	+	0.960	0.774
Positive regulatory domain I binding factor	-1410	-1392	-	1.000	0.865
Activator/repressor binding to transcription initiation site	-1410	-1392	+	1.000	0.962
Zinc binding protein factors	-1410	-1398	+	1.000	0.858
Pancreatic and intestinal homeodomain trascription factor	-1395	-1375	+	0.826	0.752
Tata-binding protein factors	-1395	-1379	-	1.000	0.908
Vertebrate caudal related homeodomain transcription factors	-1394	-1376	+	1.000	0.886
HOX - MEIS1	-1394	-1380	+	0.750	0.826
Serum response element binding factor	-1385	-1367	-	0.786	0.800
snRNA-activating protein complex	-1384	-1366	+	1.000	0.760
HOX - PBX complexes	-1373	-1357	+	1.000	0.840
EVI1-myleoid transforming protein	-1368	-1352	-	1.000	0.898
Interferon regulatory factors	-1364	-1344	-	1.000	0.941
EVI1-myleoid transforming protein	-1363	-1347	-	1.000	0.898
Interferon regulatory factors	-1359	-1339	-	1.000	0.941
EVI1-myleoid transforming protein	-1358	-1342	-	1.000	0.791
CAS interacting zinc finger protein	-1352	-1342	-	1.000	0.970
EVI1-myleoid transforming protein	-1352	-1336	-	1.000	0.898
Human and murine ETS1	-1349	-1329	-	0.857	0.833
Interferon regulatory factors	-1348	-1328	-	1.000	0.941
EVI1-myleoid transforming protein	-1347	-1331	-	1.000	0.808
Onecut homeodomain factor HNF6	-1324	-1308	-	1.000	0.822
Ccaat/Enhancer binding protein	-1286	-1272	+	1.000	0.941
RXR heterodimer binding sites	-1285	-1261	+	0.790	0.810
C/EBP homologous protein (CHOP)	-1282	-1270	+	1.000	0.923
C/EBP homologous protein (CHOP)	-1266	-1254	+	1.000	0.973

Pur-alpha binds both single-stranded and double stranded DNA in sequence-specific manner	-1260	-1248	-	1.000	0.985
Signal transducer and activtor of transcription	-1249	-1231	-	1.000	0.895
Zinc binding protein factors	-1249	-1225	+	0.813	0.788
POZ domain zinc finger expressed in B-cells	-1248	-1232	-	0.756	0.772
Signal transducer and activtor of transcription	-1247	-1229	+	0.759	0.801
MAF and AP1 related factors	-1234	-1214	+	0.866	0.857
Growth factor independent transcription repressor	-1212	-1198	-	1.000	0.960
Sine oculis (SIX) homeodomain factors	-1206	-1198	+	1.000	1.000
Zinc binding protein factors	-1199	-1177	+	1.000	0.793
myoblast determining factors	-1188	-1172	-	1.000	0.986
Two-handed zinc finger homeodomain	-1187	-1175	+	1.000	0.988
Brn POU domain	-1185	-1167	+	1.000	0.925
MEF2, monocyte-specific enhancer binding factor	-1173	-1151	-	1.000	0.802
CLOX and CLOX homology (CDP) factors	-1148	-1126	-	1.000	0.944
AARE binding factors	-1147	-1139	+	1.000	0.952
snRNA-activating protein complex	-1145	-1127	+	0.892	0.735
CCAAT binding factors	-1143	-1129	-	1.000	0.910
SWI/SNF related nucleophosphoproteins with a RING finger DNA binding motif	-1143	-1133	+	1.000	0.961
PAX-3 binding sites	-1135	-1117	-	1.000	0.826
Camp-responsive element binding proteins	-1132	-1112	+	1.000	0.945
Camp-responsive element binding proteins	-1129	-1109	+	1.000	0.907
EVI1-myleoid transforming protein	-1126	-1110	+	1.000	0.973
LEF1/TCF	-1093	-1077	-	1.000	0.927
PAR/bZIP family	-1067	-1051	-	0.860	0.794
Nuclear factor 1	-1056	-1036	+	1.000	0.834
Nuclear factor 1	-1056	-1036	-	0.750	0.877
C/EBP homologous protein (CHOP)	-1047	-1035	+	1.000	0.973
Ccaat/Enhancer binding protein	-1027	-1013	+	1.000	0.956
PAR/bZIP family	-1027	-1011	-	0.860	0.803
RBPJ-kappa	-1005	-991	-	1.000	0.940
Signal transducer and activtor of transcription	-1004	-986	+	1.000	0.842
Vertebrate homologues of enhancer of split complex	-981	-967	-	0.750	0.872
myoblast determining factors	-981	-965	-	1.000	0.983
myoblast determining factors	-969	-953	-	1.000	0.985
Two-handed zinc finger homeodomain	-968	-956	+	1.000	0.981
Brn POU domain	-966	-948	+	1.000	0.925
MEF2, monocyte-specific enhancer binding factor	-962	-940	-	0.780	0.750
Heterogeneous PAX-4 binding sites	-959	-949	-	1.000	1.000
AARE binding factors	-928	-920	+	1.000	0.952
Vertebrate SMAD family of transcription factors	-915	-907	-	1.000	0.991
RXR heterodimer binding sites	-905	-881	-	1.000	0.972
v-ERB and RAR-related orphan receptor	-902	-880	-	1.000	0.921

alpha					
Camp-responsive element binding proteins	-899	-879	+	1.000	0.992
NKX homeodomain	-892	-878	+	1.000	1.000
HOX-PBX complexes	-887	-871	+	1.000	0.832
Factors with moderate activity to homeo domain consensus sequence	-886	-870	-	1.000	0.911
AP1, Activating protein 1	-885	-875	+	0.833	0.946
AP1, Activating protein 1	-885	-875	-	0.880	0.960
SOX/SRY-sex/testis determinig and related HMG box	-885	-869	+	0.916	0.906
CLOX and CLOX homology (CDP) factors	-864	-842	-	1.000	0.752
Factors with moderate activity to homeo domain consensus sequence	-858	-842	-	0.878	0.941
Brachyury gene mesoderm developmental factor	-853	-833	+	1.000	0.999
C/EBP homologous protein (CHOP)	-837	-825	-	1.000	0.917
Metal induced transcription factor	-834	-820	+	1.000	0.900
myoblast determining factors	-834	-818	-	1.000	0.984
Two-handed zinc finger homeodomain	-833	-821	+	1.000	0.962
Serum response element binding factor	-827	-809	-	0.786	0.842
snRNA-activating protein complex	-826	-808	+	1.000	0.771
GATA binding factors	-814	-802	+	1.000	0.916
PAX-2 binding sites	-805	-783	+	0.789	0.780
MYT1 C2HC zinc finger protein	-801	-789	+	0.750	0.798
OCT1 binding factor (POU-specific domain)	-799	-787	+	1.000	0.864
NKX homeodomain	-797	-783	-	1.000	0.976
Tata-binding protein factors	-797	-781	-	1.000	0.874
Lim homeodomain factors	-796	-780	-	1.000	0.814
PAX-2/5/8 binding sites	-796	-784	-	0.800	0.887
NKX homeodomain	-794	-780	+	1.000	0.928
Mouse Krueppel like factor	-767	-747	+	0.750	0.741
Neuron-restrictive silencer factor	**-738**	**-718**	**-**	**0.782**	**0.707**
				0.782	**0.721**
E2F-myc activator/cell cycle regulator	**-737**	**-721**	**+**	**0.809**	**0.859**
				Absent in U937PM	
NKX homeodomain	-726	-712	+	1.000	0.974
Heat shock factors	-706	-682	-	1.000	0.864
Neuron-specific-olfactory factor	-696	-674	-	1.000	0.922
Neuron-specific-olfactory factor	-695	-673	+	1.000	0.882
EGR/nerve growth factor induced protein C & related factors	-687	-671	+	1.000	0.883
GC-Box factors SP1/GC	-687	-673	+	0.876	0.900
Myc associated Zinc fingers	-685	-673	+	1.000	0.886
Nuclear factor 1	-683	-663	-	1.000	0.820
PAX-2 binding sites	-678	-656	-	1.000	0.793
NeuroD, Beta2, HLH domain	-672	-660	+	0.767	0.841
Octamer binding protein	-671	-657	-	1.000	0.816
Cellular and viral myb-like transcriptional regulators	-670	-658	+	1.000	0.904
TEA/ATTS DNA binding domain factors	-665	-653	+	1.000	0.692
Nuclear receptor subfamily 2 factors	-662	-638	-	1.000	0.886
Peroxisome proliferator-activated receptor	-659	-637	-	0.784	0.872

Camp-responsive element binding proteins	-642	-622	+	0.750	0.834
Winged helix binding sites	-638	-628	-	1.000	0.960
Interferon regulatory factors	-626	-606	+	0.774	0.850
Nuclear factor of activated T-cells	-626	-608	+	1.000	0.994
CLOX and CLOX homology (CDP) factors	-621	-599	+	0.929	0.956
HOX-PBX complexes	-621	-605	+	0.833	0.852
PAX-4/PAX-6 paired domain biding sites	-614	-596	+	1.000	0.879
Metal induced transcription factor	-603	-589	-	0.949	0.947
PAX-3 binding sites	-590	-572	+	1.000	0.872
NKX homeodomain	-587	-573	-	1.000	0.902
SWI/SNF related nucleophosphoproteins with a RING finger DNA binding motif	-586	-576	+	1.000	0.985
OCT6 binding factors-astrocytes + glioblastoma cells	-582	-570	-	1.000	0.916
GLI zinc finger family	-567	-553	-	1.000	0.876
RXR heterodimer binding sites	-564	-540	+	0.952	0.809
Zinc binding protein factors	-563	-541	-	1.000	0.753
CCAAT binding factors	-550	-536	-	0.750	0.838
PBX1-MEIS1 complexes	-550	-534	+	1.000	0.797
GC-Box factors SP1/GC	-523	-509	-	1.000	0.841
PAR/bZIP family	-509	-493	+	0.781	0.849
MEF2, monocyte-specific enhancer binding factor	-492	-470	+	0.763	0.891
Zinc binding protein factors	**-474**	**-452**	**-**	**1.000**	**0.925**
				Absent in U937EC	
Zinc binding protein factors	**-471**	**-449**	**-**	**1.000**	**0.943**
				Absent in U937EC	
Activator protein 2	**-469**	**-455**	**+**	**0.881**	**0.925**
				0.830	**0.912**
GLI zinc finger family	-466	-452	-	1.000	0.981
MAF and AP1 related factors	-463	-443	-	0.813	0.924
Brachyury gene mesoderm developmental factor	-456	-436	+	0.750	0.706
Hypoxia inducible factor, bHLH/PAS protein factor	-454	-439	+	1.000	0.960
Vertbrate homologue of enhancer of split complex	-453	-439	-	1.000	0.968
Hypoxia inducible factor, bHLH/PAS protein factor	-453	-437	-	1.000	0.967
E-box binding factors	-452	-440	+	1.000	0.988
Vertebrate homologues of enhancer of split complex	-452	-438	+	1.000	0.961
E-box binding factors	-451	-439	-	1.000	0.977
SWI/SNF related nucleophosphoproteins with a RING finger DNA binding motif	-402	-402	-	1.000	0.987
RXR heterodimer binding sites	-401	-377	+	1.000	0.822
RXR heterodimer binding sites	-391	-367	+	0.952	0.820
C/EBP homologous protein (CHOP)	-372	-360	-	1.000	0.917
PAX-5 B-cell-specific activator protein	-372	-344	-	0.904	0.800
Ccaat/Enhancer binding protein	-370	-356	-	1.000	0.960
Selenocysteine tRNA activating factor	-361	-339	-	1.000	0.781
X-box binding factors	-354	-336	+	1.000	0.939
HOX-PBX complexes	-334	-318	-	0.764	0.801

EVI1-myleoid transforming protein	-300	-284	+	1.000	0.833
Activator/repressor binding to transcription initiation site	-300	-282	-	1.000	0.857
bHLH transcription factor dimer of HAND2 and E12	-294	-280	-	1.000	0.794
E2F-myc activator/cell cycle regulator	-292	-276	+	1.000	0.849
Serum response element binding factor	-289	-271	-	0.885	0.870
Serum response element binding factor	-288	-270	+	0.888	0.903
RXR heterodimer binding sites	-283	-259	+	1.000	0.902
Interferon regulatory factors	-279	-259	+	1.000	0.736
Estrogen response element	-276	-258	+	1.000	0.890
p53 tumor suppressor	-274	-252	+	1.000	0.783
Vertebrate steroidogenic factor	-274	-262	+	1.000	0.992
Mouse Krueppel like factor	-273	-253	-	1.000	0.796
GC-Box factors SP1/GC	-260	-246	+	1.000	0.851
MAF and AP1 related factors	**-258**	**-238**	**+**	**0.875**	**0.948**
				0.750	**0.905**
CP2-erythrocyte factor related to drosophila	-244	-226	+	0.791	0.861
Human and murine ETS1	-244	-224	-	1.000	0.895
Heat shock factors	-243	-219	-	0.763	0.768
Nuclear factor kappa B/c-rel	**-243**	**-231**	**+**	**1.000**	**0.902**
Nuclear respiratory factor 1	-222	-206	+	1.000	0.793
PAX-5 B-cell-specific activator protein	-220	-192	-	0.952	0.843
CTCF BORIS gene family, transcription regulators with 11 highly conserved zinc finger domains	-216	-192	-	0.789	0.805
Autoimmune regulatory element binding factor	-204	-178	+	1.000	0.860
EGR/nerve growth factor induced protein C & related factors	-204	-188	+	0.832	0.817
Neuron-specific-olfactory factor	-184	-162	+	1.000	0.927
GC-Box factors SP1/GC	-176	-162	+	0.750	0.878
Basic and erythroid krueppel like factors	-214	-158	+	1.000	0.950
Nuclear receptor subfamily 2 factors	-159	-135	-	0.779	0.863
Peroxisome proliferator-activated receptor	-156	-134	-	0.784	0.765
RXR heterodimer binding sites	-150	-126	-	1.000	0.895
Selenocysteine tRNA activating factor	-139	-117	+	1.000	0.830
CLOX and CLOX homology (CDP) factors	-129	-107	+	1.000	0.957
CCAAT binding factors	**-126**	**-112**	**+**	**1.000**	**0.966**
v-ERB and RAR-related orphan receptor alpha	-105	-83	+	1.000	0.910
Nuclear factor of activated T-cells	**-102**	**-84**	**-**	**1.000**	**0.970**
Homolog to deformed epidermal autoregulatory factor-1 from D. melangoaster	-101	-83	+	0.761	0.761
Human and murine ETS1	-99	-79	-	1.000	0.934
TCF11 transcription factor	-95	-89	+	1.000	1.000
Neuron-specific-olfactory factor	-81	-59	+	1.000	0.972
Tata-binding protein factors	-72	-56	-	1.000	0.901
Tata-binding protein factors	-67	-51	+	1.000	0.908
human actute myelogenous leukemia factors	-63	-49	-	1.000	0.980
Activator protein 2	-53	-39	-	0.905	0.924
Nuclear receptor subfamily 2 factors	-42	-18	-	0.750	0.761
RXR heterodimer binding sites	-40	-16	-	0.782	0.700

Neron-restrictive silencer factor	-32	-12	+	0.782	0.700
X-box binding factors	-21	-3	+	0.875	0.957

9.5 The C5aR 3'UTR sequences aligned

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                          1131                                                1180
3'UTR EC          (1)     GCGACAGCCTCATGGGCCACTGTGGCCCGATGTCCCCTTCCTTCCCGGCC
3'UTR PM          (1)     GCGACAGCCTCATGGGCCACTGTGGCCCGATGTCCCCTTCCTTCCCGGCC
gen 3'UTR         (1)     GCGACAGCCTCATGGGCCACTGTGGCCCGATGTCCCCTTCCTTCCCGGCC
C5aR mRNA HUMC5AAR (1078) GCGACAGCCTCATGGGCCACTGTGGCCCGATGTCCCCTTCCTTCCCGGCC
mRNA C5aR NM_001736 (1103) GCGACAGCCTCATGGGCCACTGTGGCCCGATGTCCCCTTCCTTCCCGGCC
C5aR mRNA BC008982 (1131) GCGACAGCCTCATGGGCCACTGTGGCCCGATGTCCCCTTCCTTCCCGGCC
3'UTR Chimp       (1)     GCGACAGCCGCATGGGCCACTGTGGCCCGATGTCCCCTTCCTTCCCGGCC
Consensus         (1131)  GCGACAGCCTCATGGGCCACTGTGGCCCGATGTCCCCTTCCTTCCCGGCC
                          1181                                                1230
3'UTR EC          (51)    ATTCTCC---CTCTTGTTTTCACTTCACTTTTCGTGGGATGGTGTTACCT
3'UTR PM          (51)    ATTCTCC---CTCTTGTTTCCACTTCACTTTTCGTGGGATGGTGTTACCT
gen 3'UTR         (51)    ATTCTCC---CTCTTGTTTTCACTTCACTTTTCGTGGGATGGTGTTACCT
C5aR mRNA HUMC5AAR (1128) ATTCTCC---CTCTTGTTTTCACTTCACTTTTCGTGGGATGGTGTTACCT
mRNA C5aR NM_001736 (1153) ATTCTCC---CTCTTGTTTTCACTTCACTTTTCGTGGGATGGTGTTACCT
C5aR mRNA BC008982 (1181) ATTCTCC---CTCTTGTTTTCACTTCACTTTTCGTGGGATGGTGTTACCT
3'UTR Chimp       (51)    ATTCTCCTTCCTCTTGTTTTCACTCCACTTTTCGTGGGATGGCGTTACCT
Consensus         (1181)  ATTCTCC   CTCTTGTTTTCACTTCACTTTTCGTGGGATGGTGTTACCT
                          1231                                                1280
3'UTR EC          (98)    TAGCTAACTAACTCTCCTCCATGTTGCCTGTCTTTCCCAGACTTGTCCCT
3'UTR PM          (98)    TAGCTAACTAACTCTCCTCCATGTTGCCTGTCTTTCCCAGACTTGTCCCT
gen 3'UTR         (98)    TAGCTAACTAACTCTCCTCCATGTTGCCTGTCTTTCCCAGACTTGTCCCT
C5aR mRNA HUMC5AAR (1175) TAGCTAACTAACTCTCCTCCATGTTGCCTGTCTTTCCCAGACTTGTCCCT
mRNA C5aR NM_001736 (1200) TAGCTAACTAACTCTCCTCCATGTTGCCTGTCTTTCCCAGACTTGTCCCT
C5aR mRNA BC008982 (1228) TAGCTAACTAACTCTCCTCCATGTTGCCTGTCTTTCCCAGACTTGTCCCT
3'UTR Chimp       (101)   TAGCTAACTAACTCTCCTCCATGTTGCCTGTCTTTCCCAGACTTGTCCCT
Consensus         (1231)  TAGCTAACTAACTCTCCTCCATGTTGCCTGTCTTTCCCAGACTTGTCCCT
                          1281                                                1330
3'UTR EC          (148)   CCTTTTCCAGCGGGACTCTTCTCATCCTTCCTCATTTGCAAGGTGAACAC
3'UTR PM          (148)   CCTTTTCCAGCGGGACTCTTCTCATCCTTCCTCATTTGCAAGGTGAACAC
gen 3'UTR         (148)   CCTTTTCCAGCGGGACTCTTCTCATCCTTCCTCATTTGCAAGGTGAACAC
C5aR mRNA HUMC5AAR (1225) CCTTTTCCAGCGGGACTCTTCTCATCCTTCCTCATTTGCAAGGTGAACAC
mRNA C5aR NM_001736 (1250) CCTTTTCCAGCGGGACTCTTCTCATCCTTCCTCATTTGCAAGGTGAACAC
C5aR mRNA BC008982 (1278) CCTTTTCCAGCGGGACTCTTCTCATCCTTCCTCATTTGCAAGGTGAACAC
3'UTR Chimp       (151)   CCTTTTCCAGCGGGACTCTTCTCATCCTTCCTCATTTGCAAGGTGAACAC
Consensus         (1281)  CCTTTTCCAGCGGGACTCTTCTCATCCTTCCTCATTTGCAAGGTGAACAC
                          1331                                                1380
3'UTR EC          (198)   TTCCTTCTAGGGAGCACCCTCCCACCCCCCACCCCCCCC--CACACACCA
3'UTR PM          (198)   TTCCTTCTAGGGAGCACCCTCCCACCCCCCACCCCCCCCA--CACACACCA
gen 3'UTR         (198)   TTCCTTCTAGGGAGCACCCTCCCACCCCCCACCCCCCCCA-CACACACCA
C5aR mRNA HUMC5AAR (1275) TTCCTTCTAGGGAGCACCCTCCCACCCCCCACCCCCCCC--CACACACCA
mRNA C5aR NM_001736 (1300) TTCCTTCTAGGGAGCACCCTCCCACCCCCCACCCCCCCCA-CACACACCA
C5aR mRNA BC008982 (1328) TTCCTTCTAGGGAGCACCCTCCCACCCCCCACCCCCCCC--CACACACCA
3'UTR Chimp       (201)   TTCCTTCTAGGGAGCACCCTCCCACCCCCCACCCCCCCCCCCACACACCA
Consensus         (1331)  TTCCTTCTAGGGAGCACCCTCCCACCCCCCACCCCCCCCC  CACACACCA
                          1381                                                1430
3'UTR EC          (246)   TCTTTCCATCCCAGGCTTTTGAAAAACAAACAGAAACCCGTGTATCTGGG
3'UTR PM          (246)   TCTTTCCATCCCAGGCTTTTGAAAAACAAACAGAAACCCGTGTATCTGGG
gen 3'UTR         (247)   TCTTTCCATCCCAGGCTTTTGAAAAACAAACAGAAACCCGTGTATCTGGG
C5aR mRNA HUMC5AAR (1323) TCTTTCCATCCCAGGCTTTTGAAAAACAAACAGAAACCCGTGTATCTGGG
mRNA C5aR NM_001736 (1349) TCTTTCCATCCCAGGCTTTTGAAAAACAAACAGAAACCCGTGTATCTGGG
C5aR mRNA BC008982 (1376) TCTTTCCATCCCAGGCTTTTGAAAAACAAACAGAAACCCGTGTATCTGGG
3'UTR Chimp       (251)   TCTTTCCATCCCAGGCTTTTGAAAAACAAACAGAAACCCGTGTATCTGGG
Consensus         (1381)  TCTTTCCATCCCAGGCTTTTGAAAAACAAACAGAAACCCGTGTATCTGGG
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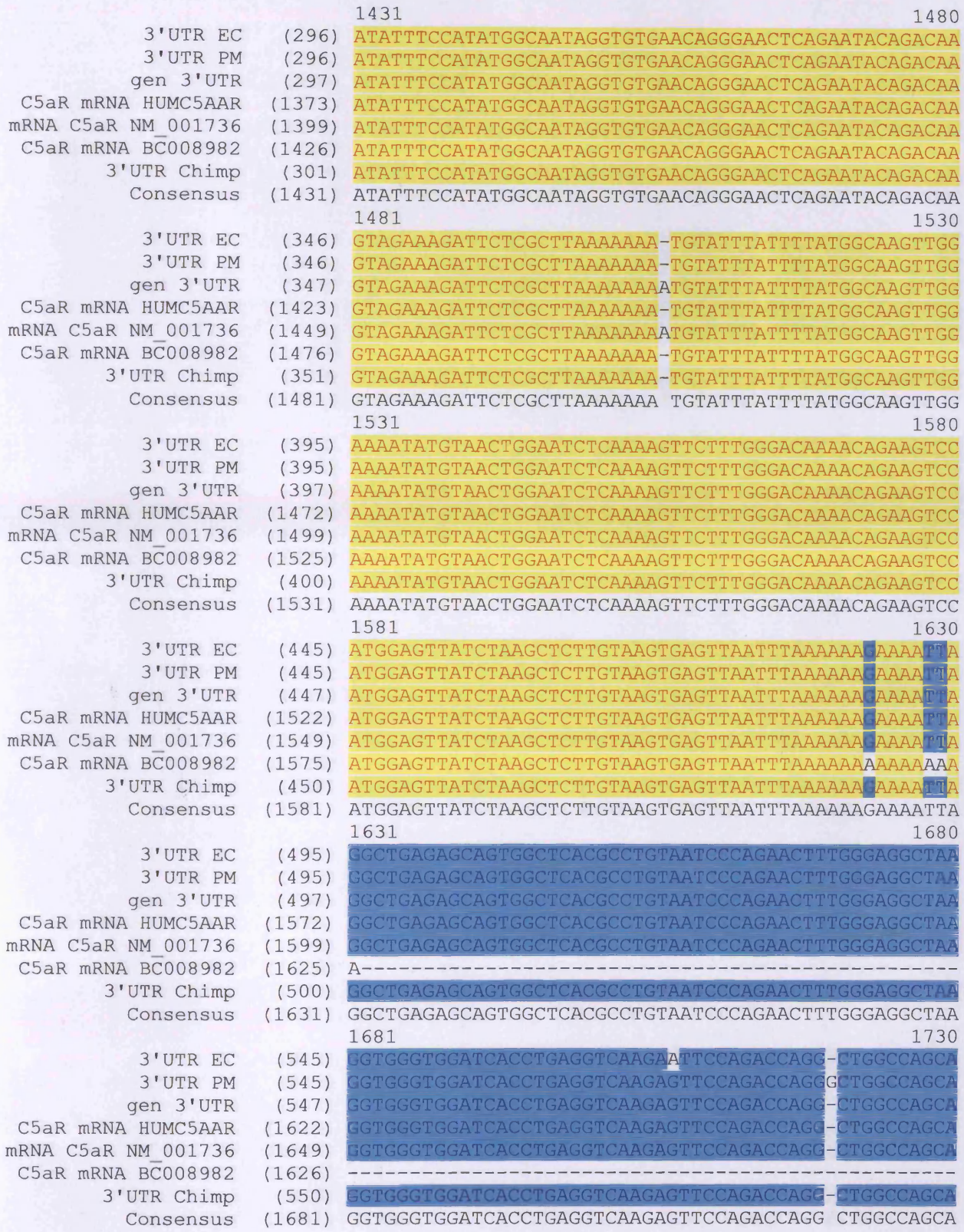
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3'UTR PM (296) ATATTTCCATATGGCAATAGGTGTGAACAGGGAACTCAGAATACAGACAA
gen 3'UTR (297) ATATTTCCATATGGCAATAGGTGTGAACAGGGAACTCAGAATACAGACAA
C5aR mRNA HUMC5AAR (1373) ATATTTCCATATGGCAATAGGTGTGAACAGGGAACTCAGAATACAGACAA
mRNA C5aR NM_001736 (1399) ATATTTCCATATGGCAATAGGTGTGAACAGGGAACTCAGAATACAGACAA
C5aR mRNA BC008982 (1426) ATATTTCCATATGGCAATAGGTGTGAACAGGGAACTCAGAATACAGACAA
3'UTR Chimp (301) ATATTTCCATATGGCAATAGGTGTGAACAGGGAACTCAGAATACAGACAA
Consensus (1431) ATATTTCCATATGGCAATAGGTGTGAACAGGGAACTCAGAATACAGACAA
1481 1530
3'UTR EC (346) GTAGAAAGATTCTCGCTTAAAAAAA-TGTATTTATTTTATGGCAAGTTGG
3'UTR PM (346) GTAGAAAGATTCTCGCTTAAAAAAA-TGTATTTATTTTATGGCAAGTTGG
gen 3'UTR (347) GTAGAAAGATTCTCGCTTAAAAAAAATGTATTTATTTTATGGCAAGTTGG
C5aR mRNA HUMC5AAR (1423) GTAGAAAGATTCTCGCTTAAAAAAA-TGTATTTATTTTATGGCAAGTTGG
mRNA C5aR NM_001736 (1449) GTAGAAAGATTCTCGCTTAAAAAAAATGTATTTATTTTATGGCAAGTTGG
C5aR mRNA BC008982 (1476) GTAGAAAGATTCTCGCTTAAAAAAA-TGTATTTATTTTATGGCAAGTTGG
3'UTR Chimp (351) GTAGAAAGATTCTCGCTTAAAAAAA-TGTATTTATTTTATGGCAAGTTGG
Consensus (1481) GTAGAAAGATTCTCGCTTAAAAAAA TGTATTTATTTTATGGCAAGTTGG
1531 1580
3'UTR EC (395) AAAATATGTAACTGGAATCTCAAAAGTTCTTTGGGACAAAACAGAAGTCC
3'UTR PM (395) AAAATATGTAACTGGAATCTCAAAAGTTCTTTGGGACAAAACAGAAGTCC
gen 3'UTR (397) AAAATATGTAACTGGAATCTCAAAAGTTCTTTGGGACAAAACAGAAGTCC
C5aR mRNA HUMC5AAR (1472) AAAATATGTAACTGGAATCTCAAAAGTTCTTTGGGACAAAACAGAAGTCC
mRNA C5aR NM_001736 (1499) AAAATATGTAACTGGAATCTCAAAAGTTCTTTGGGACAAAACAGAAGTCC
C5aR mRNA BC008982 (1525) AAAATATGTAACTGGAATCTCAAAAGTTCTTTGGGACAAAACAGAAGTCC
3'UTR Chimp (400) AAAATATGTAACTGGAATCTCAAAAGTTCTTTGGGACAAAACAGAAGTCC
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1581 1630
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C5aR mRNA HUMC5AAR (1522) ATGGAGTTATCTAAGCTCTTGTAAGTGAGTTAATTTAAAAAAGAAAATTA
mRNA C5aR NM_001736 (1549) ATGGAGTTATCTAAGCTCTTGTAAGTGAGTTAATTTAAAAAAGAAAATTA
C5aR mRNA BC008982 (1575) ATGGAGTTATCTAAGCTCTTGTAAGTGAGTTAATTTAAAAAAAAAAAAAA
3'UTR Chimp (450) ATGGAGTTATCTAAGCTCTTGTAAGTGAGTTAATTTAAAAAAGAAAATTA
Consensus (1581) ATGGAGTTATCTAAGCTCTTGTAAGTGAGTTAATTTAAAAAAGAAAATTA
1631 1680
3'UTR EC (495) GGCTGAGAGCAGTGGCTCACGCCTGTAATCCCAGAACTTTGGGAGGCTAA
3'UTR PM (495) GGCTGAGAGCAGTGGCTCACGCCTGTAATCCCAGAACTTTGGGAGGCTAA
gen 3'UTR (497) GGCTGAGAGCAGTGGCTCACGCCTGTAATCCCAGAACTTTGGGAGGCTAA
C5aR mRNA HUMC5AAR (1572) GGCTGAGAGCAGTGGCTCACGCCTGTAATCCCAGAACTTTGGGAGGCTAA
mRNA C5aR NM_001736 (1599) GGCTGAGAGCAGTGGCTCACGCCTGTAATCCCAGAACTTTGGGAGGCTAA
C5aR mRNA BC008982 (1625) A----------
3'UTR Chimp (500) GGCTGAGAGCAGTGGCTCACGCCTGTAATCCCAGAACTTTGGGAGGCTAA
Consensus (1631) GGCTGAGAGCAGTGGCTCACGCCTGTAATCCCAGAACTTTGGGAGGCTAA
1681 1730
3'UTR EC (545) GGTGGGTGGATCACCTGAGGTCAAGAATTCCAGACCAGG-CTGGCCAGCA
3'UTR PM (545) GGTGGGTGGATCACCTGAGGTCAAGAGTTCCAGACCAGGGCTGGCCAGCA
gen 3'UTR (547) GGTGGGTGGATCACCTGAGGTCAAGAGTTCCAGACCAGG-CTGGCCAGCA
C5aR mRNA HUMC5AAR (1622) GGTGGGTGGATCACCTGAGGTCAAGAGTTCCAGACCAGG-CTGGCCAGCA
mRNA C5aR NM_001736 (1649) GGTGGGTGGATCACCTGAGGTCAAGAGTTCCAGACCAGG-CTGGCCAGCA
C5aR mRNA BC008982 (1626) ----------
3'UTR Chimp (550) GGTGGGTGGATCACCTGAGGTCAAGAGTTCCAGACCAGG-CTGGCCAGCA
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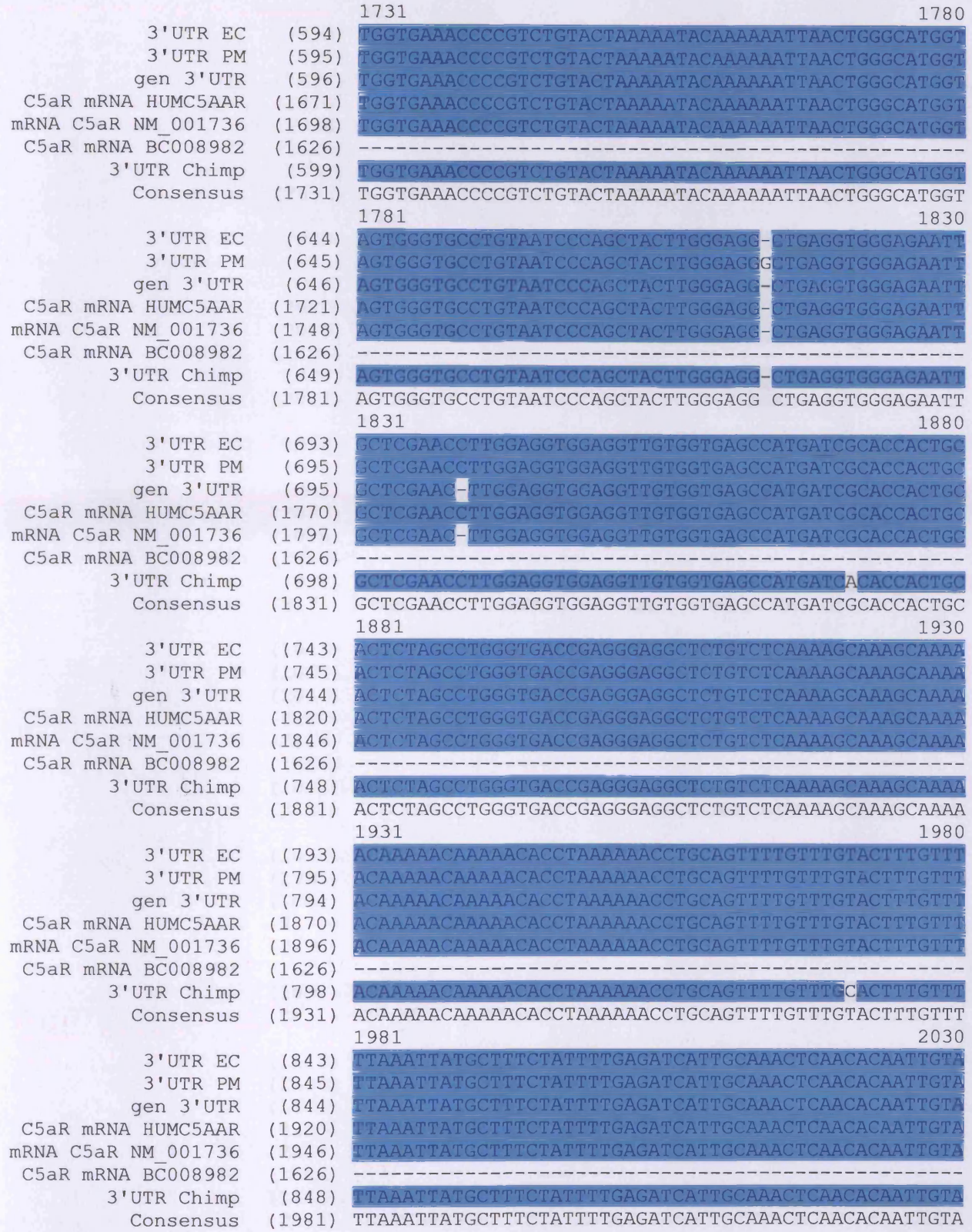
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gen 3'UTR (596) TGGTGAAACCCCGTCTGTACTAAAAATACAAAAAATTAACTGGGCATGGT
C5aR mRNA HUMC5AAR (1671) TGGTGAAACCCCGTCTGTACTAAAAATACAAAAAATTAACTGGGCATGGT
mRNA C5aR NM_001736 (1698) TGGTGAAACCCCGTCTGTACTAAAAATACAAAAAATTAACTGGGCATGGT
C5aR mRNA BC008982 (1626) ----------
3'UTR Chimp (599) TGGTGAAACCCCGTCTGTACTAAAAATACAAAAAATTAACTGGGCATGGT
Consensus (1731) TGGTGAAACCCCGTCTGTACTAAAAATACAAAAAATTAACTGGGCATGGT
1781 1830
3'UTR EC (644) AGTGGGTGCCTGTAATCCCAGCTACTTGGGAGG-CTGAGGTGGGAGAATT
3'UTR PM (645) AGTGGGTGCCTGTAATCCCAGCTACTTGGGAGGGCTGAGGTGGGAGAATT
gen 3'UTR (646) AGTGGGTGCCTGTAATCCCAGCTACTTGGGAGG-CTGAGGTGGGAGAATT
C5aR mRNA HUMC5AAR (1721) AGTGGGTGCCTGTAATCCCAGCTACTTGGGAGG-CTGAGGTGGGAGAATT
mRNA C5aR NM_001736 (1748) AGTGGGTGCCTGTAATCCCAGCTACTTGGGAGG-CTGAGGTGGGAGAATT
C5aR mRNA BC008982 (1626) ----------
3'UTR Chimp (649) AGTGGGTGCCTGTAATCCCAGCTACTTGGGAGG-CTGAGGTGGGAGAATT
Consensus (1781) AGTGGGTGCCTGTAATCCCAGCTACTTGGGAGG CTGAGGTGGGAGAATT
1831 1880
3'UTR EC (693) GCTCGAACCTTGGAGGTGGAGGTTGTGGTGAGCCATGATCGCACCACTGC
3'UTR PM (695) GCTCGAACCTTGGAGGTGGAGGTTGTGGTGAGCCATGATCGCACCACTGC
gen 3'UTR (695) GCTCGAAC-TTGGAGGTGGAGGTTGTGGTGAGCCATGATCGCACCACTGC
C5aR mRNA HUMC5AAR (1770) GCTCGAACCTTGGAGGTGGAGGTTGTGGTGAGCCATGATCGCACCACTGC
mRNA C5aR NM_001736 (1797) GCTCGAAC-TTGGAGGTGGAGGTTGTGGTGAGCCATGATCGCACCACTGC
C5aR mRNA BC008982 (1626) ----------
3'UTR Chimp (698) GCTCGAACCTTGGAGGTGGAGGTTGTGGTGAGCCATGATCACACCACTGC
Consensus (1831) GCTCGAACCTTGGAGGTGGAGGTTGTGGTGAGCCATGATCGCACCACTGC
1881 1930
3'UTR EC (743) ACTCTAGCCTGGGTGACCGAGGGAGGCTCTGTCTCAAAAGCAAAGCAAAA
3'UTR PM (745) ACTCTAGCCTGGGTGACCGAGGGAGGCTCTGTCTCAAAAGCAAAGCAAAA
gen 3'UTR (744) ACTCTAGCCTGGGTGACCGAGGGAGGCTCTGTCTCAAAAGCAAAGCAAAA
C5aR mRNA HUMC5AAR (1820) ACTCTAGCCTGGGTGACCGAGGGAGGCTCTGTCTCAAAAGCAAAGCAAAA
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C5aR mRNA BC008982 (1626) ----------
3'UTR Chimp (748) ACTCTAGCCTGGGTGACCGAGGGAGGCTCTGTCTCAAAAGCAAAGCAAAA
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1931 1980
3'UTR EC (793) ACAAAAACAAAAACACCTAAAAAACCTGCAGTTTTGTTTGTACTTTGTTT
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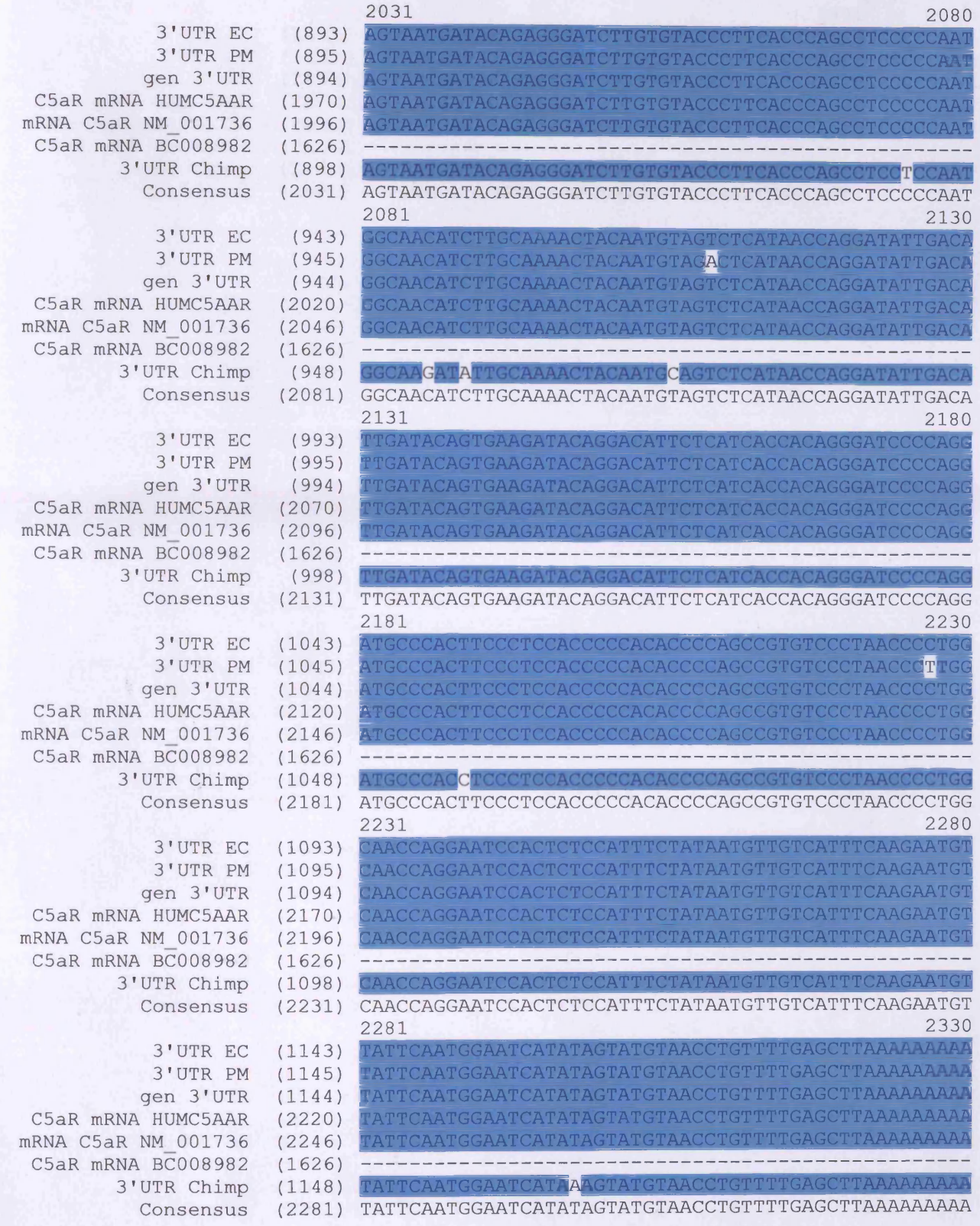
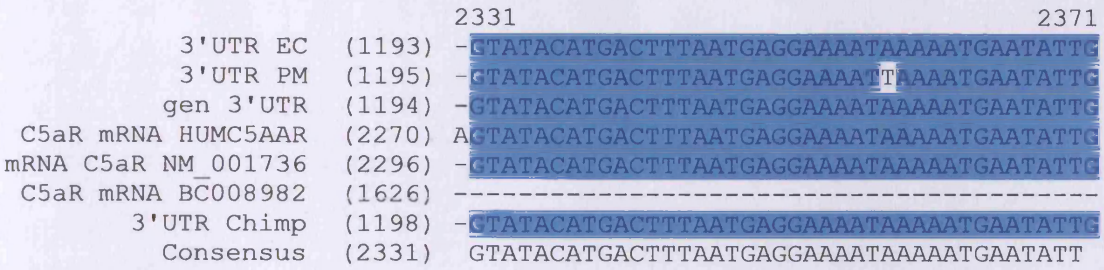
gen 3'UTR (794) ACAAAAACAAAAACACCTAAAAAACCTGCAGTTTTGTTTGTACTTTGTTT
C5aR mRNA HUMC5AAR (1870) ACAAAAACAAAAACACCTAAAAAACCTGCAGTTTTGTTTGTACTTTGTTT
mRNA C5aR NM_001736 (1896) ACAAAAACAAAAACACCTAAAAAACCTGCAGTTTTGTTTGTACTTTGTTT
C5aR mRNA BC008982 (1626) ----------
3'UTR Chimp (798) ACAAAAACAAAAACACCTAAAAAACCTGCAGTTTTGTTTGCACTTTGTTT
Consensus (1931) ACAAAAACAAAAACACCTAAAAAACCTGCAGTTTTGTTTGTACTTTGTTT
1981 2030
3'UTR EC (843) TTAAATTATGCTTTCTATTTTGAGATCATTGCAAACTCAACACAATTGTA
3'UTR PM (845) TTAAATTATGCTTTCTATTTTGAGATCATTGCAAACTCAACACAATTGTA
gen 3'UTR (844) TTAAATTATGCTTTCTATTTTGAGATCATTGCAAACTCAACACAATTGTA
C5aR mRNA HUMC5AAR (1920) TTAAATTATGCTTTCTATTTTGAGATCATTGCAAACTCAACACAATTGTA
mRNA C5aR NM_001736 (1946) TTAAATTATGCTTTCTATTTTGAGATCATTGCAAACTCAACACAATTGTA
C5aR mRNA BC008982 (1626) ----------
3'UTR Chimp (848) TTAAATTATGCTTTCTATTTTGAGATCATTGCAAACTCAACACAATTGTA
Consensus (1981) TTAAATTATGCTTTCTATTTTGAGATCATTGCAAACTCAACACAATTGTA


2031 2080
3'UTR EC (893) AGTAATGATACAGAGGGATCTTGTGTACCCTTCACCCAGCCTCCCCCAAT
3'UTR PM (895) AGTAATGATACAGAGGGATCTTGTGTACCCTTCACCCAGCCTCCCCCAAT
gen 3'UTR (894) AGTAATGATACAGAGGGATCTTGTGTACCCTTCACCCAGCCTCCCCCAAT
C5aR mRNA HUMC5AAR (1970) AGTAATGATACAGAGGGATCTTGTGTACCCTTCACCCAGCCTCCCCCAAT
mRNA C5aR NM_001736 (1996) AGTAATGATACAGAGGGATCTTGTGTACCCTTCACCCAGCCTCCCCCAAT
C5aR mRNA BC008982 (1626) ----------
3'UTR Chimp (898) AGTAATGATACAGAGGGATCTTGTGTACCCTTCACCCAGCCTCCTCCAAT
Consensus (2031) AGTAATGATACAGAGGGATCTTGTGTACCCTTCACCCAGCCTCCCCCAAT
2081 2130
3'UTR EC (943) GGCAACATCTTGCAAAACTACAATGTAGTCTCATAACCAGGATATTGACA
3'UTR PM (945) GGCAACATCTTGCAAAACTACAATGTAGACTCATAACCAGGATATTGACA
gen 3'UTR (944) GGCAACATCTTGCAAAACTACAATGTAGTCTCATAACCAGGATATTGACA
C5aR mRNA HUMC5AAR (2020) GGCAACATCTTGCAAAACTACAATGTAGTCTCATAACCAGGATATTGACA
mRNA C5aR NM_001736 (2046) GGCAACATCTTGCAAAACTACAATGTAGTCTCATAACCAGGATATTGACA
C5aR mRNA BC008982 (1626) ----------
3'UTR Chimp (948) GGCAAGATATTGCAAAACTACAATGCAGTCTCATAACCAGGATATTGACA
Consensus (2081) GGCAACATCTTGCAAAACTACAATGTAGTCTCATAACCAGGATATTGACA
2131 2180
3'UTR EC (993) TTGATACAGTGAAGATACAGGACATTCTCATCACCACAGGGATCCCCAGG
3'UTR PM (995) TTGATACAGTGAAGATACAGGACATTCTCATCACCACAGGGATCCCCAGG
gen 3'UTR (994) TTGATACAGTGAAGATACAGGACATTCTCATCACCACAGGGATCCCCAGG
C5aR mRNA HUMC5AAR (2070) TTGATACAGTGAAGATACAGGACATTCTCATCACCACAGGGATCCCCAGG
mRNA C5aR NM_001736 (2096) TTGATACAGTGAAGATACAGGACATTCTCATCACCACAGGGATCCCCAGG
C5aR mRNA BC008982 (1626) ----------
3'UTR Chimp (998) TTGATACAGTGAAGATACAGGACATTCTCATCACCACAGGGATCCCCAGG
Consensus (2131) TTGATACAGTGAAGATACAGGACATTCTCATCACCACAGGGATCCCCAGG
2181 2230
3'UTR EC (1043) ATGCCCACTTCCCTCCACCCCCACACCCCAGCCGTGTCCCTAACCCCTGG
3'UTR PM (1045) ATGCCCACTTCCCTCCACCCCCACACCCCAGCCGTGTCCCTAACCCTTGG
gen 3'UTR (1044) ATGCCCACTTCCCTCCACCCCCACACCCCAGCCGTGTCCCTAACCCCTGG
C5aR mRNA HUMC5AAR (2120) ATGCCCACTTCCCTCCACCCCCACACCCCAGCCGTGTCCCTAACCCCTGG
mRNA C5aR NM_001736 (2146) ATGCCCACTTCCCTCCACCCCCACACCCCAGCCGTGTCCCTAACCCCTGG
C5aR mRNA BC008982 (1626) ----------
3'UTR Chimp (1048) ATGCCCACCTCCCTCCACCCCCACACCCCAGCCGTGTCCCTAACCCCTGG
Consensus (2181) ATGCCCACTTCCCTCCACCCCCACACCCCAGCCGTGTCCCTAACCCCTGG
2231 2280
3'UTR EC (1093) CAACCAGGAATCCACTCTCCATTTCTATAATGTTGTCATTTCAAGAATGT
3'UTR PM (1095) CAACCAGGAATCCACTCTCCATTTCTATAATGTTGTCATTTCAAGAATGT
gen 3'UTR (1094) CAACCAGGAATCCACTCTCCATTTCTATAATGTTGTCATTTCAAGAATGT
C5aR mRNA HUMC5AAR (2170) CAACCAGGAATCCACTCTCCATTTCTATAATGTTGTCATTTCAAGAATGT
mRNA C5aR NM_001736 (2196) CAACCAGGAATCCACTCTCCATTTCTATAATGTTGTCATTTCAAGAATGT
C5aR mRNA BC008982 (1626) ----------
3'UTR Chimp (1098) CAACCAGGAATCCACTCTCCATTTCTATAATGTTGTCATTTCAAGAATGT
Consensus (2231) CAACCAGGAATCCACTCTCCATTTCTATAATGTTGTCATTTCAAGAATGT
2281 2330
3'UTR EC (1143) TATTCAATGGAATCATATAGTATGTAACCTGTTTTGAGCTTAAAAAAAAA
3'UTR PM (1145) TATTCAATGGAATCATATAGTATGTAACCTGTTTTGAGCTTAAAAAAAAA
gen 3'UTR (1144) TATTCAATGGAATCATATAGTATGTAACCTGTTTTGAGCTTAAAAAAAAA
C5aR mRNA HUMC5AAR (2220) TATTCAATGGAATCATATAGTATGTAACCTGTTTTGAGCTTAAAAAAAAA
mRNA C5aR NM_001736 (2246) TATTCAATGGAATCATATAGTATGTAACCTGTTTTGAGCTTAAAAAAAAA
C5aR mRNA BC008982 (1626) ----------
3'UTR Chimp (1148) TATTCAATGGAATCATAAAGTATGTAACCTGTTTTGAGCTTAAAAAAAAA
Consensus (2281) TATTCAATGGAATCATATAGTATGTAACCTGTTTTGAGCTTAAAAAAAAA


2331
2371
3'UTR EC (1193) -GTATACATGACTTTAATGAGGAAAATAAAAATGAATATTG
3'UTR PM (1195) -GTATACATGACTTTAATGAGGAAAATTAAAATGAATATTG
gen 3'UTR (1194) -GTATACATGACTTTAATGAGGAAAATAAAAATGAATATTG
C5aR mRNA HUMC5AAR (2270) AGTATACATGACTTTAATGAGGAAAATAAAAATGAATATTG
mRNA C5aR NM_001736 (2296) -GTATACATGACTTTAATGAGGAAAATAAAAATGAATATTG
C5aR mRNA BC008982 (1626) ----------
3'UTR Chimp (1198) -GTATACATGACTTTAATGAGGAAAATAAAAATGAATATTG
Consensus (2331) GTATACATGACTTTAATGAGGAAAATAAAAATGAATATT