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C5L2, A NONSIGNALLING C5A RECEPTOR

IS UNCOUPLED TO G PROTEINS

A thesis submitted for the Degree

of Doctor of Philosophy (Ph.D.)

by

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at

University of Dublin, Trinity College

2007

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DECLARATION

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Signed Dubhfeasa Maire Slattery

Date 8th April 2008

SUMMARY: C5L2, A NONSIGNALLING C5A RECEPTOR IS UNCOUPLED TO G PROTEINS

The complement anaphylatoxins are prominent mediators of host defence and inflammation. The small subunit of the fifth component of complement, C5a has complex effects *in vivo* including potential anti- and pro-inflammatory effects in acute pancreatitis with associated lung injury, and sepsis respectively. Deletion of the classical C5a receptor C5aR, does not fully explain all these effects. Secondly, expression of receptors for C5a on parenchymal cells in the lung, liver, smooth muscle and endothelial cells serve as yet uncharacterised functions. We suspected that an additional receptor for C5a might exist. Ohno and colleagues (Ohno *et al.* 2000) had just cloned a novel human gene encoding the putative orphan receptor "C5a like 2 receptor", C5L2, which they found on immature but not mature dendritic cells. We cloned C5L2 from man and mouse and characterised this receptor. Sequence alignment of murine and human C5L2 revealed a high identity in the deduced protein and nucleotide sequences (>60%). C5L2 unlike C5aR, has a natural substitution of leucine for arginine at position 132 in the normally highly conserved 'DRY' sequence at the end of the third transmembrane sequence. We demonstrated almost congruent expression of both C5aR and C5L2 in human and murine tissues studied including heart, spleen and brain etc. but bone marrow and peripheral blood leukocytes are the most abundant sources of message for both receptors. Regarding the biophysical properties of C5L2 we demonstrated that C5L2 is a high affinity binder of C5a (Kd = 2.5 ± 0.4nM), similar to that of the classical C5a receptor, C5aR (Kd = 3.4 ± 0.7 nM). C5L2 binds the less active derivative of C5a, C5a des Arg with almost the same affinity as it does C5a (Kd = 12.0 ± 2.3 nM). In contrast C5aR binds C5a des Arg with

~200 fold less avidity than that of C5a (Kd = 660 ± 153 nM). Human blood leukocytes bind C5a with a similar affinity as either C5aR/L1.2 or C5L2 /L1.2 cells but bind C5a des Arg less well than C5aR/L1.2 cells (Kd 1900 ± 126nM). Regarding kinetics of binding, C5aR has a very rapid "association rate" essentially at equilibrium within a few minutes of addition of C5a ligand. In contrast C5L2 requires 60 minutes to reach equilibrium (~ 100 fold slower). The dissociation rate for C5aR is three fold faster than that of C5L2. We provide evidence that C5aR is robustly phosphorylated in response to five minutes exposure to C5a agonist but only a modest ligand dependant phosphorylation is demonstrated for C5L2. C5aR is rapidly internalised as a function of C5a ligand concentration to a level of ~50% within 5 minutes, while the surface expression of C5L2 is virtually unchanged over 1 hour. We demonstrate that C5L2 unlike C5aR is uncoupled to G proteins and is non-signalling. C5L2 does not activate mitogen activated protein kinase in response to agonist binding, does not mediate calcium flux, nor stimulate chemotaxis. Introduction of a point mutation replacing leucine with arginine is associated with a partial gain of function in coupling to G proteins. We demonstrated co-expression of C5aR and C5L2 on human granulocytes. We examine changes in gene transcription by microarray analysis when bone marrow cells from both wild type and C5aR deficient mice are exposed to mouse C5a. Wild type bone marrow cells respond robustly to C5a with induction and suppression of a number of inflammation related genes in contrast to C5aR deficient mice, (expressing C5L2 alone) which do not respond. We postulate that C5L2 modulates the biological functions of C5a/ C5a des Arg *in vivo* or acts as a decoy receptor. Recently, C5L2 has been shown to be critical for the biological activities of C5a and C3a and may be a target for treating diseases like asthma, sepsis and pneumonia.

This thesis is dedicated to:

my wonderful parents, Mairead and Michael,

my ever-supportive and loving husband, Mick

and to "smiling Michael" and "laughing Lochlainn"

- our two great boys!

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Abbreviations

bHLH	*Basic helix loop helix*
bp	Base pair
BSA	Bovine serum albumen
$CaCl_2$	Calcium chloride
C5a	Complement fragment 5a
C5aR	Complement fragment 5a receptor
C5L2	C5a-like receptor 2
cDNA	Complimentary deoxyribonucleic acid
CLP	Caecal ligation and puncture
DARC	Duffy antigen receptor for chemokines
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulphoxide
FACS	Fluorescent activated cell sorter
FCS	Foetal calf serum
fMLP	formyl-Met-leu-Phe
GPCR	G protein-coupled receptor
HBSS	Hanks balanced salt solution
HEK293T	Human embryonic kidney 293T cells
HEPES	N-(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonic acid
KCl	Potassium chloride
K_D	Dissociation constant
L1.2 cells	Murine pre-B-lymphoma cell line
MAC	Membrane attack complex
MAP kinase	Mitogen activated protein kinase
mC5a	Mouse C5a
$MgCl_2$	Magnesium chloride
mRNA	Messenger ribonucleic acid
MOF	Multi organ failure
NaCl	Sodium chloride

Abbreviations continued

NaN_3	Sodium azide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PSA	Pseudomonas aeruginosa
RNA	Ribonucleic-acid
RT- PCR	Reverse transcriptase polymerase chain reaction

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3) Chemokine Receptors–chapter 2.3

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PRESENTATIONS of work related to this thesis

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Abstract published in American Review of Respiratory and Critical Care Medicine

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CHAPTER I

GENERAL INTRODUCTION TO COMPLEMENT

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1.1 AN OVERVIEW OF COMPLEMENT

1.1.1 The complement system

The complement system plays an important role in host defence against infectious agents. It was discovered by Jules Bordet as a heat labile component of normal plasma, which augments the opsonisation of bacteria by antibodies and allows some antibodies to kill bacteria. This activity was said to "complement" the antibacterial activity of antibody, hence its name. Although first discovered as an effector arm of the antibody response, complement first evolved as part of the innate immune response, where it still has a very important role.

The complement system is made up of more than thirty five plasma proteins that react with one another to opsonise pathogens and induce a series of inflammatory responses that help to fight infection. Several complement proteins are proteases that are themselves activated by proteolytic cleavage. These enzymes (or "zymogens") were first discovered in the gut. In the complement system the precursor zymogens are widely distributed throughout body fluids and tissues without adverse effects. At sites of infection however, they are activated locally and trigger a series of potent inflammatory events. The complement system activates through a triggered–enzyme cascade. In such a cascade, an active complement enzyme generated by cleavage of its zymogen precursor then cleaves its substrate, another complement zymogen, to its active enzymatic form. This in turn cleaves and activates the next zymogen in the complement pathway. In this way, the activation of a small number of complement proteins at the start of the pathway is hugely

amplified by each successive enzymatic reaction, resulting in the rapid generation of a disproportionately large complement response. The complement proteins are synthesised mainly in hepatocytes, however, significant amounts are produced by monocytes, macrophages and epithelial cells in the gastrointestinal and genitourinary tracts. As expected, the complement system contains many regulatory membrane proteins that function to prevent uncontrolled complement activation and protect host cells from accidental complement attack. In addition, the complement system includes multiple distinct cell-surface receptors that exhibit specificity for the physiological fragments of complement proteins and that occur on inflammatory cells and cells of the immune system.

Complement protects against infection in three ways (Tomlinson S. 1993).#First it generates large numbers of activated complement proteins that bind covalently to pathogens, opsonising them for engulfment by phagocytes bearing receptors for complement. Second, the small fragments of some complement proteins act as chemoattractants to recruit more phagocytes to the site of complement activation and in addition to activate these phagocytes. Third, the terminal complement components damage and directly kill certain bacteria by creating pores in the bacterial membrane.

There are three pathways of complement activation: the classical pathway which is initiated by the binding of C1q, the first protein of the complement cascade, to the pathogen surface. The mannose-binding lectin (MBL) pathway, which is initiated by binding of the mannose-binding lectin, (a serum protein whose level increases during the acute-phase response), to mannose–containing carbohydrates on bacteria or viruses. Thirdly, the alternative pathway, which can be initiated by the binding of spontaneously activated C3 in plasma to the surface of a pathogen. While these pathways depend on different molecules for their initiation, all three converge to generate a crucial enzymatic activity that, in turn generates the effector molecules of complement (Figure 1.1).

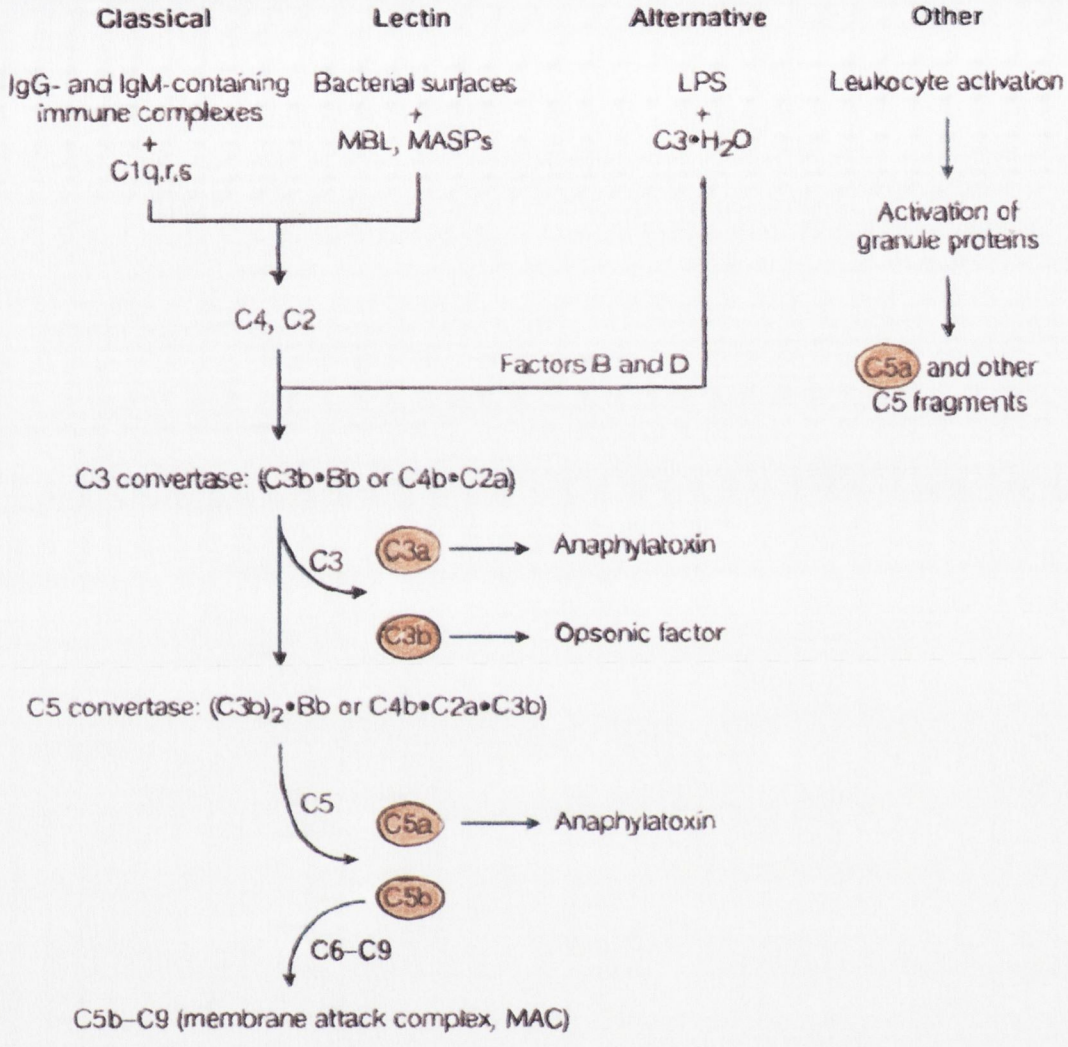


Figure 1.1 Overview of the main components and effector actions of complement.

(Ward P.A.,2004 *Nature Reviews Immunology*, 4, 133-142)

The events of the complement cascade can be divided into two sequences of reactions called "early" and "late". The early events culminate in the generation of a protease called C3 convertase. The "early" events of complement activation consist of triggered-enzyme cascades in which inactive complement zymogens are successively cleaved to yield two fragments, the larger of which is an active serine protease. The active protease is retained at the pathogen surface, and this ensures that the next complement zymogen in the pathway is also cleaved and activated at the pathogen surface. By contrast, the small peptide fragment is released from the site of the reaction and often mediates the inflammatory response.

The nomenclature of complement requires explanation. All components of the classical complement pathway and the membrane attack complex are designated by the letter C followed by a number. The native components have a simple number designation, e.g. C1 and C2 but the components were numbered in the order of their discovery rather than the sequence of reactions. The reaction sequence is C1, C4, C2, C3, C5, C6, C7, C8, and C9. The products of the cleavage reactions are designated by added lower case letters, the larger fragment being designated "b" and the smaller "a": thus, for example, C4 is cleaved to C4b, the large fragment of C4 that binds covalently to the surface of the pathogen and C4a, a small fragment with weak pro-inflammatory properties. The components of the alternative pathway, instead of being numbered, are designated by different capital letters, for example factor B and factor D. As with the classical pathway, their cleavage products are designated by the addition of lower case "a" and "b": thus the large fragment of B is called Bb and the small fragment, Ba. Finally, in the mannose-binding lectin pathway, the first enzymes to be activated are known as the mannose-binding lectin associated serine proteases, MASP-1 and MASP-2, after which the pathway is essentially the same as the classical pathway.

The formation of C3 convertase activity is pivotal in complement activation leading to the production of the principal effector molecules, and initiating the late events. In the classical and MB-lectin pathways the C3 convertase is formed from membrane bound C4b complexed with C2b, designated C4b2b. In the alternative pathway, a homologous C2 convertase is formed from membrane bound C3b complexed with Bb, C3bBb. The alternative pathway can act as an amplification loop for all three pathways, because it is initiated by the binding of C3b (Frank *et al.*1991).

The C3 convertases formed by the early events of complement activation are bound covalently to the pathogen surface. Here they cleave C3 to generate large amounts of C3b, the main effector molecule of the complement system and C3a, a peptide mediator of inflammation. The most well-known function of C3a is its ability to cause degranulation of basophils and mast cells, resulting in the release of vasoactive amines (e.g. histamine) which cause oedema and constriction of smooth muscle, especially in the gut and upper airways of the lungs (Schellenberg *et al.*1984, del Balzo *et al.* 1989, Erdei *et al.* 1997). The C3b binds covalently to the bacterial cell membrane, imparting the signature of "foreignness" to bacteria, which leads to rapid opsonisation and phagocytic uptake of these organisms through receptors for C3b and related C3b products on phagocytic cells (Schlesinger *et al.* 1990, Payne *et al.* 1987, Pryzwansky *et al.* 1985) and ultimate destruction of these organisms. The main function of C3b seems to be the containment of bacteria *in vivo*. In addition, C3b binds to the C3 convertase to form a C5 convertase that produces the most important small peptide mediator of inflammation, C5a, as well as a large active fragment C5b, that initiates the "late" events of complement activation. These comprise a sequence of polymerisation reactions in which the terminal components interact to form a membrane attack complex, which creates a pore in cell membranes of certain pathogens that can lead to their death.

The complement pathway which leads to potent inflammatory and destructive effects and which has a series of built-in amplification steps, is potentially dangerous and must be subject to tight regulation. One important safeguard is that key activated complement components are rapidly inactivated unless they bind to the pathogen surface on which

their activation was initiated. In addition there are several points in the pathway at which regulatory proteins act on complement components to prevent inadvertent activation of complement on host cells, thereby protecting them from accidental damage. These regulatory mechanisms will be discussed later.

1.1.2 The classical pathway

The classical pathway is initiated by the binding of C1q, the first protein in the complement cascade, to the pathogen surface. C1q can bind to the surface of pathogens in one of three ways. It can bind directly to surface components of some bacteria, including certain proteins of bacterial cell walls and polyanionic surface structures, such as lipoteichoic acid on Gram–positive bacteria. Secondly, C1q binds to C-reactive protein, an acute phase protein of human plasma that binds to phosphocholine residues in bacterial polysaccharides, such as pneumococcal C polysaccharide: hence the name C-reactive protein. Thus complement proteins can act in the absence of adaptive immunity and represent the humoral arm of innate immunity. Thirdly, C1q is a key link between the effecter mechanisms of innate and adaptive immunity by binding to antibody-antigen complexes/ immunoglobulin-G (IgG) and IgM containing immune complexes (Cooper N.R. 1985). Thus the classical pathway has a role in both innate and adaptive immunity.

1.1.2.1 C1 complex

C1q is part of the C1 complex, which comprises a single C1q molecule bound to two molecules each of the zymogens C1r and C1s. C1q has six globular heads, linked together by a collagen like tail which surround the $(C1r:C1s)_2$ complex (Figure 1.2) Binding of more than one of the C1q heads to a pathogen surface or to the Fc portions of antibodies in an immune complex causes a conformational change in the $(C1r:C1s)_2$ complex, which leads to the activation of an autocatalytic enzymatic activity in C1r; the active form of C1r then cleaves its associated C1s to generate an active serine protease.

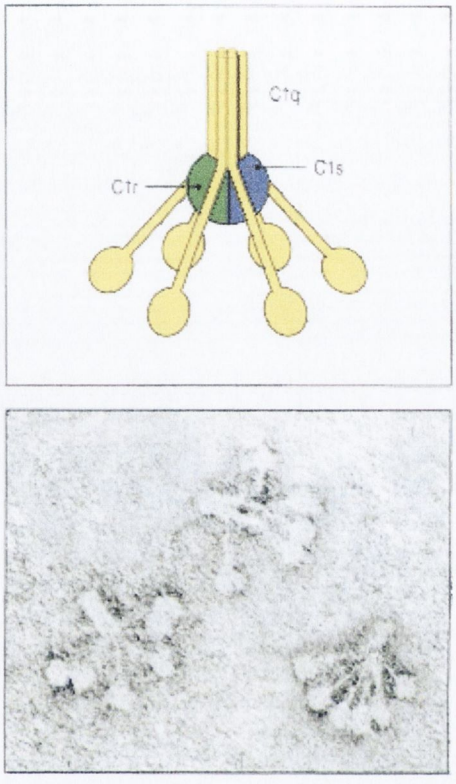


Figure 1.2 C1: the first protein in the classical pathway of complement activation is a complex of C1q, C1r and C1s.

(Janeway *et al.* Immunobiology, 2005, 59)

Once activated, the C1s enzyme acts on the next two components of the classical pathway, cleaving C4 and then C2 to generate two large fragments, C4b and C2b, which together form the C3 convertase of the classical pathway (Arlaud *et al.* 2002). In the first step, C1s cleaves C4 to produce C4b, which may bind covalently to the surface of the pathogen. The covalently attached C4b then binds one molecule of C2, making it susceptible, in turn to cleavage by C1s. C1s cleaves C2 to produce the large fragment C2b, which is itself a serine protease. C4b2b, the complex of C4b with the active serine protease C2b, remains covalently linked to the surface of the pathogen as the C3

convertase of the classical pathway. Its most important activity is to cleave large numbers of C3 molecules to produce C3b molecules that coat the pathogen surface. At the same time the other cleavage product C3a, initiates a local inflammatory response. These reactions comprise the classical pathway of complement activation.

1.1.3 The mannose-binding lectin (MB-lectin) pathway

The MB-lectin pathway uses a protein very similar to C1q to trigger the complement cascade. This serum protein, called mannose binding lectin (MBL), is a member of the collectin family of proteins, of which surfactant proteins A and D (SP-A and SP-D) are also members. The lectin pathway involves interaction of MBL with mannose residues on bacterial surfaces, and with ficolins, resulting in the activation of MBL-associated serine protease I (MASP1)-MASP3 in the serum (Lu *et al.* 2002). MBL binds specifically to mannose residues, and to certain other sugars, which are accessible and arranged in a pattern that allows binding on many pathogens. On vertebrate cells however, these are covered by other sugar groups, especially by sialic acid. Thus MBL is able to initiate complement activation by binding to pathogen surfaces while avoiding activation on host cell surfaces (Jack *et al.* 2001). It is present at low concentrations in normal plasma of most individuals and its production is increased by the liver during the acute phase reaction driven by the innate immune response.

Mannose-binding lectin, like C1q, is a two to six headed molecule that forms a complex with two protease zymogens, which in the case of the MBL complex are MASP-1 and MASP-2. MASP-2 is closely homologous to C1r and C1s and MASP-1 is a slightly more distantly related cousin; all four enzymes are likely to have evolved from gene duplication of a common precursor (Gal *et al,* 2001, Schwaeble *et al.* 2002). When the MBL complex binds to a pathogen surface, MASP-2 is activated. At this point, the classical and lectin pathways converge, the outcome being limited cleavage of C4 and C2, generating C4bC2a, a C3 convertase. The role if any, of MASP-1 in complement activation is uncertain. Thus the MB lectin pathway initiates complement activation in a

closely similar way to the classical pathway, forming a C3 convertase from C2b bound to C4b. People deficient in MBL experience a substantial increase in infections during early childhood, indicating the importance of the MB-lectin pathway for host defence. The age window of susceptibility to infections associated with MBL deficiency illustrates the particular importance of innate host defence mechanisms in early childhood, which is a time before the child's adaptive immune responses are fully matured and after maternal antibodies transferred across the placenta and in colostrum have been lost.

1.1.4 The alternative pathway

The alternative pathway is so named because it was discovered as a second, or "alternative" pathway for complement activation after the classical pathway had been defined. This pathway can proceed on many microbial surfaces in the absence of specific antibody, and it leads to the generation of a distinct C3 convertase designated C3bBb (Xu *et al.* 2001). In contrast to the classical and MB-lectin pathways of complement activation, the alternative pathway does not depend on a pathogen-binding protein for its initiation; instead it is initiated through the spontaneous hydrolysis of C3. Alternatively, lipopolysaccharide (LPS) derived from Gram-negative bacteria may trigger the alternative pathway. The distinctive components of the pathway include C3, Factor B (B), Factor D (D), and Properdin (P), with active fragments being C3b, Ba, Bb, D and P. Several mechanisms ensure that the activation pathway will only proceed on the surface of the pathogen.

C3 is abundant in plasma, and C3b is produced at a significant rate by spontaneous cleavage (also known as "tickover"). This occurs through the spontaneous hydrolysis of the thioester bond in C3 to form $C3(H_2O)$, which has an altered conformation, allowing binding of the plasma protein factor B. The binding of B by $C3(H_2O)$ then allows a plasma protease called factor D to cleave factor B to Ba and Bb, the latter remaining associated with $C3(H_2O)$ to form the $C3(H_2O)Bb$ complex. This complex is a fluid–phase C3 convertase, and although it is only formed in small amounts it can cleave many

molecules of C3 to C3a and C3b. Much of this C3b is inactivated by hydrolysis, but some attaches covalently, through its reactive thioester group, to the surfaces of pathogens or of host cells. C3b bound in this way is able to bind factor B, allowing its cleavage by factor D to yield the small fragment Ba and the active protease Bb. This results in the formation of the alternative pathway C3 convertase, C3Bb.

When C3b binds to host cells, several complement regulatory proteins, present in the plasma and on host cell membranes, combine to prevent complement activation from proceeding (Xu *et al.* 2001). These proteins interact with C3b and either prevent the convertase from forming or promote its rapid dissociation. Thus, the complement receptor 1 (CR1) and a membrane–attached protein known as decay-accelerating factor (DAF or CD55) compete with factor B for binding to C3b on the cell surface, and can displace Bb from convertase that has already formed. Convertase formation can also be prevented by cleaving C3b to its inactive derivative iC3b. This is achieved by a plasma protease, factor I, in conjunction with C3b-binding proteins that can act as cofactors, such as CR1 and membrane cofactor of proteolysis (MCP or CD46), another host cell membrane protein. Factor H is yet another complement-regulatory protein in plasma that binds C3b and like CR1 it is able to compete with factor B to displace Bb from the convertase in addition to acting as a cofactor for factor I. Factor H binds preferentially to C3b bound to vertebrate cells because it has an affinity for the sialic acid residues present on these cells. In contrast, because pathogen surfaces lack these regulatory proteins and sialic acid residues, the C3bBb convertase can form and persist. Indeed this process may be favoured by a positive regulatory factor, called properdin or factor P, which binds to many microbial surfaces and stabilises the convertase. Deficiencies in factor P are associated with a heightened susceptibility to infection with *Neisseria* species (Fijen *et al.* 1999).

Once formed the C3bBb convertase rapidly cleaves yet more C3 to C3b which can bind to the pathogen and either act as an opsonin or reinitiate the pathway to form another molecule of C3bBb convertase. Thus the alternative pathway activates through an amplification loop that can proceed on the surface of a pathogen but not on a host cell.

This same amplification loop enables the alternative pathway to contribute to complement activation initially triggered through the classical or MB-lectin pathways. The C3 convertases resulting from activation of the classical and MB-lectin pathways (C4b2b) and from the alternative pathway (C3bBb) are apparently distinct. Understanding the complement system is simplified by recognition of the close evolutionary relationships between the different complement proteins. The complement zymogens factor B and C2 are closely related proteins encoded by homologous genes located in tandem within the major histocompatibility complex (MHC) located on human chromosome 6. Their respective binding partners, C3 and C4 both contain thioester bonds that provide the means of covalently attaching the C3 convertases to a pathogen surface. Only one component of the alternative pathway seems entirely unrelated to its functional equivalents in the classical and MB-lectin pathways: the initiating serine protease, factor D. Factor D can also be singled out as the only activating protease of the complement system to circulate as an active enzyme rather than a zymogen. This is both necessary for the initiation of the alternative pathway through spontaneous cleavage and safe for the host because factor D has no other substrates than factor B when bound to C3b. This means that factor D finds its substrate only at a very low level in plasma and at pathogen surfaces where the alternative pathway of complement activation is allowed to proceed.

Although not commonly classified as a complement-activation pathway, proteases from neutrophils and macrophages can cleave C5. In the case of neutrophils, this is associated with elastase (Vogt *et al.* 2000); in macrophages, the C5-cleaving enzyme seems to be a serine protease that has not been further characterised (Huber-Lang *et al.* 2002).

Recently a new complement activation pathway has been proposed (Huber-Lang *et al.* 2006). The authors suggest that in the genetic absence of C3, thrombin substitutes for the C3-dependent C5 convertase and that this linkage between the complement and coagulation pathways may represent a new pathway of complement. They demonstrated that acute lung inflammatory injury in both wild type and C3 deficient mice after deposition of IgG immune complexes was of equivalent intensity and was C5a dependant, but injury was greatly attenuated in Hc deficient mice, where Hc encodes C5.

Injury in lungs of C3 deficient mice and C5a levels in bronchoalveolar lavage fluids from these mice were greatly reduced in the presence of antithrombin III or hirudin but were not reduced in similarly treated C3 sufficient mice. Plasma from C3 deficient mice contained threefold higher levels of thrombin activity compared to plasma from C3 sufficient mice. There were higher levels of F2 messenger RNA (encoding prothrombin) as well as prothrombin and thrombin protein in liver of C3 deficient mice compared to C3 sufficient mice. A potent solid-phase C5 convertase was generated using plasma from either C3 sufficient or C3 deficient mice. Human C5 incubated with thrombin generated C5a that was biologically active in the absence of C3, suggesting a new complement activation pathway. Further work needs to be performed in this area.

Comparison of the different pathways of complement activation illustrates the general principle that most of the immune effector mechanisms that can be activated in a nonclonal fashion as part of the early non-adaptive host response against infection have been harnessed during evolution to be used as effector mechanisms of adaptive immunity. It is almost certain that the adaptive response evolved by adding specific recognition to the original non-adaptive system.

1.1.5 Role of C3 convertase

The formation of C3 convertase is the point at which the three pathways of complement activation converge, because both the classical and MB-lectin pathways convertase, C4b2b and the alternative pathway convertase C3bBb have the same activity, and they initiate the same subsequent events. They both cleave C3 to C3b and C3a. C3b binds covalently through its thioester bond to adjacent molecules on the pathogen surface; otherwise it is inactivated by hydrolysis. C3 is the most abundant complement protein in plasma, occurring at a concentration of 1.2mg/ml and up to 1000 molecules of C3b can bind in the vicinity of a single active C3 convertase. The main effect of complement activation is to deposit large quantities of C3b on the surface of the infecting pathogen,

where it forms a covalently bonded coat that can signal the ultimate destruction of the pathogen by phagocytes.

The next step in the cascade is the generation of the C5 convertases. In the classical and MB-lectin pathways, a C5 convertase is formed by the binding of C3b to C4b2b to yield C4b2b3b. The C5 convertase of the alternative pathway is formed by binding of C3b to the C3 convertase to form $C3b_2Bb$. C5 is captured by these C5 convertase complexes through binding to an acceptor site on C3b and is then rendered susceptible to cleavage by the serine protease activity of C2b or Bb. This reaction, which generates C5b and C5a is much more limited than cleavage of C3, because C5 can be cleaved only when it binds to C3b that is part of the C5 convertase complex. Thus complement activated by all three pathways leads to the binding of large numbers of C3b molecules on the surface of the pathogen, the generation of a more limited number of C5b molecules and the release of C3a and C5a.

1.1.6 Complement receptors

The most important action of complement is to facilitate the uptake and destruction of pathogens by phagocytic cells. Ingestion of complement-tagged pathogens by phagocytes is mediated by receptors for the bound complement proteins (Ehlers *et al.* 2000). This occurs by the specific recognition of bound complement components by complement receptors on phagocytes. These complement receptors bind pathogens opsonised with complement components: opsonisation of pathogens is a major function of C3b and its proteolytic derivatives. C4b also acts as an opsonin but has a relatively minor role, largely because so much more C3b than C4b is generated.

Six types of receptor for bound complement components have been identified (Ravetch *et al.* 2002 and Ehlers *et al.* 2000). The best characterised is the C3b receptor CR1 (CD55), which is expressed on both macrophages and polymorphonuclear leukocytes. Binding of C3b to CR1 cannot by itself stimulate phagocytosis, but it can lead to phagocytosis in the

presence of other immune mediators that activate macrophages. For example, the small complement fragment C5a can activate macrophages to ingest bacteria bound to their CR1 receptors. C5a binds to another receptor expressed by macrophages, the C5a receptor C5aR, which has seven membrane-spanning domains. Receptors of this type couple with intracellular guanine-nucleotide-binding proteins called G proteins and the C5a receptor signals in this way. Proteins associated with the extracellular matrix, such as fibronectin, can also contribute to phagocyte activation: these are encountered when phagocytes are recruited to connective tissue and activated there. C3a, which has inflammatory activities similar to those of C5a, although it is a less potent chemoattractant, binds to its own specific receptor the C3a receptor, which is homologous in structure to the C5a receptor (Linehan *et al.* 2000). Three other complement receptors, CR2 (also known as CD21), CR3 (CD11b:CD18), and CR4 (CD11c:CD18) bind to inactivated forms of C3b that remain attached to the pathogen surface (Table 1.1).

CR1 and CR3 are especially important in inducing phagocytosis of bacteria with complement components on their surface. CR2 is found mainly on B cells, where it is also part of the B cell co-receptor complex and the receptor by which Epstein-Barr virus selectively infects B cells, causing infectious mononucleosis. CR1 and CR2 share structural features with the complement regulatory proteins that bind C3b and C4b. CR3 and CR4 are integrins; CR3 is known to be important for leukocyte adhesion and migration, whereas CR4 is only known to function in phagocytic responses. The C5a and C3a receptors are seven transmembrane spanning G-protein coupled receptors.

Receptor	Specificity	Functions	Cell types
CR1 (CD35)	C3b, C4b iC3b	Promotes C3b and C4b decay Stimulates phagocytosis Erythrocyte transport of immune complexes	Erythrocytes, macrophages, monocytes, polymorphonuclear leukocytes, B cells, FDC
CR2 (CD21)	C3d, iC3b, C3dg Epstein–Barr virus	Part of B-cell co-receptor Epstein–Barr virus receptor	B cells, FDC
CR3 (Mac-1) (CD11b/ CD18)	iC3b	Stimulates phagocytosis	Macrophages, monocytes, polymorphonuclear leukocytes, FDC
CR4 (gp150, 95) (CD11c/ CD18)	iC3b	Stimulates phagocytosis	Macrophages, monocytes, polymorphonuclear leukocytes, dendritic cells
C5a receptor	C5a	Binding of C5a activates G protein	Endothelial cells, mast cells, phagocytes
C3a receptor	C3a	Binding of C3a activates G protein	Endothelial cells, mast cells, phagocytes

Table 1.1 Distribution and function of receptors for complement proteins on the surface of cells. (Janeway *et al.*, Immunobiology, 2005, 67).

1.1.7 Small fragments of complement

The small complement fragments C3a, C4a and C5a act on specific receptors to produce a local inflammatory response. When produced in large amounts or injected systemically they induce a generalised ciculatory collapse, or anaphylactic shock, therefore these small fragments of complement are termed anaphylatoxins.

C5a is more active than C3a which is more active than C4a. They cause local inflammatory responses by acting directly on local blood vessels, stimulating an increase

in blood flow, an increase in vascular permeability, and an increase in binding of phagocytes to endothelial cells (Kohl J. 2001, Schaufstatter *et al.* 2002, Monsinjon *et al.* 2001). C5a activates mast cells to release mediators such as histamine and TNF-α, that contribute to the inflammatory response. The increase in vessel diameter and permeability leads to the accumulation of fluid and protein. Fluid accumulation increases lymphatic drainage, binding pathogens and their antigenic components to nearby lymph nodes. C5a acts directly on neutrophils and monocytes to increase their adherence to vessel walls, their migration towards sites of antigen deposition and their ability to ingest particles. C5a increases the expression of CR1 and CR3 on the surfaces of these cells. In this way C5a, and to a smaller extent C3a and C4a, act in concert with other complement components to hasten the destruction of pathogens by phagocytes. The antibodies, complement, and cells thus recruited participate in pathogen clearance by enhancing phagocytosis. In addition, the small complement fragments directly increase the activity of phagocytes. C5a and C3a signal through transmembrane receptors that activate G proteins, thus the action of C5a in recruiting neutrophils and monocytes is analogous to that of chemokines which also act via G proteins to control cell migration.

1.1.8 Membrane attack complex (MAC)

One of the important effects of complement activation is the assembly of the terminal components of complement to form a membrane attack complex. The end result is a pore in the lipid bilayer membrane that destroys membrane integrity (Figure 1.3) (Bhakdi *et al.* 1991). This is thought to kill the pathogen by destroying the proton gradient across the pathogen's cell membrane, causing uncontrolled outward flow of electrolytes, (especially potassium), resulting in rapid cell lysis. Interestingly, a form of MAC can be generated by oxidation of C5, followed by the addition of C6 and then C7-C9 (with C9 being added in limiting amounts) (Vogt *et al.* 1992). Under such conditions, this complex does not induce cell lysis. This complex has been shown to activate endothelial cells, resulting in transcriptional up regulation of mediators such as interleukin-8 (IL-8) and the CC-chemokine ligand 2 (CCL2, also known as monocyte chemotactic protein 1, MCP1)

The first step in the formation of the membrane attack complex is the cleavage of C5 to a C5 convertase to release C5b. C5b initiates the assembly of the later components and their insertion into the cell membrane. First one molecule of C5b binds one molecule of C6, and the C5b6 complex then binds one molecule of C7. This reaction leads to a conformational change in the constituent molecules, with the exposure of a hydrophobic site on C7, which inserts into the lipid bilayer. Similar hydrophobic sites are exposed on the later components C8 and C9 when they are bound to the complex, allowing these proteins to insert into the lipid bilayer. C8 is a complex of two proteins, C8β and C8α-γ. The C8β protein binds to C5b and the binding of C8β to the membrane associated C5b67 complex allows the hydrophobic domain of C8α-γ to insert into the lipid bilayer. Finally C8α-γ induces the polymerisation of 10 to 16 molecules of C9 into a pore forming structure called the membrane attack complex (MAC) (Parker *et al.* 2002, Scibek *et al.* 2002, Wang *et al.* 2000). The MAC has a hydrophobic external face allowing it to associate with the lipid bilayer but a hydrophilic internal channel (diameter of 100 A). This channel allows the free passage of solute and water across the lipid bilayer. The distribution of the lipid bilayer leads to the loss of cellular homeostasis and the proton gradient across the membrane, the penetration of enzymes such as lysozyme into the cell, and the eventual destruction of the pathogen. Deficiencies in the complement component C5-C9 are associated with susceptibility to *Neisseria* species that may cause meningitis and gonorrhoea.

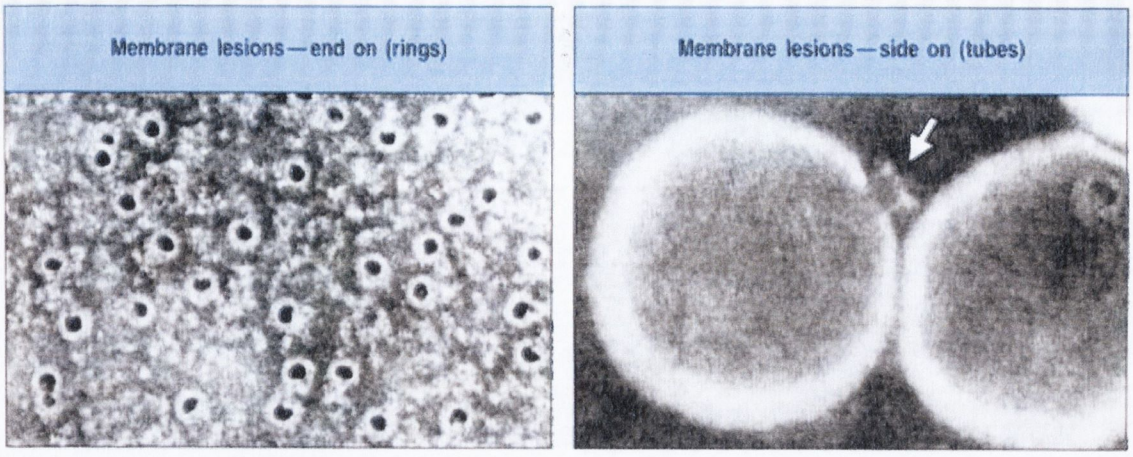


Figure 1.3 Assembly of the membrane-attack complex generates a pore in the lipid bilayer membrane (Janeway *et al.*, Immunobiology, 2005, 71).

1.1.9 Regulatory proteins of the complement system

Given the destructive effects of complement and the way in which its activation is rapidly amplified through a triggered-enzyme cascade, many mechanisms are in place to prevent its uncontrolled activation. The effecter molecules of complement are generated through the sequential activation of zymogens, which are present in plasma in the inactive form. The activation of these zymogens usually occurs on a pathogen surface and the activated complement fragments produced in the ensuing cascade of reactions usually bind nearby or are rapidly inactivated by hydrolysis. These two features act as safeguards against uncontrolled activation. In addition, eight regulatory proteins exist which allow complement to distinguish self from non self (Liszewski *et al.* 1996, Suankratay *et al.* 1999, Smith *et al.* 2001). These act at different stages of the complement cascade, dissociating complexes or catalysing the enzymatic degradation of covalently bound complement proteins (Table 1.2). C1 inhibitor (C1INH), binds to the active enzymes C1r:C1s causing them to dissociate from C1q (Jiang *et al.* 2001). The active C3

convertase, C4b2b cleaves C3 to C3a and C3b but the decay-accelerating factor (DAF), C4 binding protein (C4BP), and complement receptor 1 (CR1) displace C2b from the C4b2b complex. C4b bound by C4BP, MCP or CR1 is cleaved by soluble protease factor I (I) to inactive forms C4d and C4c. C5 convertases cleave C5 to C5a and C5b. CR1 and factor H (H) displace C3b. CR1 and factor H act as cofactors in the cleavage of C3b by factor I. CD59 (protectin) prevents final assembly of the membrane attack complex at the C8 to C9 stage (Pangburn M.K. 2000). The alternative pathway C3 convertase is similarly regulated by DAF, CR1, MCP and factor H.

Regulatory proteins of the classical and alternative pathways	
Name (symbol)	**Role in the regulation of complement activation**
C1 inhibitor (C1INH)	Binds to activated C1r, C1s, removing them from C1q
C4-binding protein (C4BP)	Binds C4b, displacing C2b; cofactor for C4b cleavage by I
Complement receptor 1 (CR1)	Binds C4b, displacing C2b, or C3b displacing Bb; cofactor for I
Factor H (H)	Binds C3b, displacing Bb; cofactor for I
Factor I (I)	Serine protease that cleaves C3b and C4b; aided by H, MCP, C4BP, or CR1
Decay-accelerating factor (DAF)	Membrane protein that displaces Bb from C3b and C2b from C4b
Membrane cofactor protein (MCP)	Membrane protein that promotes C3b and C4b inactivation by I
CD59 (protectin)	Prevents formation of membrane-attack complex on autologous or allogenic cells. Widely expressed on membranes

Table 1.2 Regulatory proteins of the classical and alternative pathway. (Janeway *et al.*, Immunobiology, 2005, 72).

1.1.10 Complement and the adaptive immune response

Antibodies are able to initiate the classical pathway of complement after binding to pathogen or after forming immune complexes of antigen and antibody. IgM is specialised to activate complement efficiently upon binding antigen and to compensate for the low affinity of a typical IgM antigen–binding site. IgM antibodies are found mainly in the blood and are pentameric in structure. IgG antibodies are usually of higher affinity and are found in blood and in extracellular fluid, where they can neutralise toxins, viruses and bacteria, opsonise them for phagocytosis and activate the complement system.

Soluble immune complexes of antigen and antibody which fix complement are cleared from the circulation via complement receptors on red blood cells. Immune complexes, made up of small soluble antigens which form antigen: antibody complexes contain too few molecules of IgG to be readily bound to the Fcγ receptors. These antigens contain toxins bound by neutralising antibodies and debris from dead micro-organisms. Such immune complexes are found after most infections and are removed from the circulation through the action of complement. The soluble immune complexes trigger their own removal by activating complement, again through the binding of C1q, leading to the covalent binding of the activated components C4b and C3b to the complex, which is then cleared from the circulation by the binding of C4b and C3b to complement receptor 1 (CR1) on the surface of erythrocytes. The erythrocytes transport the bound complexes of antigen, antibody and complement to the liver and spleen. Here macrophages bearing CR1 and Fc receptors remove the complexes from the erythrocyte surface without destroying the cell, and then degrade them. Even larger aggregates of particulate antigen and antibody can be made soluble upon activation of the classical complement pathway and the consequent binding of C3b to the aggregates; these can then be removed by binding to complement receptors. Immune complexes that are not removed tend to deposit in the basement membranes of small blood vessels, most notably those of the renal glomerulus, where the blood is filtered to form urine. Immune complexes that pass

through the basement membrane of the glomerulus, bind to the complement receptor CR1 on the renal podocytes which lie just underneath the basement membrane. In the autoimmune disease systemic lupus erythematosis, excessive levels of circulating immune complexes cause huge deposits of antigen, antibody and complement on the podocytes damaging the glomerulus resulting in kidney failure.

Antibody, independent of its role in activating the classical complement pathway, is able to function in the alternative complement pathway (Ratnoff *et al.* 1983). Metastable C3b is capable of binding directly to immunoglobulin G (IgG). The role of immunoglobulin in the alternative pathway function is important because C3b covalently bound to IgG displays relative resistance to inactivation by factor H and I when compared to free C3b. The resistance appears to be entirely due to the reduced affinity for factor H and this confers on the complex an enhanced capacity to activate C3 in serum (Fries *et al.*1984). This complex of C3b with bactericidal IgG was found to be much more effective than IgG alone in killing *Escherichia coli* in serum (Joiner *et al.* 1985). A number of immunoglobulins are able to activate the alternative complement pathway and play an important role in host defence in the infective process. Aggregated IgG and aggregated IgM both activate the pathway as well as some aggregated IgA myeloma proteins and some IgE myelomas: although the immunoglobulin concentration required for such activation is actually relatively high.

C3b is subject to regulatory mechanisms and can be cleaved into derivatives that cannot form an active convertase. One of the breakdown products of C3b is C3dg which binds only to CR2. CR2 is found on B cells as part of the co-receptor complex that can augment the signal received through the antigen-specific immunoglobulin receptor. Thus a B cell whose antigen receptor is specific for a given pathogen will receive a strongly augmented signal on binding this pathogen if it is also coated with C3dg. The B cell co-receptor is expressed on mature B cells as a complex of the cell surface molecules CD19, CD21 and CD81. One way in which this complex can be co-ligated with the B cell receptor is through recognition of an antigen that has activated complement. Complement receptor two, CR2 (also known as CD21), is a receptor for the C3dg fragment of complement, so

antigens, such as pathogens, that have activated complement directly or through the activation of antibody can cross-link the B cell receptor with CD21 and its associated proteins. This induces phosphorylation of the cytoplasmic tail of CD19 by B cell receptor associated tyrosine kinases, which in turn leads to the binding of Src-family kinases and the recruitment of a lipid kinase called phosphatidylinositol 3-OH kinase (PI3-kinase). (Denny *et al* 2000). Co-ligation of the B cell receptor with its CD19/CD21/CD81 co-receptor increases signalling 1000 to 10,000 fold. The activation of complement can contribute to producing a strong antibody response i.e. innate humoral immune response can contribute to activating adaptive humeral immunity.

1.1.11 Complement's role in danger recognition

The complement system is one of the most important humoral systems to sense danger i.e. to recognise conserved patterns on pathogens and on altered/damaged self. In addition to this important role in danger recognition, the complement system has the ability to translate the danger information into an adequate cellular innate or adaptive immune response (Kohl J. 2006). This is accomplished by two distinct mechanisms: a) danger sensors that have recognised altered cells or pathogens can directly activate cell-bound receptors (e.g. C1q/C1q receptor activation) and/or b) danger sensors initiate cleavage of complement factors C3 and C5, the fragments of which acquire the ability to bind to complement receptors and/or regulators. It is the specific interaction of the danger sensors and of the cleavage fragments with distinct cell-bound receptors/regulators that directs the immune response toward an innate or an adaptive phenotype. Further, the expression pattern of the complement receptors critically impacts the shape of the immune response. Complement has the ability to discriminate between physiological and pathological danger, i.e. between physiological cell death and death in response to injury. In the former case, cells are merely flagged for enhanced phagocytosis (by C3 fragments) without accompanying inflammation (through CR3) whereas in the latter case inflammatory signals are accessorily triggered (e.g. by the release of anaphylatoxins, which recruit and activate neutrophils, eosinophils etc.). This function is of major

importance for apoptotic cell clearance and tissue repair but in addition plays important roles in fibrotic tissue remodelling in response to chronic tissue injury. Further, complement cleavage fragments may prevent the development of maladaptive immune responses at the mucosal surface. Here, complement fragment C5a does not act as a danger transmitter but as a "homeostasis transmitter", as its interaction with the C5a receptor on dendritic cells provides a signal that prevents dendritic cells from activating CD4+ T cells. The generation of regulatory T cells in response to CD46 ligation may have a similar function, as injured cells lose CD46 expression, which may lead to decreased proliferation of Tregs and consecutively increased production of T effector cells. Although we are still at the beginning of understanding the complex interaction patterns within the complement system, recent data suggest substantial crosstalk between the signalling pathway downstream of complement receptors and other receptors of the innate immune system that function as immune sensors and/or transmitters (i.e. toll like receptors, FcgammaRs130 and 131). Thus complement is important as both a sensor and effector system of innate and adaptive immune responses.

1.1.12 Effector functions of complement

The complement system acts in several ways to mobilise defence mechanisms and involves both innate and adaptive immunity. Its many functions include cell lysis, opsonisation by C3b which targets foreign particles for phagocytosis, chemotaxis by C5a which attracts phagocytic cells to the site of damage, and increase in the permeability of the capillary beds by both C5a and C3a. The early components of complement are important for solubilising antigen-antibody complexes assisting in their catabolism and elimination from the body. Failure of this function can lead to immune complex disorders. Lysis of antibody–coated cells is an important function that can be problematic and cause Rhesus disease, immune haemolytic anaemia or immune thrombocytopenic purpura. Complement promotes antibody formation. Breakdown of C3b generates a fragment C3d that binds to antigens enhancing their uptake by dendritic cells and B cells. The C3d antigen complex binds to the same receptor on B cells that the Epstein Barr

virus uses to gain entry into B cells, where it may cause infectious mononucleosis and sometimes Burkitt's lymphoma.

Taken overall, complement has three critical physiological activities: i) defending against microbial infections by triggering the generation of a membranolytic complex (C5b9 complex) at the surface of the pathogen and complement fragments (named opsonins, i.e. C1q, C3b, VR3 and CR4) to promote phagocytosis. Soluble complement anaphylatoxins (C5a, C3a and C4a) greatly control the local pro-inflammatory response through the chemotaxis and activation of leukocytes, ii) bridging innate and adaptive immunity (essentially through CR2 expressed on B cells) and iii) disposing of immune complexes and the products of the inflammatory injury (i.e. other danger signals, e.g. toxic cell debris and apoptotic corpses) to ensure the protection and healing of the host (Frank *et al.* 1991). The regulatory mechanisms of complement are finely balanced so that on one hand, the deposition of complement is focused on the surface of invading microorganisms and on the other hand, the deposition of complement on normal cells is limited by several key complement inhibitors (e.g. CD46, Cd55 and Cd59).

1.1.13 Defects in complement components cause defective humoral immune function

The spectrum of infections associated with complement deficiencies overlaps substantially with that seen in patients with deficiencies in antibody production. Defects in the activation of C3 and in C3 itself, are associated with a wide range of pyogenic infections, emphasising the important role of C3 as an opsonin, promoting the phagocytosis of bacteria. In contrast, defects in the membrane attack components of complement (C5-C9) have more limited effects and result exclusively in susceptibility to *Neisseria* species, the causative agents of meningitis and gonorrhoea. This indicates that host defence against these bacteria, which are capable of intracellular survival is mediated by extracellular lysis by the membrane-attack complex of complement. Accurate data from large population studies in Japan, where endemic *Neisseria meningitidis* infection is rare, show that the risk each year of infection with this organism

is approximately 1 in 2,000,000 to a normal person. This compares with a risk of 1 in 200 in the same population to a person with inherited deficiency of one of the membrane-attack complex proteins: a 10,000-fold increased risk compared with a person with normal complement activity. The early components of the classical complement pathway (C1, C2 and C4) are particularly important for the elimination of immune complexes and apoptotic cells, which can cause significant pathology in autoimmune diseases such as systemic lupus erythematosis. Defects in the early components of the alternative pathway (Factor D and Factor P) and in C3 lead to susceptibility to extracellular pathogens, particularly pyogenic bacteria. Deficiency of mannose-binding lectin (MBL), the recognition molecule of mannose-binding pathway, is associated with bacterial infections, mainly in early childhood (Frank M.M. 2000).

Another set of diseases are caused by defects in complement control proteins. People lacking decay-accelerating factor (DAF) and CD59 which protects a person's own cell surfaces from complement activation, destroy their own red blood cells resulting in paroxysmal nocturnal haemoglobinuria. A more striking loss of a regulatory protein is seen in patients with C1-inhibitor defects. C1 inhibitor irreversibly inhibits the activity of several serine proteinase enzymes. These include two enzymes that participate in the contact activation system of blood clotting: factor XIIa (activated Hageman factor) and kallikrein, in addition to the two enzymes that together initiate the classical pathway of complement, C1r and C1s. Deficiency of C1 inhibitor leads to a failure of regulation of these two pathways resulting in the excessive production of vasoactive mediators that cause fluid accumulation in the tissues and epiglottal swelling that can lead to suffocation. These mediators are bradykinin, produced by cleavage of high molecular weight kininogen by kallikrein and the C2 kinin, produced by the activity of C1s on C2a. This deficiency of C1 inhibitor is known as hereditary angioneurotic oedema.

1.1.14 Anaphylatoxin peptides derived from C3, C4 and C5

Anaphylatoxins are low-molecular weight, biologically active peptides that are defined functionally by their actions on small blood vessels, smooth muscle, mast cells and peripheral blood leukocytes. The small complement fragments C3a, C4a and C5a act on specific receptors to produce local inflammatory responses. They are generated enzymatically from C3, C4 and C5. Analyses of the complete amino acid sequences of C3a, C4a and C5a from man and a variety of animal species have revealed striking similarities among these peptides, suggesting a common evolutionary origin. When produced in large amounts or injected systemically they induce a generalised circulatory collapse, producing a shock like syndrome similar to that seen in a systemic allergic reaction involving IgE antibodies. This reaction is termed anaphylactic shock, and the small fragments of complement are referred to as anaphylatoxins. C3a was the first anaphylatoxin to have its complete primary structure elucidated (Hugli *et al.* 1975). C4a has a pentapeptide structure and contracts smooth muscle, although it is approximately 500 fold less active in this respect than C3a pentapeptide. Of the three, C5a has the highest specific biological activity. All three induce smooth muscle contraction and increase vascular permeability, but in addition C5a and C3a act on the endothelial cells lining blood vessels to induce adhesion molecules. C3a and C5a activate mast cells that populate submucosal tissues to release mediators such as histamine and tumour necrosis factor α (TNF α) that cause similar effects. The changes induced by C3a and C5a recruit antibody, complement and phagocytic cells to the site of infection and increased fluid in the tissues hastens the movement of pathogen-bearing antigen-presenting cells to local lymph nodes, contributing to the prompt initiation of the adaptive immune response.

In addition C5a acts directly on neutrophils and monocytes to increase their adherence to vessel walls, their migration towards sites of antigen deposition and their ability to ingest particles. C5a increases the expression of CR1 and CR3 on the surfaces of these cells. In

this way C5a and to a smaller extent C3a and C4a, act in concert with other complement components to hasten the destruction of pathogens by phagocytes. C5a and C3a signal through transmembrane receptors that activate G proteins.

1.2 THE CLASSICAL C5A RECEPTOR, C5aR, (CD88)

1.2.1 History of C5aR

Inflammatory response-related leukocyte chemoattractants are activated by a number of soluble mediators that include various classes of chemokines, leukotrienes, complement factors (C3a and C5a) and bacterial peptides (formyl-Met-Leu-Phe [fMLP]).The receptors that mediate the action of these factors belong to a large family of G protein coupled receptors, possessing a seven transmembrane domain motif and sharing sequence similarities and common transduction pathways. These include the chemokine receptors CCR1-CCR9, CXCR1-CXCR6 and CX_3CR1 (Premack *et al.*1996, Power *et al.* 1997, Roos *et al.* 1997, Imai *et al.* 1997, Tiffany *et al.* 1997, Yoshida *et al.* 1997) the C5a and C3a receptors (Gerard *et al.* 1991, Sozzani *et al.* 1999, Ames *et al.* 1996, Crass *et al.* 1996) the fMLP receptor (Boulay *et al.* 1990) and the leukotriene B4 (LTB4) receptors (Steinman *et al.* 1991, Yokomizo *et al.* 1997). This family of G protein coupled chemoattractant receptors is known to mediate the transport and activation of neutrophils and macrophages.

Early studies in the seventies and eighties established the presence of a high affinity receptor for C5a on polymorphonuclear leukocytes, with a K_d of ~1nM and relatively abundant expression of 100,000 to 200,000 sites per cell (Chenoweth *et al.* 1978, Chenoweth *et al.* 1982, Van Epps *et al.* 1984). Through the use of bi-functional cross linking agents, [^{125}I]-labelled C5a was covalently bound to its receptor in polymorphonuclear membranes, and sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis identified a predominant molecular species at 52kDa. Correction for the contribution of the ligand indicated the C5a-receptor molecular weight was ~42kDa

(Rollins *et al.* 1985, Johnson *et al.* 1985, Johnson *et al.* 1987, Huey *et al.* 1987, Gerard *et al.* 1989).

In 1991, molecular cloning of the C5a-receptor from a U937 cell complementary DNA (cDNA) library indicated that it is a member of the seven transmembrane-segment superfamily of G protein coupled receptors (GPCR) (Gerard *et al.* 1991, Boulay *et al.* 1991). Following transfection into human embryonic kidney cells, (HEK293T), the cloned C5a-receptor cDNA expresses binding sites for C5a with a dissociation constant, (Kd) of ~1nM. The deduced amino acid sequence was found to have a 34% homology with the f-Met- Leu-Phe receptor (Boulay *et al.* 1990). Since its identification, C5aR appeared to be the sole receptor for C5a. Expression of C5aR has been found on a variety of cells in many different organs, including epithelial cells, endothelial cells, myeloid cells and T cells (Zwirner *et al.* 1999, Haviland *et al.* 1995, Floreani *et al.* 1998, Schieferdecker *et al.* 2000, Wetsel 1995, Gasque *et al.* 1997, Chenoweth *et al.* 1978 and Nataf *et al.* 1999). C5aR is especially prevalent on neutrophils (Chenoweth *et al.* 1978), indicating that in the presence of high concentrations (10nM or greater) of C5a, neutrophils are particularly susceptible to adverse consequences. Monocytes have far fewer binding sites (van Epps *et al.* 1984) and might, therefore, be more resistant to the effects of high levels of C5a. In 1992 Gerard *et al.* localised the C5a-receptor gene to human chromosome 19q13.3-13.4 along with the f-MLP receptor and two f-MLP receptor homologues, suggesting they may have evolved by gene duplication (Lu *et al.* 1992).

1.2.2 Structure of C5aR: binding and activation sites

C5aR is a typical 45-kDa G-protein coupled rhodopsin-type receptor with seven trans-membrane regions and three extracellular loop regions (Gerard *et al.* 1991). It has a relatively flexible carboxy-terminal region. Evidence for distinct binding and activation sites in the C5a-receptor has been derived from mutagenesis and monoclonal antibody studies. In the 1990s, the extracellular N-terminal region of the receptor was shown to

provide one domain required for high affinity ligand interaction or "docking" (Oppermann *et al.* 1993, Mery *et al.* 1994, De Martino *et al.* 1994, Siciliano *et al.* 1994). Interaction with a monoclonal anti-peptide antibody raised against the N-terminal sequence was felt to completely block ligand binding and activation (Oppermann *et al.* 1993). Treatment with a spider venom metalloproteinase indicated subsequently that specific cleavage of the receptor blocks binding of intact C5a, but not C-terminal C5a analogues, further supporting the predicted existence of a second interaction site.

In 1994, it was thought that ligands for many G-protein–coupled receptors, including chemokine receptors bind at least in part, in a pocket formed by the transmembrane helices and induce in the receptor a conformational change that promotes guanine nucleotide exchanges in G–proteins (Gerard *et al.* 1994). Chemokines were thought to bind this pocket at their N termini, and chemokines N terminal truncations function as receptor antagonists. A paradigm for receptor–ligand interactions among low molecular weight agonists such as retinal and catecholamines has the ligands binding to their receptors at a single site within the pocket formed by the transmembrane segments (Nakayama *et al.* 1990, Tota *et al.* 1990). In contrast, peptidgeric agonist-receptor systems such as those for C5a, interleukin-8 and pituitary glycoprotein hormones appear to display two regions of interaction: one receptor domain largely confers binding affinity, while a second site mediates ligand biological activity (Leong *et al.* 1994, Valove *et al.* 1994). C5a, a cationic peptide of 72 amino acids derived from the C5 component of complement, has been shown to have a number of structural requirements for interaction with the receptor. Previous studies demonstrate that human C5a can bind and activate the murine C5a receptor with potency almost identical to the human receptor, suggesting that elements in common for both species provide the agonist site. Comparison of the murine C5a receptor to the human molecule demonstrates that the transmembrane segments are well conserved, while extracellular sequences are quite divergent, thus providing a dramatic "mutagenesis" result (Gerard *et al.* 1991).

Because data suggested that the address site for C5a was largely extracellular, site directed mutagenesis of hydrophilic and charged residues within the transmembrane

segments was undertaken, to investigate potential message sequences (Kolakowski *et al.* 1995). In 1995, site-directed mutagenesis of the human C5a-receptor additionally identified cysteine residues within transmembrane sequences four and five (TM4 and TM5) as involved in binding and signal transduction, and suggested it is an important interaction site with the ligand C-terminus (Kolakowski *et al.* 1995),(Figure 1.4). In addition, the relative orientation of the transmembrane domains appears important for maintenance of ligand binding, not only for the C5a-receptor but for other members of this receptor family. Mutation of a leucine residue to proline in the third extracellular loop appeared to completely block ligand binding with no effect on expression of the receptor (Kolakowski *et al.* 1995). In 2001, Farzan *et al.* demonstrated that the NH_2 terminal tyrosines at positions 11 and 14 of the C5aR are post-translationally modified by the addition of sulphate groups and that the sulphate moieties of each of these tyrosines are critical to the ability of the C5aR to bind C5a and to mobilise calcium (Farzan *et al.* 2001). G-protein coupled receptors appear to have evolved a common strategy for the recognition of large peptide ligands. Ligands binding to elements in the NH_2 terminal and extracellular loop regions, constituting a "high affinity binding site" lead to the presentation of ligand sequences which activate receptors.

However, recent studies have indicated a more markedly complex interaction between C5a and C5aR (Huber-Lang *et al.* 2003) which has important implications. There might be at least three targets each on C5a and C5aR for interception that would be effective in interfering with ligand-receptor interactions (Figure 1.4). The first binding site is between the negatively charged amino residues in region 21-30 of C5aR (Chen *et al.* 1998, Siciliano *et al.* 1994) and four positively charged residues in the D1 region of C5a. The negatively charged amino-terminal residues (10-18) of C5aR seem not to be directly involved in binding with C5a, but it has been suggested that they interact with the positively charged residues in the hydrophobic regions of residues in the extracellular C5aR loop II, which is close to the fifth transmembrane helix, presumably forming a stable C5a-binding pocket (Chen *et al.* 1998). On the other end of the extracellular C5aR loop II (close to the fourth transmembrane region), there is an additional positively charged region that could interact with the negatively charged D2 region of C5a, which

might represent a second, newly recognised binding site. The disulphide bond between cysteine 189 in the extracellular loop II might stabilise what seems to be the second binding site. There is also fairly good evidence for the existence of a third site of C5a-C5aR interaction (Raffetseder *et al.* 1996, Kola *et al.* 1996), in which arginine 206 in the fifth transmembrane domain of C5aR has an important role in binding the carboxyl terminus of C5a by acting as a counter-ion for arginine 74 of C5a. The interaction of C5a with C5aR contrasts with the interaction between C3a and C3aR, in which the only known interacting site on C3a is the carboxy-terminal peptide region.

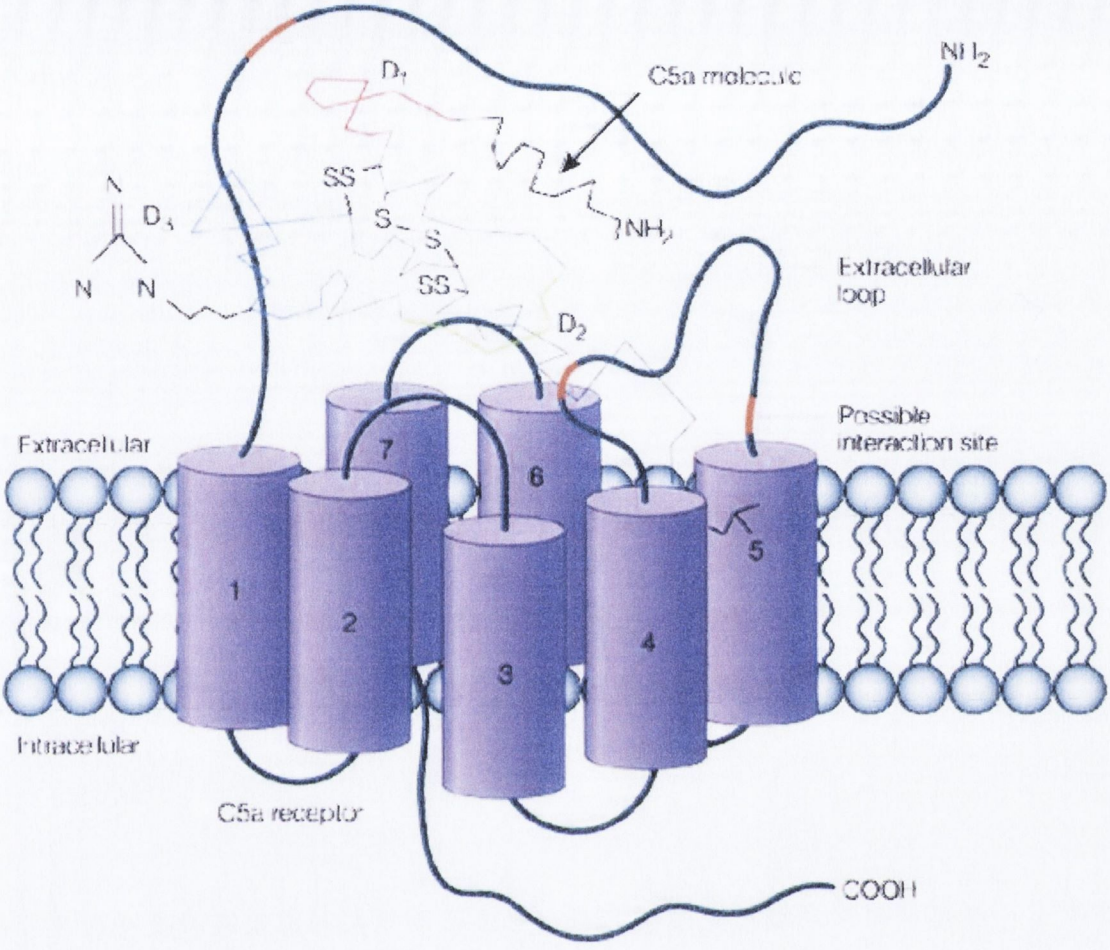


Figure 1.4 Schematic structure of human C5a receptor, C5aR.

The three interacting sites on C5aR (shown in red) include the amino-terminal peptide region and loop II of the transmembrane regions (defined as the extracellular loop between transmembrane regions 4 and 5), in which C5a interacts with two regions of this loop.(Ward P.A. 2004, Nature Reviews, Immunology 4, 133-141)

1.2.3 Role of hydrophilic and cysteine residues in the C5a receptor, C5aR

Results from other studies of G-protein coupled receptors (G.P.C.R.s) have demonstrated that charged or other polar side chains are crucial for the maintenance of receptor conformation and ligand binding. In the β2 adrenergic receptor, the conserved aspartic acid residue in transmembrane segment-3 (TMS-3) is required to provide a counter ion to the catecholamine charge. In rhodopsin, retinal is linked via a Schiff base to Lys^{296} and the resulting charge is thought to be balanced by Glu^{113} (Cohen *et al.* 1992, Nathans *et al.* 1992). Some mutations at these sites in rhodopsin are active in the dark. Thus charged residues within the TMS can be involved in direct interaction with the ligand and/or play a role in the equilibrium between active and inactive forms.

The C5a receptor encodes an asparagine residue (Asn^{119}) at the position where the amino group of catecholamine drugs bind to Asp^{113} of β2 adrenergic receptors (Strader *et al.* 1998). Conservative mutations at these sites demonstrate that Asp^{132} is essential for the proper expression and trafficking of the C5a receptor. Asp^{82} and Asn^{119} residues have been shown to be crucial components for the generation of the active form of the receptor. The results from mutagenesis of all transmembrane cysteine residues showed that three are required for binding (Cys^{109}, Cys^{188} and Cys^{221}). It had been previously determined for other G.P.C.R.s such as rhodopsin, that cysteines in extracellular loops one and two are linked in a disulfide bond which stabilises the structure (Karnik *et al.* 1990, Davidson *et al.* 1994). Others demonstrated that data from mutants was consistent with these residues participating in a similar disulfide bond in the C5a receptor (Kolakowski *et al.* 1995).

1.2.4 Role of proline residues in the C5a receptor, C5aR

Structural studies of bacteriorhodopsin show that proline residues within the transmembrane segments introduce kinks in the helices, as the amine hydrogen is not available for stabilisation (Henderson *et al.* 1990). Mutation of helical proline residues results in a number of changes in C5a receptor function, e.g. proper trafficking to the cell surface. A general defect in the processing of opsin in *Drosophila* (ninaA) results from mutations in a "*cis-trans-propyl*" isomerase, indicating that proline residues in the TMS play a specific role in the ability of these receptors to form proper structures (Shieh *et al.* 1989). Studies from mutations of proline residues in the C5a receptor in the rat M3 muscarinic receptor (Wess *et al.* 1993) were confirmed by similar results from Kolakowski's work on C5aR. Proline mutations and intracellular loop mutations of the receptor have distinct behaviours, for when they are defective in function, the number of binding sites is also decreased, suggesting a processing defect. Studies of rhodopsin and the β2–adrenergic receptor indicate that TMS6-7 are tightly associated in the membrane and are able to function in "split" receptor experiments caused by cleavage of rhodopsin with trypsin (Bayramashvili *et al.* 1984) or by co-expression of partial receptors. Kolakowski *et al.*'s results suggested that the structure of the extracellular loop is critical for adopting the necessary structure for activating G-proteins (Kolakowski *et al.* 1995).

1.2.5 Other mutations: mutations in the intracellular loops of C5aR

Mutations in the intracellular loops were unlikely to affect the direct interaction of C5a with the receptor, but were thought to be of importance regarding the contact points of G-proteins with the C5a receptor. Results from two point mutations (Thr $^{235}\rightarrow$Ala and Thr $^{238}\rightarrow$Arg) suggest that this region of the third intracellular loop is important for efficient coupling to G-proteins (Kolakowski *et al.* 1995).

Therefore using site directed mutagenesis, and subsequent binding and signalling studies of the mutant C5a receptors, amino acids located in the transmembrane site have been identified as being involved in the activity of the message site namely Asp^{82}, Asn^{119}, Cys^{157} and Cys^{221}. The nature of these functional groups (acidic, thiol or amide) suggested that hydrogen bonding or ionic interactions within the 7TMS bundle are crucial for providing ligand binding and G-protein activation. Proline mutations and intracellular loop mutations of the receptor have distinct behaviours leading to a reduction in the number of binding sites. Mutation of arginine in the DRY sequence is associated with uncoupling to G proteins

1.3 THE COMPLEMENT FRAGMENT C5A

1.3.1 History of C5a

The most important small peptide mediator of inflammation, C5a is formed when C3b binds to the C3 convertase to form a C5 convertase that produces C5a, in addition to a large active fragment C5b that initiates the "late" events of complement activation. C5a is released from the amino-terminal region of the α-chain of C5 by C5 convertase. Human C5a is a 74-amino-acid glycoprotein with complex anti-parallel helical structures (Shin *et al.* 1968). This complex, glycosylated molecule has four helices that are connected by three loops (D_1-D_3) and the helices are cross linked by disulphide bridges (Figure 1.5). The effects of C5a are mediated by binding to its receptor, the C5a receptor, C5aR (CD88) present on myeloid cells (in particular neutrophils), and non-myeloid cells such as bronchiolar and alveolar epithelial cells and endothelial cells (Haviland *et al.* 1995, Monsinjon *et al.* 2003, Schraufstatter *et al.* 2002, Riedemann *et al.* 2002). It has been appreciated as a biological principal for many years. It is both an anaphylatoxin, in that it amplifies the inflammatory process, and a chemoattractant for neutrophils and macrophages. The purification of C5a from complement activated sera from several species in the 1970's allowed for a much fuller characterisation of its varied functions (Gerard *et al.* 1991, Franke *et al.* 1988).

1.3.2 Structure function relationship of C5a

Classical structure function studies of the C5a ligand had demonstrated two regions of the protein that are required for high affinity binding and potent signalling via the heterotrimeric G-proteins (Nakayama *et al.* 1990, Vogt *et al.* 1986, Yancey *et al.* 1989, Gerard *et al.* 1985, Mollison *et al.* 1989). The conformationally critical disulfide bonds present in C5a knit a core domain essential for high affinity receptor binding. Chemical and exopeptidase cleavages demonstrate that the ligand COOH-terminal region encodes the receptor activating sequence, Met-Gln-Leu-Gly-Arg (Kawai *et al.* 1991, Gerard *et al.* 1979). Further studies using site directed mutagenesis have located distinct receptor contact points within the ligand NH_2-terminal and the disulfide core (Mollison *et al.* 1989). Subsequently, evidence has been described from mutageneisis and monoclonal antibody studies showing the importance of the NH_2 terminus for high affinity ligand binding (Oppermann *et al.* 1993, Mery *et al.* 1994, and De Martino *et al.* 1994). Similarly to C5a, the cationic chemokine interleukin-8 recognises acidic NH_2-terminal receptor sequences and selectivity in ligand binding for these highly homologous receptors can be transferred with molecules chimeric at the NH_2 terminus (La Rosa *et al.*, G.J., Thomas, K.M., 1992, Gayle *et al.* 1993). Subsequent work with the C5a/C5a receptor pair substituting Gα16 for the GαI subunit (Gerard *et al.* 1991, Rollins *et al.* 1991, Camps *et al.* 1992, Amatruda *et al.* 1993) suggests that C5a, (like other peptidgeric ligands that utilise G.P.C.R.s) conform to the message: address model of Schwyzer (Schwyzer, R., 1980) where the "address" domain binds "recognition" sequences in the receptor and presents the "message" domain to the agonist site in the receptor. The distinct binding and activation sites in the C5a-receptor mirror the duality of C5a ligand interaction sites. Recently as discussed above, further studies suggest that three potential sites on C5a are involved in interactions with C5aR (Figure 1.5), including the four positively charged residues in the loop region D1, the negatively charged loop region D2 and the carboxy-terminal region of C5a (Huber-Lang *et al* .2003)

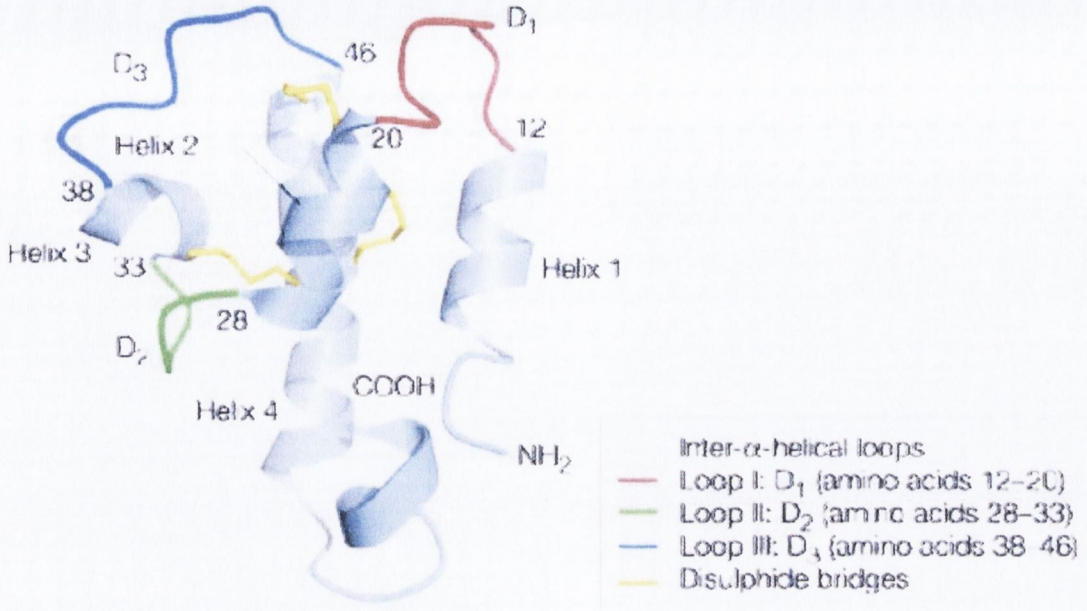


Figure 1.5 Schematic structure of human C5a (Ward P.A. 2004, *Nature Reviews Immunology* 4, 133-142)

1.3.3 Functions of C5a under regulated conditions

1.3.3.1 C5a and normal signalling pathways in neutrophils leading to assembly of NADPH oxidase

Interaction of C5a with C5aR triggers a complex signalling cascade in neutrophils (Huber-Lang *et al.* 2002), as shown in a simplified manner in Figure 1.6. The events shown in the figure lead to phosphorylation and translocation of cytosolic subunits of NADPH oxidase (p40 ^{phox}, p47 ^{phox} and p67 ^{phox}) to the cell membrane, where assembly of catalytically active oxidase occurs, due to the formation of a heteropolymeric complex containing both translocated cytosolic subunits and pre-existing cell-membrane subunits. Simultaneously, activation of cytoplasmic phospholipase A_2 ($cPLA_2$) occurs (Huber-Lang *et al.* 2002) followed by production of leukotriene B_4 (LTB_4), which is a powerful

chemotactic attractant for neutrophils, and thromboxane$_4$ (TX_4), which has a potent aggregation-inducing effects on platelets (Zaitsu *et al.* 2000). Arachidonic acid is also generated. Arachidonic acid enhances the assembly of NADPH oxidase (Pompeia *et al.* 2003). NADPH oxidase facilitates electron and proton release from the interior of the neutrophil with subsequent formation of superoxide anion (O_2-) which is rapidly converted to H_2O_2. H_2O_2 is vital for killing gram-negative bacteria, especially in the presence of myeloperoxidase. However, excessive amounts of C5a block this activation sequence in neutrophils as will be discussed later.

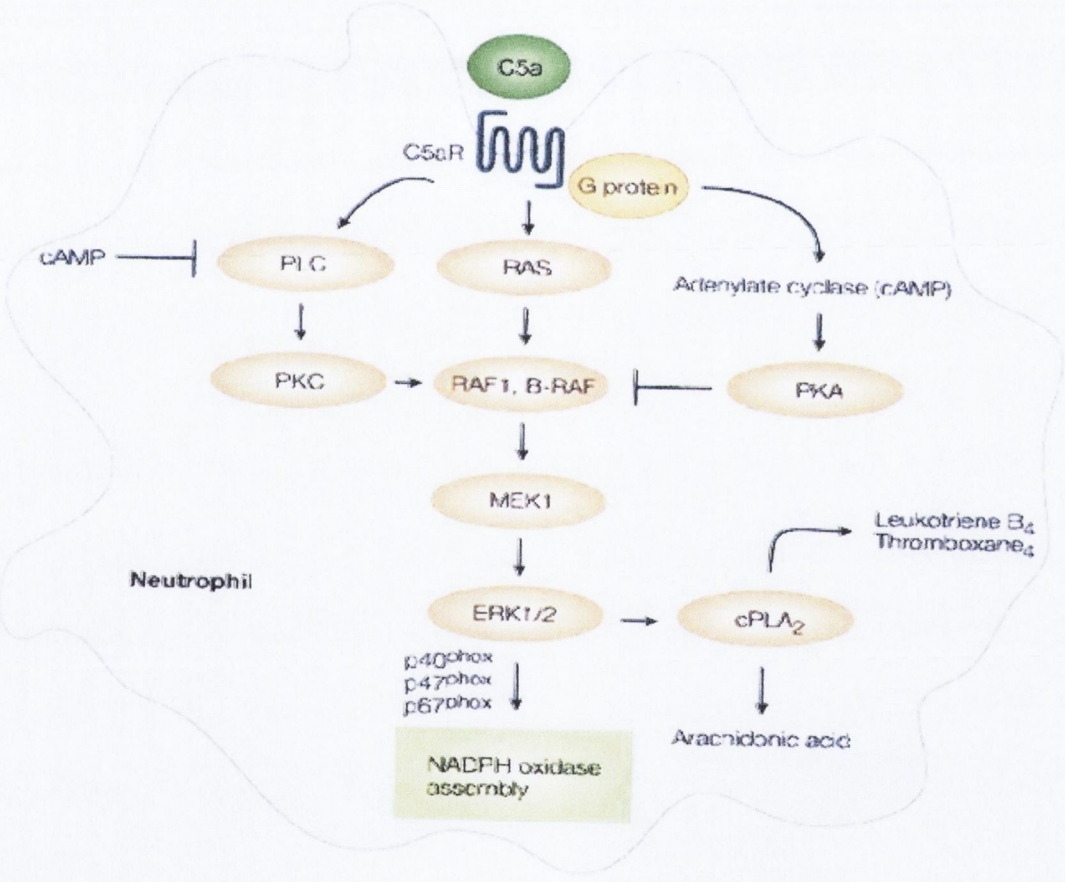


Figure 1.6 Signalling pathways for C5a interaction with C5aR on neutrophils.

(Ward P.A. 2004, *Nature Reviews Immunology*, 4,133-142)

1.3.3.2 Biological effects of C5a

There is good evidence that C5a interacting with C5aR on neutrophils, macrophages and endothelial cells leads to a series of protective events that induce a localised and contained inflammatory response (Figure1.7). The end result is removal by phagocytosis or lysis (by the MAC) of infectious agents (especially bacteria) and restoration of the tissue to its original state. These carefully orchestrated events are tightly regulated and are ordinarily confined to a small area in the tissue. Appearance of complement–activation products (C3a, C5a and C5b-C9) in the plasma is tentative evidence for the loss of control of the inflammatory response, hyper-activation of the complement system or both. C5a causes various types of responses in different cell types. C5a causes a local inflammatory response by acting directly on local blood vessels, increasing blood flow, vascular permeability and binding of phagocytes to endothelial cells. This increased fluid leak from blood vessels leads to extravasation of complement and immunoglobulin. In addition, increased vascular diameter and permeability leads to increased accumulation of fluid and protein, increased lymph drainage delivering antigen and pathogens to the lymph nodes, together with the antibodies, complement and cells recruited, augmenting pathogen clearance by enhancing phagocytosis. C5a causes many pro-inflammatory effects such as the release of granular enzymes from phagocytic cells, activation of mast cells causing the release of mediators e.g. histamine and tumour necrosis factor–alpha (TNF-α), production by neutrophils of superoxide anion, and induction of thymocyte apoptosis during sepsis.

C5a has important protective functions for phagocytic cells (neutrophils and macrophages) in that it "primes" them for enhanced functional responses (Goldstein *et al.* 1974, Molines *et al.* 2002) through the signalling pathways described in figure 1.7. In the priming process, cell contact with C5a causes markedly enhanced phagocytic responses and increased assembly of NADPH oxidase when neutrophils come into contact with a second agonist (such as bacteria or LPS). The ability of neutrophils to engulf, ingest and

kill bacteria is enhanced by prior contact with C5a. In addition, C5a initiates limited functional responses in leukocytes such as granule enzyme release and the generation of superoxide anion (Goldstein *et al* 1974, Molines *et al.* 2002, Sacks *et al.* 1978). In these aspects, C5a enhances the innate immune functions of phagocytic cells. C5a also induces chemotactic response of neutrophils, which accumulate at sites of C5a generation. Collectively, these events result in increased protection against locally present infectious agents. Another interesting effect of C5a on neutrophils is its ability to confer resistance to apoptosis (Perianayagam *et al.* 2002). It has been known for some time that neutrophils are short lived, with an *in vivo* life span of about 24 hours. When neutrophils are subjected to prolonged in vitro incubation (12-24 hours) at 37°C, they undergo apoptosis, but if they are first exposed to C5a, (or other mediators such as granulocyte colony stimulating factor) they become resistant to apoptosis (Perianayagam *et al.* 2002). It has been demonstrated that C5a activates the phosphatidylinositol 3-kinase (PI3K) pathway, because blockade of this pathway with a selective PI3K inhibitor abolishes resistance of neutrophils to apoptosis after exposure to C5a (Perianayagam *et al.* 2002). The implications of C5a-induced resistance of neutrophils to apoptosis are unclear. Theoretically this could prolong the life span of neutrophils at inflammatory sites, enhancing protection against infectious agents. However it could also be argued that apoptosis of neutrophils is an important mechanism for their removal (therefore containing the inflammatory response) and that, if the apoptosis mechanism is diminished this could prolong neutrophil life span, implying that the inflammatory response will be exaggerated.

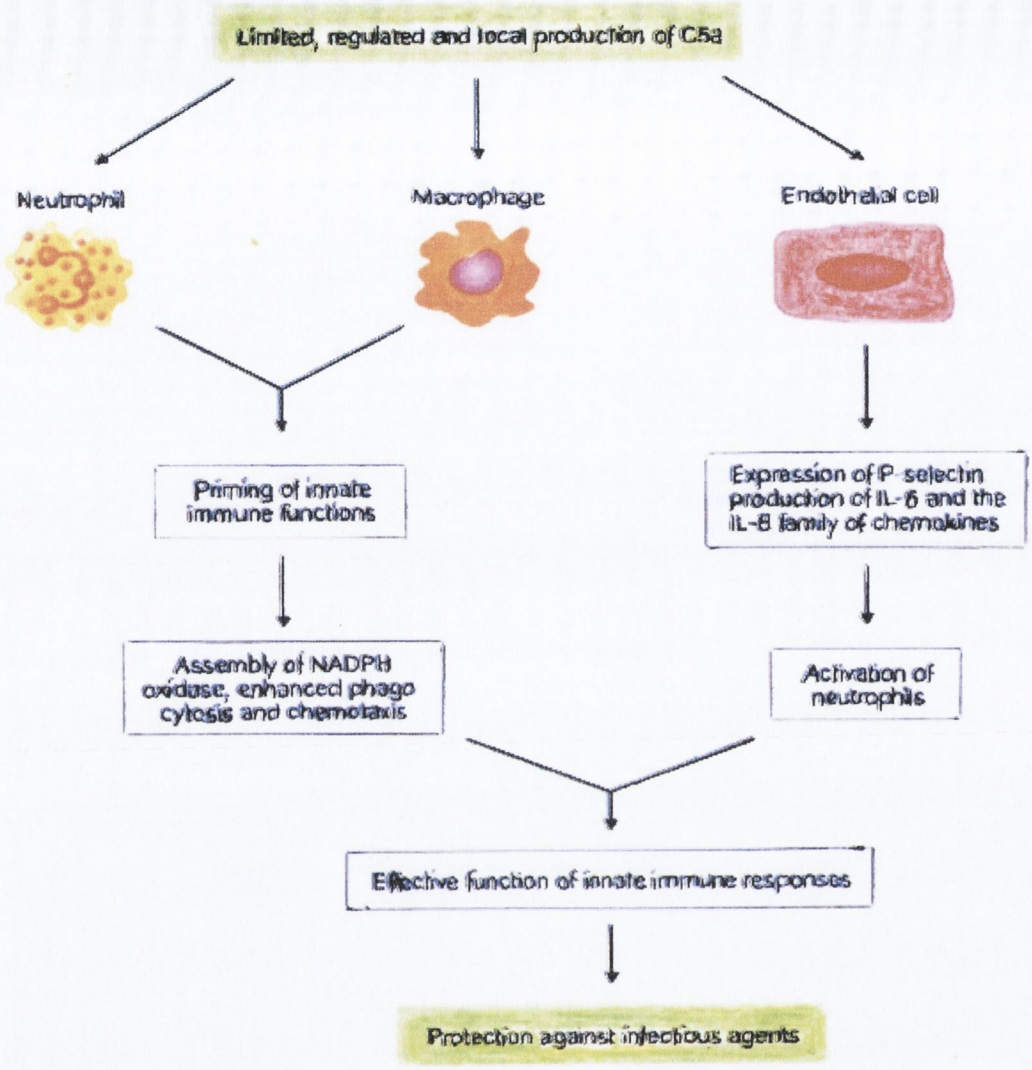


Figure 1.7 Protective host defence effects of local, limited and regulated production of C5a. (Ward P.A. 2004, *Nature Reviews Immunology,* 4, 133-142)

Endothelial cells have a key role in the inflammatory response by undergoing an activation process that facilitates adhesion of neutrophils to vascular luminal surfaces and the subsequent activation of these adherent neutrophils (Jagels *et al.* 2000, Molad *et al.*

1994). C5a causes translocation of Weible-palade granules in endothelial cells. These are cytoplasmic granules that contain the adhesion molecule P-selectin and the clotting protein, von-Willebrand factor (vWF). When endothelial cells are exposed to C5a or stimuli such as histamine, these granules fuse with the cell membrane, causing cell-surface expression of P-selectin and secretion of vWF (Foreman *et al.* 1994). Cell surface bound P-selectin interacts with its ligand, P-selectin glycolipid 1 (PSGL1), on neutrophils to initiate rolling of neutrophils, due to intermittent adhesive interactions between neutrophils and endothelial cells. This is the first in a series of adhesive interactions before transmigration of neutrophils into extravascular sites occurs. In addition, C5a enhances the function of endothelial–cell agonists, such as LPS, causing increased endothelial–cell production of the IL-8 family of CXC-chemokines, CC-chemokines and Il-6 (Riedemann *et al.* 2002, Laudes *et al.* 2002, Hopken *et al.* 1996, Strieter *et al.* 1992). IL-8 related chemokines react with CXC-chemokines receptors (CXCRs) on adherent neutrophils to prime them for enhanced functional responses. C5a can also induce the expression of C5aR by endothelial cells (Laudes *et al.* 2002).

1.3.4 C5a and the adaptive immune response

Immunoglobulins activate complement on binding antigen via the classical pathway. In addition they can activate complement via the alternative pathway by binding C3b. Immune complexes of antigen and antibody can fix complement and are cleared from the circulation via complement receptors on red blood cells. Complement bridges the innate and adaptive immune system through the complement receptor 2, CR2 expressed on B cells. A degradation product of C3b called C3dg binds CR2, which is part of the co-receptor complex on B cells, leading to the augmentation of signal received through the antigen specific immunoglobulin receptor.

The complement system and the Toll-like receptors (TLRs) are two central arms of innate immunity that are critical to host defence as well as development of adaptive immunity. Most pathogens activate both complement and TLRs, suggesting the potential

for crosstalk between the two systems. For example, it has recently been shown that C5a negatively regulates toll like receptor 4 (TLR-4) and CD40-induced synthesis of IL-12 family cytokines (IL-12, IL-23 and IL-27) from inflammatory macrophages by extracellular signal-regulated kinase and phosphoinositide 3 kinase-dependent pathways (Hawlish *et al.* 2005). This decreased cytokine response translates into a decreased T helper type 1 (Th1) response *in vitro* and *in vivo*. Interestingly, an enhanced Th1 immunity was found in C5a receptor-deficient mice, something that conferred protection against Leishmania major infection. This work identifies the negative impact of C5a on IL-12 family cytokines as an important mechanism for regulating Th1 polarisation in response to innate and adaptive immune network activation.

C5a/C5aR is linked to the important pro-and anti-inflammatory cytokine interleukin-6 (IL-6) which is known to be up-regulated in sepsis. We know that interception of C5a and C5aR results in improved outcomes in experimental sepsis and that IL-6 plays a role in the up-regulation of C5aR in sepsis. Using the caecal ligation and puncture (CLP) model in mice, treatment with IL-6-sepecific or C5aR specific antibody results in significantly improved survival, dependent on the amount of antibody infused. Blockade of C5aR also greatly attenuates the rise of plasma tumour necrosis factor (TNF) and IL-6 after CLP, indicating that C5a, C5aR and IL-6 are linked in pathogenic events and are associated with adverse outcomes of sepsis. CLP animals showed significantly increased binding of ^{125}I-labelled anti-C5aR to organs when compared to either control mice at 0 hours or CLP animals infused with normal rabbit ^{125}I-labelled IgG (Riedemann *et al.* 2003). Binding of ^{125}I-labelled anti-C5aR to lung, liver, kidney and heart was significantly decreased in anti-IL-6-treated animals 6 hours after CLP. Reverse transcriptase polymerase chain reaction (RT-PCR) experiments with mRNA isolated from various organs obtained 3, 6 and 12 hours after CLP demonstrated increased C5aR mRNA expression during the onset of sepsis, which was greatly suppressed in CLP mice treated with anti-IL-6. The authors suggest that IL-6 plays an important role in the increased expression of C5aR in lung, liver, kidney and heart during the development of sepsis in mice and that interception of IL-6 leads to reduced expression of C5aR and improved survival. The onset of sepsis is associated with the early appearance of IL-6 in the plasma, resulting in transcriptional up-

regulation of C5aR in various organs. This indicates that in sepsis, generation of IL-6, by increasing C5aR expression in the vasculature, enhances the ability of C5a to cause harmful effects in various organs. Indeed one can speculate that production of IL-6 during sepsis causes up-regulation of C5aR expression in the vasculature of various organs putting them at increased risk of C5a induced injury. Thus in experimental sepsis, C5a appears to be a major force that leads to adverse outcomes, whereas IL-6 seems to function as an enhancer of such unwanted outcomes.

C5a plays a critical role together with C3a in linking the innate and adaptive immune response in allergic asthma. Data suggests a model in which C5a and C3a play important but opposing roles during allergen-induced T cell polarisation: C5a prevents Th2 polarisation whereas C3a promotes Th2 responses (Hawlisch *et al* 2004). During the effector phase, both anaphylatoxins trigger the inflammatory response and contribute to bronchoconstriction. Subsequently Kohl *et al.* (Kohl *et al.* 2006) suggested a dual role for C5a in allergic asthma (see later). Thus C5a has a dual role, being an important player in both innate and adaptive immunity

1.4 PRO-INFLAMMATORY ROLES OF C5A/C5AR

1.4.1 Role of C5a in host defence

The inflammatory response is considered to include margination and infiltration of leukocytes, release of granule bound proteolytic enzymes and activated oxygen-and nitrogen-derived radicals, changes in blood flow, increased vascular permeability and contraction of smooth muscle. C5a is capable of all these functions. Thus C5a anaphylatoxin is a complete pro-inflammatory molecule. At subnanomolar to nanomolar levels, C5a elicits chemotaxis of all myeloid lineages (neutrophils, eosinophils, basophils, macrophages and monocytes), causes an increase in vascular permeability that is markedly potentiated by prostaglandins and circulating leukocytes and induces the expression of endothelial adhesion molecules (Mourshargh *et al.* 1990, Werfel *et al.*

1992, Schulman *et al.* 1988, Yancey *et al.* 1989, Mac Glashan *et al.* 1991, Foreman *et al.* 1994). C5a induces release of intra-cellular granules and activation of NADPH oxidase for the respiratory burst. Thus, C5a has a broad breadth of bioactivity, in contrast to chemotactic cytokines of the α- and β- chemokine families (e.g. interleukin-8, monocyte chemotactic protein [MCP]-1and eotaxin).

In 1991, two groups demonstrated that the anaphylatoxins function on a wide variety of bone marrow derived cells via coupling to G protein coupled receptors (Gerard *et al.* 1991, Boulay *et al.* 1991). It was known that activation of anaphylatoxin receptors mediates signal transduction pathways triggering a variety of pro-inflammatory events. However, by interacting with the cytokine and chemokine network C3a and C5a also exhibit anti- inflammatory properties.

1.4.2 Role of C5a/C5aR in sepsis

1.4.2.1 General overview of complement and C5a in sepsis

Sepsis in humans is a difficult condition to treat and is associated with a high mortality rate. By 1999 there was abundant evidence that in sepsis, complement activation, production of cytokines and unregulated inflammatory responses occur (Bone 1996, Nichlos *et al.* 1984, Border *et al.* 1987, Dinarello *et al.* 1993, Deitch *et al.* 1998, Wichtermann *et al.* 1980, Nakae *et al.* 1996). It was and still is generally accepted that the complement system provides essential protection against bacteria through generation of the opsonic factor C3b, which promotes phagocytosis and intracelllar killing of bacteria by H_2O_2 and the myeloperoxidase-dependant pathway (Quezado *et al.* 1994, Kubens *et al.* 1988, Koehl *et al.* 1993, Klebanoff *et al.* 1975, Elsbach *et al.* 1983). The loss of C3 in genetically manipulated mice considerably increases the death rate after challenge with live bacteria or with bacterial liposaccharide (Wessels 1995, Fischer *et al.* 1997). The membrane attack complex (C5-9) is important in the lysis of Gram-negative bacteria (Kubens *et al.* 1988, Keohl *et al.* 1993, May *et al.* 1972). By the early 1990s C5a

was known to be a potent agonist for neutrophils and endothelial cells (Koehl *et al.* 1993) and to have vasopermeability and vasodilating activities (Koehl *et al.* 1993, Schumacher *et al.*1991). In humans with sepsis, blood levels of C3a, C5a and C5b-C9 are increased whereas the whole complement haemolytic activity, i.e. the ability of serum complement to lyse antibody coated red-blood cells is diminished (Gerard *et al.* 2003). Ward and colleagues had demonstrated that neutrophils stimulated with C5a become refractory or "deactivated" to further stimulation with this peptide but after exposure to high doses of C5a, global deactivation to chemotactic peptides occurs (Ward *et al.* 1986). At that time there was clinical evidence that blood neutrophils from humans with early sepsis lose functional responsiveness to C5a and in the later phases of sepsis, lose responsiveness to structurally different chemotaxins such as bacterial chemotactic factor (Solomkin *et al.* 1981). In patients with sepsis, C5a might reach levels in the serum as high as 10-100nM (Solomkin *et al.* 1981). Such concentrations were shown to rapidly suppress the ability of blood neutrophils to respond chemotactically not only to C5a, but also to a structurally unrelated chemotactic peptide, fMLP, produced by bacteria, (Tomhave *et al.* 1994). However the exact role of C5a in sepsis was still unknown at that time.

Currently there is no general consensus regarding the precise pathway(s) of complement activation in sepsis. It is postulated that the lectin and alternative pathways are activated following release *in vivo* of lipopolysaccharide from Gram-negative bacteria and/or interaction of bacteria with mannose-binding lectin through mannose-containing residues on bacterial surfaces, but these assumptions require validation. Whatever the pathway(s), it is agreed that marked activation of complement and release of C5a occurs in sepsis.

1.4.2.2 C5aR in sepsis

Soon after the findings in sepsis which demonstrated benefits of blockade of C5a with antibodies, C5aR became the focus of interest. Blockade of C5aR by a small cyclic C5aR antagonist as well as by antibodies to the N-terminal end of C5aR resulted in significantly improved survival in caecal ligation and puncture (CLP) mice (Riedemann *et al.* 2002

and Huber-Lang *et al.* 2002). A strong increase in C5aR surface expression was found early (6 hours) after CLP in mouse lung, liver, kidney and heart (Riedemann *et al.* 2002). This increase was found to be strongly dependant on plasma IL-6, because IL-6 blockade in CLP mice inhibited the C5aR increase in all four organs and resulted in improved survival in a dose-dependent manner (Riedemann *et al.* 2003). C5aR blockade with antibodies, in turn resulted in decreased IL-6 plasma levels at 6 hours after sepsis (Riedemann *et al.* 2002). This data suggests a complex interaction and positive feedback between IL-6 generation and C5aR up-regulation and between C5aR activation (via C5a) and IL-6 production. As C5a is believed to be activated within the first few hours of CLP-induced sepsis in rodents (and IL-6 levels are peaking at ~ 6 hours), it is tempting to hypothesise that the early generation of C5a may be the driving force for up-regulation of IL-6. Earlier findings in alveolar epithelial cells demonstrated that LPS-induced generation of mediators, such as tumour necrosis factor α (TNF-α) and chemokines (macrophage inflammatory protein-2, cytokine-induced neutrophil chemoattractant) were strongly enhanced in the co-presence of C5a. *In vitro* data indicated that C5a may also enhance LPS-induced IL-6 production in granulocytes (Riedemann *et al.* 2003). The same has been suggested for peripheral blood mononuclear cells (PBMC) (Montz *et al.* 1991). In such a scenario, early C5a generation during sepsis would boost mediator-induced production of IL-6 in the serum resulting in fast up-regulation of C5aR in various organs which in turn, could make these organs more susceptible to C5a effects but also result in reducing circulating C5a levels by binding and internalising C5a as a potential negative feedback (Figure1.8 and 1.9). Increased C5aR gene expression was found during various kidney diseases (Abe *et al.* 2001). The effect however, of C5aR increase and activation in the various organs is unclear so far. In addition, IL-6 is a strong inducer of C-reactive protein which is a known activator of the classical pathway of complement (Banks *et al.* 1995). This is a further potential function of IL-6 in sepsis.

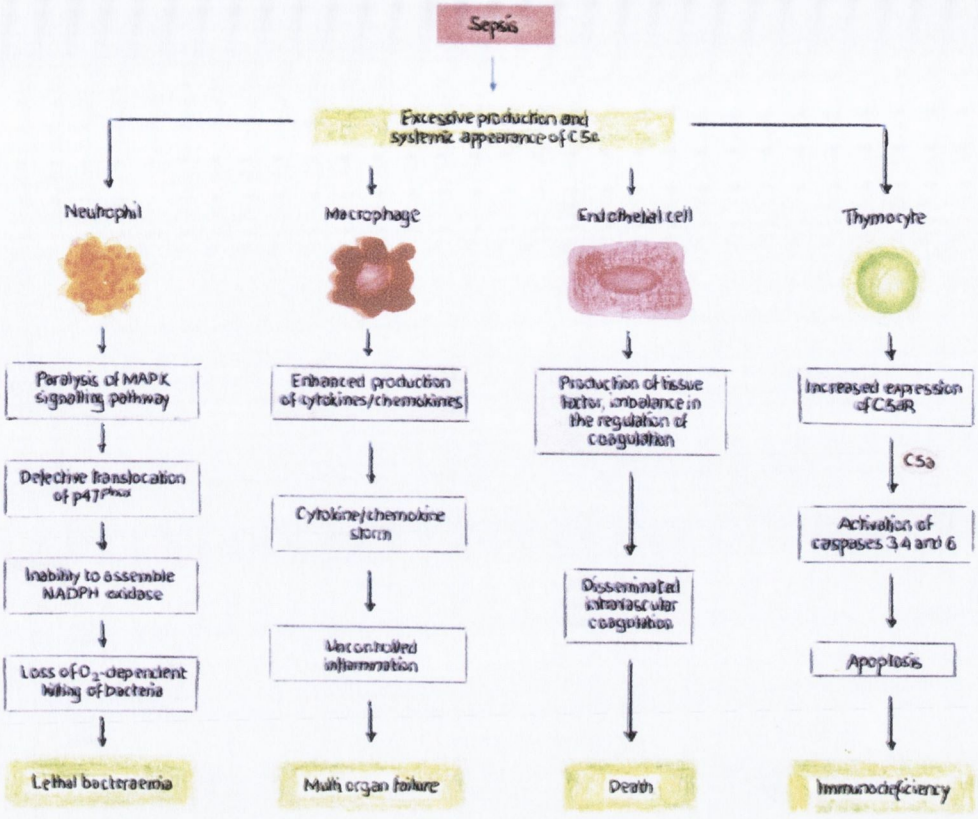


Figure 1.8 Outcomes of excessive generation of C5a, as occurs in sepsis.

Neutrophils become functionally paralysed, whereas macrophages and endothelial cells produce excessive pro-inflammatory mediators. Thymocytes undergo C5a-mediated caspase activation and apoptosis. (Ward P.A., 2004 *Nature Reviews Immunology* 4, 133-142)

1.4.2.3 C5aR and neutrophils during sepsis

In contrast to organs, there is evidence that C5aR expression on neutrophils may be regulated differently during sepsis, because binding of radiolabelled C5a to PMN has been demonstrated to be vastly down-regulated during CLP- induced sepsis in rodents

(Huber-Lang *et al.* 2001) which was reversed by blockade of C5a with antibodies. In excess of 80% reduction in cell-surface expression of C5aR on blood neutrophils within 24 hours of CLP in rats was documented (Guo *et al.* 2003). The loss of C5aR on blood neutrophils appears to be a result of internalisation and translocation of C5a/C5aR complexes (Guo *et al.* 2003) to the golgi apparatus, where under acidic conditions the ligand is hydrolysed and the receptor is recycled to the cell-surface. When neutrophils are incubated *in vitro* with C5a, by 30 minutes at least half of the cell-surface C5aR is lost (Huber-Lang *et al.* 2002). Confocal microscopy shows a change in C5aR from a cell surface/cortical pattern to a diffuse granular, cytoplasmic pattern, all of which is consistent with internalisation of the receptor. It has been suggested that C5aR expression on PMN during experimental sepsis may be an indicator of outcome and therefore may have a prognostic use in the clinic. An earlier study suggested similar findings in a model of LPS infusion in rabbits (Goldman *et al.* 1986). Down regulation of C5aR receptors has subsequently been documented in humans with sepsis (Furebring *et al.* 2002).

1.4.2.4 Evidence for harmful effects of C5a on neutrophil function in sepsis

Neutrophils seem to play a special role in the context of C5a effects during sepsis. Neither IL-6 nor LPS regulate C5aR in PMN at the transcriptional level, and the surface content on PMN of C5aR has been demonstrated to decrease during sepsis although an increase has been observed in lungs, liver kidney and heart (Riedemann *et al.* 2003). Upon contact with C5a, LPS–induced tumour necrosis factor-α (TNF-α) production appears to be significantly inhibited by C5a in PMN and the opposite effect could be observed in macrophages (Riedemann *et al.* 2003). C5a has been shown to provoke a transient leukocytosis after injection into rabbits (Kajita *et al.* 1990), supporting the concept that C5a plays an important role for innate immunity via neutrophil recruitment. An earlier study had suggested that exposure of high levels of C5a could lead to non-specific, chemotactic "deactivation" thereby causing broad neutrophil dysfunction (Solomkin *et al.* 1981). Subsequently, a group discovered that blood neutrophils from CLP rats showed reduced phagocytosis and defective assembly of reduced nicotinamide

adenine dinucleotide phosphate oxidase (Huber-Lang *et al.* 2002). Blockade of C5a during sepsis prevented these effects, suggesting a harmful role of C5a for neutrophil innate-immune functions during sepsis. The chemotactic ability in neutrophils is compromised during sepsis as a result of generation of C5a (Huber-Lang *et al.* 2002). Subsequently these finding were extended by demonstrating that the production of reactive oxygen species in neutrophils is compromised during CLP-induced sepsis in rats related to C5a generation (Guo *et al.* 2003). Others suggested that C5a was also involved in altered neutrophil trafficking during sepsis as a result of differential regulation of β1-integrins (Guo *et al.* 2002). Thus excessive C5a generation during sepsis appears to impair crucial innate-immune functions of neutrophils, leading to a state of "immune paralysis" in which the first line of defence such as phagocytosis, H_2O_2 production, ability to bind C5a, and chemotactic responses to both C5a and fMLP are lost (Huber-Lang *et al.* 2002, Huber-Lang *et al.* 2001 Seely *et al.* 2002), (Figure 1.10). Thus blood neutrophils from rats display paralysis of the signalling pathways. Perturbations in neutrophils isolated from septic rats or normal human neutrophils exposed to C5a *in vitro* demonstrate an inability to phosphorylate extracellular signal regulated kinase 1/ 2 (ERK1/2 or p42/p44) which is a crucial factor in the mitogen-activated protein kinase (MAPK) signalling cascade involved in neutrophil activation. Under these conditions, neutrophils cannot phosphorylate $p47^{phox}$ a key cytosolic factor that must be translocated to the cell surface for the assembly of the NADPH oxidase (Huber-Lang *et al.* 2002).

1.4.2.5 Other harmful effects of C5a during sepsis

In addition to the impaired innate-immune functions in neutrophils, other harmful effects of C5a during experimental sepsis have been described. Laudes and colleagues demonstrated that C5a has an eminent role for disordered coagulation and fibrinolysis

during sepsis (Laudes *et al.* 2002). This was the first evidence of a relationship between the complement and coagulation system in a setting of experimental sepsis. A consumptive coagulopathy occurs frequently in patients with sepsis, indicates a loss in control of the clotting and fibrinolytic systems (Aird *et al.* 2003) and contributes to high mortality rates. In addition to enhancing endothelial cell production of pro-inflammatory mediators such as the IL-8 family of chemokines, high levels of C5a cause endothelial cell generation of a damaging product by the name of tissue factor which has potent pro-thrombotic activity that can lead to intravascular coagulopathy (Ikeda *et al.* 1997, Muhlfelder *et al.* 1979), (Figure 1.9).

Furthermore, C5a can induce thymocyte and perhaps other lymphoid cell apoptosis during sepsis. Sepsis is a state of immunodeficiency especially involving B cells and CD4+ T cells (Hotchkiss *et al.* 2001). Programmed cell death of lymphoid cells during sepsis (Hotchkiss *et al.* 1999, and Hotchkiss *et al.* 2000) is thought to be related to loss of immune functions during sepsis. In these studies, blockade of C5a during CLP-induced sepsis resulted in potent inhibition of the previously described rapid thymus involution during sepsis (>70% loss of thymic mass) and almost completely abolishes apoptosis of thymocytes and evidence of caspases 3, 6 and 9 activation (Guo *et al.* 2000, Riedemann *et al.* 2002) . Therefore, it is hypothesised that a massive C5a generation during the onset of sepsis could lead to a rapid reduction of immunocytes increasing susceptibility to infection (Figure 1.9).

Contrary to neutrophils, macrophages are not paralysed in the presence of high levels of C5a but actually have potentiated responses (Riedemann *et al.* 2003). This may be related to the lower numbers of the C5aR present on macrophages in comparison to neutrophils (Figure 1.9). Macrophages stimulated by LPS in the presence of C5a show considerably increased production of pro-inflammatory mediators such as tumour-necrosis factor and many chemokines which may be responsible for the cytokine/chemokine storm that is documented in sepsis and systemic inflammatory response syndrome (the latter indicating loss of regulation of the inflammatory response).

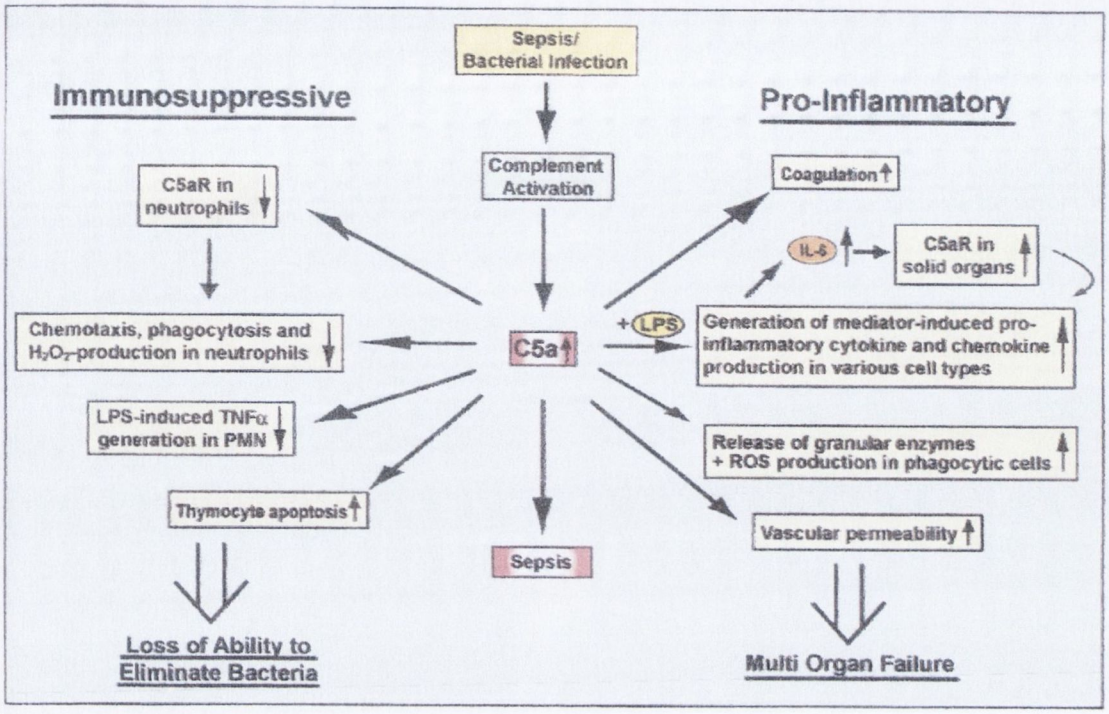


Figure 1.9 The key role of C5a/C5aR activation in sepsis

(Riedemann, *et al.*, 2003, *J. of Leuk. Biol.*, 74, 966-970)

1.4.2.6 Effects on cytokine production of co-stimulation of cells with C5a and LPS

In vitro results with C5a stimulation of various cell types did not result in significant mediator generation in most cases. When alveolar epithelial cells were co-stimulated with LPS and C5a, a much higher production of TNF-α and chemokines could be observed when compared with cells stimulated only with LPS (Riedemann *et al.* 2002), suggesting a strong, stimulatory role of C5a for the mediator-induced production of various pro-inflammatory cytokines. Earlier studies in PBMC had demonstrated that C5a stimulation resulted in gene transcription (but not translation) for IL-1β, because significant increases

in protein expression for IL-1β could not be detected (Schindler *et al.* 1990, Kaspar *et al.* 1994). Other work had demonstrated that pre-stimulation of granulocytes with C5a or other chemoattractants resulted in significantly reduced LPS-induced IL-8 production, and co-stimulation with C5a and LPS resulted in a synergistic increase in IL-8 production as compared with LPS-only-stimulated granulocytes (Strieter *et al.* 1992). In the case of LPS-induced production of the pro-inflammatory cytokine IL-6 by PBMC, similar findings have been made when they were co-stimulated with C5a and LPS (Montz *et al.* 1991). Riedemann and colleagues (Riedemann et al. 2003) suggested that this was true for granulocytes too and suggested it may have systemic implications during CLP-induced sepsis. Other in vitro work demonstrated that C5a strongly reduced mediator (LPS, interferon-γ)-induced production of IL-12 in human monocytes (Witmann *et al.* 1999). Others demonstrated that LPS-induced TNF-α production was significantly reduced in neutrophils in the co-presence of C5a, and the opposite effect (increased TNF-α production) could be seen in macrophages (Riedemann *et al.* 2003). The authors interpreted these findings to suggest that C5a inhibits the production of an important pro-inflammatory mediator in neutrophils, reflecting evidence for a harmful inhibition of innate-immune functions. In conclusion C5a in most of the in vitro experiments seems to significantly enhance mediator-induced production of cytokines and chemokines with the exception of IL-12 in PBMC and TNF-α in PMN. In the context of sepsis, a boost of pro-inflammatory mediator production early during sepsis may explain the significant contribution of C5a in the onset of sepsis. For specific cell types (e.g. neutrophils) contact with C5a may results in harmful reduction of certain immune functions.

1.4.2.7 Role of C5a in animal models of sepsis

Early experimental studies in monkeys suggested that blockade of C5a by antibodies could significantly attenuate *Escherichia coli (E. coli)* -induced septic shock and adult respiratory distress syndrome (Stevens *et al.* 1986, Hangen *et al.* 1989). Studies in rats suggested that LPS-induced septic shock could be mimicked by injection of C5a and blockade of C5a via antibody attenuated LPS-induced responses (Smedegard *et al.* 1989).

The caecal ligation and puncture (CLP) model of sepsis in rodents closely reflects the clinical phases found in humans with sepsis, with an early hyper-dynamic phase (tachycardia, increased cardiac output, fever, leukocytosis etc.) and a late hypo-dynamic phase (bradycardia, reduced cardiac output, hypothermia, leucopaenia etc.). It results in peritonitis and bacteraemia. Czermak and colleagues (Czermak *et al.* 1999) investigated the role of complement in sepsis, in particular the role of C5a. He demonstrated that rats depleted of the complement factor C3 died within ~4 days after CLP. Rats that were C3 intact which underwent CLP and were treated with pre-immune IgG, most (92%) were dead by 7 days. Blood neutrophils from these rats contained on their surface C5a. This group had high levels of bacteraemia, and their blood neutrophils when stimulated in vitro had greatly reduced production of H_2O_2, which is known to be essential for the bactericidal function of neutrophils. In contrast, when companion CLP rats were treated with IgG antibody against C5a, survival rates were significantly improved, levels of bacteraemia were considerably reduced and the H_2O_2 response of blood neutrophils was preserved. Bacterial colony forming units in spleen and liver were very high in CLP rats treated with pre-immune IgG and very low in CLP rats treated with IgG antibody against C5a, similar to values obtained in rats that underwent "sham" operations (without CLP). This was the first evidence that supported the hypothesis that sepsis results in excessive production of C5a, which leads to deactivation of neutrophils, compromising the respiratory burst (H_2O_2 production) of these cells and the closely linked bactericidal function, which is dependant on H_2O_2 generation and participation of myeloperoxidase (Klebanoff 1975, Elsbach *et al.*1983). Czermak *et al.* concluded from this work that C5a may be a useful target in the treatment of sepsis. Follow up studies aimed to determine the efficacy of blocking different regions of C5a by antibodies, revealed that blockade of the middle or C-terminal region of C5a was an effective strategy in terms of improving survival in CLP rats (Huber-Lang *et al.* 2001). In addition, it was demonstrated that delayed infusion (6 and 12 hours after CLP) of antibodies to C5a still resulted in significant survival benefits for the animals. Furthermore, C5a blockade during sepsis greatly reduced appearance of multi-organ failure in rats (Huber-Lang *et al.* 2001).

1.4.2.8 Role of C5a in sepsis in humans

In humans with sepsis, the onset of multiorgan failure, especially involving liver, lungs and kidneys is a well known complication that is associated with a high mortality rate. Persistent elevation of the anaphylatoxins C5a, C3a and C4a appears to correlate with development of multiorgan failure and is inversely correlated with survival (Nakae *et al.* 1996, Bengtson *et al.* 1986). In 2001, Huber-Lang and colleagues (Huber-Lang *et al.* 2001) examined the role of C5a in multiorgan failure in an attempt to explain the protective effects of anti-C5a in the CLP model of sepsis. In these studies multiorgan failure occurred during the first 48 hours after CLP with development of liver dysfunction, and pulmonary dysfunction (falling arterial partial pressure of O_2 and rising partial pressure of CO_2). In this model an early respiratory alkalosis developed, followed by a metabolic acidosis with increased levels of blood lactate. During these events, blood neutrophils lost their chemotactic responsiveness both to C5a and the bacterial chemotaxin, fMLP. Neutrophil dysfunction was associated with virtually complete loss of binding of C5a but binding with fMLP remained normal. If CLP animals were treated with anti-C5a, indicators of multiorgan failure and lactic acidosis were greatly attenuated. Under the same conditions, C5a binding to blood neutrophils remained intact: in tandem *in vitro* chemotactic responses to C5a and fMLP were retained. The authors suggested that treatment with anti-C5a prevents development of multiorgan failure and the accompanying onset of blood neutrophil dysfunction and that this may explain the protective effects of anti C5a in the CLP model of sepsis. They hypothesise that *in vivo* contact of neutrophils with C5a induces a down stream defect in signal transduction beyond a point at which C5aR and fMLP-receptor (fMLPR) pathways merge. In other words, although blood neutrophils from CLP rats appear to demonstrate normal binding of fMLP, this binding is unable to effectively engage a post receptor signalling pathway that is common to both C5aR and fMLPR. This is consistent with earlier studies in which neutrophils exposed to low levels of C5a (present in activated serum), acquire a chemotactic defect specific to C5a, but when exposed to a higher concentration of C5a, they acquire a broad defect in chemotactic function characterised by loss of responsiveness to C5a and to bacterial chemotactic peptides (Solomkin *et al.* 1981). *In*

vivo, the chemotactic responses of neutrophils obtained from patients with sepsis or multi-system trauma with adult respiratory distress syndrome were found to be specifically deactivated to C5a or deactivated to both C5a and fMLP depending on clinical severity and time after trauma or onset of sepsis. In contrast, no alteration in neutrophil function was found in patients who did not develop adult respiratory distress syndrome (Solomkin *et al.*1985). These studies with the CLP model suggest that the H_20_2 response of neutrophils is impaired suggesting a broad defect in post-receptor signalling pathways.

Interestingly, Huber-Lang *et al.* document that binding sites for rat C5a (but not fMLP) on neutrophils had been virtually lost (<5% of binding in control neutrophils) during the first 24 hours of sepsis. By 48 hours, neutrophils still demonstrated <50% of C5a binding, in striking contrast to nearly normal binding values of C5a for neutrophils obtained from CLP rats treated with anti-C5a. Under these latter conditions, chemotactic responsiveness to C5a and to fMLP was totally preserved. This data was in accord with findings of specific loss of C5a binding (but intact fMLP binding) to neutrophils in patients with sepsis (Solomkin *et al.*1981). They proposed that the mechanism by which anti-C5a may protect against development of multi-organ failure during sepsis is its ability to cause preservation in the chemotactic and respiratory burst function of blood neutrophils by intercepting C5a before it has a chance to bind C5aR on neutrophils. Retention of these functional responses would facilitate effective *in vivo* recruitment of neutrophils and maintenance of bactericidal function in these cells.

More recently, it has been shown that during experimental sepsis blood neutrophils have diminished ability to bind C5a because of down regulation of C5aR (Guo *et al.* 2003). In contrast lungs, kidneys, liver, heart and vascular C5aR is up-regulated in an IL-6 dependant manner (Riedemann *et al.* 2003). Increased expression of C3aR has been described on both epithelial and smooth muscle cells (Drouin *et al.* 2001) suggesting an important role for complement receptors during the systemic inflammatory response of sepsis. Despite aggressive management of sepsis and sepsis related complications, the lethality rate remains at 40-50% for patients in septic shock (Drouin *et al.* 2001, Ohno *et*

al. 2000). Randomised clinical trials have been performed with different anti-inflammatory approaches (enrolling >12,000) patients) all of which have so far failed to show improvement in survival (overall lethality of 38% in each group) (Astiz *et al.* 1998, Vincent 1998). Taken overall, the evidence suggests that an imbalance of complement activation is central to the development of sepsis and that C5a is a key player.

1.4.3 Pneumonia and the role of C5

Activation of the complement system is generally considered a protective mechanism. The sensitivity of a naturally occurring strain of mice genetically deficient in C5, (the precursor of C5a), to lethal infection by *Pseudomonas aeruginosa* (*P. aeruginosa*) was compared with that of wild type animals (Larson *et al.* 1982). Mortality was considerably higher in C5-deficient animals following intrapulmonary instillation of live bacteria. This data indicates that in the absence of C5, the mice are unable to clear the organisms, suggesting a protective role for the protein. Because of the C5 deficiency, mice could neither generate C5a anaphylatoxin nor C5b, to act as a nidus for assembly of the membrane attack complex (MAC) and studies were initially unable to resolve the relative contribution of each.

1.4.3.1 Pneumonia and the role of C5a-C5aR

Subsequently, targeted disruption of the murine C5a-receptor (C5aR) gene provided selective elimination of C5a-C5aR interactions (Hopken *et al.* 1996, Bozic *et al.* 1996). C5aR-deficient mice bred normally and displayed no gross defects when maintained under barrier conditions. To assess the contribution of the C5aR in a model of inflammatory lung reactions, mice were challenged with intra-tracheal *P. aeruginosa,* (3.5×10^6 organisms) and were studied for lethality for six days. Most (10 of 11), C5aR-deficient mice succumbed in the second day after challenge, whereas none of the wild-type mice died. Some mice were killed immediately, some at 6 hours and the remainder

at 24 hours. Wild type mice had begun to clear organisms at 6 hours but the number of *P. aeruginosa* in the C5aR deficient mice increased. When examined at 24 hours up to 99% of the initial bacterial burden was cleared in wild type mice but C5aR deficient mice continued to demonstrate bacterial survival and growth. Thus the C5a receptor was proven to be essential for clearance of micro-organisms in the lung (Hopken *et al.* 1996).

To evaluate whether this phenotype represented differential susceptibility to the inflammatory mediators of the micro-organism (such as lipopolysaccharide, elastases and formyl peptide chemoattractant factors) the authors challenged mice with equal numbers of formalin and heat killed bacteria. All C5aR deficient mice survived this treatment. This data suggested that the viability of the microorganism is a significant factor in mortality and suggested that the C5aR-deficient mice may not be able to clear the live microorganism.

The authors hypothesised that in the absence of a chemoattractant receptor, an inadequate inflammatory response might account for the lack of bacterial clearance. The lungs from wild type and C5aR deficient mice assessed for bacterial survival were lavaged, the neutrophils counted and the lungs processed for histological examination. A surprising and paradoxical increase in the number of neutrophils recovered from the C5aR deficient mice was observed. These results were supported by histological analysis which revealed a dense neutrophil consolidation in the C5aR deficient mice that exceeded the response seen in the wild type littermates. Vascular permeability was assessed by quantification of Evans blue dye, an enhanced microvascular leak was found in the C5aR deficient mice. Despite a brisk neutrophil infiltrate mediated through redundant chemoattractants and enhanced microvascular leak, C5aR deficient mice do not clear the instilled *P. aeruginosa* organisms, resulting in pulmonary injury and death. Further evidence for the importance of C5aR in mucosal defence was obtained when mice were challenged with a sub lethal inoculum of *Pseudomonas* organisms (3.5×10^5). Quantitative culture of lung homogenates from C5aR deficient mice at six days post infection indicated a new pattern of bacterial super infection with Gram positive cocci, including two species of *Streptococcus* and a strain of *Staphylococcus*, whereas lung homogenates from wild type

mice showed no growth of microorganism at all. Thus although the acute infection with *P. aeruginosa* was cleared at lower inocula, the deletion of the C5a receptor selectively predisposed mice to colonisation or super infection with secondary pathogens. This data uncovered an unexpected role for the C5a chemoattractant receptor in mucosal host defence and demonstrates that the influx of neutrophils is not on its own sufficient to clear the lung of bacteria (Hopken *et al.* 1996).

To assess the *in vitro* phagocytic response of neutrophils from C5aR deficient and wild type mice, a sterile peritoneal inflammation was initiated with glycogen. When C5aR deficient mice were tested, a decreased number of neutrophils were elicited: the number of neutrophils that migrated to the peritoneum was one sixth that of wild type mice. Thus this "non-specific" inflammatory stimulus depends partly on intraperitoneal complement activation to extravasate blood neutrophils. However, when such cells were assessed using a phagocytosis assay for their ability to kill viable *P. aeruginosa* in vitro, no significant differences were observed (Hopken *et al.* 1996).

Next the authors investigated whether the defect in bacterial clearance was limited to the lung mucosa or represented a more generalised defect. Mice were challenged with intra-peritoneal *P. aeruginosa* (10^7 organisms) and the bacterial clearance and polymorphonuclear influx were assessed at 0, 6 and 24 hours. No differences were observed in the influx of inflammatory cells between wild type and C5aR deficient mice. After 24 hours essentially the entire inoculum was cleared in both groups. Therefore it was concluded that in the mucosal surface of the lung, the clearance of micro-organisms requires the C5a receptor but in the peritoneum this receptor is accessory or redundant.

Pseudomonas species colonise the lung in cystic fibrosis and incite a vigorous inflammatory response that results in organ damage (Berger *et al.* 1989, Hornick *et al.* 1990). Mice lacking C5aR recapitulate the phenotype seen in cystic fibrosis patients: ineffective killing of *P. aeruginosa* despite a vigorous and ultimately toxic neutrophil inflammatory response. Earlier studies with C5-deficient mice which lack both the ligand as well as the lytic C5b-9 complex, indicated that mice were susceptible to toxicity with the bacteria *Staphylococcus, Haemophilus influenza* and *Pseudomonas* species (Larsen *et*

al. 1982, Toews *et al.* 1985, Cerquetti *et al.* 1986). These results represent a phenocopy of the infection seen in cystic fibrosis patients most of whom harbour the same infective species. The C5a receptor appears to be important in mediating polymorphonuclear–dependant clearance of the mucosal bacterial organisms. The authors concluded that this information maybe useful in preventing *Pseudomonas* colonisation in cystic fibrosis patients, the majority of whom become chronically colonised with *P. aeruginosa* and die of respiratory failure (~90% of patients).

1.4.4 Immune complex injury and C5aR

The formation of immune complexes and their local position induces an acute inflammatory response. Diseases such as systemic lupus erythematosis, rheumatoid arthritis, immune glomerulonephritis, certain vasculitides and Goodpasture's syndrome are examples where immune complexes are injurous to the host. The cascade of events leading to tissue injury has been studied for nearly a century (Arthus M. 1903). The Arthus reaction is now a classical immune complex model.

The effect of C5aR deficiency in immune-complex induced inflammation in the peritoneal cavity and skin was studied and compared with results from immune complex mediated inflammation in the lung (immune complex alveolitis model). Taking advantage of transgenic mice genetically deficient in the C5aR, involvement of this receptor in several models of immune complex injury was made possible. Assessment of the reverse Arthus reaction in the lung, peritoneum and in the skin demonstrates that the complement C5a receptor is implicated in the Arthus reaction but quantitatively to different degrees in the various tissues. In the lung, immune complex-mediated activation of complement and subsequently the release of the anaphylatoxin C5a have been shown to be the main event triggering the IgG-mediated hypersensitivity reaction (Bozic *et al.* 1996). The lung tissues of the immune complex-challenged C5aR deficient mice appear to be indifferent from those of the controls challenged with antibody or saline, as quantitated by measuring extravasation of Evans blue dye into alveolar space and

exudation of neutrophils into the airways and lung alveoli. Thus the authors conclude that C5a acting through its specific G protein coupled receptor is essential for the formation of the full injury seen in this model of immune complex-mediated lung injury. However, when C5aR deficient mice were assayed in the immune complex mediated peritonitis and skin injury, the C5aR was less central to the inflammatory response. The data demonstrates that while Ca5R deficiency significantly reduces the inflammatory response in both immune complex mediated peritonitis and dermal reverse Arthus reaction, C5aR independent pathways occur.

In the peritoneal reverse passive Arthus reaction, a significantly reduced neutrophil infiltration in the C5aR deficient mice compared to their wild type controls at both 4 hours (74%) and 8 hours (51%) after challenge was documented. The decrease in neutrophil infiltration was more profound after 4 hours than 8 hours suggesting that C5a is important for neutrophil recruitment in the early phase of the inflammatory response. Studies in rabbits investigating the generation of neutrophil chemoattractants in ischaemic myocardium reperfusion showed that C5a was generated within 5 minutes whereas other neutrophil chemoattractants such as IL-8 were released after a delay (Ivey *et al.* 1995). Intradermal injections of the same chemoattractants into the skin of guinea pigs demonstrated C5a-induced eosinophil accumulation compared to C5a (Collins *et al.* 1993). The parallel reduction of TNF-α levels in the C5aR deficient mice suggests that the decrease in neutrophil migration in the peritoneal cavity in the C5aR deficient mice may be partially due to the decrease in TNF-α secretion from macrophages and mast cells. The reduction in neutrophil infiltration in the peritoneal cavity in C5aR deficient mice of 50-70% is similar to the findings published previously (Ramos *et al.* 1994), which aimed to elucidate the role of complement in mast cell activation and neutrophil recruitment in immune complex-induced peritonitis in mast cell deficient, C5-deficient and decomplemented mice. In all three deficiencies a reduced neutrophil influx of 40-60% and a reduced TNF-α release of 40-70% were observed. Complete inhibition of both parameters was observed in decomplemented, mast cell deficient mice suggesting a close link *in vivo* between complement and mast cell activation and neutrophil recruitment in the peritoneal reverse passive Arthus reaction. As well as decreased TNF-α levels, they

observed a significant decrease in IL-6 levels in the peritoneal lavage samples of C5aR deficient animals compared to their wild type controls at 4 hours and 8 hours after challenge. IL-6 is a pro-inflammatory cytokine which induces the synthesis and release of acute-phase proteins by hepatocytes and serves as a growth factor for activated B cells. Il-6 is detectable in the circulation following gram-negative bacterial infection or TNF-α infusion.

Other publications at that time had shown that C5a enhances LPS induced IL-6 release *in vitro* and *in vivo* (Montz *et al.* 1991, Hopken *et al.* 1996). However, IL-6 may also function as an anti-inflammatory mediator causing inhibition of acute neutrophilic efflux and LPS-induced TNF-α release (Akira *et al.* 1993). Hopken *et al.* demonstrated decreased IL-6 levels in C5aR deficient mice during the course of immune complex-induced inflammation. This data provided direct *in vivo* evidence for an immuno-regulatory role of the C5aR and its ligand through enhancement of IL-6 secretion, a cytokine which may function as both a pro-inflammatory and anti-inflammatory mediator.

In the reverse passive Arthus reaction in the skin (Bozic *et al.* 1996), the absence of the C5aR reduced neutrophil infiltration by ~40% and permeability changes with associated oedema by ~55%. Thus in comparison to the IgG mediated Arthus reaction in the lung, where essentially quantitative inhibition was observed, the inflammatory reaction in the skin showed partial abrogation through disruption of the C5aR gene. To demonstrate partial abrogation of a dramatic inflammatory response as observed in the C5aR deficient mice in the IgG mediated Arthus reaction in the skin, sensitive and quantitative measurements were required. The authors complemented their histologic observations with quantitative measurement of neutrophil migration [myelo-peroxidase (MPO) data] and quantitative measurement of permeability changes (Evans blue dye extravasation). By simple microscopic interpretation through histological scoring of haemorrhage, oedema and neutrophil infiltration, one may overlook partial contribution of other inflammatory mediators such as complement in the IgG mediated Arthus reaction in the skin. These data supported a model where immune complex mediated

injury has multiple redundant components. The degree of dependence on the complement C5aR, and mast cells appears to be tissue specific. With regard to C5aR, the contribution in lung is greater than in peritoneum, which is in turn greater than in skin. Additionally, a kinetic contribution occurs, with C5aR more important in early inflammation in peritoneum and skin.

1.4.5 The role of C5a in asthma

Asthma is associated with an abnormal inflammatory response in the lungs. It has been demonstrated that C5a is generated not only via the classical and alternative pathways of complement activation but can be activated directly by neutral tryptase released from mast cells and pulmonary macrophages (Schwartz *et al.* 1983, Mulligan *et al.* 1996). It has been shown that the C5 component can be synthesised locally in the lungs by alveolar type II macrophages (Strunk *et al.* 1988). C5a and C3 are known to promote many pathogenic features of asthma and COPD: apart from their chemotactic role, they trigger smooth muscle contraction, enhance airway hyper-responsiveness and vascular permeability, regulate vasodilatation in the lungs, stimulate the release of histamine from basophils and mast cells, and regulate the synthesis of eosinophilic cation protein and adhesion to endothelial cells by eosinophils. A study with C3aR deficient mice and human bronchoalveolar lavage samples demonstrated a role for the C3a anaphylatoxin receptor in the effector phase of asthma (Humbles *et al.* 2000).

One clinical trial demonstrated that C5a and C3a are increased in bronchoalveolar lavage (BAL) fluid 24 hours after segmental allergen provocation in patients with asthma compared with controls and a strong correlation was found between anaphylatoxin and number of eosinophils in BAL fluid (Krug *et al.* 2001). Subsequently, concentration of C5a and C5a des Arg in addition to C3a and C4a was measured in induced sputum from stable asthmatics. Significantly increased levels of C5a and C5a des Arg were documented in the asthmatics versus healthy non-smoking controls. There was no

increase in C3a documented, possibly due to the stable nature of the asthma at the time of study, the authors proposed.

More recently, a dual role has been suggested for C5a in allergic asthma from studies on both C5aR deficient mice and mice that underwent local pharmacological targeting of the C5aR prior to initial allergen sensitisation in murine models of allergic asthma. (Kohl *et al.* 2006). They demonstrated that local pharmacological targeting of the C5a receptor (C5aR), prior to initial allergen sensitisation in murine models of inhalation tolerance or allergic asthma resulted in either induction or marked enhancement of Th2 polarised immune responses, airway inflammation, and airway hyper-responsiveness. Importantly, C5aR deficient mice exhibited a similar increased allergic phenotype. Pulmonary allergen exposure in C5aR targeted mice resulted in increased sensitisation and accumulation of CD4+ CD69+ T cells associated with a marked increase in pulmonary myeloid but not plasmacytoid, dendritic cell numbers. Pulmonary dendritic cells from C5aR deficient mice produced large amounts of CC-chemokine ligand 17 (CCL17), and CCL22 *ex vivo*, suggesting a negative impact of C5aR signalling on pulmonary homing of Th2 cells. In contrast, C5aR targeting in sensitised mice led to suppressed airway inflammation and airway hyper-responsiveness but was still associated with enhanced production of Th2 effector cytokines. These data suggest a dual role for C5a in allergic asthma i.e. protection from the development of maladaptive type 2 immune responses during allergen sensitisation at the dendritic cell /T cell interface but enhancement of airway inflammation and airway hyper-responsiveness in an established inflammatory environment.

1.4.6 Role of C5a in chronic obstructive pulmonary disease

Analysis and comparison of the induced sputum of patients with chronic obstructive pulmonary disease (COPD) compared with healthy non smokers revealed significant increases in C5a/C5a des Arg concentrations in supernatants of the induced sputum in patients with COPD compared with the control group. In patients with COPD the

C5a/C5a des Arg concentrations were significantly negatively correlated with lung diffusion coefficient (Mateja *et al.* 2004). Patients with COPD in this study all had a history of heavy cigarette smoking which may be one of the factors responsible for C5a elevation, because previous studies have found cigarette smoke to be one of the activators of the alternative pathway (Kew *et al.* 1985, Kew *et al.* 1987, Kihira *et al.* 1989, Perricone *et al.* 1983) and *in vitro* cigarette smoke has been shown to cause an increase in the population of human bronchial epithelial cell that express C5aR receptors (Floreani *et al.* 1998).

1.4.7 Role of C5 and C5 antibody in cardiac disease

1.4.7.1 Role of C5 and C5 antibody in myocardial infarction and reperfusion injury

Myocardial ischaemia and reperfusion (MI/R)–induced tissue injury involves necrosis and apoptosis. The precise contribution of apoptosis to cell death, as well as the mechanism of apoptosis induction, has not been delineated. Vakeva and colleagues (Vakeva *et al.* 1998) sought to define the contribution of the activated terminal complement components to apoptosis and necrosis in a rat model of MI/R injury. Monoclonal antibodies raised against the rat C5 complement component bound to purified rat C5 and effectively blocked C5b-9 mediated cell lysis and C5a induced chemotaxis of rat polymorphonuclear leukocytes. A single dose of this antibody blocked >80% of serum haemolytic activity for >4 hours. Administration of anti-C5 therapy 30 minutes before myocardial ischaemia and 4 hours before reperfusion significantly reduced (by 91%) left ventricular free wall polymorphonuclear infiltration compared with monoclonal antibody with no complement inhibitor activity. Treatment with antibody 1 hour before ischaemia or 5 minutes before reperfusion significantly reduced infarct size. A 42% reduction in infarct size was observed in rats treated with anti-C5 therapy after 30 minutes of ischaemia and 7 days reperfusion. Thus, anti C5 therapy in the setting of MI/R significantly inhibits cell apoptosis, necrosis and polymorphonuclear infiltration in the

rat, despite C3 deposition. The authors concluded that the terminal complement components C5a and C5b-9 are key mediators of tissue injury in MI/R.

1.4.7.2 Pexelizumab, a humoral monoclonal antibody to C5 and its role in myocardial infarction

Pexelizumab, a humanised monoclonal antibody single-chain fragment that binds to the human C5 complement component was studied as adjunctive therapy to primary percutaneous coronary intervention (PCI) in acute myocardial infarction: the COMplement inhibition, Myocardial infarction, treated with Angioplasty (COMMA) trial (Granger *et al.*2003). This prospective, randomised, placebo controlled trial randomised patients with ST-segment-elevation myocardial infarction (MI) to either placebo, bolus pexelizumab or bolus plus infusion of pexelizumab. Pexelizumab had no measurable effect on infarct size (creatine kinase-MB area under the curve). However, the 90 day mortality rate was significantly lower in the pexelizumab bolus plus infusion treated group. The mechanism of this reduced mortality was not clear. In 2006, a further study demonstrated that mortality benefit was not related to established modulators of clinical benefit such as baseline demographics, time to treatment from symptom onset, myocardial perfusion post-percutaneous coronary intervention or extent of ST elevation or infarct size (Armstrong *et al.* 2006). The authors raise the possibility that the clinical benefit of pexelizumab is mediated through novel pathways.

Reperfusion with percutaneous transluminal coronary intervention is effective in improving outcomes in patients with acute ST elevation myocardial infarction. However, in patients without prompt re-establishment of brisk coronary flow and tissue perfusion, mortality remains high, providing an opportunity for more novel treatments such as anti-inflammatories. Disappointingly, Pexelizumab administration did not affect 30 day mortality in a multicentre, prospective, double blind, placebo–controlled trial of these high risk patients (Armstrong *et al.* 2007).

1.4.7.3 Pexelizumab, C5 antibody and its role in coronary artery bypass graft surgery (CABG)

Inflammation and ischaemia–reperfusion injury coronary artery bypass graft (CABG) surgery requiring cardiopulmonary bypass is associated with postoperative myocardial infarction and mortality. During cardiac surgery requiring cardiopulmonary bypass, pro-inflammatory complement pathways are activated by exposure of blood to bio-incompatible surfaces of the extracorporeal circuit and reperfusion of ischaemic organs. Complement activation promotes the generation of additional inflammatory mediators thereby exacerbating tissue injury. In a large trial across North America and Western Europe administration of pexelizumab was not associated with a significant reduction in mortality within 30 days (Verrier *et al.* 2004) but it did reduce blood loss during on-pump cardiac surgery and completely suppressed complement dependant serum haemolytic activity within 1 hour of bolus pexelizumab treatment (Chen *et al.* 2005).

1.4.7.4 C5 antibody (Pexelizumab) and its role in a heart transplantation animal model

Regarding the xenograft heart transplantation animal model, while anti-C5 monoclonal antibody prevents hyperacute rejection it does not prevent acute vascular rejection (also known as delayed xenograft rejection). Unfortunately the latter cannot be overcome by conventional immune-suppression (Wang *et al.* 1999). Thus the role of C5-antibody in the xenograft heart transplantation animal model and therefore its future role in human heart transplantation is limited

1.4.8 C5 and C5 antibody and their role in Paroxysmal nocturnal haemoglobinuria

Paroxysmal nocturnal haemoglobinuria (PNH) is a haematologic disorder characterised by clonal expansion of red blood cells lacking the ability to inhibit complement mediated haemolysis. Eculizumab, a humanised monoclonal antibody that binds C5 complement protein, blocks serum haemolytic activity. Eculizumab treatment every fortnight resulted in a dramatic reduction in haemolysis, reduction in lactate dehydrogenase, and reduction in number of days of gross haematuria culminating in a statistically significant reduction in blood transfusions required and an improved quality of life score (Hill *et al.* 2005). This initial study has since been followed by a larger, multicentre, placebo controlled trial confirming previous results and demonstrating a sustained benefit from this treatment (Hillmen *et al.* 2006).

1.4.9 Role of C5a in other autoimmune disorders

In patients with rheumatoid arthritis, (but not osteoarthritis), synovial mast cells express significant amounts of C5aR (CD88) and release histamine in response to anaphylatoxin rHuC5a (Kiener *et al.* 1998). Regarding psoriasis, surprisingly high levels of C5a des Arg are extractable from psoriatic scales (Bergh *et al.* 1993) and intradermal administration of C5a induces skin changes similar to those observed in cutaneous hypersensitivity vasculitis that occurs through immune complex–mediated complement activation (Tagami H. 1992).

1.5 ANTI-INFLAMMATORY ROLE OF C5A/C5AR

Although C5a is a well documented, potent, pro-inflammatory mediator, C5a has been shown to exert anti-inflammatory effects in some disease states, for example acute pancreatitis and associated lung injury (Bhatia *et al.* 2001). C5aR deficient mice and mice

that genetically lack C5 were injected twelve hourly with a supra-maximally stimulating dose of caerulein. The severity of the pancreatitis was quantified by measuring serum amylase activity, pancreatic myeloperoxidase (MPO) (a reflection of neutrophil sequestration within the pancreas) and the extent of acinar cell necrosis. Morphological changes of pancreatitis include acinar cell vacuolisation, intralobular oedema, an inflammatory infiltrate and acinar cell necrosis. An associated lung injury occurs in this model, as evidenced by a rise in lung MPO activity and a rise in pulmonary microvascular permeability. Genetic deletion of C5aR resulted in an increased severity of both the pancreatitis for each of the parameters used to quantitate the severity of the pancreatitis (i.e. serum amylase level, neutrophil sequestration in the pancreas and pancreatic acinar cell necrosis) and the associated lung injury. Similarly, the morphological changes of pancreatitis as seen on haematoxylin and eosin stained sections of pancreas examined by light microscope were worse in the C5aR deficient mice. Lung MPO activity and pulmonary microvascular permeability during pancreatitis are also increased in the C5aR deficient mice compared with wild type littermates. In mice that do not express C5 and are therefore incapable of generating C5a, the severity of the pancreatitis and associated lung injury was increased compared with C5 sufficient controls. C5a did not alter in vitro caerulein-induced amylase secretion as evidenced by an identical biphasic relationship between caerulein concentration and amylase secretion either in the presence or absence of C5a. These results indicate that C5a exerts an anti-inflammatory effect in pancreatitis and are in conflict with previous reports by Merriam and colleagues (Merriam *et al.* 1997). However, the latter used a milder model of pancreatitis caused by bile-pancreatic duct ligation in C5 sufficient and deficient mice.

Proposed explanations by the authors for this unexpected finding include that C5a might act via other receptors that differ from the classical C5aR or that neutrophil chemoattractants act in a hierarchical manner as proposed initially by Foxman and colleagues (Foxman *et al.* 1997). The latter studied complex gradients of the chemoattractants C5a, formyl peptide, leukotriene B4 (LTB4) and IL-8 and observed that C5a and fMLP were both capable of arresting the migration of neutrophils towards

gradients of LTB4 or IL-8 when cells encountered the former ligand in advance of sensing the latter. Mechanistically, it had been previously demonstrated that C5a and fMLP are capable of inducing a heterologous desensitisation response to each other and to so-called secondary chemoattractants such as lipid mediators and chemokines. Thus, cells that sense C5a (or formyl peptide) in advance of chemokines or arachidonic acid metabolites would be less responsive to the latter. By extension of this logic to their observation, it follows that in an *in vivo* inflammatory response, early generation of C5a could limit the cellular recruitment and subsequent pathology induced by chemokines and other secondary mediators. Evidence pertaining to this had already been documented in ischaemia-reperfusion injury in rabbits and dogs which had been shown to lead to an initial recruitment in the first several hours that is C5a dependant for both monocytes and neutrophils (Birdsall *et al.* 1997, Ivey *et al.*1995). After this initial phase, chemokines such as IL-8 and monocyte chemoattractant protein-1 appear. Thus the kinetics of a developing acute inflammatory response has C5a as perhaps the earliest mediator, with later phase influx being controlled by chemokines and other mediators. Thus, the authors hypothesised that the absence of C5a or its receptor in an acute inflammatory event such as pancreatitis allows the so-called secondary mediators (such as chemokines and arachidonic acid metabolites), to recruit cells that have not been partially desensitised by C5a and thus a more robust inflammatory response ensues.

1.6 SUGGESTIVE EVIDENCE THAT A SECOND C5A RECEPTOR EXISTS

In 1991, identification of the leukocyte receptor for C5a appeared to account for almost all of the known biological functions of the anaphylatoxin (Gerard *et al.* 1991, Boulay *et al.* 1991). However, some of the complex effects of C5a, both pro and anti-inflammatory effects, lead us to consider the possibility that other receptor(s) for C5a might exist. Secondly, expression of receptors for C5a on parenchymal cells in the lung, liver, smooth muscle and endothelial cells serve as yet uncharacterised functions (Drouin *et al.* 2001, Haviland *et al.* 1995). Thirdly, differential effects of agonists and antagonists on

leukocyte populations suggested the possibility of receptor(s) that may be either uniquely post-translationally modified (Gerard *et al.* 1989) or are perhaps coupled differently to intracellular signalling proteins (Paczkowski *et al.* 1999, Eglite *et al.* 2000) Based on the above we decided to actively seek an additional receptor for C5a anaphylatoxin.

1.7 C5L2: C5A LIKE RECEPTOR 2

In 2000, a group cloned a novel human gene encoding the putative orphan receptor C5a like receptor 2, C5L2 (Ohno *et al.* 2000). This novel gene has an open reading frame of 337 codons (genebank ABO38237), encoding a protein with a predicted mass of 37 k Da. The hydropathy profile of the deduced amino acid sequence was felt to be consistent with the prediction of a seven transmembrane motif. Alignment of the C5L2 amino acid sequence with that of other functionally characterised human chemoattractant receptors demonstrated a similarity to C5aR (Gerard and Gerard 1991), C3aR (Ames *et al.* 1996, Crass *et al.* 1996) and the formyl Met-Leu-Ph (fMLP) receptor (Boulay *et al.* 1990). After transient transfection of C5L2 into human embryonic kidney (HEK293T) cells, fluorescent activated cell sorter (FACS) analysis revealed abundant C5L2 bound to immature dendritic cells but not to mature dendritic cells. This group speculated that C5L2 may regulate the activation of immature dendritic cells and play a role in the chemoattraction of leukocytes to inflammatory regions. We decided to study C5L2 in greater detail.

1.8 OBJECTIVES OF THIS THESIS

The objectives of this thesis were to clone the putative orphan receptor, C5L2 from both mouse and man and to characterise it. This involved assessment of tissue expression of C5L2 in mouse and man and comparing results with those of the classical C5a receptor C5aR, cloning C5L2 into the vector pcDNA3.1 and inserting a point mutation in C5L2 to assess the importance of the natural substitution of leucine for arginine in the normally highly conserved "DRY" sequence. We aimed to reveal the biophysical properties of C5L2, in particular to identify its ligand(s), study its kinetics of binding, assess if C5L2, like C5aR is phosphorylated and internalised following the addition and binding of ligand in C5L2 transfected cells and if so, to what extent and at what speed. It was important to elucidate if C5L2, unlike C5aR was uncoupled to G proteins, by virtue of the amino acid alteration in the DRY sequence and assess if addition and binding of ligand to C5L2 transfected cells stimulates chemotaxis or mediates calcium flux. Results would be compared to those of C5aR and "mutated C5L2" i.e. C5L2(L132R) transfected cells. We planned to identify if C5L2, like C5aR is a signalling receptor, by assessing if addition and binding of ligand to C5L2 transfected cells stimulates activation of the Mitogen Activated Protein (MAP) kinase pathway. Furthermore, we aimed to examine bone marrow cells from both C5aR deficient mice, (expressing C5L2 alone) and their wild type littermates, (expressing both C5L2 and C5aR) and use microarray analysis to study the response (i.e. induction or suppression) in a number of inflammation related genes to the addition and binding of ligand. C5aR is highly expressed on human peripheral blood leukocytes and in bone marrow and has a well documented, important role in sepsis. With this in mind we planned to investigate if C5L2 is co-expressed with C5aR on human granulocytes in the hope of elucidating the role of C5L2 clinically. In summary we planned to clone this putative receptor C5L2, characterise it and determine its function *in vivo*.

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CHAPTER II

MATERIALS AND METHODS

CHAPTER II

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2.1 MATERIALS

2.1.1 Chemicals

Bovine serum albumen (BSA), dimethylsulphoxide (DMSO), ampicillin, Luria-Bertani medium, agar (Select), human recombinant C5a and C5a des Arg, tris-hydrochloride HCl, *n*-dodecyl-β-D-maltoside (Anatrace), ethyleneglycol bis-(aminoethlether) tetra-acetic acid (EGTA)NaF, Na_3VO_4, *p*-nitrophenyl phosphate, benzamide, N-(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), foetal bovine serum (FBS), 4% paraformaldehyde and human recombinant C5a were purchased from Sigma (St. Louis, MO, U.S.A.) ^{125}I-C5a and^{125} I-C3a was from Dupont/NEN Life Science Products, Boston, U.S.A. C3a was from Advanced Research Technologies, (C.A.,U.S.A.), C5a hexapeptide was synthesised commercially by New England Peptide, (C.A., U.S.A.), ^{32} P-labelled orthophosphoric acid was obtained from Dupont/NENLife Science Products, Boston, U.S.A. Calcium chloride ($CaCl_2$), potassium chloride (KCl), magnesium chloride ($MgCl_2$), sodium chloride ($NACl_2$), sodium azide (NaN_3), phosphate buffered saline (PBS) and formaldehyde were bought from B.D.H. Ltd., (Poole, U.K.). Agarose was obtained from GIBCOBRL (Life Technologies Renfrewshire, Paisley, Scotland). Indicator dye Fura-2AM was purchased from Molecular Probes, (Eugene, O.R., U.S.A.). All buffer reagents for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were prepared in deionised water (Elga Prima Purelab Ultra). Electrophoresis grade agarose, ethanol, were obtained from GIBCOBRL (Life Technologies, Paisley, Scotland). Other reagents were analytical or general preparative grade and were from Sigma (St. Louis, M.O., U.S.A.)..

2.1.2 Kits

Nucleospin RNA Preparation kit (Clontech, C.A., U.S.A.), QIAquick gel extraction kit, QIAprep spin miniprep kit, QIAprep high speed plasmid, maxi prep kits were purchased from Qiagen (Valencia, C.A., U.S.A.).

2.1.3 Plasmid vectors

The vector pcDNA3.1C5aR$_{C9}$ included a nine amino acid C-terminal tag corresponding to the C-terminus of bovine rhodopsin (C9 tag) to facilitate recognition by the antibody 1D4, and was kindly loaned to us by Prof. Hyeryun Choe (Ina Sue Permutter Lab, Boston, M.A. U.S.A.).The vector pEGFP-N1 was purchased from Clontech (Palo Alto, Ca. U.S.A.)

2.1.4 Antibodies- primary and secondary

Primary antibodies: a synthetic peptide corresponding to the predicted N-terminal extracellular sequence of human C5L2 (MGNDSVSYEYGDYSDLSDRPVDC) was coupled to KLH and utilised for production of rabbit antiserum (Alpha Diagnostics, Minneapolis U.S.A.). Mouse anti-human C5aR was purchased from Serotec (San Calbiochem, C.A., U.S.A.) while isotype mouse antibody and amyloid 1-α40 were purchased from Biosciences (San Diego, C.A., U.S.A.).

Secondary antibodies: FITC-conjugated goat anti-rabbit IgG and PE conjugated goat anti-mouse IgG were purchased from Sigma (St. Louis, CA. U.S.A.). 1D4 antibody was bought from National Cell Culture (Minneapolis, MN, USA) while rabbit anti-active MAPK antibody was from Promega (Madison, W.I., U.S.A.), and peroxidase-conjugated goat anti-rabbit IgG from Jackson Immuno-Research (C.A.,U.SA).

2.2 CELL CULTURES

2.2.1 Cell Lines

Human embryonic kidney 293T cells, (ATCC, CRL11554) and murine pre-B-lymphoma line, L1.2, were available at the laboratory and human peripheral blood leukocytes were drawn from myself. Transfected cells included $C5aR_{-C9}$/L1.2, $C5L2_{-C9}$/L1.2, C5L2(L132R)/L1.2, C5aR/293T, C3aR/293T, C5L2/293T and C5L2(L132R)/293T cells.

2.2.2 Cell culture reagents

Phosphate free Dulbecco's modified eagle medium (DMEM), foetal calf serum (FCS), penicillin, streptomycin, Hank's balanced salt solution (HBSS), phosphate buffered saline (PBS) were purchased from GIBCOBRL (Life Technologies Renfrewshire, Paisley, Scotland).

2.2.3 Maintenance of cell cultures

2.2.3.1 The murine pre-B- lymphoma line (L1.2. cells)

L1.2 cells were grown in medium (RPMI) supplemented with 10% foetal calf serum (FCS), 100 units/ml penicillin and 100mg/ml streptomycin in cell culture flasks. The cells were maintained at 37°C in a humidified incubator containing 5% CO_2. Media was renewed every third day and cells split ~every fifth day after visual examination had been undertaken and cells had reached ~70-80% confluence. Supplemented medium was stored at 4°C. FCS was heat inactivated (56°C, 1 h) to inactivate complement and stored at -20°C.

2.2.3.2 Human embryonic kidney cells: HEK293T cells

HEK293T cells were routinely maintained in 75cm^2 cell culture flasks (Nunclon, Roskilde, Denmark) at 37°C in 5% CO_2 in a humidified incubator. Visual examination was undertaken and cells were split upon reaching ~80% confluence. Cells are confluent in the flask at ~12-15 x 10^6cells/flask. To maintain HEK293T cells, wash twice every two days with 10 mls of Dulbecco's Phosphate Buffered Saline (DPBS) with added calcium, magnesium and sulphate. Then add 10 mls of Dulbecco's modified eagle medium (DMEM) with added 10% foetal bovine serum (FBS) and added penicillin and streptomycin (100 units/ml penicillin and 100mg/ml streptomycin) and replace in humidified incubator.

2.2.3.2.1 Protocol for plating HEK293T Cells

Remove media and wash with 10mls of PBS (remove PBS swiftly). Add 1ml of trypsin (0.2%) to the flask and place in a 37°C humidified incubator for 30 seconds. Remove the flask from the incubator and gently tap to detach cells (turns cloudy). Examine under the light microscope to ensure that cells are detached and moving. Add 14mls of Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS with added Penicillin and Streptomycin to inactivate the trypsin. Gently pass cells through a pipette to avoid clumping. Cells are counted using a Neubauer haemocytometer. For HEK293T cells plate ~ 0.7 x 10^6 cells/ flask and place in a 37°C humidified incubator. 24 hours later visualise cells to ensure they are spread evenly over the flask. Replace media and return flask to humidified incubator at 37°C with 5% CO_2.

2.2.4 Storing cells

Stocks of viable L1.2 cells were maintained by cryopreservation. Cells were suspended in sterile FBS containing 10% dimethylsulphoxide (DMSO) and 10% RPMI, with aliquots of 10 x 10^6 cells/ml in sterile cryo-resistant tubes/ cryovials (Nunclon, Roskilde, Denmark) at

-70°C overnight and transferred to liquid nitrogen the following day for long term storage. For cell revival, cryovials removed from storage were quickly thawed and cells washed in medium. Gentle centrifugation (1,000rpm, 3 min) was used to pellet the cells which were subsequently re-suspended in 10 ml of medium and transferred to tissue culture flasks placed in a humidified incubator at 37°C with 5% CO_2.

2.2.5 Cell counting

Aliquots of cell were counted on a Neubauer haemocytometer under a light microscope. Enumerations were performed in duplicate. Cells were used when ~80% confluent.

2.3 METHODS

2.3.1 Cloning C5L2 from man and mouse

2.3.1.1 Preparation and purification of recombinant human C5a and C5a des Arg

Human recombinant C5a was purchased from Sigma (St. Louis, M.O., U.S.A.). Human C5a and C5a des Arg cDNAs were prepared by reverse transcriptase polymerase chain reaction (RT-PCR) from human lung based upon previously published sequence data (Wetsel *et al.* 1988), (accession number NM0017350). The polymerase chain reaction (PCR) primers included a *Bam* HI site at the 5' end and two in frame stop codons at the 3' end. The PCR product was inserted into the expression vector pQE-80L (Qiagen, Valencia, C.A., U.S.A.) such that a 12 amino acid N-terminal tag containing an initiating methionine and a 6x histidine metal-binding domain was in frame with the C5a or C5a des Arg coding sequence. Recombinant peptides were produced using the QIA express system, purified by affinity chromatography, and re-natured as previously described (Franke *et al.* 1988). This was performed by Neurogen Corporation (Branford Connecticut, U.S.A.) and kindly loaned to us. The C5a hexapeptide analogue Phe-Lys-Ala-dCha-Cha-dArg (DeMartino *et al.* 1994) was synthesised commercially (New England Peptide, C.A. U.S.A.).

2.3.1.2 The coding sequence of human C5L2 was obtained by reverse transcriptase polymerase chain reaction (RTPCR) using human brain cDNA

The coding sequence of human C5L2 was obtained by RT-PCR using human brain cDNA (Clontech) as a template and the following primers.

Sense Primer

rest is the
coding sequence

Nhe I ↓Kozac ↓ ↓

5'CAA CTG CAC GCT AGC CCA CC ATG GGG AAC GAT TCT GTC AGC TAC 3'

We calculate the annealing temperature at 72°C, but we use 60°C using the formula

GC = 4°C, AT = 2°C (GC x 12) +(AT x12) = 72°C

Antisense Primer

rest is the
coding sequence

*Asp*718

5' CAG TGA CCA GGT ACC ACC TCC ATC TCC GAG ACC AGGT 3 '→

↑

We cut out stop codon here

Annealing temperature for antisense primer is 64°C, we use 60°C

Having made the oligonucleotide primers we eluted and purified them and measured their optical density using the ultra violet (U.V.) spectrophotometer and converted results to pmol/µl.

We calculated the concentration of the sense primer using the following formula

$$\frac{\textbf{Optical density x 33 x dilution factor}}{\textbf{1000}} = \frac{0.413 \times 33 \times 100}{1000} = 1.3629 \mu g/\mu l$$

$= 1362.9\ \mu g/ml$

Sense $= 13.629\ \mu l/ml$ (computer read 13.78 $\mu l/ml$)

Oligo = single stranded, 44 mer, molecular weight = 325

so (44) x (325) = 14300 $\mu g/\mu mol$

$$[DNA] = (1.3629) \times \frac{(1\ \mu mol)}{14300} \times \frac{10^6}{1L} = 95.3\ \mu l/ml$$

Concentration of sense primer = 95.3 pmol/ μl

We calculated the concentration of the anti-sense primer in a similar fashion.

2.3.1.2.1 Reverse transcriptase Polymerase chain reaction (RTPCR)

The following reagents were placed in a microfuge tube i.e. buffer, the four deoxynucleoside-triphosphates, (dNTPs i.e. dTTP, dCTP, dGTP, and dATP), an excess of the 2 oligonucleotide primers above, Taq DNA polymerase and the template, human brain cDNA (Clontech) as outlined below. We used the following RTPCR protocol: step 1, 94°C x 30 minutes, step 2, 50°C x 30 minutes, step 3, 72°C x 1 minute then steps 1-3 inclusive are repeated x 34 cycles. Then step 4, 92°C x 10 minutes and step 5, 4°C until stored in a -20°C fridge.

RTPCR	µl	µl
2 x reaction mix	25	50
Template human brain (< 1 µg)	1	2
Primer sense (10 µM)	1	1
Primer anti sense (10 µM)	1	1
RT/Taq	1	2
ddH_2O	21	42
	50	100 (50 x 2 tubes)

RTPCR protocol

94°C x 30 seconds}

50°C x 30 seconds} repeat steps 1-3 inclusive x 34 cycles

72°C 1 minute}

92°C 10 minutes

4°C forever or store in -20°C fridge

Then we run the uncut RTPCR product, C5L2 on an agarose gel, (1.75%) together with a 1kb molecular weight ladder. We cut out the relevant band, and purified the DNA using the QIAquick Gel extraction kit (appendix). This protocol uses a micro centrifuge and is designed to extract and purify DNA of 70 bp to 10bp from standard or agarose gels in TAE or TBE buffer. We confirmed our product by performing a digestion with *Pst* I and subjected it to agarose gel electrophoresis which revealed appropriately sized bands. C5L2 is confirmed by sequencing (see details below).

2.3.1.3 Molecular characterisation of the Murine C5L2 Gene

A murine genomic library (Stratagene) was screened using ^{32}P-labelled cDNA corresponding to human C5L2 and yielded a ~15 kb clone encoding mouse C5L2. Genomic analysis was performed using DNA from FVB/NJ mice, restricted with *Eco* RI, *Eco* RV, *Stu* I or *Hind* III. Southern blots were hybridised with ^{32}P-labelled cDNA. (This work was performed by Dr. Shoji Okinaga).

2.3.1.3.1 Comparing aligned sequences of human C5L2, mouse C5L2 and human C5aR

The aligned sequences of mouse C5L2 and human C5L2 were analysed and the percentage identity in the deduced protein and in the nucleotide sequences was noted. In particular the usually highly conserved "DRY" sequence was analysed. The aligned sequence of human C5aR was compared with that of C5L2 for similarities and differences.

2.3.2 Insertion of C5L2 into pEGFP-N1 (enhanced green fluorescent protein)

2.3.2.1 pEGFP-N1

pEGFP-N1 (Clontech) encodes a red-shifted variant of wild type GFP which has been optimised for brighter fluorescence and higher expression in mammalian cells.(Excitation maximum = 488 nm; emission maximum = 507nm.) pEGFP-N1 encodes the GFPmut1 variant which contains the double-amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr. The coding sequence of the EGFP gene contains more than 190 silent base changes which correspond to human codon-usage preferences. Sequences flanking EGFP have been converted to a Kozak consensus translation initiation site to further increase the translation efficiency in eukaryotic cells. The multiple cloning site (MCS) in pEGFP-N1 lies between the immediate early promoter of CMV and the EGFP coding sequences. Genes cloned into the multiple cloning site (MCS) will be expressed as fusions to the N-terminus of EGFP if they are in the same reading frame as EGFP and there are no intervening stop codons. A neomycin-resistance cassette allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of this cassette expresses kanamycin resistance in *E. coli*. Fusions to the N terminus of EGFP retain the fluorescent properties of the native protein allowing the localisation of the fusion protein *in vivo*. The target gene should be cloned into pEGFP-N1 so that it is in frame with the EGFP coding sequences with no intervening in-frame stop codons. The inserted gene should include the initiating ATG codon. The recombinant EGFP vector can be transfected into mammalian cells using any standard transfection method. If required stable transformants can be selected using G418.

pEGFP-N1 can also be used simply to express EGFP in a cell line of interest (e.g. as a transfection marker).

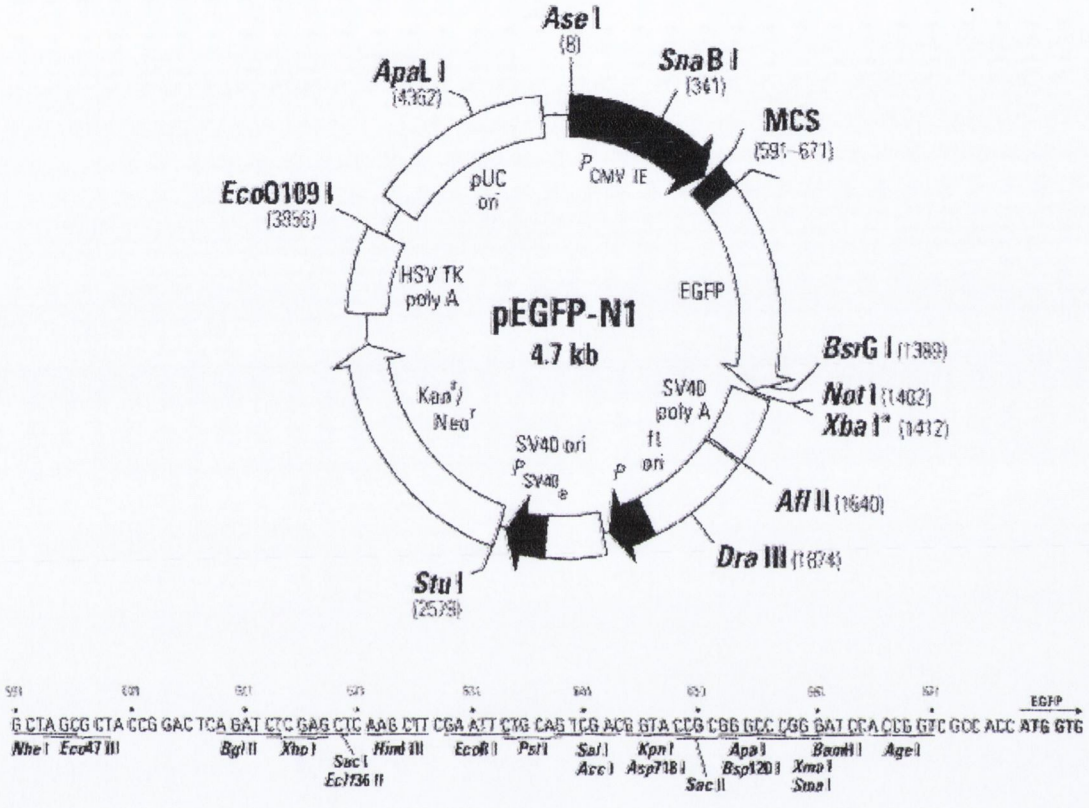


Figure 2.1 Restriction map and multiple cloning site of pEGFP-N1

2.3.2.2 Preparation of insert and vector by digestion

Having confirmed the sequence of our RTPCR product C5L2, we prepared our insert and vector by digesting both with restriction enzymes *Nhe* I and *Asp*718, (37°C x 2 hours) and running both digestion products on an agarose gel to check the size, integrity and intensity of the fragments. We expected a 4.7 kb band for vector pEGFP-N1 and a 1.1 KB band for the insert, C5L2. We gel purified the relevant bands with QIA quick gel extraction kit (appendix) and quantified the amount of DNA present by both agarose gel and by U.V. spectrophotometer.

2.3.2.3 Ligation of insert and vector

Next we ligated our insert and vector at 16°C x 3 hours as outlined below. One ligation reaction contains both insert and vector, the other acts as a control with vector only and no insert.

Ligation mix	Vector + Insert µl	Control µl
Vector (4.7 kb) EGFP	5	5
Insert (1.1 kb)C5L2	5	0
ddH_2O	7	12
Ligation B x 10	2	2
T4 ligase	1	1
	20	20

2.3.2.4 Transformation into DH5α competent cells

Next we transformed the ligated DNA (20 µl) into DH5α competent cells (100 µl). DH5α competent cells were available in the laboratory and were stored in the -80°C freezer. A sample was removed and placed on ice. Using the 10ml translucent tubes, 20µl of DNA was added to 100µl of DH5α cells and "heat shocked" at 42°C (by placing in a water bath) for 45 seconds and returned to ice. 500µl of LB (without antibiotic) was added at room temperature and incubated in a 37°C shaker for 0.5-1 hour to allow the bacteria to recover. We plated (500-600µl) onto Luria-Bertani (LB) with selective antibiotic (kanamycin) plates and incubated overnight in a humidified incubator at 37°C. Single colonies of bacteria had grown by the following morning.

2.3.2.5 Growth and storage of transformants

We transferred single colonies from a transformation plate and incubated in LB culture broth (containing selective antibiotic) overnight to stationary phase (~12 tubes). The broth must include the antibiotic used to select the transformants on the original plate to maintain the selection for the presence of the plasmid. The plasmid DNA is purified from overnight cultures of DH5α/*E.coli* in LB with selection antibiotic medium using the QIAprep Spin Miniprep Kit Protocol. We prepared a glycerol stock of each culture at this point to enable the same strain/plasmid to be re-grown and prepared again if required.

2.3.2.6 Preparation of glycerol stocks

We transferred single colonies from a transformation (LB with selection antibiotic) plate and grew up fresh colonies (< 1 week old). We transferred 1 colony per tube using a pipette tip. We grew overnight to stationary phase in a 37°C shaker in a 15ml tube containing 5mls of LB with selection antibiotic. Next morning, we added 850µl of overnight culture (DH5α / *E. coli*) to labelled eppendorf tubes and mixed well. We maintained at room temperature x 5 minutes, then froze in -80°C freezer overnight and the following day, stored stocks in liquid nitrogen.

2.3.2.7 Purification of plasmid DNA: mini-prep

We used the QIAprep Spin Miniprep Kit Protocol (appendix) which is designed for purification of up to 20 µg of high-copy plasmid DNA from our ~5 ml overnight cultures of DH5α competent cells in LB with antibiotic. To confirm our products we digested our minipreps with *Nhe* I and *Asp* 718 and subjected to agarose gel electrophoresis obtaining the expected vector band, EGFP of 4.7 kb and insert band, C5L2 of 1.1 kb. We selected one clone /miniprep to grow up in LB medium with selective antibiotic.

2.3.2.8 Obtaining good quality DNA: Maxi prep

Next we picked a single colony from a freshly streaked plate and incubated a starter culture of 2-5 ml LB medium, containing the appropriate selective antibiotic. We incubated this for ~8 hours at 37^0C with vigorous shaking (~300rpm). We used a tube or flask with a volume of at least 4 times the volume of the culture. We diluted the starter culture 1/500 or 1/1000 into selective LB medium. For high copy plasmids inoculate 50 ml or 150 ml medium. We grew at 37^0C for 12-16 hours with vigorous shaking (~300 rpm) using a flask or vessel with a volume at least 4 times the volume of the culture. The culture should reach a cell density of approximately 10^9 cells/ml which typically corresponds to a pellet wet weight of approximately 3g/l. Then we obtained good quality DNA by employing the QIA prep high speed plasmid Maxi prep kit (Qiagen, Valencia, C.A., U.S.A), (appendix).

2.3.2.9 Sequencing

Next we performed sequencing with 3 forward and 3 backward primers (we prepared earlier) to confirm our construct C5L2-EGFP. Sequencing was performed at the MRRC DNA Sequencing Core facility at Children's Hospital. To each sequencing tube we added 300ng DNA, 5pmole (5pmole/µl) of primer and 8 µl of ddH20 which gave a final volume of 12 µl. We did this with each of 3 forward and backward primers. Sequencing computer printouts were checked for errors by visual examination.

2.3.3 Cloning the coding sequence of C5L2 and C5aR into vector pcDNA3.1-$_{C9}$

2.3.3.1 pcDNA3.1(+)

pcDNA 3.1 is used as a vector. It contains 5428 base pairs (without inserts). It contains many important features e.g. the CMV promoter for efficient high-level expression of cloned cDNAs in eukaryotic cells, neomycin resistance gene for selection of stable transfectants in mammalian cells and ampicillin resistance gene for selection of transformants of mRNA among others.

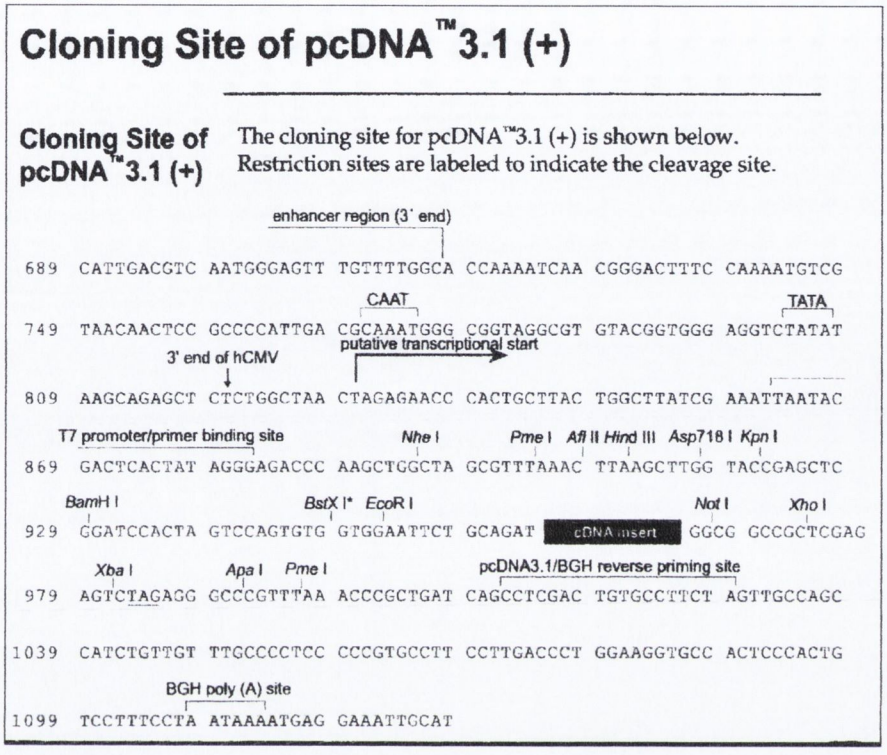


Figure 2.2 Multiple cloning site of pcDNA3.1(+)

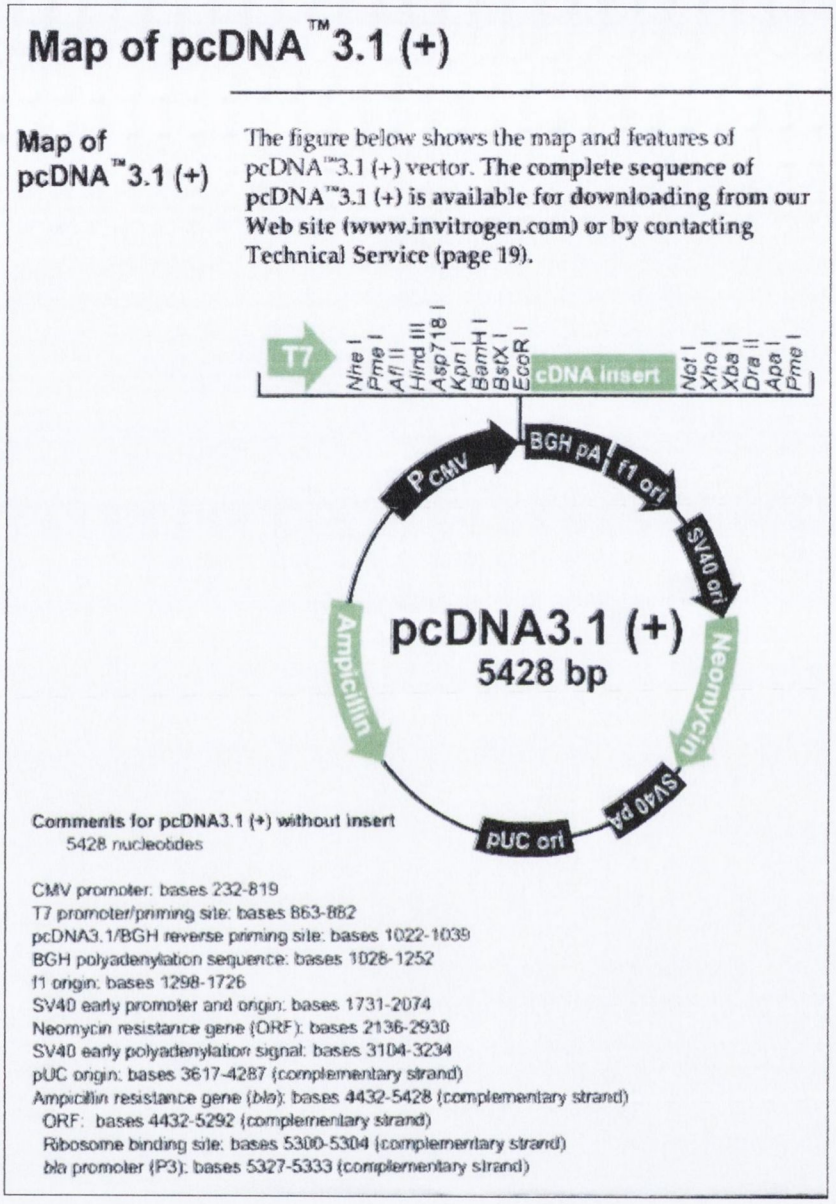


Figure 2.3 Restriction map of pcDNA 3.1(+)

2.3.3.2 Cloning C5L2 into pcDNA3.1$_{-C9}$

The coding sequence of human C5L2 was cloned into pcDNA3.1 to include a nine amino acid C-terminal tag corresponding to the C-terminus of bovine rhodopsin (C9 tag) to facilitate recognition by the antibody 1D4 (National Cell Culture Centre, Minneapolis, M.N.). Our lab already had C5aRpcDNA3.1-$_{C9}$ which was kindly loaned to us by Prof. Hyeryun Choe. We digested both C5aRpcDNA3.1$_{-C9}$ and C5L2-EGFP-N1 with *Nhe* I and *Asp* 718 as described earlier in a 37°C water bath for 2 hours. We ran both on an agarose gel and obtained the expected relevant bands of vector pcDNA3.1$_{-C9}$ (5.4kb) and insert C5L2 (1.1kb). We cut out the relevant bands and purified the DNA with QIAquick gel extraction kit (appendix). Next we quantified the amount of DNA present by comparing different volumes of vector pcDNA3.1$_{-C9}$ and insert C5L2 to known concentrations of molecular weight ladder λHindIII. Next we ligated our vector and insert as described previously (room temperature x 1 hour), one ligation mixture containing both vector and insert the other acting as a control containing vector but no insert (vector C5L2 2μl, x 10 buffer 0.8 μl, T4 ligase 0.5 μl, ddH_2O 7.7 μl). Next we transformed into DH5α competent cells and plated on LB plates with selective antibiotic. We purified the high copy plasmid DNA from our ~5 ml overnight cultures of DH5α competent cells in LB with antibiotic (as previously described) and subjected to agarose gel electrophoresis. Next we digested C5L2pcDNA3.1$_{-C9}$ and C5aRpcDNA3.1$_{-C9}$ with *Nhe* 1 and *Asp* 718 and quantified concentration of C5L2pcDNA3.1$_{-C9}$ by comparing to C5aRpcDNA3.1$_{-C9}$ because both plasmids are very similar. In addition we compared digested C5L2pcDNA3.1$_{-C9}$ with 1 kb ladder on an agarose gel. We confirmed our findings by U.V. spectrophotometry. After selection of 1 miniprep we obtained good quality DNA using the maxi prep protocol and subsequently sequenced C5L2pcDNA3.1$_{-C9}$ all as described earlier.

2.3.4 Tissue expression of C5L2 and C5aR in man and mouse using Northern blot analysis

2.3.4.1 Preparation of total RNA

For Northern blot analysis total RNA was prepared from tissues derived from BALB/c mice using RNA preparation kit (Ambion), (appendix). All mouse protocols were approved by the Animal Care and Use Committee of Children's Hospital. Mice were matched for age and gender (female, 10-12 weeks). Mice were culled with chloroform and thoracotomies performed. Mice lungs were cut with a blade on a petri dish into lobes. We homogenised the lung tissue with a commercial glass homogeniser x 30 seconds in the presence of liquid nitrogen. We proceeded with the standard RNA isolation procedure as outlined by suppliers in the RNA isolation kit (Ambion) (appendix).

2.3.4.2 Northern blot technique

We made up sample, running and blotting buffer, (appendix) and agarose gel (appendix). We loaded the RNA, 10 µg per lane, and stained with acridine orange. The RNA was electrophoresed on 1.1% denaturing agarose gel and nucleic acid molecules separated. We ran the gel at 50 volts for ~45 minutes. We wanted ~2 inches of separation between the 2 bands. (Our target is between the 2 bands of 28s and 18s). The gel was photographed and washed with x 10 SSC (blotting buffer). Nucleic acid molecules were transferred to a nitrocellulose membrane (by capillary action). Set up the apparatus as outlined below in diagram. We soaked the membrane in x10 SSC and ensured there were no bubbles which would interfere with the current of the solution. Filter paper and tissues were placed on the membrane with a weight on top. This arrangement was left overnight at room temperature. Nucleic acid must be bonded to the membrane, hybridised to the labelled probe (i.e. ^{32}P-labelled mouse C5L2 or C5aR cDNA probes) and washed extensively (with 0.5 x SSC). The hybridisation and washing conditions are critical. Human RNA blots were purchased

from Clontech and hybridised with human C5L2 or C5aR probes in the same manner as the mouse blots. Hybridised probes were then detected by autoradiography.

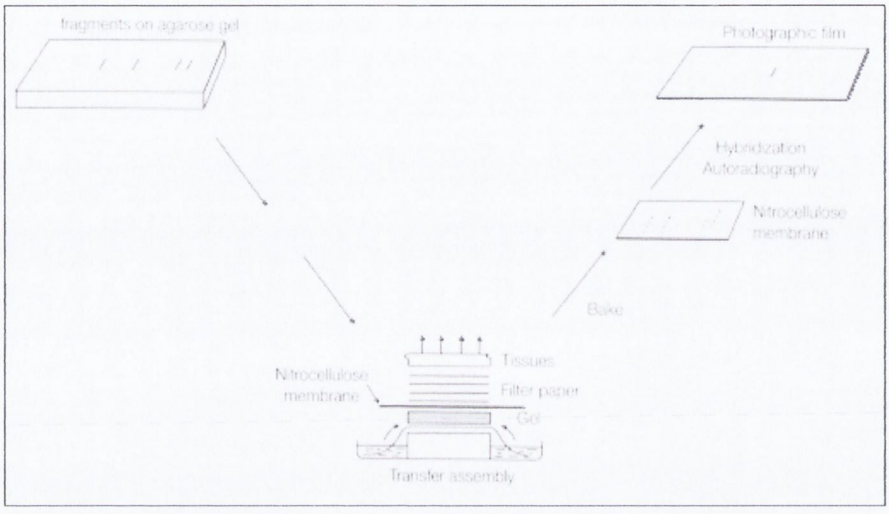


Figure 2.4 Northern blot technique-to detect which of the many RNA molecules on an agarose gel hybridise to a particular probe (From Turner *et al.* Molecular Biology, 2003)

2.3.5 Transfection: transient and stable

HEK293T cells were transiently transfected with C5aR, C5L2, "mutated C5L2" [i.e. C5L2 (L132R)] and C3aR with and without Gα16, using calcium phosphate, and were used for experiments 36-48 hours later (Gerard *et al.* 1993). The murine pre-B- lymphoma line, L1.2, was stably transfected by electroporation (Wu *et al.*1996) and stable clones were selected with 0.8mg/ml G418. C5aR/L1.2 and C5L2/L1.2 cells were frozen for re-use later.

2.3.5.1 Transient Transfection using the calcium phosphate method

Transient transfection was performed using the calcium phosphate protocol (Chen *et al.* 1997). We added 450µl of double distilled water (ddH_2O) to each 15ml sterile tube 1 and 2. We added 30µg of DNA to each tube: 15µg of Gα16 (a G protein), and 15µg C5L2, C5aR, C3aR, C5L2(L132R) DNA. The DNA must be dissolved in the ddH_2O before adding the calcium. We mixed by pipetting and by gently tapping the side of the tube. We added 50µl $CaCl_2$ (2.5M) to tube 1 and 2 and mixed by pipetting. To the second set of 15mls tubes (1a and 2a) we added 500µl of 2 x Hepes Buffer Solution (HBS). We remixed H_2O, DNA and calcium in tubes 1 and 2. We drop transferred this to tubes 1a and 2a containing the 2 x HBS. Be careful not to shake. We allowed tube contents to sit for 25-45 minutes at room temperature. We aspirated the culture media from the cells. Using a 1 ml glass pipette, we pipetted the diluted DNA drop wise onto the HEK293Tcells. Slowly, we placed the flask back on its side, gently tilting the flask forward and back and right and left to ensure the DNA covers all the cells. We placed the flask in 37°C humidified incubator with 5% CO_2 for ~7 hours (minimum of 7 hours, maximum of 15 hours) i.e. transfect in the morning and wash the cells in the evening or vice versa.

2.3.5.2 Stable Transfection by electroporation

"Electric field mediated membrane permeabilisation" or "electroporation" is an efficient transformation technique. The procedure involves subjecting bacteria to a high strength electric field in the presence of DNA and inducing the bacteria to uptake the free DNA. The mechanism of DNA uptake during electroporation is not fully understood but is deduced to be by the formation of transient pores in the cell wall as a result of the electroshock and that after contact with the lipid bilayer of the cell membrane, the DNA is taken into the cell.

Two days prior to electroporation of L1.2 cells, we split L1.2 cells in the ratio of 1:5 to approximately 300,000 cells/ml. They were in log phase ~48 hours later. We counted L1.2 cells using the Neubauer haemocytomer. We needed ~1 x 10^7 cells per reaction. We centrifuged the cells (in 50mls sterile capped tubes) for 5-10 minutes at 1400 rpm in Sorvall. We washed cells twice in 10mls of Hepes buffer solution (HBS) and re-suspended

cells in 1ml of HBS per reaction and transferred 1 ml of cells to sterile 0.4cm electroporation cuvette. We added 20μg DNA (e.g. from maxi prep) and pipetted gently to mix. Cells were incubated in cuvette for 10 minutes at room temperature. The mix was electroporated at 250V, 960uFD (using capacitance extender). The time constant was recorded. Cells were transferred using a 1ml sterile pipette to T 75 flask containing 10ml medium without selection (RPMI, 10% FCS with Pen/Strep). Cells were incubated at 37°C for 48 hours. Selection antibiotic was added to cells 48-72 hours after electroporation. Cells were incubated at 37°C for 24-48 hours. Cells were counted. Cells were plated at 25,000 cells per well in a total volume of 200μl selection media (FCS, Pen/Strep/G418 RPMI) in each well of a 96 well plate. Cells were incubated at 37°C for 2-4 weeks and inspected for colonies. When colonies had formed, we split the wells and transferred to new wells of a 6 well plate containing 2mls of selection media. We expanded cells further into T 25, then T 75 flasks. At this point one can assay for function of transfected gene. We cloned cells by limited dilution. Clones were chosen to expand from the least dense well possible. Stable clones were selected with 0.8mg/ml G418 (appendix).

2.3.6 Site directed mutagenesis

We had previously inserted C5L2 into the plasmid vector pcDNA3.1$_{-C9}$ and made C5L2 pcDNA3.1$_{-C9}$ (see earlier). Next we made sense and antisense oligonucleotides with the desired mutation i.e. replacing leucine with arginine at position 132 in the DRY sequence. The plasmid encoding the human C5L2 variant, in which leucine 132 is mutated to arginine, was generated by the QuickChange method (Stratagene). The specifically written computer programme informs us that the enzyme at the mutation site is *Pvu* 1 (also called *Xor* II). The enzyme site is C GAT CG (see underline).

D L

C5L2 : 3'-CTC CTG GCA GCT CTC AGT GCC GAC CTC TGC-5'

*

Sense oligonucleotide primer

D R

3'-CTC CTG GCA GCT CTC AGT GCC GAT CGC TGC TTC CTG GCT CTC GGG

*

CCT GCC-5'

* marks mutation site of leucine to arginine, enzyme site is underlined, D = aspartic acid, L = leucine, R = arginine

Antisense oligonucleotide primer

5'-GGC AGG CCC GAG AGC CAG GAA GCA GCG ATC GGC ACT GAG AGC

*

TGC CAG GAG-3'

We calculated the annealing temperature as outlined previously. Next we performed a PCR reaction as outlined below using the protocol below. Steps 2 to 4 inclusive are repeated 24 times before proceeding to step 4, 5 and 6.

PCR mix	2tubes µl	Control µl
C5L2pcDNA3.1– $_{C9}$ template DNA	100ng	100ng
Oligo primer sense	10pmole	-
Oligo primer antisense	10pmole	-
10x cloned PFU buffer	5µ l	5µl
10mM dNTP	2µ l	2µl
ddH_2O	37µ l	39 µl
Total	50µl	50µ l

We added 1.5µl turbo PFU after 2.5 minutes of first cycle at 94°C, then restarted programme.

PCR condition

Step 1	94° C	3 minutes	
Step 2	94°C	30 seconds}	x 25 cycles
Step 3	55°C	20 seconds }	
Step 4	72° C	6.5 sec}:	
Step 5	72° C	5 minutes	
Step6	4° C		

Next we performed *Dpn* I digestion and visualised the PCR products. When the PCR is completed change the programme to 37° C x 1 hour. For 100 ng of template, add 1.5µl of the restriction endonuclease *Dpn* 1 at 37° C for 1 hour, then end programme. *Dpn*I (recognition sequence 5'-GATC-3' where the A residue is methylated) is an enzyme which recognises methylated DNA. We removed 15µl and run on an agarose gel, together with molecular weight marker and checked for appropriate size bands i.e. visualised the PCR products as bands (i.e. DpnI-digested , *Pfu* DNA polymerase-polished PCR products) -in our case, the vector was 5.4 kb and the insert was 1.1 kb, which combined was 6.5 kb.

We transformed DNA into DH5α competent cells as described earlier. We transformed 3 plates with "mutated C5L2" (i.e. C5L2(L132R)pcDNA3.1-$_{C9}$) and a control plate with "wild type C5L2 " (i.e. C5L2pcDNA3.1-$_{C9}$). As previously described we grew up and stored transformants, prepared glycerol stocks, purified DNA using the miniprep protocol, performed maxi-prep to obtain high quality DNA and sequenced C5L2(L132R)DNA3.1-$_{C9}$ as described previously.

2.3.7 Preparation of human peripheral blood neutrophils

Draw 18 mls of human blood into a 30mls sterile syringe containing 2 mls 0.2M EDTA, pH 7.4 and place in a sterile 50ml tube. Add 6mls of dextran (pre-warmed to room temperature) (60% dextran T-500 in 0.9% sodium chloride, NaCl) and mix by inverting. Stand the 50 ml tube in ice. This prevents the red blood cells from clumping. The red blood cells settle to the bottom of the tube. Aspirate the plasma, (containing the white blood cells) by pipette and transfer to a 50 ml sterile tube. Spin at 1000 rpm for 10minutes (Sorvall). Discard supernatant of plasma. Re-suspend cells in 1ml of PBS. Lyse remaining red blood cells by re-suspending in 27mls of double distilled water (ddH_2O) followed immediately by 9mls of 3.6% NaCl. This restores the solution to 0.9% NaCl. Spin at 1000rpm for 10 minutes. Discard supernatant and wash pellet (white blood cells) twice with phosphate buffered saline (PBS) (spin at 1000 rpm for 10 minutes). For second wash, re-suspend in 10mls of 0.9% NaCl. Count cells using a Neubauer haemocytometer. Wash and spin human neutrophils at 1000 rpm x 10 minutes and re-suspend in PBS with FCS.

2.3.8 Competition binding studies: experimental technique

Competition binding experiments were performed using L1.2 cells stably transfected with the human C5aR or with human C5L2, HEK293T cells transiently transfected with C5L2, C5aR or C3aR plus Gα16 (as described previously) or with human peripheral blood neutrophils (PMNs). 2 x 10^5 HEK293T cells or 1 x 10^6 L1.2 cells or human PMNs were incubated with 0.1nM ^{125}I-C5a or 0.5 nM ^{125}I-C3a and 0-300nM unlabelled C5a, C3a, or C4a, 0-13.2μM C5a des Arg, or 0-1mM C5a hexapeptide analogue for 30 minutes at 37°C in binding buffer (appendix). Cells were washed by centrifugation leaving a pellet in the bottom of each snap cap tube. After the addition of scintillation fluid, bound ligand was quantified by gamma counting. All points were determined in duplicate and at least three independent experiments were performed for each. Results were expressed as the % maximal binding (± SEM), and data were analysed using PRISM software, testing one and two–site models of competition binding.

2.3.9 Kinetics of binding C5a to C5L2 compared with C5aR

C5aR/L1.2 and C5L2/L1.2 cells were incubated in binding buffer (appendix) containing 0.02% NaN_3 (sodium azide prevents internalisation) at 1 x 10^7/mL with 0.1 nM ^{125}I-C5a at 0°C for ~1 hour. Duplicate aliquots were removed as a function of time for up to 60 minutes. They were filtered on glass fibre filters (GF/F, Whatman) which had been soaked in 1% polyethyleneimine for hours: this blocks non specific binding. They were washed three times with ice-cold binding buffer (appendix). Filters were placed in scintillation tubes and subjected to liquid scintillation counting, i.e. the "on rate" was assessed i.e. amount of ligand bound over time. The remaining samples were divided into equal parts: 250nM C5a was added to one and buffer to the other, and samples were removed as a function of time for an additional 60 min i.e. at equilibrium, excess unlabelled C5a was added to displace the radioligand as an estimate of the "off-rate". All points were determined in duplicate, and at least three independent experiments were performed for each. Results were expressed as the mean cpm bound (± SEM) and data analysed using PRISM software.

2.3.10 Phosphorylation of C5L2 and C5aR after C5a binding

C5L2/L1.2 and C5aR/L1.2 cells were washed in phosphate free DMEM and suspended at 3 x 10^7/ml with 1mCi carrier free ^{32} P-labelled orthophosphoric acid (Dupont/ NEN Life Science products) for 3 hours at 37°C. Cells were then stimulated with 200 nM C5a for 5 minutes, quenched with 9 ml of ice-cold PBS, centrifuged and lysed in 500µl of ice cold lysis buffer (appendix). The lysates were cleared by centrifugation at 18,000g at 4°C for 10 minutes. Receptors were immunoprecipitated with 1D4 antibody covalently cross-linked to protein A Sepharose beads (Sigma-Aldrich) for 1 hour at 4°C with gentle agitation. Samples were washed, eluted with SDS sample buffer under reducing conditions for 10 minutes at 60°C, and applied to 10% SDS polyacrylamide gels. Following electrophoresis, gels were fixed, dried and exposed to BioMax MR film (Kodak). Dr. Shoji Okinaga

performed the immunoprecipitation and SDS polyacrylamide gel electrophoresis component of this experiment.

2.3.11 Receptor Internalisation Experiment

Assessment of receptor internalisation was performed utilising human polymorphonuclear cells (PMN) incubated with the synthetic C5a hexapeptide agonist at increasing concentrations. Human PMN (0.5 x 10^6 cells) were prepared as described above in Dulbecco's phosphate buffered saline (DPBS) with 2% goat serum. Increasing concentrations of C5a hexapeptide was added: 0-10 μM C5a hexapeptide and incubated at 37°C for 5 minutes. Cells were subsequently transferred to ice (0°C) and receptor expression evaluated by flow cytometric analyses (see below). Spin cells to pellet and re-suspend in first antibody while on ice i.e. amyloid1-α 40, isotype mouse antibody, rabbit anti-human C5L2 or mouse anti-human C5aR. Leave for 1 hour at 0°C (on ice). Wash after 1 hour (i.e. 2 spins in PBS with 10% FCS). Add FITC conjugated goat anti-rabbit IgG or PE conjugated goat anti-mouse IgG and leave for 1 hour at 0°C. Wash twice in PBS with 10% FCS. The final wash is in PBS which contains 2% formaldehyde. Receptor expression is evaluated by flow cytometric analysis.

2.3.12 Fluorescent activated cell sorter (Facs) analysis of human granulocytes

Primary and secondary antibodies were made up in ice-cold phosphate buffered saline (PBS) containing 2% foetal bovine serum (FBS). Human peripheral blood neutrophils were prepared (as described earlier) and suspended in ice-cold PBS-2%FBS at 1 x 10^7/ml. Aliquots of 100μl were incubated with primary antibodies: 10 μg/ml rabbit anti-human C5L2 and mouse anti-human C5aR, for 1 hour at 0°C (on ice). PBS-2%FBS without antibody was used as a negative control. Cells were spun (1000rpm x 3 minutes), supernatant removed completely with slow, gentle vacuum and secondary antibodies added : FITC-conjugated goat anti-rabbit IgG and PE-conjugated goat anti-mouse IgG. Cells were left on ice for 1 hour covered with aluminium foil (antibodies are light

sensitive). Cells were spun and washed twice in PBS-2%FBS (1ml). For third wash PBS was used. Cells were re-suspended in 4% formaldehyde in PBS (0.5ml) and transferred to Facs labelled tubes for flow cytometry. Flow cytometric analyses were performed utilising a dual laser cytometer (FACS Scan) and data was analysed with Cellquest software (Becton Dickinson). We gated on tightly on granulocytes and assessed staining pattern. All samples were performed in duplicate and each experiment was performed at least three times.

2.3.13 Calcium mobilisation by C5aR/293T, C5L2/293T and C5L2(L132R)/293T cells

HEK293 T cells were transiently transfected with plasmids encoding human C5aR, C5L2 or C5L2 (L132R) with or without the plasmid encoding the G protein subunit Gα16, that has been shown to greatly facilitate C5a mediated signal transduction (Amatruda *et al.* 1993). Cells were harvested at 48 hours (i.e. vacuum off the media from HEK293T cell flask. PBS (5mls) without calcium, magnesium was added so that the cells detached. Flask was placed in a humidified, 37°C incubator, with 5% CO_2 for 30 seconds then removed. We pipetted the contents of the flask i.e. 5mls PBS and cells, into a 50mls sterile tube. We washed the tissue culture flask with a further 5mls PBS (without Ca/Mg^{2+}) and pipetted the contents into the sterile 50 ml tube). Cells were counted using a Neubauer haemocytometer. Cells were spun x 5 minutes at 1000 rpm and PBS removed by pipette. Cells were re-suspended in 12.5mls of buffer : 20mM HEPES, pH 7.4, 125mM NaCl, 1nM $CaCl_2$, 1nM $MgCl_2$, 5nM KCl, 0.5 nM glucose and 0.2% BSA buffer. Dimethylsulphoxide (DMSO) was added to indicator dye FURA-2-AM (Molecular Probes). Cells were incubated with the indicator dye Fura-2 for 1 hour at 37°C in a water bath in buffer (20mM HEPES, pH 7.4, 125mM NaCl, 1nM $CaCl_2$, 1nM $MgCl_2$, 5nM KCl, 0.5 nM glucose and 0.2% BSA).

Cover with aluminium foil (dye is light sensitive). Cells were spun at 1000 rpm for 5 minutes, then re-suspended in 12.5mls buffer (as above). This wash step was repeated. Cells were re-suspend in the volume we wanted e.g. 2 x 10^6 293T cells in 12mls. Changes in intracellular calcium concentration, in response to C5a were determined by monitoring

the fluorescence emission at 510nm with excitation at 340 and 380 nm as a function of time. Responses were quantified as the peak of the ratio of 340/380nm wavelengths.

2.3.14 Chemotaxis

Chemotaxis was measured using a modification of the Boyden technique (Aggett *et al.* 1979). Cells transfected with C5L2 and C5aR (1 x 10^7/ml) were fluorescently labelled with calcein-AM (Molecular Probes), suspended in 20 mM HEPES, pH 7.5, 125mM NaCl, 5mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 0.5mM glucose, 0.2% bovine serum albumin and tested for chemotactic activity in modified Boyden chambers. Cells (0.1ml) were placed in the upper wells of 3-micron, 6.4mm FlurorBlok filter inserts (Falcon) with 0.6 ml of buffer containing 0 to 10 mM C5a or 8μMfMLP in the lower wells. Chambers were incubated at 37°C for 45 minutes and chemotaxis determined by measuring the fluorescence intensity excitation: 485nm, emission: 535nM passing to the underside of the filter. Measurements were determined in duplicate for three independent experiments.

2.3.15 Analysis of mitogen activated protein kinase (MAPK)

Phosphorylation of MAP kinase was investigated using $C5L2_{C9}$ /L1.2 and $C5aR_{C9}$ /L1.2 cells. Cells were washed twice with serum free RPMI and pre-incubated at 2 x 10^6/ml for 30 minutes at 37°C with gentle agitation (bench rocker). Cells were then treated with 0 or 200 nM C5a for 0.5, 1, 3, 5, 10, or 30 minute periods. Cells were immediately collected in ice-cold lysis buffer (appendix) and frozen in liquid nitrogen. After three cycles of freezing (liquid nitrogen) and thawing (cold running water), lysates were centrifuged at 2500g for 10 minutes at 4°C. The supernatants were further centrifuged at 97000g for 1 hour at 4°C and subjected to Western blot analysis. PC12 cell extract "treated" acted as our positive control with MAP kinase phosphorylated, while our negative control was PC12 cell extract "untreated". Following electrophoresis on 10% polyacrylamide gels, proteins were

transferred to nitrocellulose and stained with rabbit anti-active-MAPK antibody as described by the supplier. Blots were incubated with peroxidase-conjugated goat anti-rabbit IgG and signals were detected by ECL Plus (Amersham Pharmacia Biotec) using BioMax MR film (Kodak) (see details below)

2.3.15.1 SDS-polyacrylamide gel electrophoresis (PAGE)

An ATTO system was used for all SDS-PAGE gels (ATTO Corporation, Japan) and protein samples were electrophoresed using a Consort electrophoresis power supply unit. Proteins were separated on reducing gels prepared using discontinuous buffer system. Resolving and stacking gels were prepared to the required percentage acrylamide in the order indicated (below). APS and TEMED were added last with gentle swirling of the mixture. Electrophoresis was carried out at 150 volts, 25 milli Amps (mA) per gel for 1-1.5 hour, or until the loading dye had run to the bottom of the gel.

Composition of 10% Separating/ Resolving Gel

Protogel	13.2 ml
H_2O	16.6 ml
30% APS	100 µl
TEMED	30 µl
Protogel buffer (lower)	10ml

Composition of Stacking gel

Ultrapure Proto-Gel	1.3 ml
Protogel Stacking buffer (upper)	2.8 ml
H_2O (add first)	7.4 ml
30% APS	30 µl
TEMED	10 µl

2.3.15.2 Western blotting

Western blotting was carried out using the semi-dry method for the transfer of electrophoresed proteins to immobilising membranes and was performed using an ATTO semi-dry transfer system (ATTO Medical Supplies, Japan). A semi-dry transfer apparatus (Sigma, St. Louis, M.O. U.S.A.) was used to transfer proteins to a nitrocellulose membrane. During SDS-PAGE, polyvinylidene fluoride (PVDF) transfer membrane (0.45µm, Pall Life Sciences) was briefly saturated in methanol for 10-15 seconds and then equilibrated in transfer buffer (appendix) for approximately 20-30 minutes prior to semi dry transfer. Whatmann 3mm filter paper of the same dimensions was also saturated in transfer buffer prior to semi-dry blot sandwich assembly, which was assembled in the order cathode, filter paper, acrylamide gel, PVDF membrane, filter paper and anode. Electrophoretic transfer was performed at 150 volts, 25 milli Amps (mA) per gel for 60-90 minutes as required. Following semi-dry transfer, the PVDF membrane was removed and processed for immunoblotting.

2.3.15.3 Immunodetection and development

Following electrophoresis on 10% polyacrylamide gels and proteins transferred to nitrocellulose, the transferred membrane was stained with Ponceau S solution for a few minutes to detect protein and assess transfer efficiency. After removing Ponceau S (wash with PBS) non specific sites on the membrane were blocked by immersing membrane in freshly prepared blocking buffer (appendix) and left overnight on a shaking table at 4°C. Membranes were then washed three times with washing buffer (appendix) and incubated with primary antibody (rabbit anti-active-MAPK antibody, as described by the supplier). Following incubation with the primary antibody, membranes were washed several times with washing buffer over 30 minutes. Blots were then incubated with the secondary antibody (peroxidase-conjugated goat anti-rabbit IgG, as described by the supplier). Then membranes were finally washed 4-5 times with washing buffer over 30 minutes with the

final wash in PBS alone. Membranes were stored in PBS at 4°C if development could not be carried out immediately. Detection of immunoblots was performed using the enhanced chemiluminesence (ECL) Plus method (Amersham Pharmacia Biotec), (Leong and Fox 1990). Membranes were exposed to BioMax MR film (Kodak) for the appropriate time period (range 10s to 1 min) and exposed films were developed using an automatic developer (CURIX 60, AGFA, Type 9462/100/140, Agfa-Gevaert AG, Munich, Germany). PC12 cell extract "treated" acted as our positive control i.e. MAP kinase is phosphorylated, while our negative control (unphosphorylated MAPkinase) was PC12 cell extract "untreated"

2.3.16 Antisera toC5L2 and flow cytometric analysis of human granulocytes

An affinity-purified polyclonal antibody against a synthetic peptide corresponding to the predicted N terminal extracellular sequence of human C5L2 (MGNDSVSYEYGDYSDLSDRPVDC) was coupled to KLH and utilised for production of rabbit antiserum (Alpha diagnostics). We used amyloid-ı-α-40 as our negative control for C5L2. For flow cytometric analyses, cells (either L1.2 transfectants or human leukocytes) were suspended in PBS containing 2% foetal bovine serum at 1 x 10^7 /ml. Aliquots of 100µL w ere incubated with 10µg/ml rabbit anti-human C5L2, mouse anti-human C5aR or irrelevant controls for 1 hour at 0°C on ice. Cells are washed by spinning for 3 minutes, shaking off antibody and re-suspending the cell pellet using a pipette in 200µl FBS/PBS. Cells were incubated with FITC-conjugated goat anti-rabbit IgG (light sensitive so cover with aluminium foil) or PE-conjugated goat anti-mouse IgG, respectively and placed on ice for 30 minutes. Cells were washed twice in PBS/FBS and re-suspended PBS x 1, and fixed in 4% paraformaldehyde in PBS for flow cytometry (Werfel *et al.* 1996). Analyses were performed utilising a dual laser cytometry and data was analysed with Cellquest software (Becton Dickinson).

2.3.17 Bone marrow cell preparation in mice

Femurs from wild type and C5aR deficient BALB/c mice were isolated. The marrow was flushed out gently and cells aspirated into RPMI containing 2% foetal calf serum (FCS) using a 25g needle. Cells were dispersed (i.e. single cell suspensions were prepared) by passing the marrow gently through a 21 gauge needle. The marrow cells were centrifuged at 400g for 10 minutes and re-suspended in RPMI at 1 x 10^7 cells/ ml. Aliquots of 1 ml were transferred to six well-dishes and pre-incubated for 30 minutes at 37°C in 5% CO_2. Cells were then stimulated with 0 or 100 nM C5a in PBS containing 0.1% BSA (low endotoxin) for 4 hours. Total RNA was extracted using RNA aqueous (Ambion) (appendix)

2.3.18 Microarray analyses

Total RNA was subjected to micro array analyses (Harvard Medical School Micro array Facility). Gene chips were analysed using MAS 5.0 software (Affymetrix) to create .chp files, which were copied to Microsoft Excel and saved as tab delimited text files. Tab delimited text files were analysed using GeneSpring software (Silicon genetics) and normalised as follows: the 50th percentile of all measurements was used as a positive control for each sample. Each measurement for each gene was divided by this synthetic positive control, assuming that this was at least 10. The bottom 10^{th} percentile was used as a test for correct background subtraction. This was never less than the negative of the synthetic positive control. Each gene was normalised to itself by making a synthetic positive control for that gene and dividing all measurements for that gene by this positive control, assuming it was at least 0.01. This synthetic control was the median of the gene's expression values over all the samples. Last, normalised values below 0 were set to 0. Analysis of the fold change in gene expression was calculated using GeneSpring. First, data file restrictions were placed on the text files to include only genes with an absolute call of

present (P). Then experimental samples were compared using the fold change analysis tool in GeneSpring. To determine the differential expression of genes, data file restrictions were placed on the text files to create lists of files with the absolute call of present and absent. The gene lists were then compared using the Venn diagram tool of GeneSpring. Annotation tables of gene lists were created using the batch analysis tool of NetAffyx (Affymetrix.com). Text files of probe set IDs were imported into NetAffyx, and resulting annotation tables were downloaded and saved as Excel files.

2.3.19 Data analysis

Competition binding experiments were conducted a minimum of three times except where otherwise stated. Statistical analysis of results involved calculation of the percentage maximal binding ± the standard error of the mean (SEM). Data was analysed using PRISM software, testing one and two site models of competition binding. Regarding kinetics of binding experiments, all points were determined in duplicate and at least three independent experiments were performed. Results were expressed as the mean counts per minute (cpm) bound ± SEM and data was analysed using PRISM software. When using the Fluorescence activated cell sorted scan (Facs-Scan), data was analysed with Cellquest software (Becton Dickinson).

2.3.20 Animal care

Wild type BALB/c mice were obtained from Charles River Laboratories (Boston, M.A. U.S.A). Our laboratory had previously disrupted the gene that encodes C5aR and had developed C5aR deficient BALB/c mice. The animals were housed in the Specific Pathogen Free animal rooms in the Ina Sue Perlmutter Cystic Fibrosis Laboratory, (Children's Hospital, Boston, MA, U.S.A.). All animal procedures and mouse protocols were approved by the Animal Care and Use Committee of Children's Hospital and were conducted according to institutional and National Institute of Health (N.I.H.) guidelines for

animal care and use. Mice used were age and sex matched: aged between 8-12 weeks. I was responsible for the care of these mice.

CHAPTER III

CLONING AND TISSUE EXPRESSION OF C5L2 IN MOUSE AND MAN

CHAPTER III

CLONING AND TISSUE EXPRESSION OF C5L2 IN MOUSE AND MAN

3.1 INTRODUCTION

Evidence was mounting that a second C5a receptor might exist. Complex effects of C5a *in vivo,* including both pro- and anti- inflammatory effects: in sepsis and acute pancreatitis with associated lung injury respectively, were not fully explained by deletion of the classical receptor, C5aR (Bhatia *et al.* 2001, Huber-Lang *et al.* 2001). Secondly, expression of receptors for C5a on parenchymal cells in the lung, liver, smooth muscle and endothelial cells serve as yet uncharacterised functions (Drouin *et al.* 2001, Haviland *et al.* 1995). Thirdly, an orphan receptor that localises to the same region of chromosome 19 as the C5a and formyl peptide receptors (Gerard *et al.* 1993) was recently identified as C5L2 (C5–like receptor 2) and was shown to be present on immature but not mature dendritic cells (Ohno *et al.* 2000). We decided to study this orphan receptor in further detail.

Before we could test for characteristics of this putative receptor C5L2, we needed to prove that we could express C5L2 on the cell surface. We employed the vector pEGFP-N1, which incorporates the enhanced green fluorescent protein (EGFP) to circumvent this problem. We inserted the coding sequence of C5L2 into pEGFP-N1 and then stably transfected L1.2 cells with C5L2-pEGFP-N1. If the cells fluoresced under the fluorescent microscope, then that would prove C5L2 expression. Then we could proceed to test for characteristics of this putative receptor C5L2, e.g. ligand binding and coupling to G proteins etc. In view of the fact that pEGFP-N1 is a bulky vector we decided to subsequently clone C5L2 into a less bulky, more user friendly vector once receptor expression was confirmed on the cell surface. pcDNA3.1 was chosen and C5aRpcDNA3.1$_{-C9}$ was kindly loaned to us by Prof Hyeryun Choe from our lab (Ina Sue Perlmutter and Cystic Fibrosis Lab, Boston,

M.A.,U.S.A.). The sense primer contained *Nhe* 1 and the anitsense primer *Asp*718. Subsequently, the coding sequence of C5L2 was cloned into pcDNA3.1 to include nine amino acid C terminal tag corresponding to the C-terminus of bovine rhodopsin (-C9 tag) to facilitate recognition by the antibody 1D4. *Asp* 718 fits at the N terminus at the 5' end, in frame with the C9 tag (chapter II).

3.2 OBJECTIVES

(Please see chapter II for methods)

1) Molecular cloning of the orphan receptor C5L2 from man and mouse and insertion of C5L2 into the enhanced green fluorescent protein vector pEGFP-N1 (to prove receptor expression on cell surface) and subsequently into pcDNA3.1$_{-C9}$.

2) To demonstrate tissue expression of C5L2 and C5aR in human and murine tissues and compare and contrast results of both receptors in these two species, using Northern blot analysis.

3) Insert a point mutation at position 132 of the normally highly conserved "DRY" sequence, (using site directed mutagenesis), replacing leucine with arginine.

3.3 RESULTS

3.3.1 Molecular cloning of C5L2 from man and mouse

The coding sequence of human C5L2 was obtained by RT-PCR using human brain cDNA (Clontech) as a template and two oligonucleotide primers incorporating *Nhe* I and *Asp* 718 (chapter II). The RTPCR product, C5L2 underwent agarose gel electrophoresis and was compared to 1kb molecular weight ladder (Figure 3.1). Digestion with *Pst* I and subsequent agarose gel electrophoresis revealed appropriately sized bands. C5L2 was confirmed by sequencing.

A murine genomic library (Stratagene) was screened using ^{32}P-labelled cDNA corresponding to human C5L2 and yielded a ~15 kb clone encoding mouse C5L2. Genomic analysis was performed using DNA from FVB/NJ mice, restricted with *Eco* RI, *Eco* RV, *Stu* I or *Hind* III. Southern blots were hybridized with ^{32}P-labelled cDNA (chapter II) (Figure 3.2). This work was performed by Dr. Shoji Okinaga.

We analysed the aligned sequences of mouse C5L2 and human C5L2 and a 61.3% identity in the deduced protein was noted and a 68.9% identity in the nucleotide sequences (Figure 3.3). The important arginine residue in the DRY sequence following the third transmembrane segment is conserved in both human and murine C5L2 as a leucyl residue.

Comparing the aligned sequences of C5aR, and C5L2, (Figure 3.4) the G protein recognition sequence, in C5L2 in the third intracellular loop (between TM5 and TM6) is shorter than exists in C5aR by several cationic residues. C5aR has been shown to have distinct ligand binding and activation sites (Siciliano *et al.* 1994). Receptor activation by ligand involves the engagement of charged and uncharged residues on the extracellular faces of the transmembrane helices. For C5aR, these include Ile ^{116}, Val ^{286}, Arg ^{175}, Glu ^{199}, Arg ^{206}, and Asp ^{282} (Geva *et al.* 2000, Crass *et al.* 1999, Cain *et al.* 2001). C5L2 shares all of these residues except for Asp ^{282}, where there is a Glu residue. The N terminus of C5L2 retains the tyrosines flanked by acidic amino acids, which have previously been demonstrated to be modified in the C5a receptor, C5aR by sulphation, and constitutes the major C5a docking site (Farzan *et al.* 2001). Thus C5L2 has a similar acidic ligand-binding N terminal domain to C5aR, and these acidic residues in the N terminus are characteristic of complement fragment receptors.

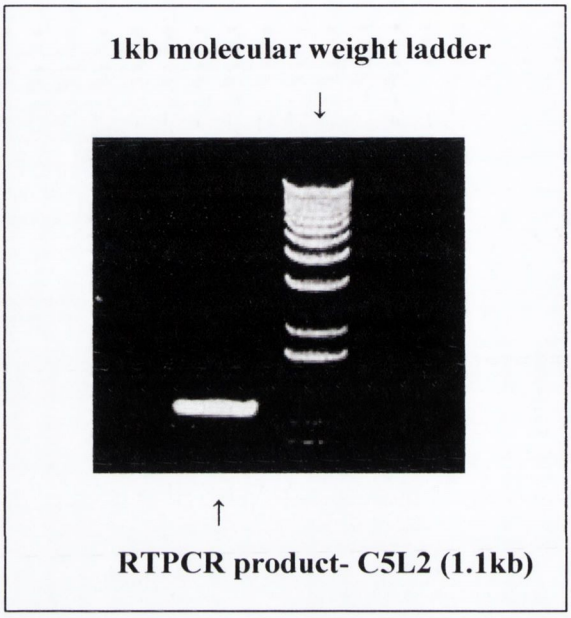


Figure 3.1 **Agarose gel electrophoresis comparing uncut RTPCR product, C5L2 with 1 kb molecular weight ladder verifying that RTPCR was successful.**

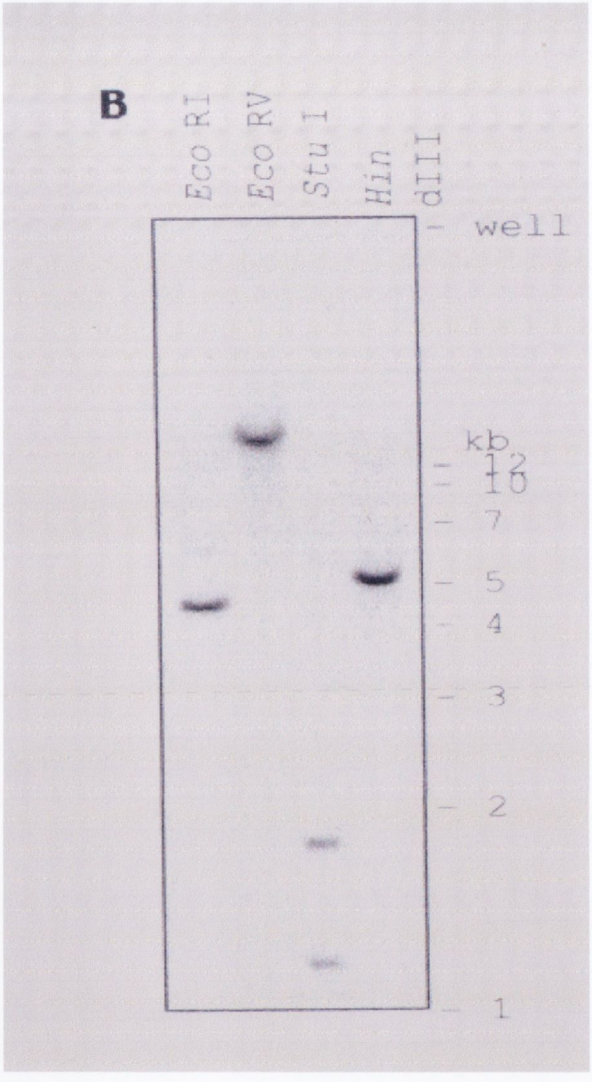


Figure 3.2 Southern blot analysis

Mouse genomic DNA was digested with restriction endonucleases as indicated above the panel (i.e. *Eco* RI, *Eco* RV, *Stu* I, *Hind* III) and hybridised with a cDNA probe corresponding to the full length coding region of human C5L2. Only *Stu* I cuts the deduced open-reading frame of mouse C5L2. Dr. Shoji Okinaga performed this Southern blot.

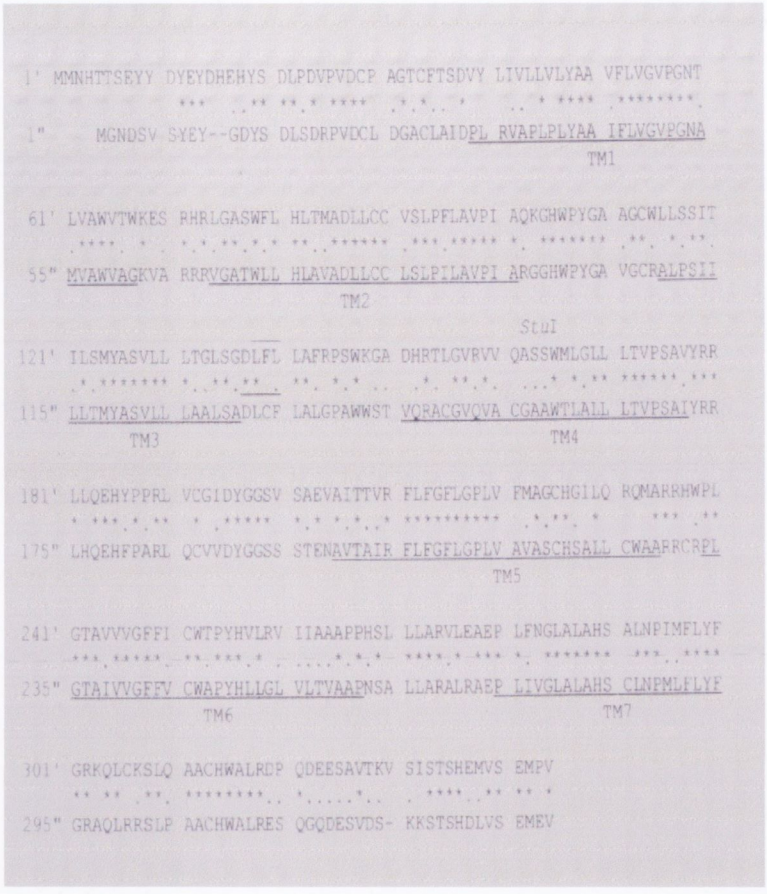


Figure 3.3 **Deduced amino acid sequence (single letter amino acid notation) of mouse C5L2 (top line) aligned with the sequence of human C5L2 (bottom line)**

Asterisks indicate identical amino acids found in both sequences, and full stops indicate chemically similar amino acids. Hyphens have been introduced to maximise sequence homology. Underlines show putative transmembrane segments with TM1-TM7 denotations. The overlines represent the sequences corresponding to the DRF sequence found in the C5a receptor.

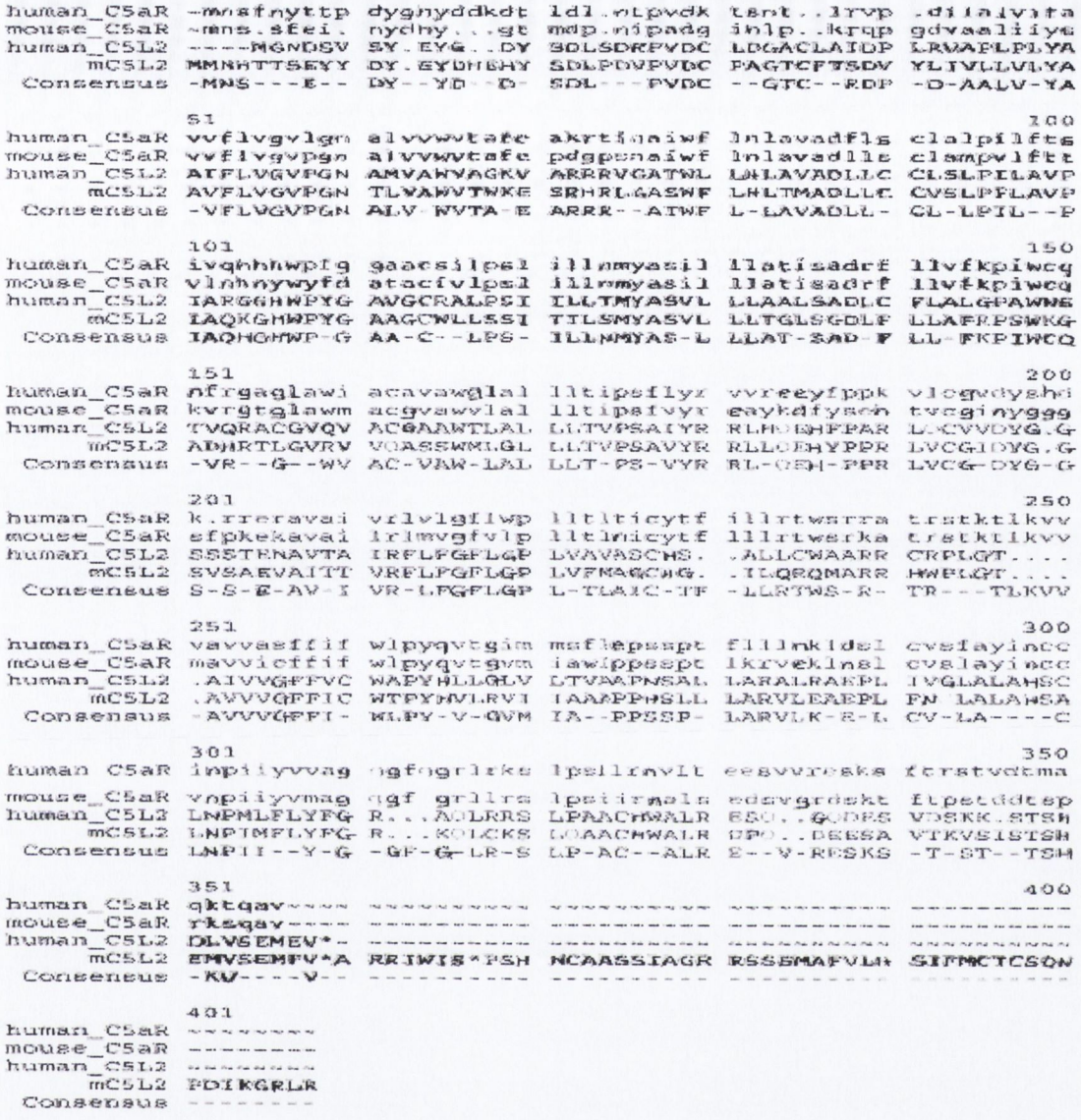


Figure 3.4 **Sequence alignment of human C5aR, mouse C5aR, human C5L2 and mouse C5L2.** (From Pretty results www.alignmentcolor.html)

3.3.1.2 Insertion of C5L2 into pEGFP-N1

Having confirmed the sequence of C5L2 to be correct (using Ohno et al.'s work as reference), we prepared our vector pEGFP-N1 (enhanced green fluorescent protein) (chapter II) and insert C5L2 by digesting both with *Nhe* I and *Asp* 718 and subjected both separately to agarose gel electrophoresis to check size, integrity and intensity of fragments prior to ligation. This revealed a 4.7 kb vector band, pEGFP-N1 and a 1.1kb insert band, C5L2 (Figure 3.5).

Both bands were cut out, the DNA purified (QIAquick gel extraction kit- appendix) and the amount of DNA present was quantified in both vector pEGFP-N1 and insert C5L2 by U.V. spectrophotometry. We subsequently ligated the vector and insert (with ddH_2O, T4 ligase and x 10 ligation buffer) at $16^{O}C$ x a few hours (chapter II), transformed into DH5α competent cells as described previously (chapter II), selected a range of transformants and made miniprep DNA (appendix). We confirmed recovery of plasmid DNA from minipreps by agarose gel electrophoresis. Next we digested minipreps of C5L2-EGFP with *Asp* 718 and *Nhe*1, revealing a 1.1 kb insert bands and 4.7 kb vector band (Figure 3.6).

We selected the brightest clone (miniprep) from gel 3.6, incubated in LB with kanamycin overnight, performed maxi-prep the following day to obtain good quality DNA and the product was sent for sequencing (Figure 3.7). After confirming our sequence was correct we used this for all subsequent experiments.

We transfected C5L2-EGFP into the murine pre-B-lymphoma line, L1.2 cells by electroporation and stable clones were selected with 0.8mg/ml G418 as previously outlined (chapter II). We subsequently demonstrated cell surface expression of the C5L2 receptor in pC5L2-EGFP-N/L1.2 cells using the fluorescence microscope (Figure 3.8).

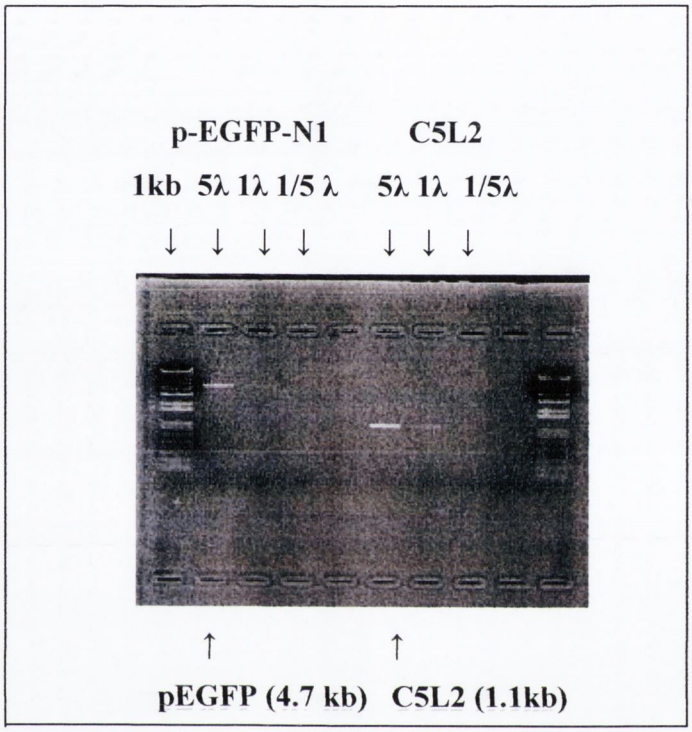
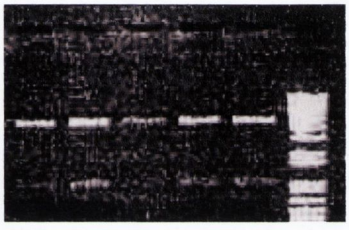


Figure 3.5 **Agarose gel electrophoresis demonstrates insert C5L2 and vector pEGFP-N1, both after digestion with *Asp* 718 and *Nhe*1 (each at three different concentrations) compared with 1kb molecular weight marker.**

EGFP vector band 4.7kb and C5L2 insert 1.1 kb bands

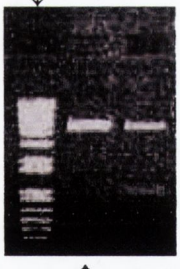


↑

1 kb molecular weight ladder

1 kb molecular weight ladder

↓



↑

No 1.1 kb C5L2 insert band seen in lane 2 so discard this miniprep

Figure 3.6 Agarose gel electrophoresis after digestion of mini-preps of C5L2-EGFPwith *Asp* 718 and *Nhe* I revealing insert C5L2 band of 1.1 kb and vector pEGFP band of 4.7 kb.

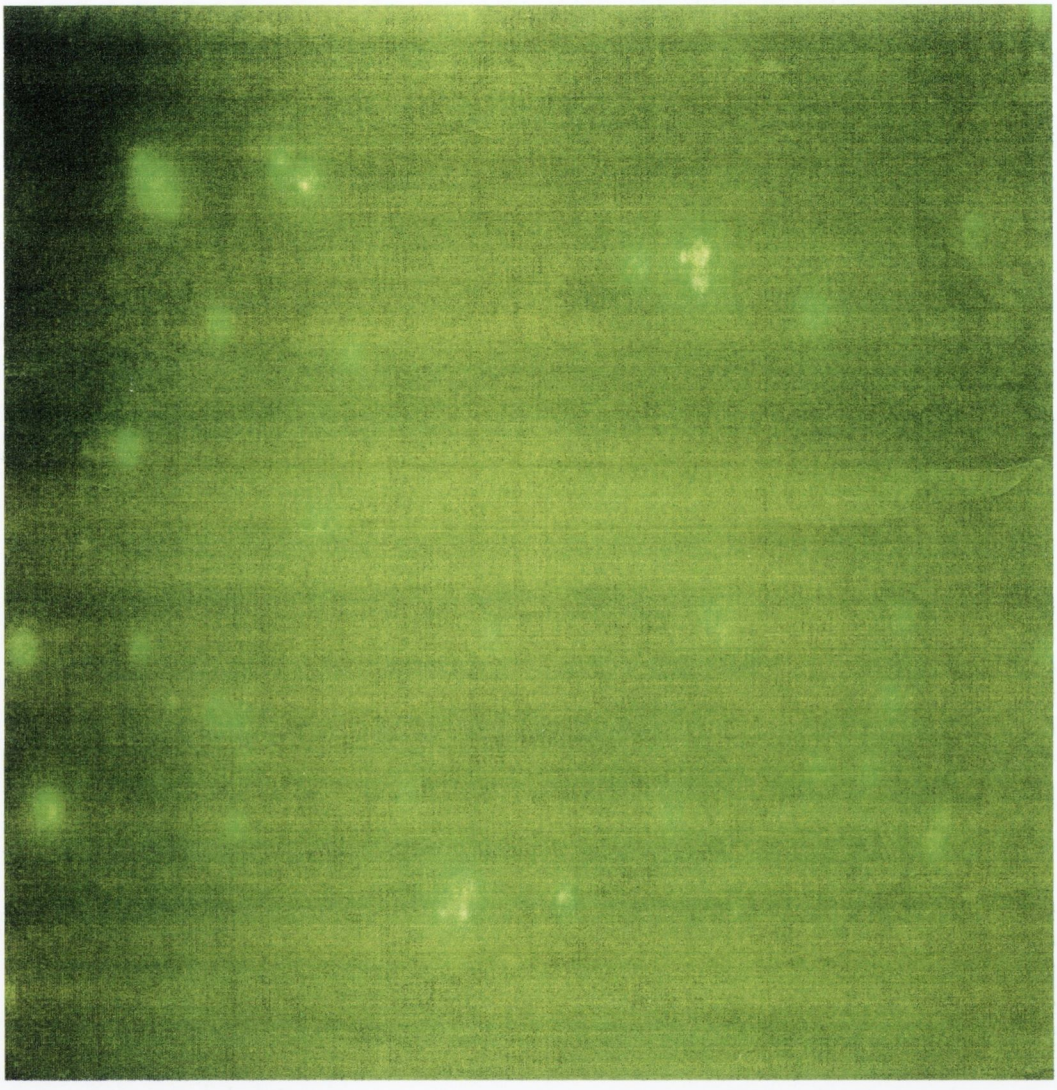


Figure 3.7 **C5L2-EGFP/L1.2 cells fluorescing under the fluorescence microscope proving expression of C5L2 receptor on cell surface.**

L1.2. cells were stably transfected by electroporation with pC5L2-EGFP-N1.

3.3.1.3 Insertion of the coding sequence of C5L2 into pcDNA3.1

Subsequently, we successfully cloned the coding sequence of human C5L2 into pcDNA3.1-$_{C9}$ which includes a nine amino acid C-terminal tag corresponding to the C-terminus of bovine rhodopsin (C9-tag) (chapter II). pcDNA3.1-$_{C9}$ is a less bulky and more user friendly vector than pEGFP-N1. C5aRpcDNA3.1$_{-C9}$ was kindly loaned to us by Prof. Hyeryun Choe at our lab (Ina Sue Perlmutter Lab., Boston).

As described in chapter II, we first digested C5aRpcDNA3.1-$_{C9}$ and C5L2-EGFP with *Nhe*1 and *Asp* 718 and subjected both to agarose gel electrophoresis (Figure 3.9). We cut out the relevant bands, vector (5.4kb) and insert (1.1kb) and purified the DNA (QIAquick gel extraction kit- appendix). We quantified the amount of DNA present by comparing vector pcDNA3.1-$_{C9}$ and insert C5L2 to the molecular weight ladder λHindIII (Figure 3.10).

We ligated C5L2 and pcDNA3.1$_{-C9}$, transformed them into DH5α competent cells, purified the DNA using a mini-prep protocol (appendix) and subjected the C5L2-pcDNA3.1$_{-C9}$ minipreps to agarose gel electrophoresis (Figure 3.11).

After digestion of C5L2pcDNA3.1$_{-C9}$ and C5aRpcDNA3.1$_{-C9}$ with *Asp* 718 and *Nhe* 1 we quantified the concentration of C5L2pcDNA3.1$_{-C9}$ using agarose gel electrophoresis by comparing with C5aRpcDNA3.1$_{-C9}$ (Figure 3.12) because C5aRpcDNA3.1$_{-C9}$ is very similar to C5L2pcDNA3.1$_{-C9}$. Secondly we compared digested C5L2pcDNA3.1$_{-C9}$ with a 1kb ladder (Figure 3.13).

We confirmed our findings with U.V. spectrophotometer. Then we selected one clone, performed a maxi-prep to obtain good quality DNA and sent this for sequencing. There were no errors and so we used this C5L2pcDNA3.1$_{-C9}$ for all subsequent experiments.

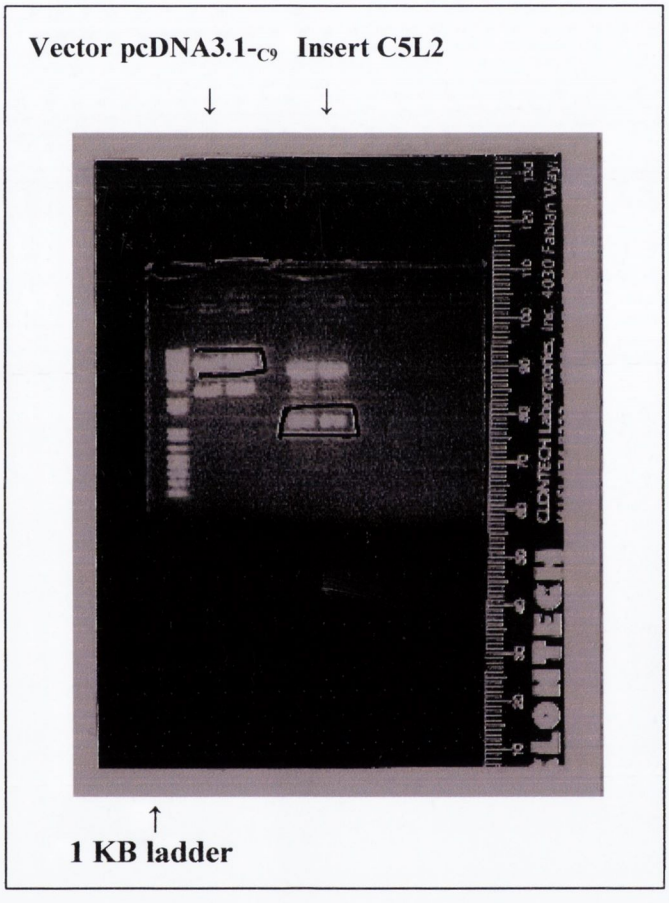


Figure 3.8 **Agarose gel electrophoresis after digestion of C5aRpcDNA3.1-$_{C9}$ and pC5L2-EGFP with *Asp* 718 and *Nhe* 1, demonstrating vector band pcDNA3.1-$_{C9}$ (5.4 kb), and insert C5L2 band (1.1 kb), and comparing with 1kb molecular weight marker.**

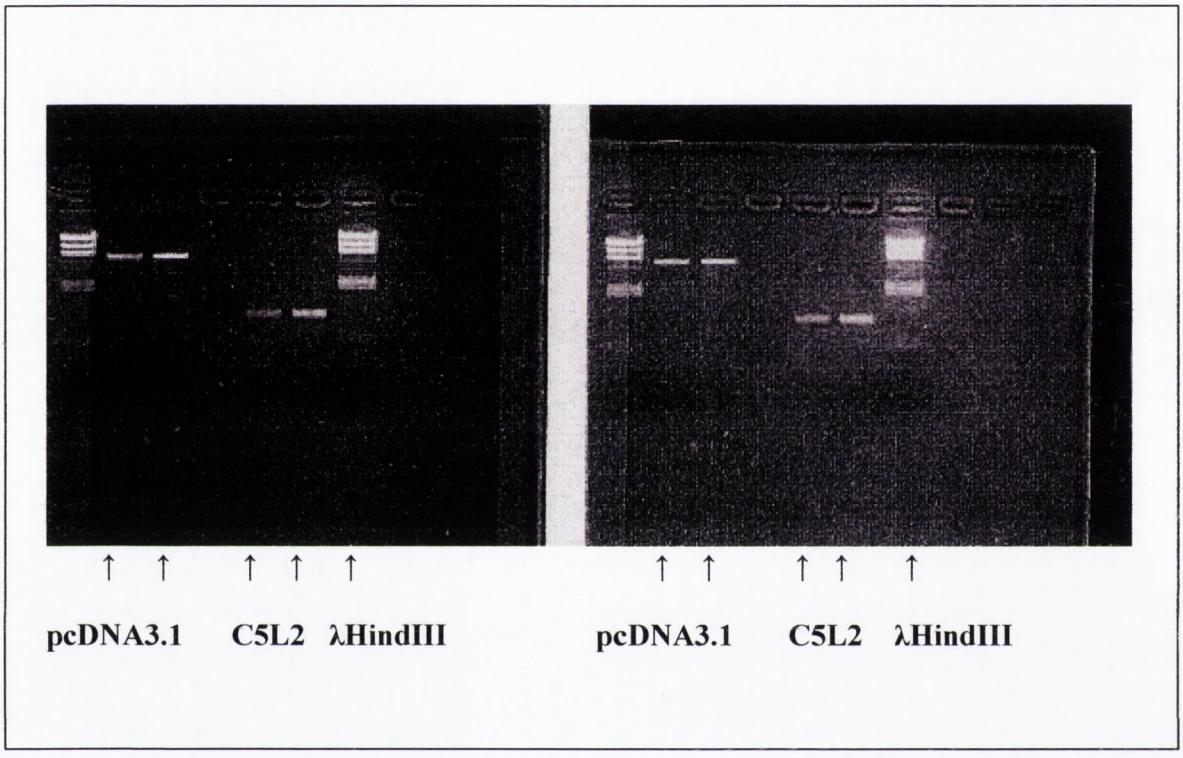


Figure 3.9 **Two agarose gel electrophoresis quantifying the amount of DNA present in vector pcDNA3.1$_{-C9}$ and insert C5L2 by comparing with λHind III.** λHind III is in lane 1 and 7, vector pcDNA3.1-$_{C9}$ at a volume of 5µl and 10µl in lane 2 and 3 respectively and insert C5L2 at a volume of 5µl and 10µl in lane 5 and 6.

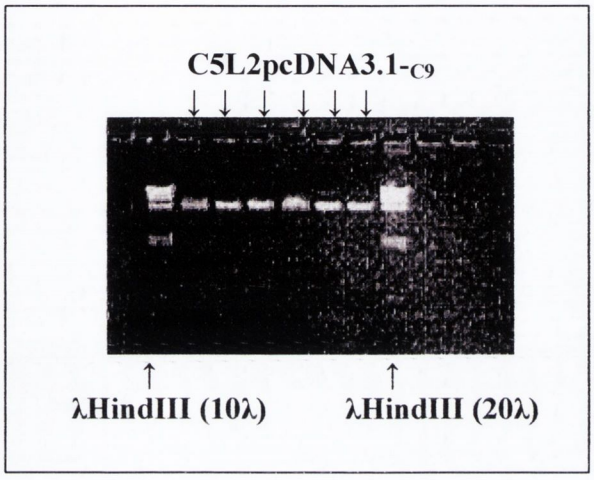


Figure 3.10 **Agarose gel electrophoresis comparing mini-preps of C5L2pcDNA3.1$_{-C9}$ to λHindIII after ligation of C5L2 and pcDNA3.1$_{-C9}$**

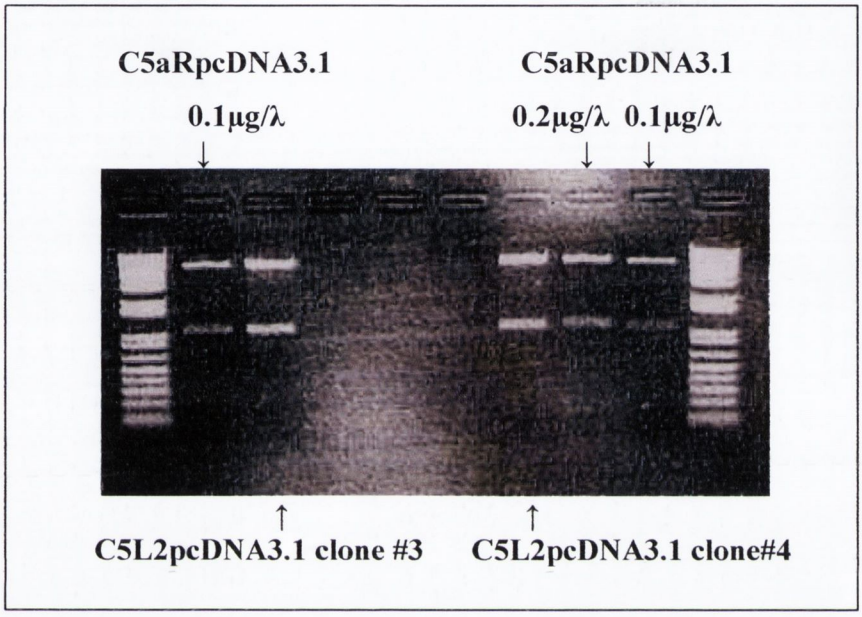


Figure 3.11 **Agarose gel electrophoresis comparing vector (5.4kb) and insert (1.1kb) bands of C5L2pcDNA3.1$_{-C9}$ with C5aRpcDNA3.1$_{-C9}$ after digestion with *Asp* 718 and *Nhe* 1 to quantify amount of DNA present.**

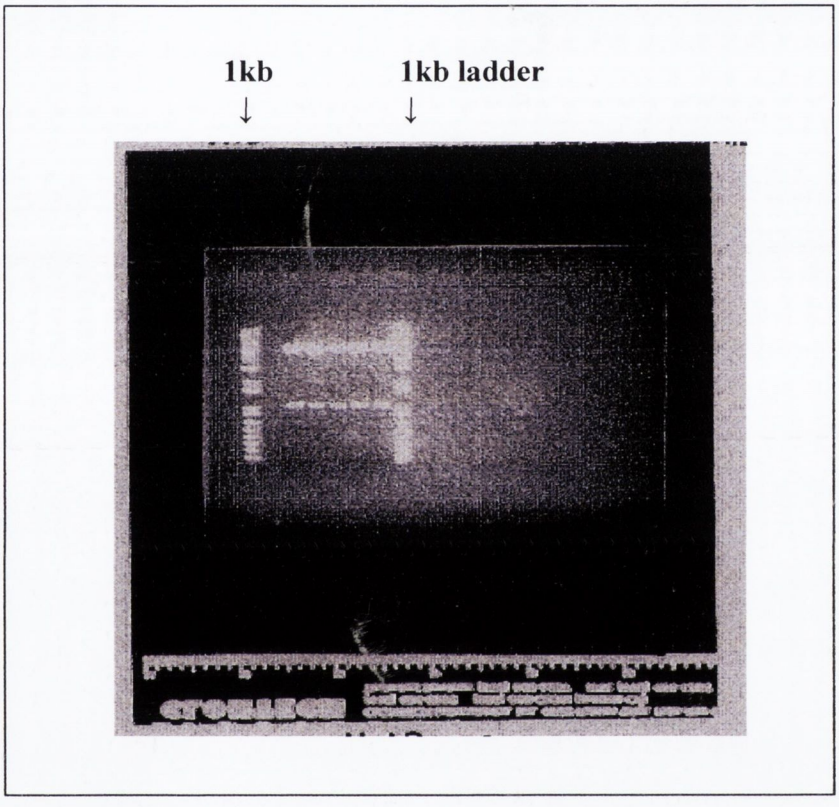


Figure 3.12 **Agarose gel electrophoresis comparing vector pcDNA3.1-C9 (5.4 kb) and insert C5L2 (1.1kb) bands with 1kb molecular weight ladder after digestion of C5L2pcDNA3.1$_{-C9}$ with *Asp* 178 and *Nhe* 1.**

3.3.2 Tissue expression of C5L2 and C5aR in man and mouse

Northern blot analysis was employed to assess tissue expression in human and murine tissues of C5L2 and C5aR (chapter II). In man, C5L2 is expressed most strikingly in human peripheral blood leukocytes, bone marrow, and spleen. C5aR is expressed most abundantly in human peripheral blood leukocytes, in addition to bone marrow, spleen, lung, placenta and heart (Figure 3.14).

In mouse, both C5L2 and C5aR are expressed in lung, spleen and heart but is highly expressed in bone marrow. (Figure3.15). Thus there is virtually congruent expression of both C5L2 and C5aR in all tissues studied.

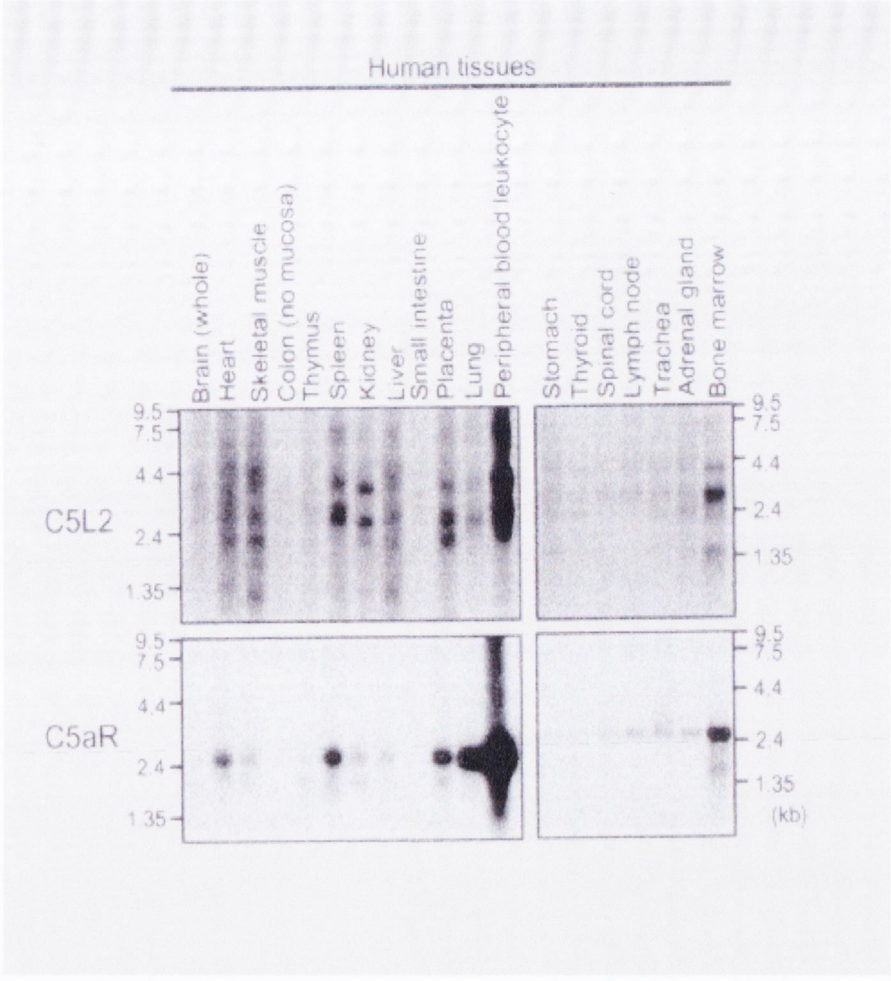


Figure 3.13 Northern blot analysis of C5L2 and the C5a receptor (C5aR) in human tissues. Human Multiple Tissue Northern (MTN) blots (Clontech) containing 2 micro gram (left panel) or 1 micro gram (right panel) of poly A+ RNA per lane. Blots were hybridised sequentially with human C5L2 (upper panel) or human C5a receptor (bottom panel) probes. Positions of RNA molecular markers are indicated.

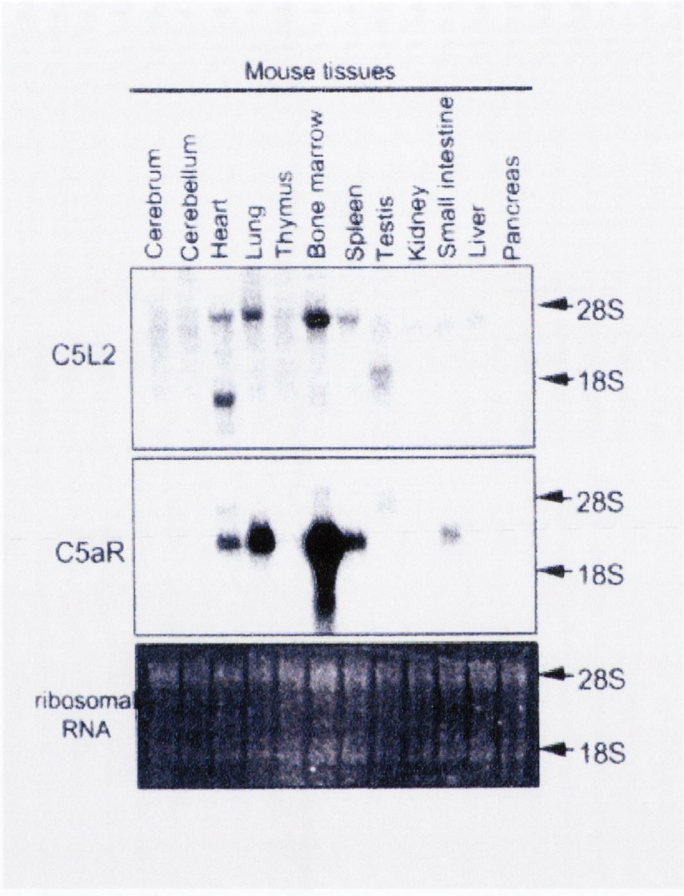


Figure 3.14 Northern blot analysis of C5L2 and the classical C5a receptor, (C5aR) in murine tissues. Northern blot analysis of C5L2 and the C5a receptor in the mouse. Ten micro grams of total RNA from murine tissues was electrophoresed on a gel, transferred to a nylon membrane, and hybridised sequentially with mouse C5L2 (upper panel) or mouse C5a receptor (middle panel) probes. Arrows indicate the positions of mouse rRNA as molecular markers. RNA stained with acridine orange is shown in the bottom panel to assess the quality of RNA.

3.3.3 Insertion of a point mutation at position 132 of DRY sequence, replacing leucine with arginine

Using site directed mutagenesis and the Quick-Change method (Stratagene), we successfully generated a plasmid encoding the human C5L2 in which the leucine at position 132 in the DRY sequence is mutated to arginine [C5L2(L132R)pcDNA3.1$_{-C9}$] (chapter II). Two oligonucleotide primers were made containing the desired mutation. PCR was performed, followed by Dpnl digestion. Appropriate sized bands were confirmed on agarose gel. The Dpnl digested PCR products were then transformed into DH5α competent cells and plated onto LB ampicillin plates and grown overnight. Glycerol stocks were made at this time point (chapter II). Plasmid DNA isolated by miniprep underwent agarose gel electrophoresis (Figure 3.16) and concentration read on U.V. spectrophotometer. C5L2(L132R)pcDNA3.1$_{-C9}$ minipreps were digested with *Pvu* I and run on an agarose gel and compared to control, C5L2pcDNA3.1$_{-C9}$ (Figure 3.17). The enzyme *Pvu* I (*Xor* II) is at the mutation site and was chosen using a pre-designed computer programme. Colonies were grown up overnight for maxi prep. Maxi prep DNA was subjected to agarose gel electrophoresis and concentration read on U.V. spectrophotometer. C5L2(L132R)pcDNA3.1$_{-C9}$ was confirmed by sequencing.

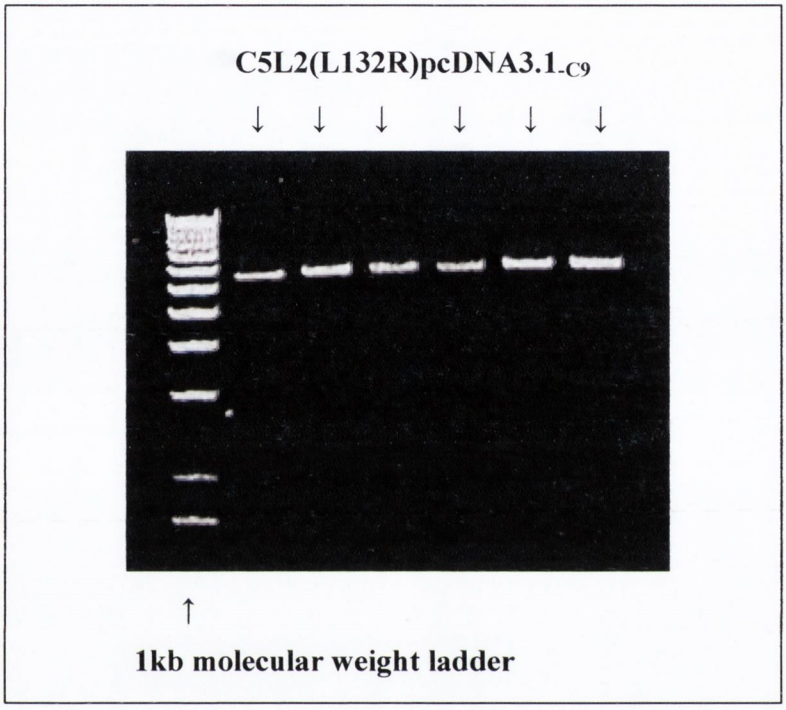


Figure 3.15 **Agarose gel comparing mini–preps of C5L2(L132R)pcDNA3.1-C9 i.e. "mutated C5L2", with 1kb molecular weight ladder**

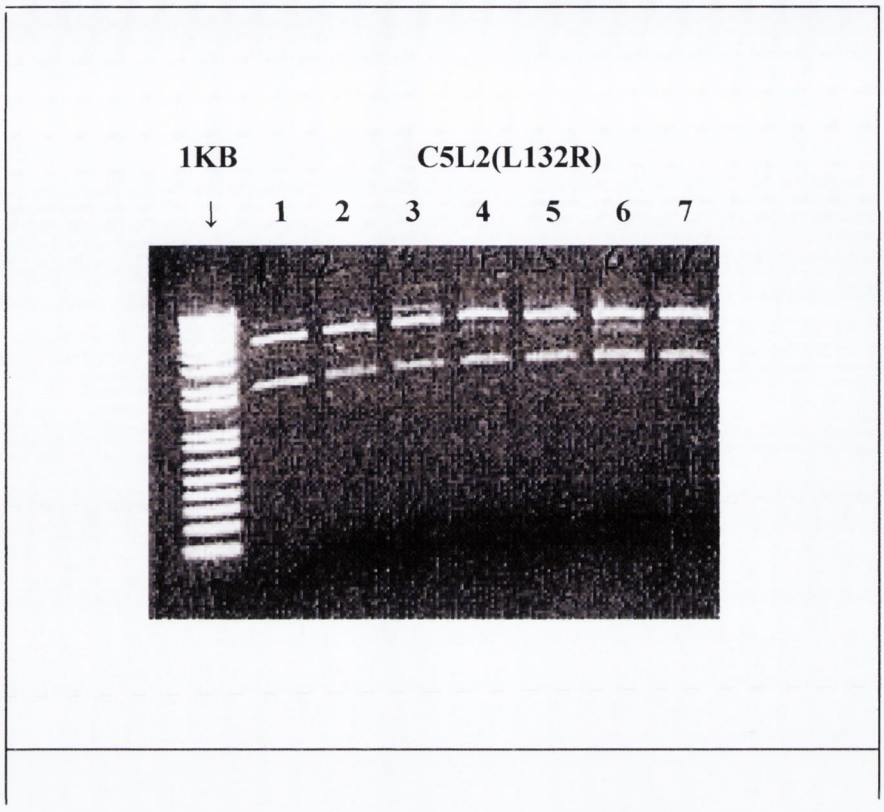


Figure 3.16 **Agarose gel electrophoresis comparing digested (*Pvu* I plus *EcoR* I) C5L2(L132R)pcDNA3.1-$_{C9}$ mini-preps with 1KB molecular weight marker.**

3.4 DISCUSSION

We suspected that a second C5a receptor existed because deletion of the known receptor, C5aR (CD88) failed to explain all of the complex effects of C5a *in vivo*. We cloned C5L2 from both mouse and man. Interestingly, comparing the aligned sequences of human and murine C5L2 we note that they possess 61.3% identity in the deduced protein and 68.9% identity in the nucleotide sequences. C5L2 and C5aR (CD88) have very similar binding and activation site-related sequences. Regarding the N terminus, we know that the binding domain of the human C5a receptor, C5aR consists of two distinct and physically separable subsites. One of these sites binds the C-terminal 8 amino acids of C5a, while the second site lies in the N terminus of the receptor and interacts with the core of C5a. By 1994, it was believed that the N terminus was responsible for 45% of the total energy for the binding of C5a (de Martino *et al.* 1994). Comparing human C5aR and human C5L2, the N termini of C5aR (CD88) and C5L2 contain several acidic residues that are characteristic of complement fragment receptors. These acidic residues for C5aR form part of the ligand binding site (Mery *et al.* 1993). C5aR has a distinct ligand binding site and activation site (Siciliano *et al.* 1994). Receptor activation by ligand involves the engagement of charged and uncharged residues on the extracellular faces of the transmembrane. For C5aR these include Ile^{116}, Val^{286}, Arg^{175}, Glu^{199}, Arg^{206}, and Asp^{282} (Geva *et al.* 2000, Crass *et al.* 1999). Interestingly, C5L2 shares all of these residues except for Asp^{282}, where there is a Glu residue. Thus C5L2 has a similar acidic ligand-binding N-terminal domain to C5aR and a similar activation domain.

We know that tyrosines at position 11 and 14 of the NH_2 terminal domain of the C5a receptor (C5aR/CD88) are post-translationally modified by the addition of sulphate groups (Farzan *et al.* 2001). The sulphate moieties of each of these tyrosines are critical to the ability of the C5aR to bind C5a and to mobilise calcium. A C5aR variant lacking these sulphate moieties efficiently mobilised calcium in response to a small peptide agonist, but not to C5a, consistent with a two–site model of ligand association in which the tyrosine–sulphated region of the C5aR mediates the initial docking interaction. A peptide based on the NH_2 terminus of the C5aR and sulphated at these two tyrosines, but not its un-sulphated analogue or a doubly sulphated control peptide, partially inhibited C5a association with its

receptor. These observations clarified structural and mutagenic studies of the C5a/C5aR association and suggested that related 7TMS receptors are also modified by functionally important sulphate groups on their NH_2-terminal tyrosines. Interestingly, we have shown that the N-terminus of C5L2 retains these important tyrosines flanked by acidic amino acids which undergo sulphation in C5aR.

Northern blot analysis demonstrated virtually congruent expression of C5L2 and C5aR in almost all tissues studied (mouse and man). Ohno and colleagues (Ohno *et al.* 2000*)* had demonstrated C5L2 to be present on immature but not mature dendritic cells. They speculated that C5L2 may regulate the activation of immature dendritic cells and play a role in chemoattraction of leukocytes to inflammatory regions. We demonstrated that both receptors are present in multiple organs including lung, heart and spleen. Importantly, in our hands, the most abundant source of message for both receptors is peripheral blood leukocytes and bone marrow. Expression of C5L2 was not detected in the latter by the Japanese group. Taking into account the major role C5a plays in host defence, it was exciting to see that human leukocytes and bone marrow were abundant sources of message for both receptors. This guided us to further investigate both regarding the role of C5L2 versus C5aR

Cloning C5L2 into pEGFP-N1 and transfecting this into a stable cell line permitted us to confirm receptor expression on the cell surface. We subsequently cloned it into the less bulky vector pcDNA3.1 to include a nine amino acid C-terminal tag (to facilitate recognition by the antibody 1D4). C5L2pcDNA3.1-$_{C9}$ was transfected into both stable and transient cell lines so that we could characterise C5L2 and study its biophysical properties, investigate if it is uncoupled to G proteins, and compare its characteristics to those of C5aR, the classical C5a receptor. In most identified G protein-coupled receptors, there is a highly conserved DRY consensus sequence. The DRY motif of G protein-coupled receptors is known to be involved in G protein activation. By 1992, it was demonstrated that Arg-^{123} is part of the highly conserved DRY sequence that is conserved in the great majority of identified G-protein coupled receptors. In addition, it was appreciated that the Asp^{71} residue is also highly conserved in most G protein coupled receptors, with only a

small number exhibiting substitutions (Probst *et al.* 1992).

In mutageneisis experiments of other receptors, the aspartic acid residues in the canonical tripeptide 'DRY' and the transmembrane segment (TMS-2) motif "LAVAD" have been implicated in G-protein interaction and conformational changes during activation (Baldwin *et al.* 1993). The C5a receptor also encodes an asparagine residue (Asn^{119}) at the position where the amino group of catecholamine drugs bind to Asp^{113} of β2 adrenergic receptors (Strader *et al.* 1998). Conservative mutations at these sites demonstrate that Asp^{132} is essential for the proper expression and trafficking of the C5a receptor.

Extensive work on two G.P.C.R.s, gonadotrophin releasing hormone receptor (Ballesteros *et al.* 1998) and the α_{1b}- adrenergic receptor (Scheer *et al.*1997) suggested that the arginine in the DRY sequence plays a key structural role in receptor activation. The crystal structure of rhodopsin clearly demonstrates the essential nature of the guanidinium group of the arginine residue in the DRY sequence for formation of multiple hydrogen bonds with residues in transmembrane helices 3 and 6 (Ballesteros *et al.* 1998). It was subsequently demonstrated that mutagenesis of this arginyl residue is associated with loss of function with respect to G protein activation, without alteration in ligand binding (Kolakowski *et al.* 1995). In 2000, it was confirmed that the DRY sequence is essential for not only G protein activation, but also phosphorylation–dependant arrestin binding (Bennett *et al.* 2000). Site directed mutagenesis has been very useful in the past at discovering detail regarding the C5a receptor and its binding to C5a.

When aligning the amino acid sequences of C5aR with mouse and human C5L2 an amino acid alteration is noted in the normally highly conserved DRY sequence, located at the end of the third transmembrane segment. Both human and murine C5L2 have a natural substitution of leucine for arginine at this site. In light of the above, we propose that C5L2 may be uncoupled to G proteins unlike the classical C5a receptor, C5aR. Mutageneisis studies have been extremely useful previously in identifying the role of certain amino acids in C5aR. We decided to utilise this technique.

Site directed mutagenesis was performed introducing a point mutation at position 132 in the DRY sequence and replacing leucine with arginine. We postulated that this might be associated with a "gain of function" and some evidence of coupling to G proteins. In view of the amino acid sequence similarity between C5L2 and C5aR, the co-location on the same region of chromosome 19, the virtually congruent expression of both receptors in many tissues in both mouse and man, and the complex effects of C5aR *in vivo,* not fully explained by deletion of the known receptor, we postulated that C5L2 may be a second C5a receptor. However, the differences between both receptors as outlined above, particularly the natural substitution of leucine for the "essential" arginine in the DRY sequence, pointed us to a receptor which may be uncoupled to G proteins and therefore may have a very different function, mode of action and biophysical properties to that of the classical C5a receptor, C5aR.

CHAPTER IV

BIOPHYSICAL PROPERTIES OF C5L2 VERSUS C5AR

CHAPTER IV

BIOPHYSICAL PROPERTIES OF C5L2 VERSUS C5AR

4.1 INTRODUCTION

4.1.1 General introduction

We wanted to characterise the orphan receptor C5L2 which we proposed may be a second C5a receptor based on its sequence similarity with C5aR (~30%), its localisation to the same region of chromosome 19 as the C5aR/formyl peptide receptor cluster (chromosome 19q13.4) (Ohno *et al.* 2000), and its widespread co-expression with C5aR in many tissues in both mouse and man. In addition, a second C5a receptor would help explain why deletion of the classical C5a receptor, C5aR does not fully account for all the known pro- and anti- inflammatory actions of C5a *in vivo*. Furthermore, a second C5a receptor would help explain the as yet uncharacterised function of C5aR expression on parenchymal cells in the lung, liver and smooth muscle.

Firstly we decided to examine the biophysical properties of C5L2. We wished to identify its potential ligands, testing in particular C5a, C3a, C4a and C5a's less potent derivative C5a des Arg. Next we wanted to determine the kinetics of binding of ligand to C5L2, followed by assessment of receptor phosphorylation and internalisation. All results would be compared to those of the classical C5a receptor, C5aR.

4.1.2 Potential ligands for C5L2

4.1.2.1 C5a and C5a des Arg

In the first stage in deactivation of anaphylatoxin activity, C5a is rapidly desarginated by serum carboxypeptidase N to the less potent derivative C5a des Arg ($C5adR^{74}$) (Bokisch *et al.* 1970). Both are potent mediators of leukocyte chemoactivity and play an important role

in the accumulation of leukocytes at sites of inflammation (Barker *et al.* 1986). The desarginated form has a different spectrum of bioactivity to intact C5a e.g. in human basophils, stimulation by intact C5a causes the release of lipid mediators (e.g. leukotriene C4) and cytokines (e.g. interleukin-4, IL-4 and IL-13), whereas C5adR^{74} stimulates only cytokine release (Egilite *et a*l. 2000). The molecular basis for the ability of different cell types to discriminate between intact C5a and C5adR74 has yet to be elucidated because until recently only a single receptor for C5a i.e. C5aR had so far been cloned (Gerard *et al.* 1991, Boulay *et al.* 1991). If C5L2 proved to be a second C5a receptor, then testing its affinity for C5a des Arg would be a logical next step.

4.1.2.2 C5a hexapeptide

The amino terminus of the human C5a receptor is required for high affinity C5a binding and for receptor activation by C5a but not by C5a analogues. C5a hexapeptide analogue Phe-Lys-Ala-dCha-Cha-dArg (De Martino *et al.* 1994) was synthesised commercially (New England Peptide). It is a full agonist of the C5a receptor and is modelled on the C-terminus of C5a. It interacts only with the pharmacophore site made up of the transmembrane helices and the juxta membrane regions of the receptor (De Martino *et al.* 1994).

4.1.2.3 C3a

C3a, like C5a has a wide range of effects in humans. It was initially described as a leukocyte chemoattractant and anaphylatoxin like C5a. Both have important roles in microbial host defence, immune regulation, (Kohl J. 2001) and protection against toxic insult (Bhatia *et al.* 2001, Mukherjee *et al.* 2000, Mastellos *et al.* 2001, and O'Barra *et al.* 2001). Both are reported to have psychopharmacological effects on feeding and drinking behaviour (Schupf *et al.* 1983, Schupf, *et al.* 1989). Both fragments are rapidly desarginated by serum carboxypeptidase, which modulates their function. Although C5a des-Arg ^{74} retains most of the activity of intact C5a, albeit with a generally lower affinity

for the C5a receptor (C5aR), C3a des-Arg ^{77} activity is profoundly reduced relative to C3a with respect to immunologic function. No binding of the C3a des-Arg^{77} form to the previously cloned and characterised C3a receptor (C3aR) is observed in transfected red blood cells or mouse macrophage/ monocytes (Zwirner *et al.* 1998). Unlike C3a, C3a des-Arg^{77} does not stimulate eosinophil chemotaxis (Daffern *et al.* 1995), prostanoid production by guinea pig peritoneal macrophages and rat kupffer cells (Puschel *et al.* 1996) or human monocyte-like U937 cell degranulation (Klos *et al.* 1992).

4.1.2.4 C4a

C4 is a protein of the classical pathway of complement activation. C4 is cleaved to an active form C4a, which is a peptide mediator of inflammation with weak activity, and C4b, which covalently binds to pathogen and opsonises it.

4.1.3 Why we used C5a hexapeptide for receptor internalisation experiments

We wanted to characterise the feature of receptor phosphorylation and internalisation following ligand binding for C5L2 compared with that of C5aR. We knew that a characteristic feature of G protein coupled receptors (G.P.C.R.s) is their rapid internalisation into endocytic vesicles following the binding of ligand. Ligand–stimulated receptor internalisation appears to function in both dephosphorylation (re-sensitisation) and signalling of activated receptor. Several endocytic mechanisms, including clathrin-mediated and caveolae-mediated endocytosis appear to play roles for G.P.C.R.s. β arrestins appear to function as endocytic adapter proteins in addition to their previously described role in the termination of G- protein coupling (Lefkowitz *et al.* 1998).

Our antisera were directed against the N-terminal sequences of both receptors. We knew that the N-terminal sequence of C5aR is important for ligand binding (Farzan *et al.* 2001). We were concerned that bound C5a might interfere with the binding of antibody by stearic competition and therefore interfere with our evaluation of internalisation. To avoid this

potential problem we used C5a hexapeptide. C5a hexapeptide analogue Phe-Lys-Ala-dCha-Cha-dArg (De Martino *et al.* 1994) was synthesised commercially (New England Peptide). C5a hexapeptide is a full agonist of the C5a receptor.

4.2 OBJECTIVES

To identify the biophysical properties of C5L2 by

1) Identifying ligand(s) for C5L2
2) Measuring the kinetics of ligand binding to C5L2
3) Assessing if C5L2 is phosphorylated after ligand binding and if so, to what extent and in what time frame
4) Determining if C5L2 is internalised after ligand binding
5) Comparing results of above experiments with those of C5aR which we would study in parallel

4.3 RESULTS

4.3.1 Competition binding studies

4.3.1.1 Competition binding studies using C5aR/L1.2.cells

L1.2 cells were stably transfected with C5aR as described earlier (chapter II) and incubated with 0.1nM ^{125}I-C5a and 0-300 nM unlabelled C5a and 0-13.2μM C5a des Arg for 30 minutes at 37°C in binding buffer (appendix). Cells were washed and bound ligand quantitated by γ-counting. All points were determined in duplicate and at least three independent experiments were performed for each. Results are expressed as the % maximal binding (± SEM) and data analysed using PRISM software testing one and two site models (chapter II). C5aR/L1.2 cells bound C5a with an apparent Kd of 3.4 $\pm$ 0.7 nM (n = 4)

(figure 4.1 and table 4.1). These results are consistent with previous reports (Gerard *et al.* 1991). Testing C5aR/L1.2 cells, C5a des Arg competed with ^{125}I-C5a with approximately 200-fold less avidity than C5a, with a Kd of 660 $\pm$ 153 nM (n = 4) (figure 4.1 and table 4.1). Similar results were obtained for C5a and C5a des Arg in C5aR/293T cells.

4.3.1.2 Competition binding studies using C5L2/L1.2 cells

L1.2 cells were stably transfected with C5L2 as described earlier (chapter II) and were studied in parallel with C5aR/L1.2 cells. C5L2/L1.2 cells bound human C5a with an affinity almost identical to the C5aR [Kd, = 2.5 $\pm$ 0.4 nM, (n = 7), (Figure 4.2 and Table 4.1). This confirmed our hypothesis and proved that C5L2 is a high affinity C5a binding protein and the second known C5a receptor. In contrast to C5aR, C5L2/L1.2 cells exhibit an affinity for C5a des Arg almost as high as for C5a with a Kd of 12.0 $\pm$ 2.3 nM (n = 4), Figure 4.2 and Table 4.1). Thus on identical cell types, C5L2 is approximately 50 fold more avid in binding C5a des Arg than the C5a receptor, C5aR (Figure 4.2 and Table 4.1).This is likely significant when identifying a potential role for C5L2. Similar results were obtained for identical experiments with C5L2/293T cells.

4.3.1.3 Competition binding studies with human peripheral blood neutrophils

Human peripheral blood neutrophils were prepared as described earlier (chapter II). When similar competition binding studies were performed using human peripheral blood neutrophils, (expressing both C5aR and C5L2, see chapter V), with increasing concentrations of C5a (0-300nM) and C5a des Arg (0-13.2μM), the apparent Kd for C5a was the same as on either L1.2 transfectant [Kd = 4.5 + 0.4 nM (n = 3)], (Table 4.1 and Figure 4.3). However, C5a des Arg was even <u>less</u> effective in competing with ^{125}I-C5a binding than the C5aR alone, exhibiting an apparent Kd of 1.9 $\pm$ 0.1 μM (n = 4), (Table 4.1 and Figure 4.3). Thus C5L2 exhibits a higher affinity for C5a des Arg, (~ equal to that of C5a) than C5aR, which in turn exhibits a higher affinity than do human neutrophils. These

results suggest a complex, potentially regulatory interplay between the C5aR and the C5L2 expressed on the same cell type.

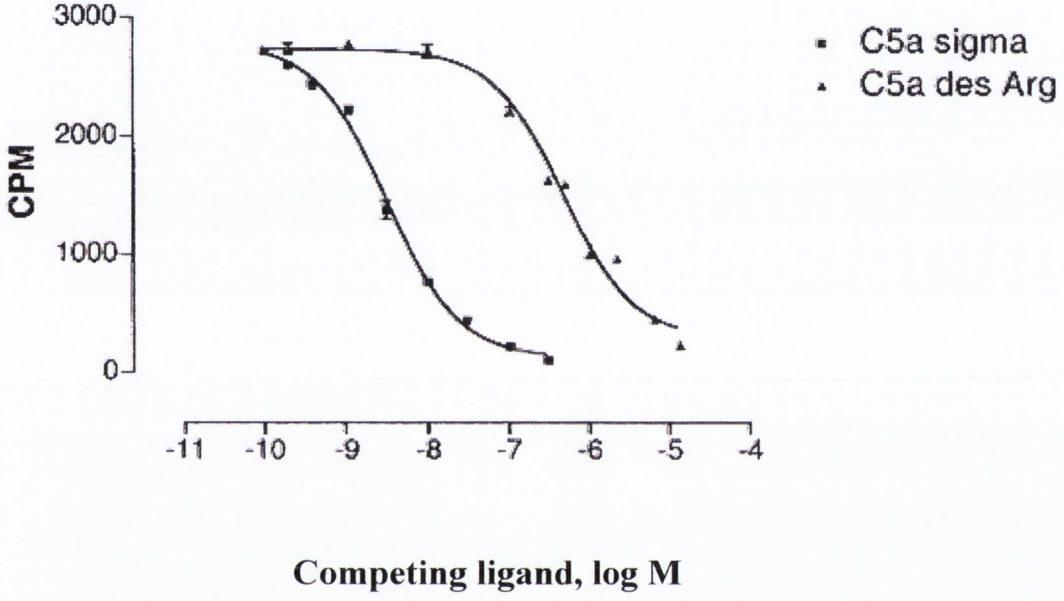


Figure 4.1 Competition binding studies with C5aR/L1.2 cells comparing binding affinities for C5a and C5a des Arg

1 x 10^6 C5aR/L1.2 cells were incubated with 0.1 nM ^{125}I-C5a and 0-300nM unlabelled C5a and 0-13.2μM C5a des Arg for 30 minutes at 37°C in binding buffer (appendix). Cells were washed and bound ligand quantitated by γ-counting. All points were determined in duplicate and at least three independent experiments were performed for each. Results are expressed as percentage maximal binding ± SEM and data analysed using PRISM software.

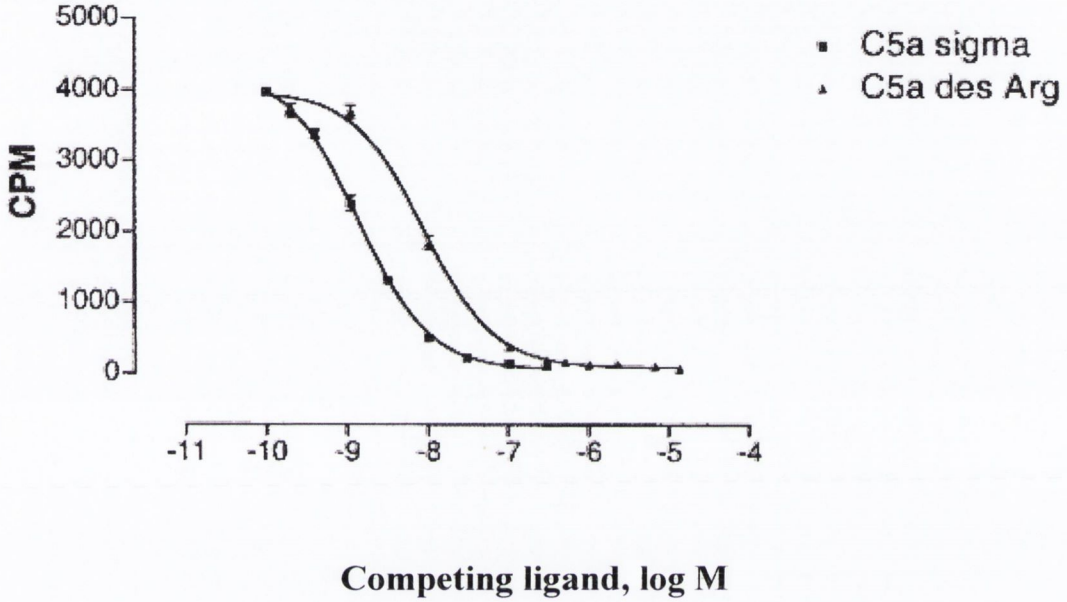


Figure 4.2 Competition binding studies with C5L2/L1.2 cells comparing binding affinities for C5a and C5a des Arg

C5L2/L1.2 cells were incubated with 0.1nM ^{125}I-C5a and 0-300 nM C5a or 0-13.2μM C5a des Arg for 30 minutes at 37°C in binding buffer (appendix). Cell were washed and bound ligand was quantitated by γ-counting. Results are expressed as percentage maximal binding ± SEM, are derived from at least three independent experiments and data analysed using PRISM software.

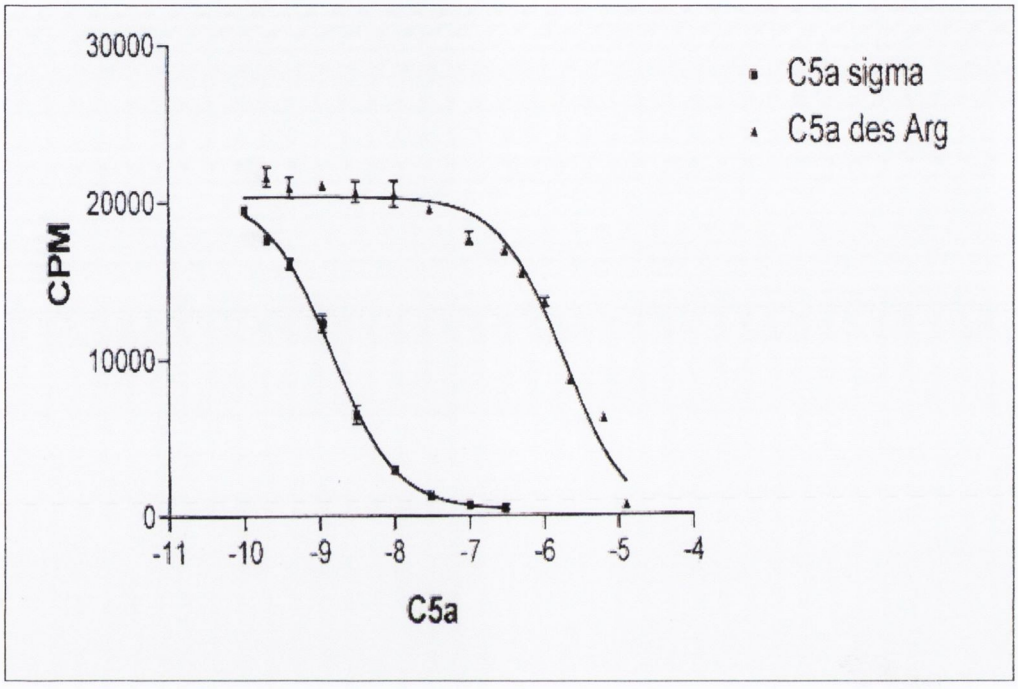


Figure 4.3 Competition binding studies with human peripheral blood neutrophils comparing binding affinities for C5a and C5a des Arg.

1 x 10^6 human neutrophils were incubated with 0.1nM ^{125}I-C5a and increasing concentrations of C5a (0-300nM) and C5a des Arg (0-13.2μM) for 30 minutes at 37°C in binding buffer (appendix). Cells were washed and bound ligand was quantitated by γ-counting. All points were determined in duplicate and at least three independent experiments were performed for each. Results are expressed as the % maximal binding ± SEM and data was analysed using PRISM software.

4.3.1.4 Competition binding studies with C3a and C3aR/293T, C5L2/293T and C5aR/293T cells

When HEK293T cells were transiently transfected with C5L2, C5aR or C3aR, (as described earlier, chapter II), only C3aR expressing cells exhibited binding of ^{125}I –C3a. The Kd was 5.1 $\pm$ 1.3 nM, concurring with previous results. No C3a binding was detected in the C5L2/HEK293T or the C5aR/HEK293T cells (Table 4.1 and Figure 4.4). Thus, in our hands C3a is not a ligand for C5L2.

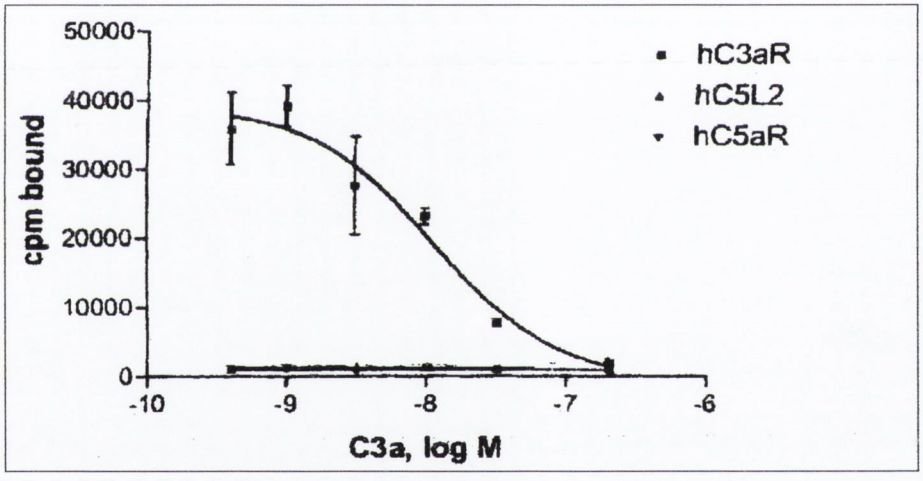


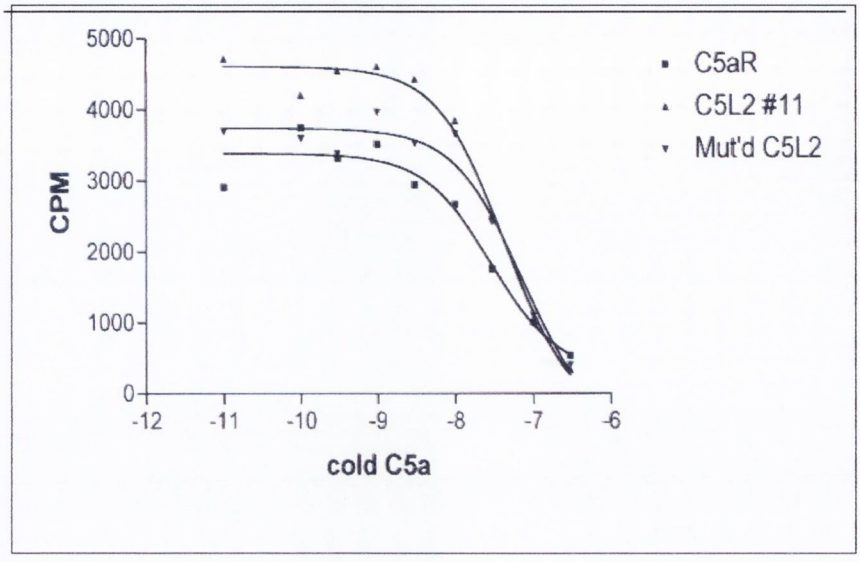
Figure 4.4 Competition binding studies measuring binding affinity for C3a in C3aR/293T, C5L2/293T and C5aR/293T cells. C3a is not a ligand for C5aR or C5L2. 1x 10^5 cells were incubated with 0.1nM ^{125}I-C3a and increasing concentrations (0-300nM) of unlabelled C5a, C3a or C5a des Arg (0- 13.2μM) for 30 minutes at 37°C in binding buffer (appendix). Cells were washed by centrifugation and bound ligand was quantitated by γ counting. Results are expressed as the % maximal binding ± SEM and data was analysed using PRISM software.

4.3.1.5 Competition binding studies with C5L2/L1.2 cells and C4a

In our hands C4a is not a ligand for C5L2 and did not compete with ^{125}I-C5a on C5L2/L1.2 cells (Table 4.1).

4.3.1.6 Competition binding with C5L2(L132R)/293T, C5L2/293T and C5aR/293T cells and C5a

Site directed mutagenesis was performed replacing the leucine at position 132 in the DRY sequence of C5L2 with arginine (chapter II). This arginine had previously been shown to be important for G protein coupling. HEK293T cells were transiently transfected as described earlier (chapter II) with "mutated C5L2" i.e. C5L2(L132R), "wild type C5L2" and C5aR. C5L2(L132R)/293T cells bind C5a well, though less well than either C5L2 or C5aR: the latter two both being high affinity binders for C5a. The Kd for C5L2(L132)/293T cells is $3.67 \times 10^{-8} \pm 1.1$ versus the Kds for C5L2 and C5aR being 2.5 nM ± 0.4 and 3.4 nM ± 0.7 respectively (Table 4.1 and Figure 4.5).



Competing ligand, log M

Figure 4.5 Competition binding study comparing binding affinities for C5a in C5aR/293T, C5L2/293T and C5L2(L132R)/293Tcells.

Cells were incubated with 0.1nM ^{125}I-C5a and increasing concentrations of unlabelled C5a. C5L2 (L132R)/293T cells bind C5a less well than C5L2/293T or C5aR/293T cells. The former has a Kd of 3.67 x 10^{-8} ± 1.1 whereas the latter two have a KD of 2.5 nM ± 0.4 and 3.4 nM ± 0.7 respectively (Table 4.1).

4.3.1.7 Scatchard analyses

Comparison of Scatchard analyses of "sites per cell" on L1.2 cells and the fluorescence intensity on both L1.2 cells and human polymorphonuclear leukocytes (PMNs) suggests similar levels of expression of C5aR and C5L2 on this cell population. Further analyses of the displacement binding curves by one and two–site models provides no compelling evidence to distinguish the two. Thus, the reduced affinity for C5a des Arg on human PMNs is not as simple as the net effect of the presence of both receptors.

4.3.1.8 Summary of competition binding experiment as in Table 4.1

Table 4.1 summaries all the competition binding experiments performed with stable and transiently transfected cells and human PMNs. C5aR bound C5a with a Kd of 3.4 $\pm$ 0.7 nM, consistent with previous findings. C5a des Arg competed with approximately 200 fold less avidity than C5a with a Kd of 660 $\pm$ 153 nM. C5L2 is a high affinity binding receptor for C5a with almost an identical Kd (2.5 $\pm$ 0.4 nM) for C5a as C5aR/L1.2 cells. Unlike C5aR/L1.2 cells, C5L2/L1.2 cells had an affinity for C5a-des Arg almost as high as that for C5a (Kd of 12.0 $\pm$ 2.3nM). In our hands, C4a did not compete with ^{125}I-C5a on C5L2/L1.2 cells. When HEK293T cells were transiently transfected with C5L2, C5aR or C3aR plus Gα16 (chapter II), only C3aR expressing cells exhibited binding of ^{125}I-C3a (Kd 5.1 $\pm$ 1.3nM). Competition binding experiments performed with human PMNs demonstrated a similar K_d for C5a as on either L1.2 transfectant. However, C5a des Arg was even less effective in competing with ^{125}I-C5a than the C5aR alone (Kd of 1900 $\pm$ 126nM). C5L2(L132R)/293T cells bound C5a with a Kd of 3.67 x 10^{-8} $\pm$ 1.1, versus C5L2/293T and C5aR/293T cells which bound C5a with a Kd of 2.5nM $\pm$ 0.4 and 3.4 $\pm$ 0.7 respectively.

Table 4.1 Binding affinities for C5a and C5a des Arg on L1.2 cells, 293T transfectants or peripheral blood neutrophils

A) 0.1nM ^{125} I-C5a[a]

Apparent affinity nm± SEM

Competing ligand

Cell Type	C5a	C5a des Arg	C4
C5L1/L1.2	2.5 ± 0.4 (7)	12.0 ± 2.3[b] (4)	Not detected (3)
C5aR/L1.2	3.4 ± 0.7 (4)	660 ± 153[b] (4)	Not detected (3)
Human PMNs	4.5 ± 0.4 (3)	1900 ± 126[b] (4)	Not done

B) 0.5nM^{125}I-C3a[a]

Apparent affinity nm± SEM

Competing ligand

Cell type	C3a
C5L2/293T	Not detected (3)
C5aR/293T	Not detected (3)
C3aR/293T	5.1±1.3 (4)

[a]A) Cells were incubated with 0.1nM ^{125} I –C5a and unlabelled C5a, C5a des Arg, or C4a in competition binding assays or B) with 0.5nM ^{125}I-C3a and unlabelled C3a, as indicated in experimental procedures, and apparent affinities (± SEM) calculated using PRISM software. All points were measured in duplicate and ≥ three separate experiments were performed for each. Results were expressed as mean cpm ± SEM and data analysed using PRISM software.

[b] significantly different at $p < 0.005$.

4.3.2 Kinetics of binding C5a to C5aR compared with C5L2

The differences in apparent affinities of C5a des Arg between C5aR and C5L2 may reflect differences in binding kinetics because apparent affinities are determined by ligand on- and off- rates. It was logical to measure the "on"- and "off- rates" of C5a on C5aR and C5L2 to help explain these differences. When the kinetics of C5a binding were assessed using L1.2 transfectants at $0^{o}C$ in the presence of sodium azide to prevent internalisation (chapter II), we observed that the C5aR has a very rapid "on- rate", essentially at equilibrium within the first minutes following addition of ligand. In contrast, under identical conditions the "on-rate" for C5L2 receptor was ~100 – fold slower, requiring at least 60 minutes to reach equilibrium (Figure 4.6). At equilibrium, addition of excess unlabelled C5a to displace the radioligand as an estimate of the "off- rate" revealed that the C5aR released ligand 3 fold faster than C5L2 (Figure 4.6).

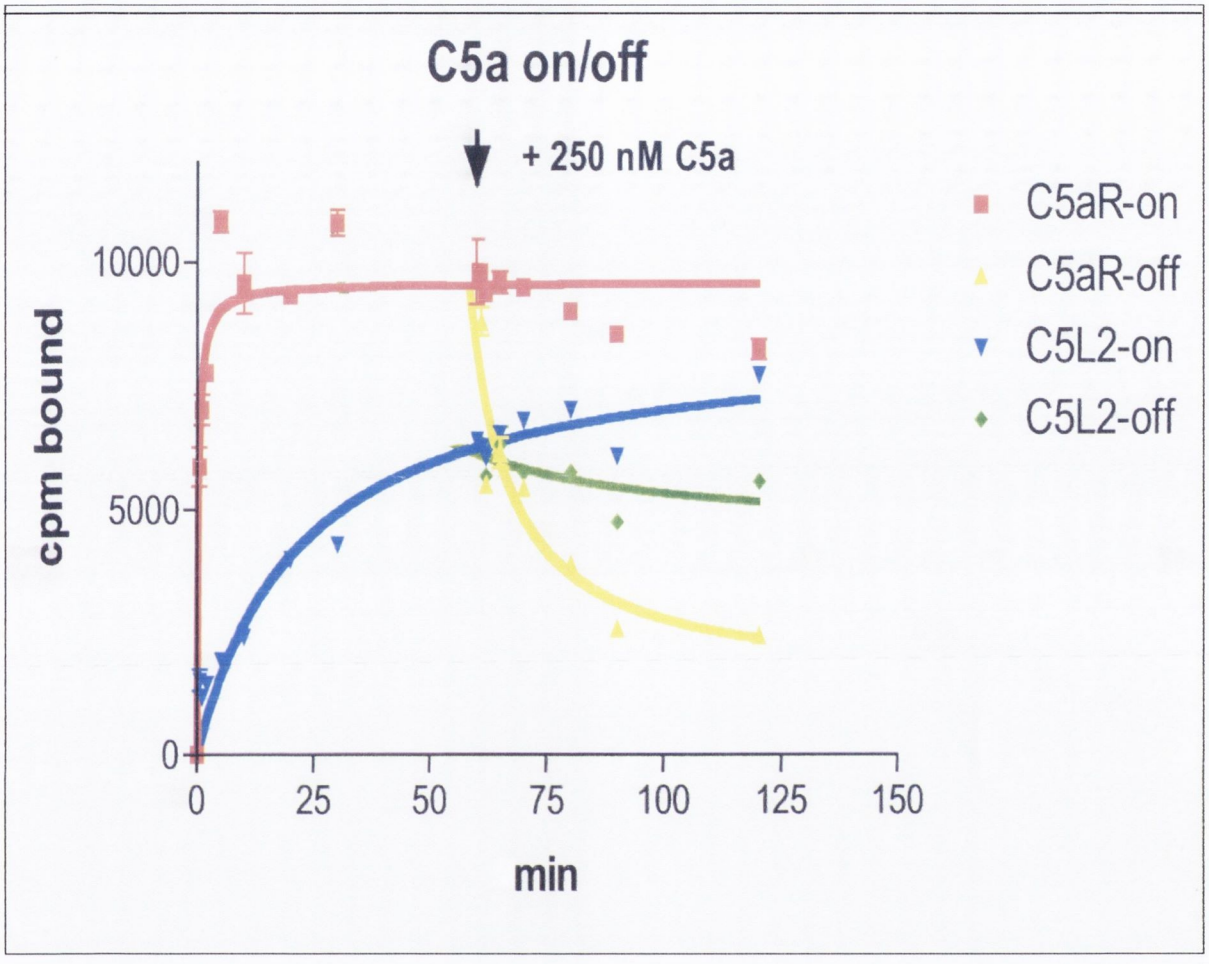


Figure 4.6: Kinetics of binding C5a to C5aR compared with C5L2

Kinetics of binding on stably transfected L1.2 cells was determined by incubating cells in binding buffer containing 0.2% sodium azide, at 1 x 10^7/ml with 0.1 nM ^{125} I–C5a at 0°C. Duplicate aliquots were removed as a function of time for up to 60 minutes and bound ligand was assessed. The remaining samples were divided into equal parts, 250 nM C5a was added to one and buffer to the other, and samples were removed with time for an additional 60 minutes. All points were determined in duplicate, and at least three independent experiments were performed for each. Results were expressed as the mean count per minute (cpm) bound (± SEM), and data analysed using PRISM software.

4.3.3 Receptor phosphorylation: C5aR compared with C5L2

It had previously been demonstrated that when C5a ligand binds the classical C5a receptor, C5aR, the receptor is phosphorylated and subsequently internalised (Naik *et al.* 1997). We wanted to investigate if C5L2, which we had just proven to be a second C5a receptor, exhibited the same characteristics. Both C5aR/L1.2 and C5L2/L1.2 cells were studied in parallel, suspended with 1mCi carrier-free ^{32}P-labelled orthophosphoric acid and stimulated with C5a. Receptors were immunoprecipitated with 1D4 antibody covalently cross-linked to protein A Sepharose beads for 1 hour at 4°C with gentle agitation. Samples were washed, eluted with SDS sample buffer under reducing conditions for 10 minutes at 60°C and analysed by 10% SDS-PAGE, fixed, dried and exposed to BioMAx MR film. Cells were stimulated with C5a for 5 minutes (Figure 4.7 top) or as a function of time (Figure 4.7 bottom) (chapter II). Dr. Shoji Okinaga performed the immuno-precipitation and SDS polyacrylamide electrophoresis component of this experiment.

Interestingly, both C5aR and C5L2 exhibit a basal level of phosphorylation in L1.2 transfectants in the absence of ligand. However, as indicated in Figure 4.7, C5aR is robustly phosphorylated after the addition of 200 nM C5a for 5 minutes, while only a modest increase in phosphorylation is seen for C5L2 studied under the same conditions. Regarding "time to maximum phosphorylation", C5aR is robustly phosphorylated in response to 5 minutes exposure to agonist. In contrast, a modest increase is seen for C5L2 which is maximal at 5-10 minutes and returns to baseline by 15 minutes.

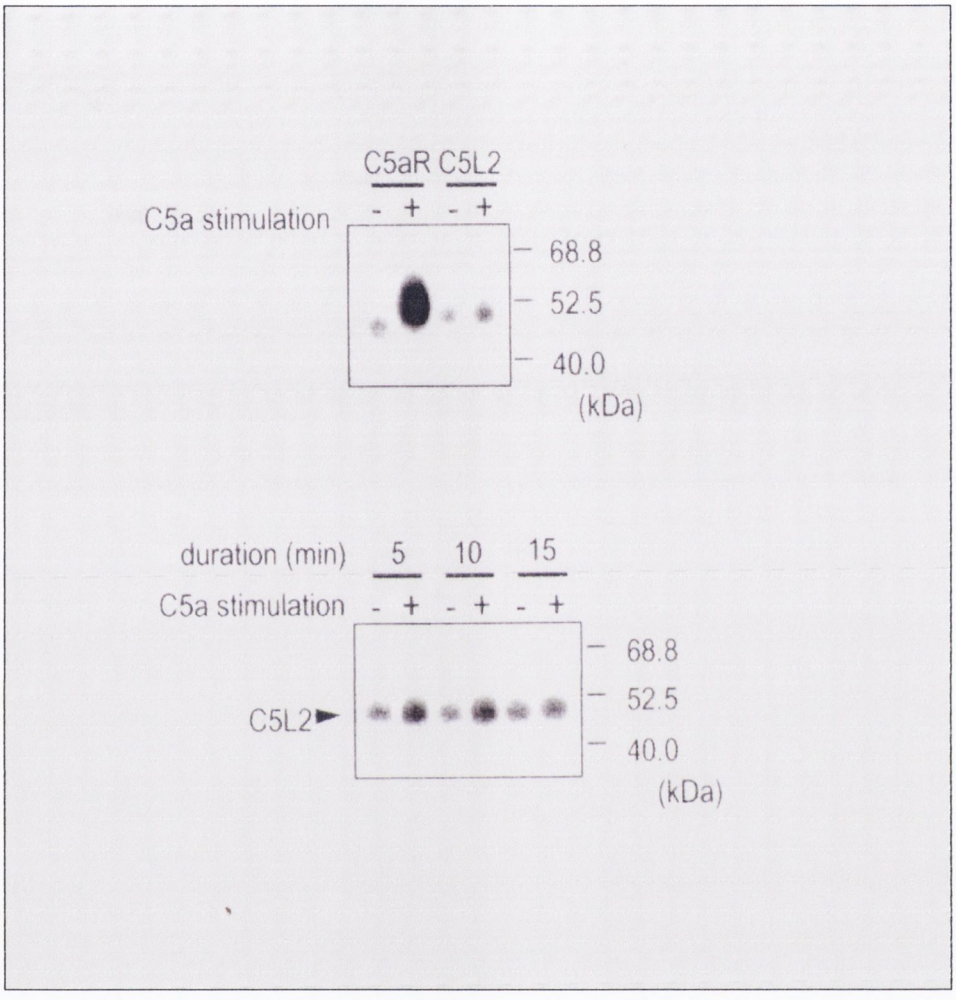


Figure 4.7 **Receptor Phosphorylation: C5aR compared with C5L2.**

Stable L1.2 transfectants were metabolically radiolabelled with [^{32}P]-orthophosphoric acid and stimulated with 0 or 200 nMC5a. Receptors were immunoprecipitated and analysed by 10% SDS-PAGE. Cells were stimulated with C5a for 5 minutes (top) or as a function of time (bottom). (Dr. Shoji Okinaga)

4.3.4 Receptor Internalisation

A characteristic feature of G protein coupled receptors is their rapid internalisation following the binding of ligands. Naik and colleagues (Naik *et al.* 1997) had previously demonstrated that C5aR is rapidly phosphorylated and internalised after ligand binding. We have demonstrated that C5L2 undergoes a modest phosphorylation after ligand binding in comparison to C5aR which is robustly phosphorylated. We wanted to characterise this feature for C5L2 compared with the C5aR.

Previously, in our laboratory Farzan and colleagues (Farzan *et al.* 2001) had demonstrated the importance of the N-terminal sequence of the C5aR for binding ligand. Our antibodies were directed against the N-terminal sequence of both receptors. We were concerned that bound C5a might interfere with the binding of antibody by stearic competition. To avoid this potential problem we used C5a hexapeptide which is modelled on the C-terminus of C5a and is a full agonist of the C5a receptor but interacts only with the pharmacophore site composed by the transmembrane helices and the juxtamembrane regions of the receptor (De Martino *et al.* 1994).

4.3.4.1 Competition Binding experiments with C5aR/L1.2 and C5L2/L1.2 cells and competing ligand C5a hexapeptide

First we demonstrated that C5a hexapeptide is capable of competing with ^{125}I-C5a, and binds to both C5aR and C5L2 with similar affinities. The apparent affinities are $27 \pm 9 \times 10^6$ and $18 \pm 7 \times 10^6$ respectively (Table 4.2).

Table 4.2 Binding affinities for C5a hexapeptide on C5L2/L1.2 and C5aR/L1.2 cells

Competing ligand cell type	C5a hexapeptide apparent affinity μM + SEM
C5L2/L1.2	18 ± 1 (n = 3)
C5aR/L1.2	27 ± 9 (n = 3)

Cells were incubated with 0.1nM ^{125} I-C5a and increasing concentrations of unlabelled C5a hexapeptide (0-1mM) for 30 minutes at 37°C in binding buffer. Cells were washed by centrifugation and bound ligand was quantitated by γ counting. All points were determined in duplicate and at least 3 independent experiments were performed for each. Apparent affinities (± SEM) calculated using PRISM software for n independent experiments (chapter II). C5a hexapeptide competes with ^{125}I-C5a and has a similar binding affinity for both C5aR and C5L2.

4.3.4.2 Receptor internalisation: C5aR compared with C5L2

Assessment of receptor internalisation was performed utilising human PMNs incubated with the synthetic C5a hexapeptide at the concentrations indicated for 5 minutes at 37°C (Figure 4.8). Samples were transferred to 0°C and receptor remaining on the cell surface was assessed by flow cytometry (chapter II).

We demonstrated that C5aR is rapidly internalised as a function of ligand concentration, to a level of ~50% within 5 minutes. In marked contrast, the surface expression of C5L2 remains virtually unchanged. Thus C5L2 appears to be anchored to the cell surface and uncoupled to internalisation in response to ligand binding. These results concur with our

previous findings that C5L2 undergoes only a modest phosphorylation after the addition of ligand in comparison to the robust response of C5aR.

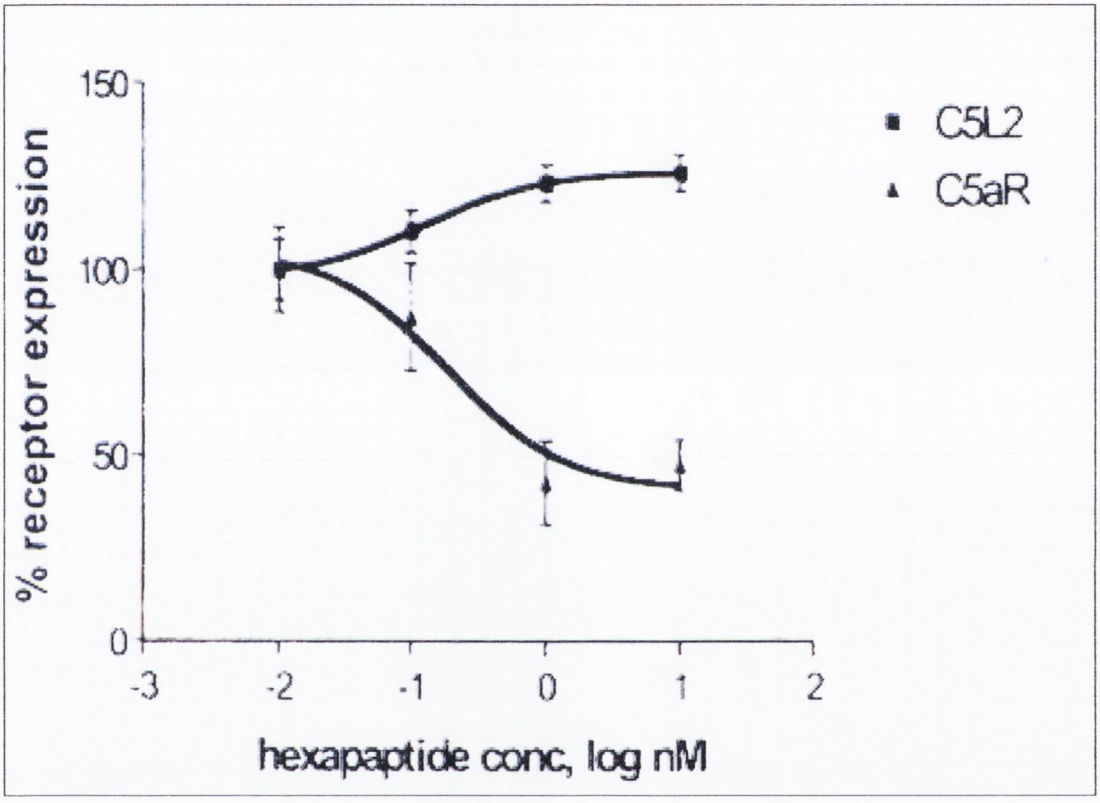


Figure 4.8 Internalisation of C5aR and C5L2 on human PMNs

Human peripheral blood neutrophils were incubated with 0-10 μM C5a hexapeptide, for 5 minutes at 37°C. Cells were subsequently transferred to ice, and receptor expression was evaluated by flow cytometric analyses. C5aR is rapidly internalised as a function of ligand concentration as expected. This reaches a level of ~50% over a period of 5 minutes. The surface expression of C5L2 remains virtually unchanged despite increasing ligand concentration.

4.4 DISCUSSION

Our results demonstrate that C5L2 is a high affinity binding receptor for C5a. In addition we demonstrated that C5L2 binds C5a des Arg (K_d, = 12.0 $\pm$ 2.3 nM) with almost the same affinity as it binds C5a (Kd = 2.5 $\pm$ 0.4 nM). This is in contrast to C5aR which binds C5a des Arg approximately 200 fold less (Kd = 660 $\pm$ 153 nM) than it does C5a (Kd = 3.4 $\pm$ 0.7 nM). C5L2(L132R)/293Tcells , i.e. "mutated C5L2" transfected cells have a similar affinity for C5a as does C5L2/293T and C5aR/293T cells. Competition binding experiments performed with human PMNs demonstrate a similar binding affinity (Kd = 4.5 $\pm$ 0.4 nM) for C5a as on either L1.2 transfectant. However, C5a des Arg was even less effective in competing for ^{125}I-C5a than the C5aR alone (Kd = 1900 $\pm$ 126 nM). These results suggest a complex, potentially regulatory interplay between the C5aR and the C5L2 expressed on the same cell type. Analyses of displacement binding curves by one or two site models were unhelpful. The reduced affinity of C5a des Arg on human PMNs is not as simple as the net effect of the presence of both receptors. In our hands neither C3a nor C4a are ligands for C5L2.

Both C3a and C5a are rapidly degraded by serum carboxypeptidase N or carboxypeptidase R to their respective des Arg derivatives (Campbell *et al.* 2001). At physiological concentrations, C3a des Arg is inactive at inducing smooth muscle contraction and increases in vascular permeability, whereas C5a des Arg retains approximately 10% of the activity of intact C5a. In a local setting where both C5a and C5a des Arg are present, with dynamically increasing quantities of C5a des Arg relative to C5a, C5L2 may serve to spare the C5a to interact with the functional receptor.

Apparent affinities are determined by ligand association binding and dissociation binding. Therefore if C5aR and C5L2 had different affinities for ligand, this may be explained by different binding kinetics. To date the association and dissociation rates (i.e. the “on” and “off rates”) for C5L2 were as yet unknown. The rapid “on rate” of C5aR, essentially at equilibrium within a few minutes after the addition of C5a ligand, which we demonstrated concurs with previous work (Naik *et al.* 1997). They demonstrated that upon challenge with

C5a, the classical C5a receptor undergoes a rapid phosphorylation on serine residues in the carboxyl–terminal region and is rapidly internalised into endosomes that cluster near the nucleus after 10 minutes. This demonstrated that the C5aR association binding ("on rate") was fast. Internalisation of a non-phosphorylable mutant was severely impaired relative to the wild type receptor. In contrast to C5aR, we found that C5L2 displays greatly retarded association kinetics with C5a, so that at low concentrations of C5a, such as might occur in a gradient *in vivo*, the classical C5aR would effectively sequester the available C5a. As higher concentrations of C5a are encountered, with the effect of reducing locomotion of the cells (chemotaxis curves are classically bell shaped), other C5a dependant effects such as degranulation and local release of products of NADPH oxidase are elicited. Under these conditions, the C5L2 may effectively sequester C5a des Arg. This would argue for C5L2 acting as a decoy receptor. In view of the retarded kinetics of C5L2 compared with C5aR, (C5L2 requires ~sixty minutes to reach equilibrium), under the conditions of binding to human neutrophils, the classical C5a receptor may be the only one detected. C5L2's quality of slow dissociation (three fold slower than that of C5aR) may be consistent with its role as a modulator of anaphylatoxin concentration, effective in limiting the inflammatory response to C5a.

In 1995, it was demonstrated that binding of C5a to its receptor triggered events leading to both stimulation of cellular responses and receptor desensitisation in myeloid cells (Giannini *et al.* 1995). It was noted that agonist occupied C5aR can be fully phosphorylated and that receptor phosphorylation was transient, with a half life of 30-40 minutes. This data suggested that internalised C5aR is recycled to the plasma membrane with a time course consistent with the kinetics of dephosphorylation. It was concluded that de-phosphorylation of C5aR might be essential to receptor recycling and re-sensitisation. Later that year, the same group demonstrated that treatment of C5aR with C5a resulted in rapid hyper-phosphorylation of the C5aR and that this phosphorylation was restricted to the six serine residues of its carboxyl-terminal tail. This segment extended from Ser ^{332} to Ser ^{338} (Giannini *et al.* 1995). By 2000, others had evaluated the precise roles that specific phosphorylation sites play in receptor signalling and found Ser ^{334} to be a key residue that controls receptor phosphorylation (Christophe *et al.* 2000). Initially, phosphorylation of

either two serine pairs, namely Ser ^{332} and Ser ^{334} or Ser ^{334} and Ser ^{338} was shown to be critical for the phosphorylation of C5aR and its subsequent desensitisation. Later studies with mutant receptors demonstrated that phosphorylation of either Ser ^{334} or Ser ^{338} alone is required for C5aR desensitisation. However, for the formation of a persistent complex, phosphorylation of Ser ^{334}/Ser ^{338} or Ser ^{327}/Ser ^{338} is necessary and sufficient. Desensitisation is followed by internalisation. A persistent complex between C5aR and β arrestins exist to the endosomal compartments. Lack of phosphorylation was found to result not only in a sustained calcium mobilisation and extracellular signal regulated kinase 2 activity but also in the enhancement of the C5a-mediated respiratory burst in neutrophil like HL-60 cells (Christophe *et al.* 2000). Others concurred and demonstrated that internalisation of receptors follows phosphorylation of serine and threonine residues in the C-terminal region (Mc Donald *et al.* 2000).

We have shown that both C5aR and C5L2 exhibit a basal level of phosphorylation in L1.2 transfectants. We have demonstrated that C5aR is robustly phosphorylated after 5 minutes exposure to the ligand C5a. These results contrast with those of C5L2 which is only modestly phosphorylated. The ligand dependent phosphorylation of C5L2 was maximal at 5-10 minutes (versus 5 minutes for C5aR) and had returned to baseline levels by 15 minutes. The modest phosphorylation of C5L2 in comparison to the robust phosphorylation of C5aR was suggestive that C5L2 may not internalise in the same manner as the classical C5a receptor, C5aR.

The binding domain of the human C5a receptor consists of at least two distinct and physically separable subsites. One of these sites binds the C-terminal 8 amino acids of C5a while the second site lies in the N terminus of the receptor and interacts with the core of C5a. In 1994, it was demonstrated that despite its importance for binding, the N terminus is not necessary for signal transduction because a C-terminal peptide analogue of C5a was able to stimulate G protein activation and to generate a calcium flux through a receptor lacking residues 2-22 (De Martino *et al.* 1994). However, intact C5a was a very poor activator of this truncated receptor. These results implied that interaction between the N terminus of the receptor and C5a produces a conformational change in C5a that allows its C

terminus to properly interact with and activate the receptor. In 1997, it was discovered that the C terminus of the human C5a receptor (C5aR) is phosphorylated after stimulation with C5a and the C terminus is required for normal ligand-dependent receptor internalisation (Boch *et al.* 1997). The same year, a separate group proved that the agonist mediated internalisation and recycling of the C5a anaphylatoxin is modulated by phosphorylation of the C-terminal domain (Naik *et al.* 1997). They demonstrated that upon challenge with C5a, the C5a receptor undergoes a rapid phosphorylation on serine residues in the carboxyl-terminal region. Using stably transfected cells with either wild type C5a receptor or mutants, they demonstrated that upon agonist binding, the wild type receptor was rapidly internalised into endosomes that cluster near the nucleus after 10 minutes. Internalisation of a non-phosphorylable mutant was severely impaired relative to the wild type receptor. Under continuous exposure to C5a and in the absence of protein synthesis, the C5a receptor was maintained in a highly phosphorylated state but was not degraded. Confocal microscopy and ligand binding studies indicated that internalised receptors were de-phosphorylated with kinetics that correlated with the kinetics of receptor recovery on the cell surface. Overall their data proved that phosphorylation plays a key role in the intracellular trafficking of the C5a receptor.

Five years later, it was shown that the tyrosines at positions 11 and 14 of the N-terminal sequence of the C5aR are post-translationally modified by the addition of sulphate groups (Farzan *et al.* 2001). The sulphate moieties of each of these tyrosines are critical to the ability of the C5aR to bind C5a and to mobilise calcium. A C5aR variant lacking these sulphate moieties efficiently mobilised calcium in response to a small peptide agonist, but not to C5a, consistent with a two-site model of ligand association in which the tyrosine-sulphated region of the C5aR mediates the initial docking interaction. In addition they demonstrated that at high concentrations a tyrosine-sulphated peptide based on the NH_2 terminus of the C5aR specifically inhibits the association of the C5aR with C5a. These observations were consistent with a physical interaction between the tyrosine-sulphated region of C5aR and C5a.

Internalisation of G protein coupled receptors has been shown to follow phosphorylation.

Our results demonstrate that ligand induced C5L2 internalisation does not occur. We studied C5L2 for 60 minutes and the receptor was essentially anchored to the surface of the cell. In contrast, C5aR was rapidly internalised (approximately 50% within 5 minutes). We propose different theories as to why C5L2 is not rapidly internalised like C5aR.

C5L2 may act as a reservoir of cell surface-associated anaphylatoxin to aid chemotaxis. Retention of ligated C5L2 at the cell surface may be important in prolonging signalling beyond the rapid responses normally stimulated by C5a. Alternatively, C5L2 may modulate the effective concentrations of C5a/C5a des Arg. In a local setting where both C5a and C5a des Arg are present, with dynamically increasing quantities of C5a des Arg relative to C5a, C5L2 could serve to spare the C5a to interact with the functional receptor, C5aR. In this way C5L2 could serve to buffer anaphylatoxin concentrations during an inflammatory response.

The concept that C5L2 may act as a "high affinity sink" can be argued in both directions. C5L2 has a very slow dissociation rate with respect to ligand and essentially is anchored at the cell surface (no internalisation after 60 minutes). In contrast, the C5aR has a more rapid dissociation rate (~ 3 fold faster than C5L2) and is rapidly internalised (approximately 50% in 5 minutes) after binding C5a. Internalisation of C5aR has been demonstrated to result from phosphorylation of the C-terminus, a feature that is not significantly shared with C5L2. The classical C5aR is recycled to the cell surface while the ligand is degraded intracellularly. C5L2's quality of slow dissociation can be argued to be consistent with its role as a modulator of anaphylatoxin concentration, effective in clearing or limiting the inflammatory response to C5a. On the other hand, one can argue against this point and state that C5L2 is unlikely to act as a sink because it is not rapidly internalised.

The Duffy antigen receptor for chemokines (DARC) on red blood cells, was until recently the only molecule in the family of G protein coupled receptors which has a significant affinity for certain chemokines (Pogo *et al.* 2000). It was demonstrated that the Duffy protein binds to chemokines of both class CXC and class CC and because of this it is referred to as "a promiscuous chemokine receptor". It does not appear to be linked to the

cytoplasmic G-protein complex (Premack *et al.* 1996). It is postulated that the function of Duffy protein in red blood cells is to serve as a "sink", or scavenger for chemokines (Darbonne *et al.* 1991, Horuk *et al.* 1993). The Duffy blood group system has clinical significance because of transfusion incompatibilities and haemolytic disease of the newborn. Secondly, its clinical significance is derived from its role as a receptor for the malarial parasite. Duffy negative human red blood cells are resistant to invasion by Plasmodium vivax (Miller *et al.* 1976). Thus C5L2 may be seen as analogous to the Duffy antigen receptor, binding proteins that may function in the buffering and presentation of chemokines (Murphy *et al.* 2000).

More recently, two further members of the chemokine receptor family, D6 and CCR binding protein (CCRbp/CCR11), have been shown to be high affinity binding receptors for β chemokines and CCR7/CCR9 ligands, respectively. D6, while binding the majority of members of the β chemokines, is however specific for this family and shows no detectable affinity for members of the α-chemokine or the C, or the CXXXC chemokines. Unlike the majority of other chemokine receptors, human D6 does not mediate calcium flux following ligand binding (Nibbs *et al.* 1997). While the concept of a decoy receptor is common for the pro-inflammatory cytokines, interleukin-1 (IL-1) and TNF-α, this was not a well established phenomenon in G.P.C.R.s. Interestingly, both D6 and the chemokine, CCRbp/CCR11 demonstrate mutations in either transmembrane segment 2 (TM2) or the conserved DRY sequence which might be predicted to disrupt G protein coupling. C5L2 displays a natural substitution of leucine for arginine at position 132 in the DRY sequence. Loss of this essential arginyl group is associated with uncoupling to G proteins.

CHAPTER V

C5L2 IS A NON-SIGNALLING C5A BINDING PROTEIN

CHAPTER V

C5L2 IS A NON-SIGNALLING C5A BINDING PROTEIN

5.1 INTRODUCTION

5.1.1 General introduction

Knowing that C5L2 displays a natural substitution of leucine for arginine at position 132 of the DRY sequence, and that loss of this "essential" arginine has been shown to be associated with uncoupling to G proteins; we inserted a point mutation replacing leucine with arginine. Our hypothesis was that this mutation may result in C5L2(L132R) coupling, at least in part, to G proteins. We assessed this by analysing the increase in intracellular calcium in response to the addition of C5a to C5L2(L132R) transfected cells and comparing this response to that obtained with wild type C5L2 and C5aR transfected cells. Uncoupling of C5L2 to G proteins would suggest inability of C5L2 to stimulate chemotaxis in response to addition and binding of C5a, unlike the C5aR. It was not clear whether addition of C5a to C5L2 transfected cells would activate mitogen activated protein (MAP) kinases. Both warranted studying. Furthermore, we have demonstrated that bone marrow and peripheral blood leukocytes are the most abundant sources of message for both C5L2 and C5aR. Virtually all signal transduction leads, either directly or indirectly to changes in mRNA expression patterns. We decided to take advantage of the C5a receptor deficient mice to probe signalling by C5L2 and examine mouse bone marrow cells in more detail regarding these two receptors and the possible role of C5L2 in inflammation. Finally, knowing the major role C5a plays in inflammation and sepsis, that C5aR is expressed on peripheral blood leukocytes and the most abundant source of mRNA for C5L2 was in peripheral blood leukocytes we investigated if C5L2 is co-expressed with C5aR on human peripheral blood leukocytes.

5.1.2 Calcium mobilisation, Gα16 and C5aR

We know that stimulation of leukocytes with chemoattractant ligands activates phospholipid turnover and calcium release, ultimately leading to chemotaxis, degranulation and the inflammatory response. The leukocyte response to these ligands is transduced by the interaction of transmembrane receptors with G-proteins. The mechanism of signal transduction by these receptors has previously been examined (Amatruda *et al.* 1993). COS-7 cells were transfected with cDNA clones encoding the receptors for C5a and platelet activating factor (PAF) and the production of inositol phosphate (I.P.) was measured in response to stimulation with these chemoattractant ligands. Cells transfected with the C5a receptor alone showed no increase in I.P. production when stimulated with ligand (5-120nM), but in cells co-transfected with these receptors and with the cDNA for Gα16 (a G protein subunit that is specific to cells of haematopoietic lineage), addition of ligand caused up to a 5 fold increase in I.P. production. This interaction was specific, because co-transfection of receptors with the G-protein Gαq or Gα11 did not allow ligand dependant increase in I.P. production. In contrast, ligand dependant activation of I.P. production was seen in COS cells solely transfected with the PAF receptor. Thus they demonstrated that the C5a receptor, C5aR utilises signalling pathways distinct from the PAF receptor and suggested that Gα16, a pertussis toxin-resistant G-protein α subunit may play a role in the leukocyte response to inflammatory ligands by facilitating C5a mediated signal transduction (Amatruda *et al.* 1993).

5.1.3 G-protein coupled receptor (G.P.C.R.) signalling e.g. C5aR and arrestins

Let us review our knowledge of G.P.C.R. signalling and the role of arrestins in a chronological fashion. We know that G.P.C.R.s constitute a family of seven-transmembrane domain proteins that transmit extracellular signals to the interior of the cell by activating a variety of signal pathways (Van Biesen *et al.* 1996, Lefkowitz *et al.* 1998, Gutkind *et al.* 1998). G.P.C.R.s lack intrinsic kinase activity but the stimulation of receptors with agonist leads to an increase in the GTPase activity of the bound

heterotrimeric G proteins. G protein activation in turn modulates downstream effectors such as the second messenger generating adenyl cyclases and phospholipases as well as ion channels. Phosphorylation of the receptors by G protein-coupled receptor kinases (GRKs), followed by recruitment of β–arrestins to agonist-occupied receptors often terminates or attenuates such signalling by blocking G proteins from further interaction with the receptors (desensitisation) (Hausdorff *et al.* 1990, Koenig *et al.* 1997, Barak *et al.* 1997, Pitcher *et al.* 1998). By 2001, evidence was accumulating to indicate that the β–arrestins also play novel roles as adapters, which recruit additional signalling molecules to ligand–activated receptors. Thus the β–arrestins appear to be involved in both termination of some aspects of G.P.C.R. signalling as well as the initiation of others. Later, work with immunofluoresence microscopy and green fluorescent protein-tagged β-arrestins demonstrated that after C5aR phosphorylation, desensitisation and internalisation, a persistent complex exists between β-arrestins and C5aR to endosomal compartments (Braun *et al.* 2003). C5aR activation is able to promote a loose association with β-arrestins, but phosphorylation of critical serines is both sufficient but necessary for the formation of a persistent complex. This suggested that C5aR is internalised via a pathway dependant on β-arrestins, clathrin and dynamin. The proteins with which the β-arrestins interact, appear to couple G.P.C.R.s to two major categories of signalling pathways. Interaction with clathrin, AP-2 and NSF mediates the ability of the β-arrestins to target ligand-activated receptors to clathrin-coated pits (Goodman *et al.* 1996, Laporte *et al.* 1999). Interaction with ASK and JNK3, as well as Src, Raf and ERK, mediate the ability of the β-arrestins to regulate the activity of the MAPKs, the c-Jun NH_2–terminal kinases (JNKs) and ERKs respectively`(Miller *et al.* 2001, Maudsley *et al.* 2000, Luttrell *et al.* 1999).

5.1.4 Mitogen-activated protein kinases (MAPKs)

In mammalian cells, at least three groups of mitogen–activated protein kinases (MAPKs) have been identified: the ERKs (1 and 2), the p38 protein kinases (α, β, γ, and δ) and the c-Jun NH_2–terminal kinases (JNKs; also referred to as stress-activated protein kinases or SAPKs) (Davis *et al.* 1999). The JNKs are encoded by at least three genes (JNK1, -2 and -3) and the transcripts of each of these genes are alternatively spliced to create mRNAs that encode 46- and 54- kD JNK isoforms (Gupta *et al.* 1996). Moreover, JNK activity is increased in settings of cell stress, mitogenesis, differentiation, morphogenesis and apoptosis (Davis *et al.* 1999, Tournier *et al.* 2000). JNK is activated by dual phosphorylation on threonine and tyrosine residues catalysed by a MAPK kinase (such as MKK4 or MKK7) which in turn, is phosphorylated and activated by a MAPKK kinase (such as MEKK1, MLK or ASK1). The functional integrity of each MAP kinase cascade is thought to be established by specific molecular interactions both between the kinases and cytoplasmic scaffolds or anchor proteins. MAPK pathways are activated by G.P.C.R.s including M1 and M2 muscarinic and angiotensin II type 1A (AT1A) receptors (Mitchell *et al.* 1995, Zohn *et al.* 1995) and by growth factor receptors with intrinsic tyrosine kinase activity. In 2000, β arrestin 2 was identified as a binding partner of JNK3 and a group described a G.P.C.R.-regulated MAPK module wherein β arrestin 2 functions as a scaffold protein (Mc Donald *et al.* 2000).

5.2 OBJECTIVES

1. To assess if C5L2 is coupled to G proteins and mediates calcium flux in its wild type form C5L2, or its mutated form C5L2 (L132R), and compare results to C5aR in both transient and stable cell lines.
2. To test if C5L2 stimulates chemotaxis.
3. To investigate if C5L2 is a signalling receptor.
4. Knowing the importance of C5aR in sepsis, we wanted to identify if C5L2 is co-expressed with C5aR on human granulocytes.

5.3 RESULTS

5.3.1 Calcium mobilisation in transfected cells: transient and stable cell lines

In our bid to assess if C5L2 is coupled or uncoupled to G proteins we decided to test our hypothesis in two cell lines: one transient, human embryonic kidney 293T (HEK293T) cells, the other stable, the murine pre-B-lymphoma line, L1.2 cells

5.3.1.1 Calcium mobilisation in transiently transfected HEK293T cells

Fura-2 loaded 293T cells transfected with wild type C5L2, C5L2(L132R) (i.e. mutated C5L2) or the C5aR with or without Gα16, as indicated in figure 5.1 were equilibrated at 37°C. 100nM C5a was added at the arrow, and increases in intracellular calcium were recorded as a function of time (seconds) by fluorescence (chapter II).

It was previously documented that cells co-transfected with C5aR and Gα16 demonstrate a clear increase in intracellular calcium on addition and binding of C5a. The G protein subunit Gα16 had been shown to greatly facilitate C5a-mediated signal transduction

(Amatruda *et al.* 1993). Therefore, HEK293T cells transfected with C5aR plus Gα16 acted as our positive control and a definite increase in intracellular calcium in response to the addition of 100nM of C5a was demonstrated, confirming previous findings (Figure 5.1). HEK293T cells transfected with pCDNA3.1 plus Gα16 acted as our negative control and as expected, did not demonstrate an increase in intracellular calcium after the addition of 100nM of C5a (Figure 5.1). We were suspicious that C5L2 was uncoupled to G proteins in part due to the natural substitution of leucine for arginine at position 132 in the DRY sequence. First we tested if HEK293T cells transfected with C5L2 without Gα16 demonstrated an increase in intracellular calcium in response to the addition of 100nM C5a: they did not (Figure 5.3). Next we co-transfected HEK293T cells with C5L2 plus Gα16 and again no increase in intracellular calcium was documented in response to 100nM C5a, confirming that C5L2 is uncoupled to G proteins (Figure 5.1).

Using site directed mutagenesis we replaced leucine with arginine and investigated if this natural substitution of leucine for the "essential arginine" was in part responsible for uncoupling to G proteins (chapter II). First we tested HEK293T cells transfected with C5L2(L132R) (mutated C5L2) without Gα16 and a mild increase in intracellular calcium in response to 100nM C5a was demonstrated (figure 5.1). Next we tested HEK293T cells co-transfected with C5L2(L132R), (i.e. "mutated C5L2") plus Gα16 and a moderate increase in intracellular calcium in response to the addition of 100nM C5a was documented (Figure5.3). The latter increase is more pronounced than that demonstrated with C5L2(L132R)/293T cells without Gα16 but is less robust than the increase seen with cells co-transfected with C5aR plus Gα16 (Figure 5.1). This confirms that the natural substitution of leucine for arginine in the DRY sequence of C5L2 is in part responsible for C5L2 being uncoupled to G proteins. As expected, replacement of the leucine with arginine results in a partial "gain of function" and evidence of partial G protein coupling.

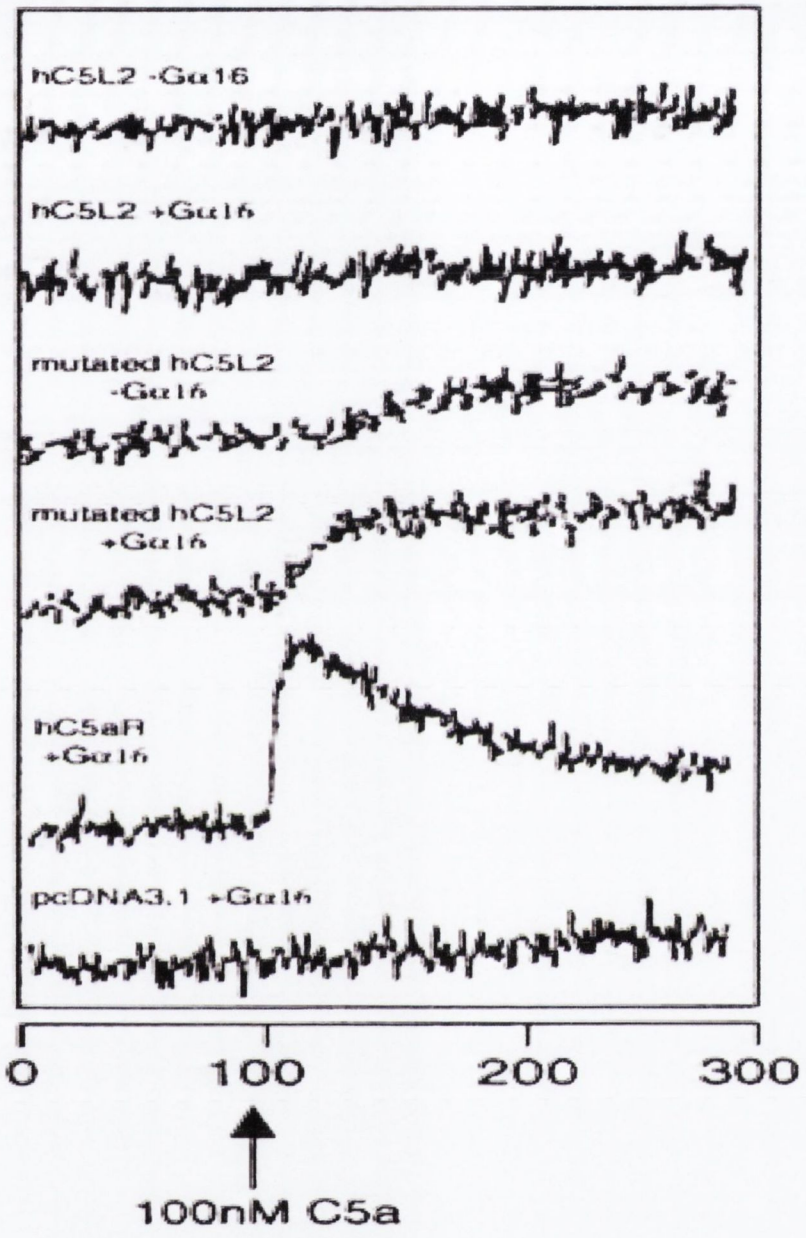


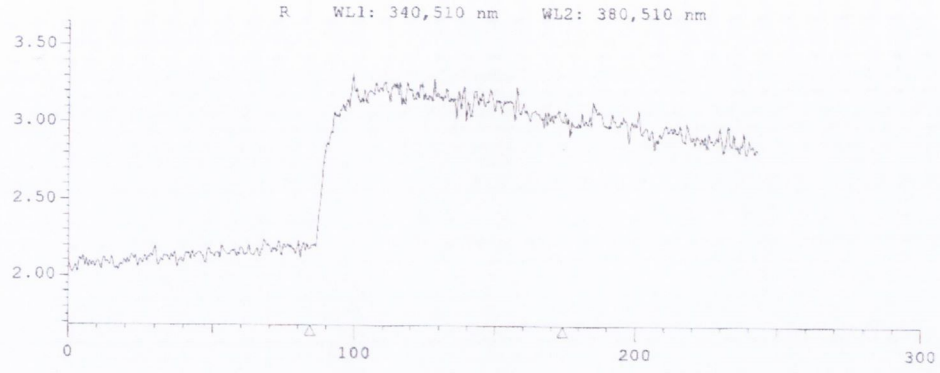
Figure 5.1 **Increases in intracellular calcium after the addition of C5a to H293T cells transiently transfected with C5aR, C5L2 or C5L2(L132R) plus or minus Gα16 as indicated. The X axis is time in seconds and Y axis, change in fluorescence.**

5.3.1.2 Calcium mobilisation in a stable cell line: L1.2 cells

We sought to confirm our transient cell line findings, in a stable cell line. Cells were Fura-2 loaded as described previously (chapter II). We used L1.2 cells as our negative control for this experiment and confirmed that addition of 100nM C5a did not increase intracellular calcium. We demonstrated and concurred with previous authors findings (Kolakowski *et al.* 1995) that C5aR/L1.2.cells exhibit a clear increase in intracellular calcium in response to the addition of 100nM C5a. C5aR/L1.2.cells acted as our positive control for our stable cell line (Figure 5.2).

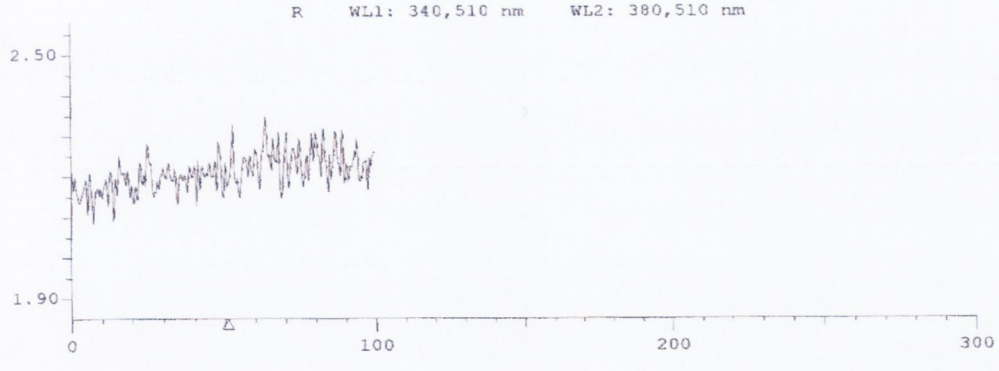
As we expected, C5L2/L1.2 cells did not mediate calcium flux as expected, thereby confirming out results from the transient cell line, that wild type C5L2 is uncoupled to G proteins unlike the classical C5a receptor, C5aR (Figure 5.3). Interestingly, the fluorescent decay characteristic of responses of the wild type C5aR (Figure 5.4) was absent with C5L2 (Figure 5.3). This is not surprising considering the minimal ligand-dependant phosphorylation of C5L2 as discussed previously.

Desensitisation of C5aR/L1.2 cells to C5a has been previously documented and was confirmed by us: i.e. despite the addition of repeated and/or increasing doses of C5a, no further increase in intracellular calcium is documented after the first increase, following the first addition of ligand (Figure 5.4). This deactivation or "desensitisation" reflects the internalisation of C5aR after ligand binding.



↑100nM C5a

Figure 5.2 Increase in intracellular calcium in C5aR/L1.2 cells after the addition of C5a. A clear increase in intracellular calcium concentration is documented in response to addition of 100nM C5a to Fura 2 loaded C5aR/L1.2 cells equilibrated at 37°C.



↑

100nM C5a

Figure 5.3 Absence of calcium mobilisation in C5L2/L1.2 cells after the addition of C5a confirming that C5L2 is uncoupled to G proteins unlike C5aR.

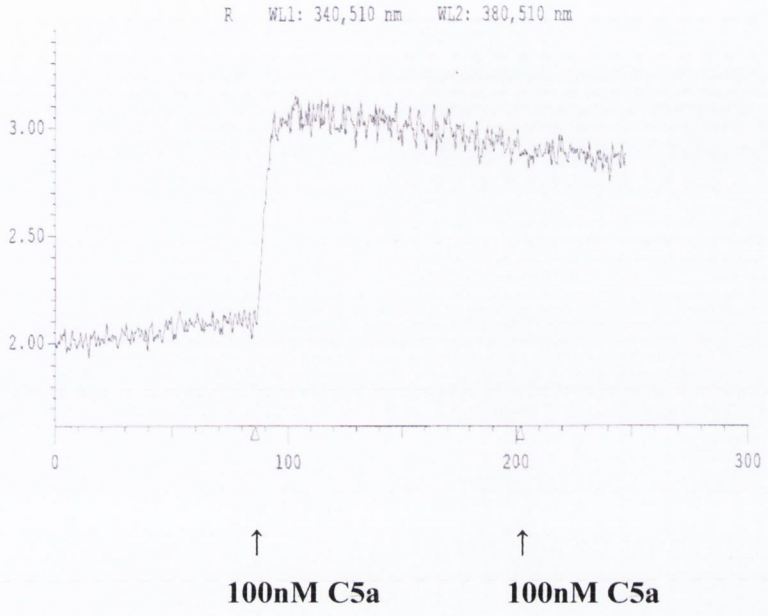


Figure 5.4 Desensitisation to C5a in C5aR/L1.2 cells.

Repeated addition of C5a does not result in a further increase in intracellular calcium in Fura-2 loaded C5aR/L1.2 cells. Changes in intracellular calcium concentration in response to C5a were determined by monitoring the fluorescence emission at 510nm with excitation at 340 and 380 nm as a function of time. Responses were quantified as the peak of the ratio of 340/380 nm wavelengths.

5.3.2 Chemotaxis

Previously it has been shown that C5aR stimulates chemotaxis in response to the addition of C5a. In our hands, no evidence of chemotactic activity was documented after the addition of ligand C5a or C3a, C4a or fMLP to fluorescently labelled C5L2-EGFP/L1.2 cells in modified Boyden chambers (chapter II). Cells were placed in the upper wells while

FluoroBlok filters with buffer (20nM HEPES,125 mM NaCl, 5nM KCl, 1nM $MgCl_2$, 1 nm $CaCl_2$, 0.5nM glucose, 0.2% Bovine serum albumin and pH 7.5), containing C5a, or C3a or C4a or fMLP were placed in the lower wells. Chambers were incubated at 37°C for 45 minutes and chemotaxis determined by measuring the fluorescence passing to the underside of the filter. Addition of buffer alone to C5L2-EGFP/L1.2 cells acted as our negative control. No statistical difference was documented in fluorescence results between addition of buffer alone versus C5a, (at 10 nm and 100 nM concentrations) versus C3a (10 nM and 100 nM) versus C4a or fMLP (100 nM). Two clones of C5L2 were tested and all measurements were determined in duplicate.

Potential Ligand	**Chemotaxis fluorescence recorded in C5L2/L1.2 cells**
Buffer/negative control	34,647/34,233
C5a 10nM	33,134/33,184
C5a 100nM	33,876/32,689
C3a 10nM	36,635/39,260
C3a 100nM	33,373/34,572
fMLP100nM	34,919/36,466
C4a 100nM	37,726/37,568

5.3.3 Assessing if C5L2 is a signalling receptor and comparing to C5aR

5.3.3.1 Activation of the mitogen activated protein (MAP) kinase cascades by C5aR but not by C5L2

Activation of the MAP kinase family has been demonstrated to occur for several G protein coupled receptors by a mechanism involving the scaffolding of the phosphorylated receptors to arrestins, which may occur independently of, and in parallel with activation of the G proteins (Mc Donald *et al.* 2000). $C5L2_{C9}$/L1.2 cells were treated with C5a for the indicated times and cytosolic proteins were subjected to Western blot analysis for

phosphorylated ERK1 and ERK2 as indicated (Figure 5.5). Phosphorylation was evaluated as a function of time (upper panels) or for the times indicated in duplicate in the presence or absence of C5a (lower panels). We demonstrate a definite activation of pERK-1 and pERK-2 after the addition of C5a in $C5aR_{C9}$/L1.2 cells. This activation of pERK-1 and pERK-2 in $C5aR_{C9}$/L1.2 cells appeared maximum between 0.5 and 1.0 minute and was no longer detectable at 30 minutes. In contrast, no activation of either pERK-1 or pERK-2 was demonstrated in $C5L2_{C9}$/L1.2 cells after the addition of C5a (Figure 5.5). Thus the relatively small extent of phosphorylation of C5L2 (chapter IV) that occurs following exposure of $C5L2_{-C9}$/L1.2 cells to C5a, is apparently mirrored by minimal/no recognition by arrestins and subsequently no activation of this transcription factor cascade. These results support our previous findings.

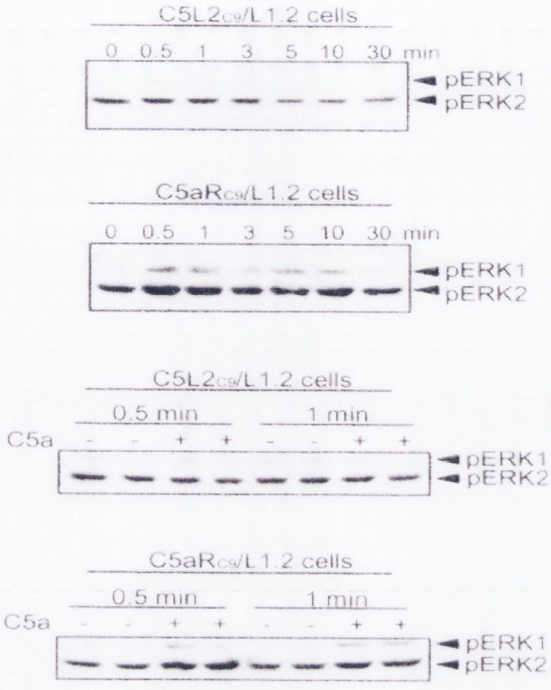


Figure 5.5 Analysis of MAP Kinase activation after the addition of C5a in $C5aR_{C9}$/L1.2 and $C5L2_{C9}$/L1.2 cells.

Phosphorylation of MAP kinase was investigated using stable L1.2 transfectants as described earlier. Cells were washed with serum free RPMI and pre-incubated at 2 x 10^6/ml for 30 minutes at 37°C with gentle agitation. Cells were then treated with 0 or 200 nM C5a for 0.5, 1, 3, 5, 10, or 30 minutes as indicated above. Cells were immediately collected in ice-cold lysis buffer (appendix) and frozen in liquid nitrogen. After three cycles of freezing and thawing, lysates were centrifuged and the supernatants were further centrifuged and subjected to Western blot analysis. Following electrophoresis on 10% polyacrylamide gels, proteins were transferred to nitrocellulose and stained with rabbit anti-active MAPK antibody. Blots were incubated with peroxidase-conjugated goat anti-rabbit IgG and signals detected by ECLPlus using Biomax MRfilm.

5.3.3.2 Results of microarray analysis of C5aR and C5L2 mediated signal transduction

We further sought to identify if C5L2, like C5aR is a signalling receptor by taking advantage of our C5a receptor deficient mice. Virtually all signal transduction leads, either directly or indirectly to changes in mRNA expression patterns. C5aR deficient mice express C5L2 alone, whereas their wild type littermates express both C5aR and C5L2. Earlier we had demonstrated that bone marrow was an abundant source of C5L2 and C5aR. Mouse bone marrow cells from C5aR deficient mice and their wild type littermates (matched for age and gender), were incubated with 0 or 100nM C5a and after 4 hours mRNA was prepared for microarray analyses, using the mouse Affymetrix 10,000 genechip (chapter II).

As shown in Table 5.1, bone marrow from wild type mice, responded to C5a with reproducible patterns of up- and down- regulated messages. In the case of up-regulated genes, many are related to inflammation or its regulation. Strikingly, C5a led to a >20 –fold induction in osteopontin-1, [also known as early T cell activated gene-1 (ETA-1)], a 3.93 fold increase in immunoregulatory gene 1 and a 2.55 fold increase in both interleukin-1 receptor antagonist (IL-1Ra) and CD14. Histidine decarboxylase cluster, basic helix-loop-helix protein and thrombospondin-1 were increased 2.24, 2.16 and 2.15 fold respectively. However, aquaporin, bone marrow cytoplasmic tyrosine kinase (BMX cytoplasmic TK) and gut enriched Kruppel like factor were suppressed 2.73, 2.39 and 2.30 fold in wild type bone marrow cells. In stark contrast, stimulation of bone marrow cells from the C5aR deficient mice, in which only C5L2 is expressed, resulted in no detectable C5a mediated changes, either induction or suppression of gene expression. These data provide powerful evidence that C5L2 is a non signalling C5a/C5a des Arg binding protein.

Table 5.1 Changes in mRNA expression in wild type (A) and C5aR deficient (B) mouse bone marrow cultures following 4 hours stimulation with 100 nM mouse C5a*

A , Wild Type C5aR

gene	fold induction	gene	fold suppression
X13986 osteopontin	20.60	LO2914 aquaporin	2.73
A1323667 immunoregulatory gene 1	3.93	A18737110 mouse hypothalamus	2.42
L32838 mouse germline IL1 RA	2.50	AF012104 BMX cytoplasmic TK	2.39
X13333 CD14	2.55	U20344 gut enriched Kruppel LF	2.30
X57437 histidine decarboxylase cluster	2.24		
Y07836 basic helix-loop-helix protein	2.16		
M62470 thrombospondin-1	2.15		
AA612450 mouse myotubes	2.13		
AV223216 type II IL1 receptor	2.12		
M34603 proteoglycan core protein	2.06		
AF061272 C-type lectin	2.03		

B. C5aR Knockout /C5aR-/-

No genes were induced or suppressed

* Average of two experiments

AF012104 BMX cytoplasmic TK = bone marrow cytoplasmic tyrosine kinase

U20344 gut enriched Kruppel LF = gut enriched Kruppel-like factor

Mouse bone marrow cells from C5aR deficient mice expressing C5L2 only and from wild type littermates, expressing both C5aR and C5L2 were incubated with 0 or 100 nM C5a and mRNA was prepared for microarray analysis using the mouse Affymetrix 10,000 genechip. Bone marrow from wild type mice responded to C5a with reproducible patterns of up- and down- regulated messages. In contrast, stimulation of bone marrow cells from C5aR deficient mice resulted in no detectable C5a mediated changes in gene expression.

5.3.4 Antisera to C5L2 and C5aR and flow cytometric analysis of human granulocytes

We had previously demonstrated that C5L2, like C5aR is highly expressed in human peripheral blood leukocytes. C5aR plays a critical role in sepsis but as yet we were unsure of the role of C5L2 *in vivo*. We decided to examine human leukocytes in more detail and assess if C5L2 is co-expressed with C5aR on these important cells which are central to fighting infection.

5.3.4.1 Demonstration of the specificity of antisera to C5L2

A synthetic peptide corresponding to the predicted N-terminal extracellular sequence of human C5L2 (MGNDSVSYEYGDYSDLSDRPVDC) was coupled to KLH and utilised for production of rabbit antiserum. We obtained this anti C5L2 antibody from Alpha Diagnostics. We confirmed the specificity of anti-C5L2 before proceeding with further experiments (Figure 5.6). Anti-C5L2 bound only to C5L2/L1.2 cells but did not recognise C5aR in stable transfectants Similarly we demonstrated the specificity of anti-C5aR.

5.3.4.2 Flow cytometric analysis of L1.2 transfectants

C5L2/L1.2 and C5aR/L1.2 cells were prepared as described previously (chapter II) and flow cytometric analysis performed (chapter II). All experiments were performed in duplicate and repeated three times. C5aR/L1.2 cells stained with mouse anti human C5aR followed by PE-conjugated goat anti mouse IgG acted as PE positive control (Figure 5.7 graph A), C5aR/L1.2 cells stained with isotype mouse IgG^1 followed by PE conjugated-goat anti mouse IgG acted as PE negative control (Figure 5.7 graph B), C5L2/L1.2 cells stained with rabbit anti human C5L2 followed by goat anti-rabbit IgG was the FITC positive control (Figure 5.7 graph C) and C5L2/L1.2 cells stained with amyloid I-40 followed by FITC conjugated goat anti rabbit IgG (Figure 5.7 graph D) was the FITC negative control.

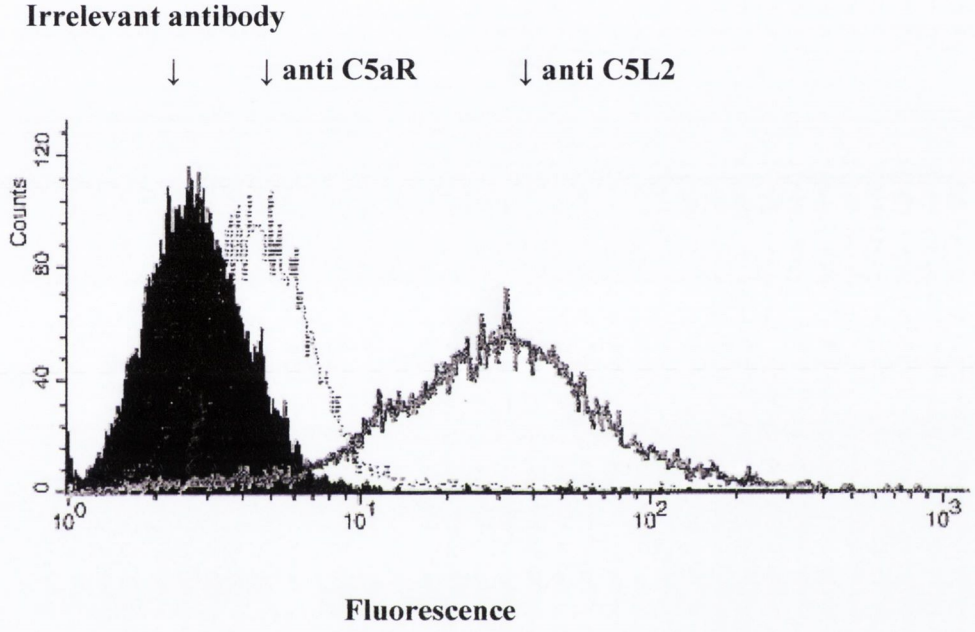


Figure 5.6 Demonstration of the specificity of anti-C5L2, in recognising C5L2 but not the C5aR in stable transfectants. Cells stained with rabbit anti-human C5L2, mouse anti-human C5aR, or an irrelevant antibody (black shaded) as indicated above by the arrows.

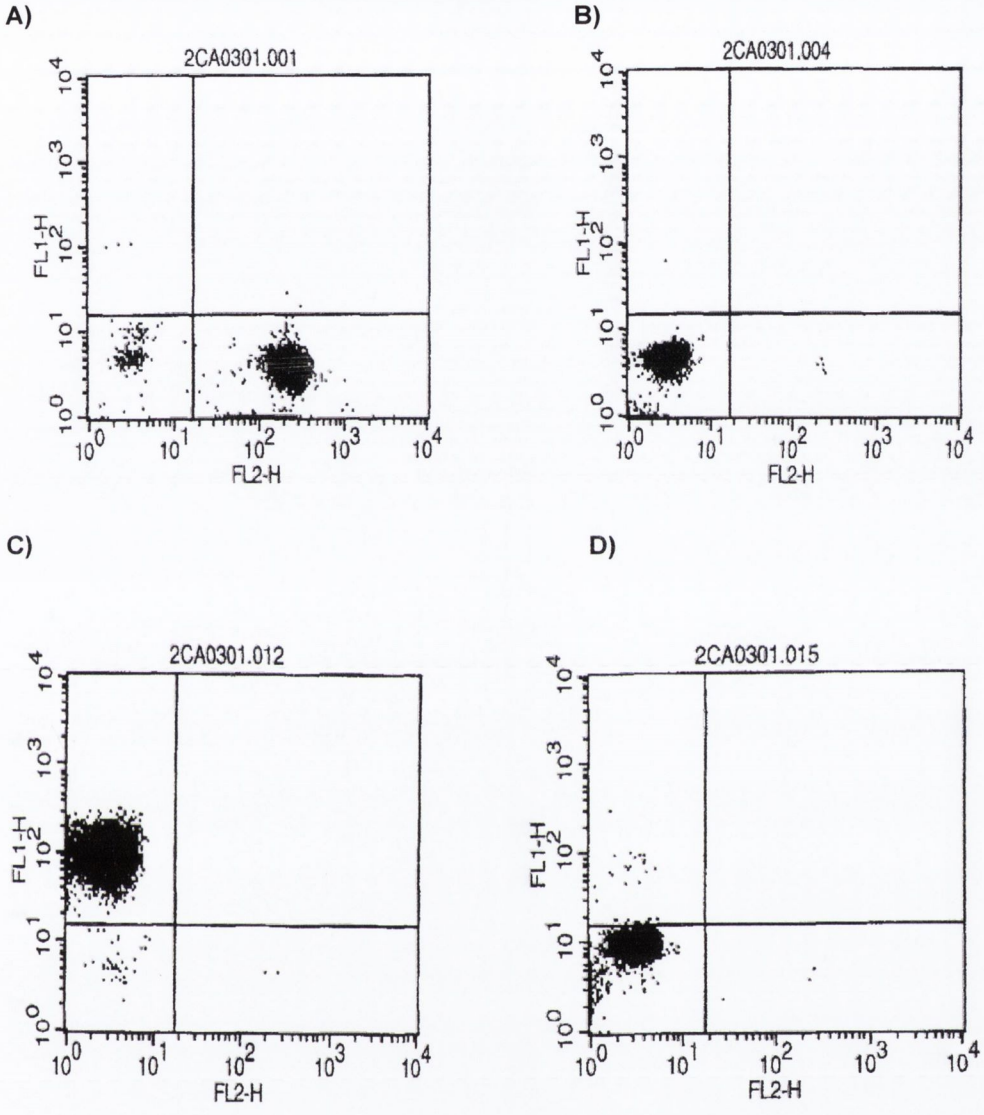


Figure 5.7 Flow cytometric analyses of C5aR/L1.2 and C5L2/L1.2 cells

C5aR/L1.2 and C5L2/L1.2 cells were suspended in PBS -2% FBS at 1 x 10^7/ ml. Aliquots of 100µl were incubated with 10µg/ml of either primary antibody for 1 hour at 0^0C. Cells were washed and incubated with either secondary antibody at 0^oC for 1 hour. After 3 washes, cells were suspended in 4% formaldehyde in PBS for flow cytometry analyses using a dual laser cytometer (FACS Scan) and data analysed with Cellquest software (Becton Dickinson). Graph A) demonstrates PE positive control, B) PE negative control, C) FITC positive control and D) FITC negative control.

5.3.4.3 Absence of cross reaction between antibodies

We examined multiple labelling with PE-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG. No cross reaction between these antibodies was demonstrated, nor between rabbit anti-human C5L2 and mouse anti-human C5aR. Therefore these antibodies do not interfere with each other.

5.3.4.4 Flow cytometric analysis of human leukocytes

Human peripheral blood leukocytes were prepared as described earlier (chapter II). Subsets of human leukocytes were analysed. Firstly, human leukocytes alone were assessed having been stained with FITC conjugated goat anti-rabbit IgG and no primary antibody (Figure 5.8). Human leukocyte subsets were assessed for expression of C5L2. In our hands C5L2 is expressed on almost all cells in the human granulocyte (Figure 5.9) and monocyte (Figure 5.10) pool: 95.5% and 98.9% respectively. The presence of C5L2 on these subsets of cells highlights the importance of C5L2.

5.3.4.5 C5L2 and C5aR are co-expressed on human granulocytes as demonstrated by two colour flow cytometric analyses

Human neutrophils were prepared as described earlier (chapter II) and cells prepared for flow cytometric analyses (chapter II). Human granulocytes were assessed for expression of the C5aR and C5L2 by two colour flow cytometric analyses using both primary and secondary antibodies: rabbit anti-human C5L2 and mouse anti-human C5aR; and PE-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG. The representative results shown in the Figure 5.11 clearly demonstrate co-expression of both C5L2 and C5aR on human peripheral blood granulocytes. This confirms the importance of C5L2 and suggests it may play a role in inflammation like C5aR. However this data does not clarify the exact role of C5L2.

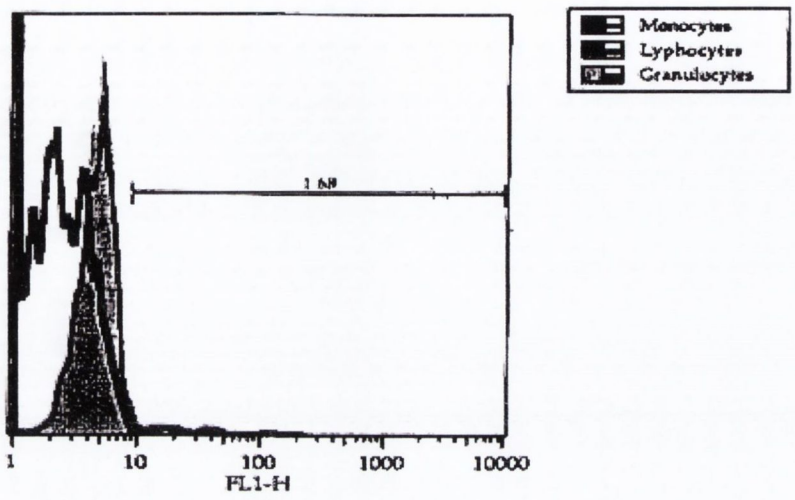


Figure 5.8 Human leukocytes alone stained with FITC conjugated goat anti-rabbit IgG and no primary antibody.

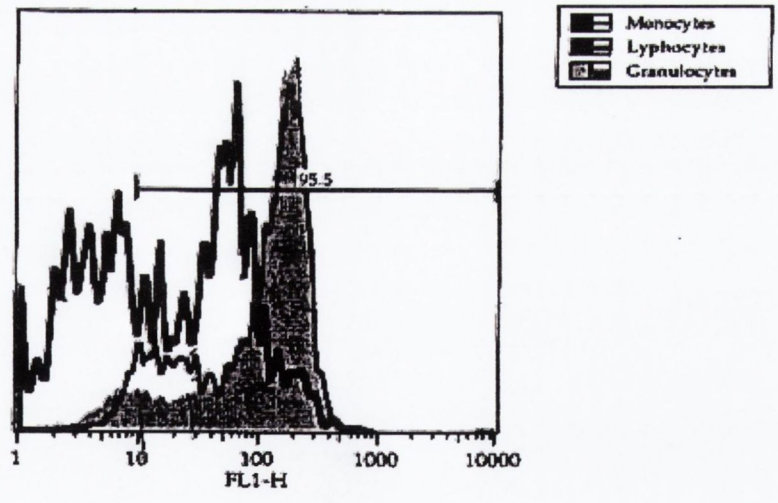


Figure 5.9 Human granulocytes stain positively for C5L2 (95.5%).

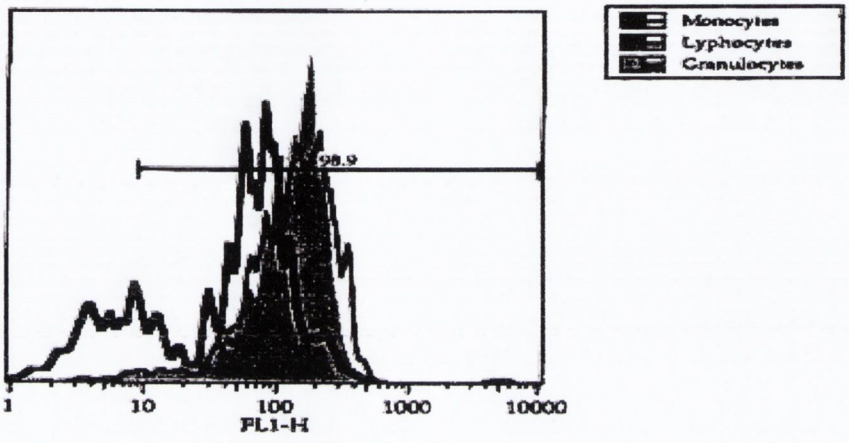


Figure 5.10 Human monocytes stain positively for C5L2 (98.9%)

For flow cytometric analyses, human neutrophils were suspended in PBS containing 2% foetal bovine serum at 1 x 10^7/ ml. Aliquots of 100µl were incubated with 10 µg/ml rabbit anti human C5L2 for 1 hour at 0°C. Cells were washed and incubated with FITC-conjugated goat anti-rabbit IgG and fixed in 4% paraformaldehyde in PBS for flow cytometry. Analyses were performed utilising a dual laser cytometer (FACS Scan) and data analysed with Cellquest software (Becton Dickinson).We gated on monocytes as shown and 98.9% stained positively for C5L2.

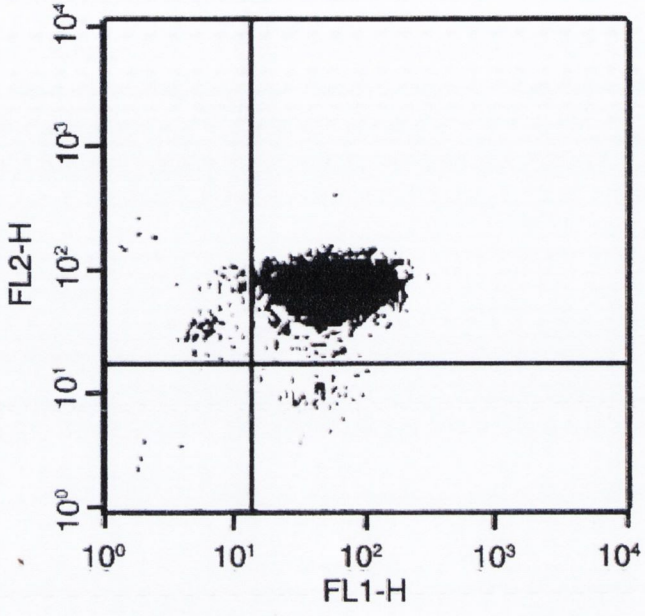


Figure 5.11 C5aR and C5L2 are co-expressed on human peripheral blood granulocytes as evidenced by these cells staining positively for both C5L2 and C5aR.

Human peripheral blood neutrophils were prepared and suspended in PBS containing 2% foetal bovine serum at 1 x 10^7/ml. Aliquots of 100µl were incubated with 10 µg/ml rabbit anti-human C5L2 and mouse anti-human C5aR for 1 hour at 0^{O}C. Cells were washed and incubated with FITC-conjugated goat anti-rabbit and PE-conjugated goat anti-mouse IgG, and fixed in 4% paraformaldehyde in PBS for flow cytometry. Analyses were performed utilising a dual laser cytometer (FACS Scan) and data were analysed with Cellquest software (Becton Dickinson). We gated on granulocytes which stain positively for both receptors indicating co-expression of C5aR and C5L2 on human granulocytes.

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5.4 DISCUSSSION

We confirmed that C5aR is obligately coupled to G proteins. We demonstrated a clear increase in intracellular calcium in C5aR transfected cells after the addition and binding of 100 nM C5a. We demonstrated that C5L2 is obligately uncoupled to G proteins: no increase in intracellular calcium is demonstrated on the addition of C5a to C5L2/293T cells (with or without G α16), or C5L2/L1.2 cells. However, introduction of a point mutation at position 132 (in the normally highly conserved DRY sequence), replacing leucine with arginine is associated with a "gain of function" regarding the ability to mediate ligand-induced changes in intracellular calcium concentration and in coupling to Gα16. The response was significantly less than for cells transfected with wild type C5aR and Gα16, nor did it decay in a manner similar to that of wild type C5aR. This result is not unexpected considering the modest C5L2 phosphorylation documented on exposure of C5L2 transfected cells to C5a in comparison to cells transfected with C5aR. The above holds true for both transient and stable transfected cells. Thus the observed leucine at position 132, conserved across species, (present in both human and murine C5L2), is a contributory factor leading to obligate uncoupling of C5L2 to G proteins.

Although C5L2 is a high affinity binding protein for C5a, addition of C5a does not stimulate chemotaxis in C5L2/L1.2 cells. This result makes logical sense based on our previous demonstration of poor C5L2 phosphorylation and lack of receptor internalisation after the addition of C5a ligand to C5L2 transfected cells. C5L2 appears uncoupled to G proteins. In contrast, the classical C5a receptor, C5aR is coupled to G proteins and stimulates chemotaxis in response to the addition of C5a to C5aR/L1.2 cells.

Treatment of $C5aR_{C9}$/L1.2 cells with 200nM C5a over a period of 0 to 30 minutes leads to activation of the MAP kinase cascade. Cytosolic proteins subjected to Western blot analysis for phosphorylated ERK-1 and ERK-2 detected phosphorylation. Activation of pERK-1 and pERK-2 in $C5aR_{C9}$/L1.2 cells reached a maximum between 0.5 and 1.0 minute and was no longer detectable at 30 minutes. Only minimal phosphorylation was detected in

$C5L2_{C9}$/L1.2 cells. These phosphorylation results are consistent with our previous finding that C5L2 is not internalised like C5aR.

Activation of the MAP kinase family has been demonstrated to occur for several G-protein coupled receptors by a mechanism involving scaffolding of the phosphorylated receptors to arrestins which may occur independently of and in parallel with activation of the G proteins. We were able to demonstrate activation of ERK-1 and ERK-2 by C5a only in cells transfected with C5aR but not in cells expressing C5L2. Thus the relatively small extent of phosphorylation of C5L2 that occurs following exposure to C5a in these cells is apparently mirrored by minimal recognition by arrestins and subsequent lack of activation of this transcription factor.

We demonstrated that C5L2 and C5aR is abundantly expressed is bone marrow and therefore this warranted further study. In our hands, bone marrow cells from wild type mice (expressing both C5aR and C5L2 receptors) respond robustly to the addition of mouse C5a with the induction and suppression of a number of genes, many related to inflammation. The most dramatic increase is in relation to osteopontin where we demonstrated a twenty fold increase.

Osteopontin (OPN), a glycosylated phosphoprotein, originally isolated from bone matrix (Butler *et al.* 1996), is produced by osteoblasts, osteoclasts and macrophages (Kido *et al.* 1995). Levels increase in active sites of bone metabolism. OPN is found in bodily fluids such as milk, blood, urine and tissues such as smooth muscle, kidney and leukocytes. It has been found in some tumours e.g. where it has been shown to promote the migration of breast cancer cells (Brown *et al.* 1992). Very high levels of osteopontin have been measured in most tumours and in the plasma of cancer patients whereas low levels are detected in healthy tissue, suggesting that blockade of osteopontin represents a highly specific and effective approach to malignancy. Osteopontin has been shown to promote cell adhesion, signalling and migration in immune cells (Liaw *et al.* 1995). It contains binding sites for several receptors, including CD44 and integrin alpha V beta 3: cell surface molecules which play a major role in mediating cell migration and adhesion.

Interestingly, while osteopontin was increased by 20 fold in wild type mice bone marrow cells, (expressing both C5aR and C5L2) stimulated with mouse C5a, C5aR deficient mice bone marrow cells, expressing C5L2 alone had no effect on this important phosphoprotein.

The cytokine mediator, interleukin 1 receptor antagonist (IL-1Ra), a natural, competitive inhibitor of IL-1α and IL-1β and a powerful endogenous anti-inflammatory molecule, was increased by 2.55 fold in bone marrow cells from wild type mice following stimulation with mC5a. IL-1Ra is produced as different isoforms; one secreted (sIL-1Ra) and three intracellular (icIL-1Ra1, icIL-1Ra2, icIL-1Ra3). As a member of the IL-1 family that binds to IL-1 receptors but does not induce any intracellular response, IL-1Ra prevents the interaction between IL-1 and its cell surface receptors, and thus competitively inhibits the biological effects of IL-1 (Dripps *et al.* 1991, Granowitz *et al.* 1991, Greenfeder *et al.* 1995). The administration of IL-1Ra ameliorates the course of arthritis in several experimental models (Gabay *et al.* 2000) and therapeutic administration of IL-1Ra has beneficial effects in patients with rheumatoid arthritis (Bresnihan *et al.* 1998). Induction of this anti-inflammatory molecule in wild type mice bone marrow cells may in part explain the anti-inflammatory role of C5aR in the acute pancreatitis and associated lung injury model previously discussed (chapter I). In contrast to wild type mice, no increase in IL-1Ra was documented in C5aR deficient mice.

Histamine influences many aspects of the immune response including antibody production following immunisation. In histidine decarboxylase deficient mice, serum levels of IgM auto antibodies are significantly higher than in wild type sera (Quintana *et al.* 2004). The histidine decarboxylase cluster was increased 2.24 fold in wild type mice bone marrow cells.

C type lectin was increased 2.03 fold in wild type mice bone marrow cells after stimulation with mouse C5a (mC5a). Lectins, carbohydrate binding proteins, are classified according to the carbohydrate-recognition domain of which there are two main types: S-type and C-type. Protein-carbohydrate interactions have been shown to mediate a variety of biological activities such as homeostasis and immune responses. Immune activities include pathogen

recognition and apoptosis. Interestingly most of the lectins involved in these immunological processes belong to the C-type animal lectin superfamily. These are classified into subgroups based on their function and structure e.g. endocytic lectins (membrane-bound receptors that mediate endocytosis of glycoproteins), colectins (represented by the soluble mannose-binding proteins of mammalian serum and liver) and selectins (membrane bound proteins involved in inflammation)

In contrast certain genes were suppressed in the wild type mice bone marrow cells after mC5a stimulation such as aquaporin. Aquaporins form a large, diverse family of proteins and have been found in bacteria, plants and animals. They form pores in the membranes of cells and selectively conduct water molecules through the membrane while preventing the passage of ions (e.g. potassium and sodium) and other small molecules. Less than a decade ago, scientists discovered the aquaporin Z gene (aqp Z) in *E. coli*, pointing to the protein's role in regulating water. Aquaporins are typically composed of identical subunit proteins. Water molecules traverse the narrowest portion of the water channel in single file. Their presence increases the permeability of membranes to water by as much as ten-fold. Aquaporins reabsorb 99% of the water in the kidney and thus prevent death from dehydration. Mutations in the aquaporin-2 gene (AQP2) cause an autosomal dominant form of nephrogenic diabetes insipidus. Aquaporin was suppressed by 2.73 fold in wild type mice bone marrow cells after mC5a stimulation but no change was documented in those of C5aR deficient mice.

Protein tyrosine kinases play a key role in the regulation of cell proliferation, differentiation, migration and survival. Bone marrow tyrosine kinase (Bmx tyrosine kinase), a member of the Tec family is expressed in haematopoietic cells of the granulocytic and monocytic lineages. It too was suppressed in wild type mice bone marrow cells (2.39 fold).

Basic Helix-Loop Helix proteins (bHLH) are a group of eukaryotic transcription factors involved in the exertion of a determinative influence in a variety of developmental and differentiation pathways in many organs. They are characterised by a highly evolutionary

conserved bHLH domain of 40-50 amino acid residues. Class A bHLH proteins (E proteins) are widely expressed while class B are tissue specific. Pod1 is a bHLH highly expressed in the mesenchyme of developing organs including lung, kidney and heart. Pod1 is essential for regulating properties of the mesenchyme that are critically important for lung and kidney morphogenesis (Quaggin *et al.* 1999). bHLH was increased by 2.16 fold in wild type mice but no change was observed in C5aR deficient mice.

Thrombospondin-1 is a large matricellular protein that acts as a pleiotropic growth factor for human vascular smooth muscle and may play a role in the progression of vascular disease. Thrombospondin-1 stimulates cell chemotaxis and proliferation but both processes are tyrosine kinase dependant. TSP has been shown to be robustly expressed in ischaemic brain (Lin *et al.* 2003). TSP was increased 2.15 fold in wild type mice bone marrow cells.

Gut enriched Kruppel-like factor is an eukaryotic zinc finger containing transcription factor expressed extensively in the gastrointestinal tract, particularly in the terminally differentiated villus cells. Its expression is associated with growth arrest. It has been shown to bind to the basic transcription element of the cytochrome P-450A1 promoter and suppress its activity (Zhang *et al.* 1998). Down regulation of GKLF expression in oesophageal squamous cancer causes the cells to become hyper-proliferative and less adhesive suggesting that decreased expression of GKLF may contribute to the malignant phenotype of oesophageal squamous cancer (Wang *et al.* 2002). GKLF was suppressed 2.30 fold in bone marrow cells in wild type mice but no change was documented in C5aR deficient mice bone marrow cells.

Analyses of the reproducible patterns of the above up- and down -regulated messages in the genes related to inflammation and its regulation has cast further light on the role of C5a *in vivo*. However the role of C5L2 in inflammation may be elucidated, when this study is repeated with C5L2 deficient mice. Work is currently underway on targeted deletion of the C5L2 gene from the mouse at our lab.

We demonstrated co-expression of C5L2 and C5aR on human granulocytes, a major player in the inflammatory process. C5a has a pro-inflammatory role in sepsis and antibody to C5a has been successful in improving survival rates in animal models of sepsis. While co-expression of C5L2 and C5aR on virtually all cells in the human monocyte, granulocyte pool certainly confirms the importance of C5L2, it does not clarify its exact role. It has been postulated that C5L2 may modulate the effective concentrations of C5a/C5desArg. In a local setting where both C5a and C5a des Arg are present with dynamically increasing quantities of C5a des Arg relative to C5a, C5L2 could spare the C5a to interact with the functional receptor, C5aR. Testing the *Pseudomonas aeruginosa* pneumonia model and the caecal ligation peritonitis model on C5L2 deficient mice (once developed) and comparing results to those from C5aR deficient mice and their wild type littermates will be useful in exploring the role of C5L2.

CHAPTER VI

DISCUSSION AND THE FUTURE OF C5L2

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6.1 GENERAL DISCUSSION OF C5A AND C5L2

During the proteolytic cascade of complement activation, small (~10 kDa) cationic fragments of C3, C4 and C5 are released, known as the anaphylatoxins C3a, C4a and C5a. The anaphylatoxins participate in host defence through initiating chemotaxis and activation of myeloid cells, enhancement of vascular permeability, contraction of smooth muscle, and as yet poorly understood functions on endothelial and epithelial cells (Guo *et al.* 2005, Gerard *et al.* 1994). Conversely, inappropriate activation of complement *in vivo* may be involved in a number of autoimmune diseases, asthma, rheumatoid arthritis and cardiac disease (Ward P.A. 2004).

Given the potential for anaphylatoxins to cause injury, mechanisms exist to limit the duration of their biologic activity. Serum carboxypeptidase N and R are recognised as anaphylatoxin inactivators (Matthews *et al.* 2004). All three anaphylatoxins contain carboxyl-terminal arginine residues. In the case of C3a and C4a, the desarginine derivatives are virtually incapable of binding to the C3a and C4a receptor, while in the case of C5a, the biologic activity decreases by more than one order of magnitude, and the binding affinity of the desarginine derivative changes from subnanomolar to micromolar. In whole serum or plasma, removal of the arginine by carboxypeptidase is close to instantaneous. Control of the residual activity of C5a des Arg is presumed to be through clearance and degradation of the ligand following receptor internalisation.

The variety, complexity and differences in the biological effects of C5a suggested existence of additional receptors for this anaphylatoxin. Studies revealed C5a has a pro-inflammatory effect in sepsis, systemic inflammatory response syndrome and myocardial reperfusion injury. In contrast, C5a exerts an anti-inflammatory effect in acute pancreatitis and associated lung injury (Bhatia *et al.* 2001).

In 2000, Ohno et al. (Ohno *et al.* 2000) described an orphan receptor with significant homology to the C5a receptor that is expressed on immature (but not mature) dendritic cells. Chromosomal analysis reveals that C5L2 is adjacent to the C5a receptor gene on human chromosome 19. Comparing the sequence alignment between C5aR, and C5L2, the N termini of C5aR and C5L2 contain several acidic residues that are characteristic of complement fragment receptors and that for C5aR at least form a part of the ligand binding site (Mery *et al.* 1993). C5aR has been shown to have distinct ligand binding and activation sites (Siciliano *et al.* 1994). Receptor activation by ligand involves the engagement of charged and uncharged residues on the extracellular faces of the transmembrane helices. For C5aR, these include Ile^{116}, Val^{286}, Arg^{175}, Glu^{199}, Arg^{206}, and Asp^{282} (Geva *et al.* 2000, Crass *et al.* 1999, Cain *et al.* 2001). C5L2 shares all of these residues except for Asp^{282}, where there is a Glu residue. Thus C5L2 has a similar acidic ligand-binding N terminal domain to C5aR, retaining the tyrosines flanked by acidic amino acids which have previously been demonstrated to be modified in the C5a receptor by sulphation (Farzan *et al.* 2001) and constitutes the major C5a docking site. C5L2 has a similar ligand activation domain to C5aR.

Comparing the aligned sequences of C5L2 from mouse and man we demonstrated that they possess 61.3% identity in the deduced protein and 68.9% identity in the nucleotide sequences. Notably, the unusual replacement of the essential arginine residue in the DRY sequence following the third transmembrane segment is conserved in mouse as in man as a leucyl residue. We demonstrated that the G protein recognition sequence in the third intracellular loop (between TM5 and TM6) is also shorter than exists in the C5aR by several cationic residues.

We demonstrated the presence of C5L2 mRNA in many human and murine tissues. Our results showed that C5L2 is particularly abundant in peripheral leukocytes and bone marrow in addition to spleen, testis, heart, brain, lung, liver, kidney, ovary and immature dendritic cells (Ohno *et al.* 2000). We demonstrated that C5L2 is actually a high affinity C5a and C5a des Arg binder, binding both ligands with ~ equal affinity (nanomolar Kd). In

contrast, C5aR binds C5a des Arg 100 fold less well than C5L2. We demonstrated that human neutrophils bind C5a as efficiently as C5aR and C5L2.

Based on data obtained using transfected HEK293T and red blood cells, Kalant *et al.* proposed that C5L2 is a functional receptor for C3a and C3a des Arg (Kalant *et al.* 2003) and suggested that C5L2 mediates the action of acylation-stimulating protein (APS, also known as C3a des Arg) and may have a role in lipid metabolism (Kalant *et al.* 2005). However, we have consistently been unable to detect binding of C3a to C5L2, using both transiently transfected HEK293T cells and stably transfected L1.2 mouse lymphoblasts. Interestingly one of the co-authors on Kalant's paper of 2003 has subsequently disagreed with his previous findings and demonstrated that using ^{125}I-ligand–binding assays and flow cytometry with fluorescently labelled ligands, that neither C3a nor C3a des Arg are ligands for C5L2 (Johswich *et al.* 2006). This concurs with our findings. Thus C5L2 is unlikely to be directly involved in lipid metabolism.

Regarding the role of C5L2, it demonstrates certain properties that are consistent with its role as a decoy or scavenger receptor for C5a. Firstly the receptor C5L2 displays greatly retarded association kinetics with C5a, so that at low concentrations of C5a, such as might occur in a gradient *in vivo*, the classical C5aR would effectively sequester the available C5a: C5aR has a very rapid "on rate", essentially at equilibrium within the first minutes following addition of ligand versus C5L2 which was ~100 fold slower, requiring at least 60 minutes to reach equilibrium. As higher concentrations of C5a are encountered, with the effect of reducing the locomotion of the cells, (chemotaxis curves are classically bell shaped), other C5a dependant effects such as degranulation and local release of products of NADPH oxidase are elicited. Under these conditions, the C5L2 may effectively sequester C5a des Arg.

C5L2 may modulate the effective concentrations of C5a and C5a des Arg. Both C3a and C5a are rapidly degraded by serum carboxypeptidase N or carboxypeptidase R to their respective des Arg derivatives (Campbell *et al.* 2001). At physiological concentrations, C3a des Arg is inactive at inducing smooth muscle contraction and increases in vascular

permeability, whereas C5a des Arg retains approximately 10% of the activity of intact C5a. In a local setting where both C5a and C5a des Arg are present with dynamically increasing quantities of C5a des Arg relative to C5a, we suggest that C5L2 could serve to spare the C5a to interact with the functional receptor.

The concept of decoy receptors is well established for the pro-inflammatory cytokines interleukin 1 (IL-1) and tumour necrosis factor alpha (TNFα). Heretofore, however, the only example of a similar molecule in the family of G protein coupled receptors is the Duffy antigen receptor for chemokines (DARC) on red blood cells, which has significant affinity for certain chemokines (Pogo *et al.* 2000). More recently, two additional members of the chemokine receptor family, D6 and CCR binding protein, (CCRbp/CCR11) (Nibbs *et al.* 1997, Schweickart *et al.* 2001, and Gosling *et al.* 2000) have been shown to be high affinity binding receptors for β chemokines and CCR7/CCR9 ligands, respectively. Curiously, both of these receptors have mutations in either TM2 or the DRY sequence that might be predicted to disrupt G protein coupling, as we have shown for C5L2. In addition, D6 is localised to lymphatic endothelium and may function to present chemokines to locally trafficking lymphocytes, macrophages or dendritic cells (Nibbs *et al.* 2001). Similarly the limited binding profile of the CCRbp (effectively CCR7 and CCR9 ligands) may portend a role for this receptor in regulating trafficking of CCR7 and CCR9 positive cells, perhaps by sequestering or presenting ligands.

Another feature of C5L2 consistent with its role as a modulator of anaphylatoxin concentration is the fact that it has a very slow dissociation rate with respect to ligand and is effectively anchored to the cell surface. In contrast, the classical C5aR, is rapidly phosphorylated and internalised (after binding C5a) a feature that is not significantly shared with C5L2. The functional C5aR is recycled to the cell surface while the ligand is degraded intracellularly. One interpretation we propose is that C5L2 could be effective in limiting or clearing the inflammatory response to C5a, acting as a "high affinity sink" for ligand. On the other hand, one can argue that interaction of C5L2 with C5a actually prolongs the inflammatory response beyond the transient effects of C5a, due to retention of the C5a-

C5L2 complex on the cell surface, by virtue of C5L2's slow dissociation rate and in the absence of internalisation of this complex.

In response to ligand C5a binding, C5L2, unlike C5aR, is not robustly phosphorylated, is not internalised and only minimally activates known distal pathways (such as MAP kinases), does not mediate calcium flux (in its wild type state), does not stimulate chemotaxis, nor mediate significant signal transduction events associated with G protein activation pathways.

The crystal structure of rhodopsin clearly demonstrates the essential nature of the guanidinum group of the arginine residue in the DRY sequence for formation of multiple hydrogen bonds with residues in transmembrane helices 3 to 6 (Okada *et al.* 2001). It has previously been demonstrated that mutagenesis of this arginyl residue is associated with loss of function with respect to G protein activation, without alteration in ligand binding (Kolakowski *et al.* 1995). Our lab used this approach, for example to dissociate G protein signalling from HIV-1 usage of CCR5 as a co-receptor for fusion and entry into cells (Farzan *et al.* 1997). We demonstrated that C5L2 is uncoupled to G proteins at least in part due to the natural substitution of leucine for arginine at position 132 in the DRY sequence. Employing site directed mutagenesis, we inserted a point mutation, (replacing leucine with arginine), and demonstrated a gain of function in coupling to Gα16. In our hands, HEK293T cells expressing C5L2(L132R) i.e. mutated C5L2, plus Gα16 are partially restored in their ability to mediate ligand induced changes in intracellular calcium concentration.

We have demonstrated abundant expression of C5L2 mRNA in human peripheral blood leukocytes and this has subsequently been confirmed (Otto *et al.* 2004). We demonstrated co-expression of C5L2 and C5aR on human granulocytes and in view of the co-location of both receptors and high affinity binding of C5L2 for C5a, we suspect that C5L2, like C5aR may have an important role in sepsis and other inflammatory conditions such as asthma, allergy and cystic fibrosis.

6.2 C5A MUTANTS

Recently C5a mutants were found to be potent antagonists of the C5a receptor, C5aR and of C5L2 (Otto *et al.* 2004). This group discovered a positively charged amino acid at position 69 as being crucial for the C5aR antagonism of the C5a mutant A8. They demonstrated that A8 and A8 $^{\Delta 71\text{-}73}$ are potent inhibitors of C5a and C5a des Arg binding to C5L2. They highlight the fact that because A8 $^{\Delta 71\text{-}73}$ is structurally very similar to C5a, blocks C5a and C5a des Arg binding to the two known receptors, (C5aR and C5L2) and is a powerful C5a receptor antagonist in animal models of inflammation, A8 $^{\Delta 71\text{-}73}$ may be a useful therapeutic agent to target C5a/ C5a des Arg activities in humans. In addition, A8 $^{\Delta 71\text{-}73}$ might be a valuable tool for studying the functions of C5L2.

6.3 SEPSIS C5L2, C5AR AND C5A

6.3.1 General discussion regarding sepsis, C5a and C5aR

A hallmark of sepsis is the uncontrolled activation of the complement system, resulting in excessive generation of anaphylatoxins, especially C3a and C5a (Goya *et al.* 1994, Hecke *et al.* 1997), which is accompanied by the ensuing dysfunction of neutrophils (Goya *et al.* 1994, Solomkin *et al.* 1981, Botha *et al.* 1995). Generation of C5a in the pathogenesis of sepsis has been described as "too much of a good thing" (Gerard C. 2003). As discussed earlier, blood polymorphs have diminished ability to bind C5a (Huber-Lang *et al.* 2001) with down-regulation of C5aR (Guo *et al.* 2003) while in lungs, kidneys, liver, and heart, vascular C5aR is up-regulated in an IL-6 dependant manner (Riedemann *et al.* 2003). Blockade of either C5a or C5aR improves survival rates of animals with sepsis (Czermak *et al.* 1999, Huber-Lang *et al.* 2002). In an LPS induced sepsis model in mice, C3aR expression has been described as being increased on both epithelial and smooth muscle cells (Drouin *et al.* 2001) suggesting an important role for complement receptors during the systemic inflammatory response of sepsis.

6.3.2 C5L2, C5aR and C5a in animal models of sepsis

It has been demonstrated that with the onset of caecal ligation and puncture (CLP)-induced sepsis, C5aR content on blood neutrophils drops, reaching its lowest point at 24 hours after CLP, presumably due to ligand-receptor internalisation (Guo *et al.* 2003). Correspondingly innate immune functions such as ligand-receptor chemotaxis and the oxidative burst of neutrophils were substantially impaired (Guo *et al.* 2003). Beyond 24 hours after CLP, blood neutrophils start to undergo a functional recovery, correlating with increased surface C5aR expression, (Guo *et al.* 2003).

Recently experimental sepsis in rodents after CLP was studied to evaluate the functional role of C5L2 (Gao *et al.* 2005). The authors concurred that C5aR content on neutrophils significantly dropped (by 2.5-fold), 24 hours after CLP and steadily increased thereafter. Interestingly, they demonstrated that C5L2 content on blood neutrophils increases significantly between 24 and 36 hours after the onset of CLP. To determine the mechanism by which C5L2 content on neutrophils is regulated during sepsis, they evaluated C5L2 mRNA levels in purified neutrophils at 0, 24, and 36 hours after CLP. The C5L2 mRNA level dramatically increases 24 and 36 hours after the onset of CLP suggesting a transcriptional mechanism is involved in increased expression of C5L2. To visualise C5L2 changes in neutrophils during sepsis, they conducted confocal fluorescence microscopy analysis. In control cells, both C5aR and C5L2 showed a uniform cortical pattern on the neutrophil periphery, indicative of membrane staining. In contrast, a diffuse pattern of staining was found in the cytoplasmic compartment in 24 hour CLP neutrophils for C5aR, but not C5L2, indicating that C5L2 does not undergo internalisation like C5aR (concurring with our results) after CLP. The authors suggest that C5L2 and C5aR may be independently regulated in blood neutrophils during sepsis.

It is well established and our results confirm that interaction of C5a with C5aR leads to rapid internalisation of the receptor-ligand complex because of phosphorylation of serine residues in the receptor, while dephosphorylation of the receptor results in it being recycled

back to the surface of the cell (Naik *et al.* 1997, Giannini *et al.* 1995, Van Epps *et al.* 1990). Although excessive C5a appears to be produced during sepsis, we propose that the lack of phosphorylation of C5L2 after contact with C5a may be linked to the absence of internalisation of the C5a/C5L2 complex. Previous work had demonstrated that neutrophils from 24 and 48 hour CLP rats showed nearly 400% increase in the surface content of C5a (Czermak *et al.* 1999). Based on these findings it is possible that the substantial amounts of C5a on the surface of blood neutrophils during sepsis are due to C5a binding in a stationary manner to surface C5L2.

It was previously demonstrated that there is an increased *in vivo* binding of C5aR and accompanying increased expression of mRNA for C5aR in mouse lung, liver, kidney and heart after CLP-induced sepsis (Riedemann *et al.* 2002). Gao and colleagues (Gao *et al.* 2005) demonstrated that the *in vivo* binding of ^{125} I-anti-C5L2 was significantly increased in mouse lung, liver and heart but not kidney, 12 hours after CLP when compared with levels at time 0. This suggests increases in C5L2 in multiple organs during sepsis. In addition real-time PCR experiments show dramatically increased mRNA expression for C5L2 in mouse liver and lung during sepsis. The biological implications of changes in C5a receptors during sepsis are not currently known but may be linked to development of multi-organ failure. Furthermore whether the regulation of C5L2 expression in the different organs during sepsis is similar to that in neutrophils remains to be defined. Because it has been shown in an animal model of CLP-induced sepsis that lungs, liver, heart and kidneys are targets for development of multi-organ failure, and blocking of C5a reduces the intensity of multi-organ failure (Huber-Lang *et al.*2001), it will be of great interest to explore if C5L2 plays a functional role in these organs during sepsis.

In addition the authors (Gao *et al.* 2005) provide evidence that exposure of rat neutrophils to C5a and LPS results in dramatically increased release of IL-6 in the presence of anti-C5L2. In contrast, blockade of C5aR significantly reduced the *in vitro* IL-6 levels after cell stimulation by C5a and LPS. Furthermore, mice injected with anti-C5L2 at the start of CLP show dramatically increased serum levels of IL-6 when compared with control animals receiving non-specific IgG. In contrast, serum levels of IL-6 are reduced to nearly

undetectable levels in anti-C5aR treated mice (Gao *et al.* 2005). These results, coupled with the fact the demonstration of increased surface expression of C5L2 on neutrophils during sepsis concur with our belief that C5L2 may have an important functional role during inflammatory events, behaving as a decoy receptor (or scavenger) receptor for C5a, thereby limiting the inflammatory response caused by C5a.

6.3.3 C5L2 during experimental and clinical sepsis in humans

Despite aggressive management of sepsis and sepsis–related complications, the lethality rate remains at 40-60 % for patients in septic shock (Rangel-Frausto *et al.* 1995, Martin *et al.* 2003). Randomised clinical trials have been performed with various anti-inflammatory approaches, (enrolling >12,000 patients) all of which failed to show improvement in survival (overall lethality of 38% in each group (Astiz *et al.* 1998, Vincent *et al.* 2002).
Recently a group (Huber-Lang *et al.* 2005) describe changes in C5L2 during experimental and clinical sepsis in humans. Three approaches were used. Firstly, excessive generation of C5a during sepsis was simulated *in vitro* by exposure of polymorphonuclear leukocytes (PMNs) to a C5a concentration found in humans with sepsis (Hecke *et al.* 1997, Solomkin *et al.*1981). Secondly, C5L2 content on PMNs was evaluated during CLP induced sepsis: CLP has been documented to closely mimic the pathophysiological conditions of sepsis in humans (Deitch *et al.* 1998, Wichterman *et al.* 1980). Thirdly, C5L2 expression was determined in human blood PMNs in patients who were suffering from severe sepsis or septic shock, as defined by consensus criteria for the definition of sepsis (Bone *et al.* 1992, Levy *et al.* 2003).

6.3.3.1 Experimental studies: exposure of PMN to C5a levels found in humans with sepsis

C5L2 was detected by Western blot analysis on both rat and human PMN. *In vitro* exposure of either rat or human PMNs to 10nM rat C5a or human C5a, respectively revealed a time-

dependant loss of C5L2 on PMNs. In simultaneously performed flow cytometry studies, C5a exposure of human PMN (up to 4 hours), also exhibited a time- dependant decrease of C5L2 on PMN surfaces. By Western blot analysis, C5L2 content was diminished in cell lysates and was not detected in supernatant fluids after incubation of PMNs with C5a, suggesting a loss of C5L2 on PMNs after exposure of PMN to C5a for 2-4 hours. Interestingly, similar results have been reported for the heptahelical CC-chemokine decoy receptor, D6, which is capable of ligand internalisation and degradation in the absence of the classical G protein-dependant signalling properties (Fra *et al.* 2003, Thelen *et al.*2001). Others have demonstrated that monocytes exposed to bacterial LPS show reduction in RNA expression of the IL-1 decoy receptor (IL-1-1RII), suggesting that under certain conditions, a down–regulation of the decoy receptor can occur, perhaps causing monocytes to be more responsive to IL-1β (Penton-Rol *et al.* 1999).

6.3.3.2 Experimental studies: evaluation of C5L2 content on PMN during caecal ligation and puncture (CLP)

Several experimental sepsis studies on rodents have indicated protective effects by blockade of either C5a or C5aR (Huber-Lang *et al.* 2001, Czermak *et al.* 1999, Huber-Lang *et al.* 2002). In 2005, Huber-Lang and colleagues investigated the effects of intravenous infused anti-C5a on C5L2 in PMN during CLP-induced sepsis. As expected, and in accordance with previous reports (Huber-Lang *et al.* 2001, Czermak *et al.* 1999, Huber-Lang *et al.* 2002, Huber-Lang *et al.* 2001) CLP rats treated with anti-C5a showed improved outcome (75% survival), whereas the survival rate in CLP animals treated with pre-immune IgG was <25%, 48 hours after CLP. In these studies, blood PMN from CLP animals treated with pre-immune IgG revealed a time-dependant loss of C5L2. In the early stage, of experimental sepsis (<12 hours after CLP), blood PMN from all animals had easily detectable C5L2 levels regardless of the experimental manipulations. Following the appearance of clinical signs of sepsis (>12hours), C5L2 in blood PMN was barely detectable by Western blot analysis, and by 36 hours had virtually disappeared. It is noteworthy that during the experimental sepsis course there was a 12-18 hour delay in

C5L2 reduction relative to changes described for C5aR (Huber-Lang *et al.* 2001). Other mediators of the complement cascade or the chemokine/cytokine network could be involved in C5L2 reduction (e.g. excessively generated C3a or its degradation products (Huber-Lang *et al.* 2002, Furebring *et al.* 2002). The loss of C5L2 during progressive CLP sepsis was in striking contrast to the condition of anti-C5a treated CLP animals that had minimal clinical signs of sepsis and exhibited preservation of C5L2 on blood PMN. These findings indicate that generation of C5a during sepsis, or *in vitro* exposure of PMN to C5a leads to loss of C5L2, whereas blockade of C5a in CLP animals not only maintains expression of C5L2, but greatly improves clinical outcome. Several studies support the concept that decoy receptors are beneficial during inflammatory responses. In a model of *Streptococcus pyogenes* induced arthritis in mice, the presence of osteoprotegerin, a decoy receptor for receptor activator of NF-kB ligand, prevented bone destruction in infected joints (Sakurai *et al.* 2003). In another inflammation model in which Fas ligand induced lung inflammatory injury was studied, the presence of the decoy receptor 3 analogue reduced PMN recruitment into the alveolar compartment and inhibited the appearance in bronchoalveolar lavage fluids of pro-inflammatory cytokines (Wortinger *et al.* 2003). The anti-inflammatory IL-10, was found to uncouple pro-inflammatory chemokine receptors and to generate functional decoy receptors that act as scavengers for inflammatory cytokines (D'amico *et al.* 2000).

When C5a binds to C5aR, serine and threonine residues in the C-terminal, cytoplasmic region of the receptor are phosphorylated, resulting in rapid internalisation involving ~60% of the receptor and subsequent recycling of C5aR to the membrane in the span of 80-90 minutes (Braun *et al.* 2003, Naik *et al.* 1997, Bock *et al.* 1997). During experimental sepsis, blockade of C5a resulted in retention (or fast recovery) of both C5a-binding capacity (Huber-Lang *et al.* 2001) and C5aR content on PMN (Guo *et al.* 2003). In contrast, we demonstrated that C5L2 does not undergo rapid internalisation in response to ligand binding. This may be due to a different pattern of serine/threonine phosphorylation in the carboxyl tail of C5L2.

6.3.3.3 Human studies: C5L2 expression determined in human blood PMN in patients with sepsis or septic shock

The *in vitro* and *in vivo* findings of Huber-Lang and colleagues (Huber Lang *et al.* 2005) demonstrate for the first time, that in humans with severe sepsis or septic shock, a significant reduction in C5L2 expression occurs on PMN. This is in contrast to the results of Gao and co-workers from animal studies of sepsis (Goa *et al.*2005). Strikingly, Huber-Lang's group demonstrated that C5L2 content in PMN obtained from patients with sepsis who survived an observation period of 30 minutes exhibited higher levels of C5L2 than patients with sepsis who failed to survive. Furthermore, reduced C5L2 expression seems to correlate with sepsis induced multi-organ failure, which experimentally has been shown to be attenuated by anti-C5a (Huber-Lang *et al.* 2001) and by C5aR antagonist (C5aRa) treatment (Harkin *et al.* 2004), although C5a receptor antagonist (C5aRa) fails to directly interact with C5L2 (Otto *et al.* 2004). This data does not explain if C5L2 functions as a decoy receptor to mitigate against the consequences of interactions between C5a and C5aR. The authors felt that it is likely that C5L2 functions as a C5a /C5a des Arg scavenging receptor in PMN during sepsis. As a recent report suggests, the IL-1β decoy receptor may be a target for potential gene therapy in arthritis (Attur *et al.* 2002). Although speculative, up regulation of C5L2 might be a novel therapeutic approach to block the harmful effects of C5a during sepsis and developing multi organ failure.

6.4 An anti-inflammatory function for C5L2: studies with C5L2 deficient mice

In addition to the work discussed two other articles in 2005 proposed an anti–inflammatory role for C5L2: the first examined C5L2 deficient mice (Gerard *et al.* 2005) and the second examined C5L2 in rat astrocytes (Gavrilyuk *et al.* 2005).

Gerard *et al.* developed mice bearing a targeted deletion of C5L2 (Gerard *et al.* 2005). They studied the functional role of C5L2 in the immune complex mediated lung injury model: this is an intrapulmonary version of a reverse passive Arthus reaction and has been

demonstrated to be dependant on C5a acting at the C5a receptor (Hopken *et al.* 1997). Mice were given ovalbumin via tail injection, anti-ovalbumin IgG was instilled into the trachea and after 4 hours animals were sacrificed and analysed for inflammatory markers. The bronchoalveolar lavage (BAL) fluid was analysed for total cells, neutrophils and monocytes. A dramatic increase in total cells and neutrophils was observed in C5L2 deficient mice versus wild type animals. This phenotype for C5L2 deficient mice is opposite to the results obtained with mice deficient in the classical C5a receptor, C5aR (Hopken *et al.* 1997). The increased cellular influx correlated with an increase in the concentration of TNF-α and IL-6 in lung homogenates and in TNF-α levels in BAL. Both cytokines were increased ~4 fold in immune complex treated C5L2 deficient mice compared to wild type animals. These findings were confirmed by histological examination of the bronchoalveolar lavage fluid and lung parenchyma. C5L2 deficient mice were also more responsive to C5a than wild type mouse cells (expressing both receptors), in *in vitro* chemotaxis assays. Bone marrow cells isolated from C5L2 deficient mice and wild type littermates were examined for their ability to migrate to 10nM C5a relative to 8μM fMLP. Chemotaxis of C5L2 deficient cells to C5a was ~40% greater relative to the response to 8μM fMLP than cells from wild type mice.

Interestingly, it has been demonstrated that the overall expression level of mRNA for C5L2 is significantly lower than that for the C5a receptor and generally does not have a 1:1 correspondence among tissues (Su *et al.* 2002). The median expression values for C5L2 are approximately one third that of the C5aR. In some murine tissues, for example pancreas and spleen, high levels of C5aR expression are associated with below median expression of C5L2, while in lactating mammary gland and skeletal muscle, the mRNA levels for these two receptors are nearly equal. Clearly, the significance of these differences is subject to evaluation of the protein levels, but it suggests that C5L2 acts independently of the C5a receptor to counteract C5a/C5a receptor mediated inflammation. Whether it does so by reducing the effective C5a levels available to act at the classical C5a receptor as suggested by Gao *et al.* (Gao *et al.* 2005), by triggering anti–inflammatory signalling pathways, or both, awaits further study.

Gavrilyuk and colleagues demonstrated that C5L2 is expressed by glia and neurons in rat brain tissue (Gavrilyuk *et al.* 2005). It is well documented that brain inflammation is regulated by endogenous substances including neurotransmitters such as noradrenaline, which can increase anti- inflammatory genes. Quantitative PCR confirmed that in astrocytes, C5L2 mRNA expression is increased by noradrenaline, whereas *in vivo* depletion of cortical noradrenaline reduced C5L2 levels. Immunocytochemical staining of adult rat brain revealed C5L2 immunoreactivity staining through out the brain co-localised to neurons and astrocytes. The *in vivo* data suggested that the normally present levels of cortical noradrenaline maintain C5L2 expression thus contributing to an "anti-inflammatory" environment. Similarly *in vitro,* in the absence of exogenously added noradrenaline, C5L2 levels are low and cells are receptive to inflammatory stimuli. Noradrenaline addition increases C5L2 levels and other anti-inflammatory genes and thereby increases the overall "anti-inflammatory" or "refractory" state of the cells. The authors suggest that changes in C5L2 levels in diseased brain could therefore contribute to the progression of inflammatory damage and cell death in neurological diseases including multiple sclerosis, Alzheimer's and Parkinson's disease.

6.5 C5L2 is critical for the biological activities of the anaphylatoxins C5a and C3a

A recent paper published in Nature illuminated some of the roles of C5L2 (Chen *et al.* 2007). Using gene targeting, this group demonstrated that C5L2 is required to facilitate C5a signalling in neutrophils, macrophages and fibroblasts *in vitro*. They showed that in certain *in-vivo* settings deficiency of C5L2 results in reduced inflammatory cell infiltration, suggesting that C5L2 is critical for optimal C5a-mediated cell infiltration. In addition they showed that C5L2 is involved in optimising C3-induced signals. Furthermore, C5L2 deficient mice are hypersensitive to lipopolysaccharide induced septic shock, show reduced ovalbumin induced airway hyper-responsiveness and inflammation and are mildly delayed in haematopoietic cell regeneration after γ-irradiation, like mice incapable of C3a/C3aR signalling (Humbles *et al.* 2000, Kildsgaard *et al.* 2000, Ratajcak *et al.* 2004). Their data

indicate that C5L2 can function as a positive modulator for both C5a- and C3a-anaphylatoxin-induced responses.

The authors provide evidence that C5L2 is an important downstream mediator of C5a functions. Furthermore, they suggest that specific aspects of LPS signalling seem to depend on the presence of C5L2. To investigate the role of C5L2 in C5a-mediated responses, neutrophils were stimulated and the surface Mac-1 (also known as Itgam) induction was monitored. Mac-1 is normally enhanced when neutrophils are activated. Neutrophils from C5L2 deficient mice (C5L2-/- neutrophils), showed impaired Mac-1 induction compared with wild type cells in response to C5a alone. This defect was magnified when neutrophils were stimulated with C5a plus LPS. They examined TNF-α and IL-6 production by neutrophils after stimulation. LPS alone induced comparable levels of TNF-α and IL-6 in wild type and C5L2-/- neutrophils. As previously reported, C5a treatment reduced TNF-α production (Riedemann *et al.* 2003) but enhanced IL-6 production (Hawlisch *et al.* 2005) in LPS-stimulated wild-type neutrophils. In contrast C5a was unable to affect LPS-induced TNF-α and IL-6 production in C5L2-/- cells. Furthermore C5a des Arg induced strong Mac-1 induction in wild-type neutrophils, but not in C5L2-/- cells. However, neither C3a nor C3a des-Arg induced Mac-1 expression in wild type or C5L2-/- neutrophils.

Mitogen activated protein kinase (MAPK) activation has recently been recognised as a key downstream event induced by C5a (Riedemann *et al.* 2004, Perianayagam *et al.* 2002). In wild type neutrophils, C5a treatment alone strongly induced ERK1/2 activation and weakly enhanced phospho-p38 expression in the author's hands. A low dose of LPS enhanced C5a-induced activation of various MAPKs. In contrast C5L2-/- neutrophils showed only weak induction of activated ERK1/2, JNK or p38 in response to C5a, either alone or with LPS.

C5a is known to have a critical role in regulating macrophage functions (Hawlisch *et al.* 2005). The authors demonstrated that C5a-induced activation of ERK1/2 was severely impaired in C5L2-/- macrophages from both C57BL/6 and BALB/c strains of mice. In addition, LPS strongly up-regulated expression of the co-stimulation molecules CD40 and CD86 in wild type macrophages, whereas C5a suppressed this induction. In contrast,

C5L2-/- macrophages showed slightly higher basal CD40 and CD86 expression. Furthermore, C5a-mediated suppression of LPS-induced CD40 and CD86 expression was impaired in C5L2-/- macrophages. Unlike in neutrophils, C5a suppressed LPS-induced TNF-α and IL-6 production in wild type macrophages: a process that is impaired in C5L2-/- cells. Thus C5L2 is an important downstream mediator of C5a functions and specific aspects of LPS signalling seem to depend on the presence of C5L2.

To determine whether C5L2 is involved in C3a-initiated signalling, Chen and co-workers stimulated neutrophils with C3a and examined downstream signals. Phosphorylated ERK1/2 were readily detected in wild type neutrophils, following stimulation, but these signals were substantially reduced in C3a stimulated C5L2-/- neutrophils. Similar reductions were detected in bone marrow derived C5L2-/- macrophages. Actin polymerisation is an early consequence of neutrophil activation induced by chemoattractants. The authors detected C3a-induced F-actin formation in wild type and C5L2-/- neutrophils. Almost 50% of wild-type cells were positive for F-actin staining after stimulation, compared with only 11% of C3a-stimulated C5L2-/- neutrophils. Thus the authors concluded that C5L2 is required for optimal C3a signalling.

As discussed earlier, recent report (Gerard *et al.* 2005) demonstrated that C5L2 deficient mice were capable of responding to intra-peritoneal injection of recombinant C5a and no significant difference was observed between wild-type and C5L2 deficient mice in terms of short term neutrophils recruitment. The authors tested the effect of C5L2 deficiency on thioglycollate (TGC)-induced peritonitis, knowing that C5a/C5aR mediated signalling is important for regulation of inflammatory responses *in vivo* (Hopken *et al.* 1996, Guo *et al.* 2004). In comparison to TGC-treated wild-type mice, TGC-treated C5L2 deficient mice showed a mild reduction (35%) in macrophage infiltration into the peritoneal cavity at 72 hours. This suggests that absence of C5L2 does not impact on *in vivo* C5a signals to the same degree as C5aR deficiency, and that certain short-term cell recruitment effects require only C5aR. To characterise long term defects of inflammatory cell migration in C5L2 deficient mice, Chen *et al.* (Chen *et al.* 2007) generated dorsal air pouches on mice six days before injection of C5a and TGC. Infiltrated cells were recovered and analysed for total cell

numbers and numbers of neutrophils and macrophages. Co-injection of C5a and TGC induced inflammatory cell infiltration that was more robust in wild type air pouches versus those of C5L2 deficient mice. Fewer neutrophils (2.3 fold) and fewer macrophages (3.5 fold) were present in the inflammatory infiltrates of C5L2 deficient mice compared with wild type controls after 24 hours of stimulation. Importantly, activation of the infiltrated neutrophils in the air pouches of C5L2 deficient mice was impaired compared with wild-type mice.

Regarding asthma, both C5a and C3a have been implicated in its pathogenesis so it was logical to test how C5L2 deficiency affects the development of asthma-like airway hyper-responsiveness and inflammation. Airway hyper-responsiveness to inhaled metacholine in ovalbumin (OVA) sensitised (multiple challenges of OVA) wild type BALB/c mice is markedly enhanced compared with normal saline treated controls. C5L2 deficient mice demonstrated markedly reduced airway hyper-responsiveness compared with their sensitised wild type littermates. This was confirmed by histological analyses demonstrating markedly reduced allergen-induced inflammatory cell infiltration in C5L2 deficient mice versus wild-type littermates (Chen *et al.* 2007). Thus C5L2 appears to be important in the pathogenesis of asthma possibly related to the involvement of C5a and C3a.

Regarding LPS challenge, C5L2 deficient mice showed increased susceptibility to lethal effects of intra-peritoneal injected LPS and higher IL-1β levels than their wild-type littermates. These results are similar to those demonstrated in C3aR deficient mice (Kildsgaard *et al.* 2000) suggesting that both C3aR and C5L2 are involved in a regulatory mechanism that normally protects against LPS–induced shock responses. Further evidence that C5L2 may be important in optimising C3a-dependent signalling *in vivo* was that peripheral blood leukocyte recovery was perturbed in C5L2 deficient mice after sub-lethal irradiation compared to wild type controls. C3a is known to initiate signalling to promote haematopoietic stem cell engraftment.

As discussed above, C5L2 has been implicated in the regulation of inflammation (Gerard *et al.* 2005, Gavrilyuk *et al.* 2005) and sepsis (Gao *et al.* 2005, Huber-Lang *et al.* 2005). C5L2 has also been reported to mediate acylation stimulating protein-induced signalling and β-arrestin redistribution (Kalant *et al.* 2005) suggesting that C5L2 is capable of transducing signals. The authors suggest that C5L2 may contribute to signalling induced by C5a or C3a by hetero-dimerising with C5aR or C3aR, though no interactions between C5L2 and C5aR or C3aR were detected in their hands. They suggest that C5L2 may be critical for up-regulating cell surface expression of C5aR particularly in neutrophils.

Knowing that ectopic expression of C5L2 in rat glioma cells can decrease inflammatory responses even without ligand stimulation (Gavrilyuk *et al.* 2005), the authors propose that ligand stimulation of C5L2 may trigger signals and contribute to C5a and C3a signalling outputs in parallel to the classical receptors. To investigate further the role of C5L2 in C5a-mediated signalling, Chen and colleagues ectopically expressed C5L2 in C5L2 deficient and C5aR deficient lung fibroblasts (Chen *et al.* 2007). Over-expression of C5L2 in C5L2 deficient cells but not C5aR deficient strongly enhanced ERK activation induced by C5a stimulation. In contrast, C5L2 deficient cells reconstituted with a truncated C5L2 without the first amino-terminal extracellular domain or with a truncated last carboxy-terminal intracellular domain, did not show enhanced ERK signals in response to C5a. These results indicate that reconstitution of C5L2 can mediate optimal C5a signal in the C5L2 -/- cells and that the presence of both C5aR and C5L2 is required for optimal signals to be induced by C5a. The authors generated stable RAW cell lines which contain endogenous C5L2 and C5aR. Over-expression of C5L2 reduced LPS-induced IL-6 production. These results are consistent with the possibility that over-expressed C5L2 can generate signals to activate ERK as well as to down regulate lipopolysaccharide/Toll like receptor 4 (LPS/TLR4)-induced responses.

Thus C5L2 can act as a positive regulator that is critical for C5a and C3a signalling. C5L2 can work co-operatively with signals mediated by C5aR and C3aR and has been shown to be involved in controlling both pro-inflammatory and anti-inflammatory responses and in particular certain aspects of LPS signalling.

6.6 Therapeutic interventions in sepsis

Sepsis is a major problem world wide from both a morbidity and mortality viewpoint (mortality rate 40-60%) and a financial viewpoint. In the United States it affects ~600,000 individuals per annum compared with ~50,000 deaths per annum due to road traffic accidents and similar numbers due to breast cancer and prostate cancer. Cardiovascular disease is one of the only disease categories with a higher mortality rate than sepsis (Sarnak *et al.* 2000). Although sepsis seems to unleash an up-regulated systemic inflammatory response, clinical trials that are designed to suppress specific inflammatory mediators in humans with sepsis have largely failed (Vincent *et al.* 2002, Riedemann *et al.* 2003). This "one by one" strategy of targeting mediators has so far worked in animals, especially rodents but not in humans (Riedemann *et al.* 2003). Two main problems in the treatment of sepsis exist: firstly it can be difficult to diagnose accurately especially in the early stages and no specific laboratory test currently exists to diagnose this early phase accurately, and secondly internationally we do not fully understand its pathogenesis.

6.6.1 Therapeutic interventions used in animal models of sepsis

Interventions that have been successful in sepsis in rodents but not in humans include tumour necrosis factor specific antibody (Beutler *et al.* 1985, Reinhart *et al.* 2001), LPS antibody (Cohen *et al.* 1999), C1 inhibitor (Liu *et al.* 2003), interleukin-1 receptor antagonist (IL-1Ra), (Fisher *et al.* 1997, Opal *et al.* 1997), platelet activating factor antagonists (Dhainaut *et al.* 1994, Dhainaut *et al.* 1998), eicosanoid inhibitors (Vincent *et al.* 2001, Yu *et al.* 1993), antioxidants (Spies *et al.* 1994), nitric oxide inhibitors (Avontuur *et al.* 1998), and thrombin III-specific antibody (Warren *et al.* 2001). Activated protein C has been successful in the treatment of sepsis in humans. This inhibits the clotting factor, factor Va and factor VIIIa, effectively blocking coagulation. Activated protein C indirectly increases fibrinolysis by inhibiting plasminogen activator inhibitor 1 (PAI1). In clinical

trials in humans with sepsis, treatment with recombinant activated protein C reduced the mortality rate from 31% to 25% (Bernard *et al.* 2001) forming the basis for approval of activated protein C use in humans with sepsis.

In vivo neutralisation of C5a with the use of specific antiserum is largely protective in a CLP model of sepsis (Czermak *et al.* 1995). An excess of intravascular C5a down regulates C5a receptors and decreases responsiveness to other neutrophil mediators, probably through heterologous desensitisation of the inflammatory G-protein coupled receptors. Oddly neutralisation of intravascular C5a by anti-C5a antibodies enhances bacterial clearance, presumably though alternative neutrophil-chemoattractant receptors such as IL-8 and leukotriene B_4 (LTB4). In primates infused intravenously with *Escherichia coli*, neutralisation of C5a also protects against the sepsis syndrome (Stevens *et al.* 1986). A concern about the clinical usefulness of this work is that neutralisation of C5 inhibits the formation of C5-C9, which is important in bacterial clearance. For this reason a selective inhibitor of C5a or C5aR is being sought. However, lack of C5, as in naturally deficient mice or in C5-deficient humans, does not seem to cause major problems with survival although the mice are susceptible to pneumonia. This indicates that blockage of C5a or C5aR in humans might not markedly jeopardise host defences.

6.6.2 Interventions that hold promise in humans with sepsis

Interventions that have demonstrated promise in rodents include C5a-specific antibody which improved survival from 20% to 70% (Czermak *et al.* 1999) as did C5a receptor antagonist (C5aRa) (Huber-Lang *et al.* 2002), and C5aR-specific antibody (Riedemann *et al.* 2002). C5aRa is a synthetic, cyclical peptide that competes with the carboxy-terminal region of C5a for binding to C5aR, effectively preventing C5a to induce signalling in C5aR-containing cells. This peptide binds to C5aR with high affinity and is resistant to hydrolysis by serum enzymes. C5aRa had previously been shown to inhibit the adverse effects in rats after LPS infusion (Short *et al.* 1999). High mobility group B1 specific protein (HMGB1) (Wang *et al.* 1991), migration inhibitory factor (MIF) specific antibody

(Calandra *et al.* 2000) and IL-6 specific antibody (Riedemann *et al.* 2003) have all been employed in rodents with sepsis. These interventions have not been utilised in humans with sepsis to date. A potential concern in blockade of C5a or C5aR would be that, if the problem of infection was local, such blockade might compromise local defences, in contrast with sepsis when excessive C5a causes paralysis of innate immune functions of neutrophils.

6.6.3 Location of C5a may be critical

It seems plausible that in the sepsis syndrome, the location of C5a may be critical in terms of its potential to protect or harm. Local generation of C5a which occurs for example in an animal model of *P. aeruginosa* (Hopken *et al.* 1996) is essential for early control of infection. In contrast, an excess of intravascular C5a cripples neutrophils, allowing unrestrained proliferation of bacteria. Simultaneously, sequestration of neutrophils in the micro-circulation injures the lungs, liver and kidney (Riedemann *et al.* 2002). In this proposed mechanism, it is leukocytes and not C5a receptors in the target organ that may be critical because, in the model of *P. aeruginosa* pneumonia, massive up-regulation of epithelial C5a receptors also occurs (unpublished data) and yet deletion of C5a receptors leads to a fatal pneumonitis (Hopken *et al.* 1996). The effect of C5a is mediated by cellular C5a receptors and blockade of these receptors both on myeloid cells and epithelial cells holds promise for the future.

In the sepsis syndrome, C3a and C5a may have opposite effects. Genetic deletion of the C3a receptor in mice increases susceptibility to endotoxin-induced shock and increases circulating levels of TNF-α and interleukin-1-β. Thus it has been proposed that C3a receptors have an anti-inflammatory effect. Whether these effects, which have been observed with injected endotoxin extend to actual sepsis in humans remains to be determined.

6.7 Future studies regarding C5L2 : laboratory, animal, and human

6.7.1 Future laboratory and animal studies regarding C5L2

Further work into the mechanism of how C5L2 affects C5a function is warranted: whether C5L2 affects C5a function through the formation of heteromeric complexes with the C5aR, or whether it utilises a G–protein independent signalling pathway would prove interesting and possibly fruitful for drug targeting.

Future animal experiments that may yield interesting results include subjecting C5L2 deficient mice to intra-tracheal *P. aeruginosa* and comparing results to those of C5aR deficient mice. This is superior to the LPS model which does not provide the challenge of a live, viable and potentially multiplying bacteria. Double knock out mice of both C5L2 and C5aR subjected to the *Pseudomonas* pneumonia model, the CLP model, the reverse Arthus reaction model and the ovalbumin induced hyper-responsiveness and inflammation model might prove interesting when compared to C5L2 deficient and C5aR deficient mice and wild type mice (already performed).

6.7.2 Future human studies regarding C5L2

It will be very interesting to explore the role of C5L2 further in patients with sepsis, pneumonia, asthma, cystic fibrosis and paroxysmal nocturnal haemoglobinuria. Two avenues could be explored. Firstly, perhaps there are different C5L2 polymorphisms which make people more susceptible to the above disorders. This could be assessed by investigating patients who are critically ill with septic shock and associated multi-organ failure requiring treatment in the intensive care unit (I.C.U.) versus non-critically ill gram-negative bacteraemic patients treated on a hospital ward versus healthy controls. Similarly, severe asthmatics with status asthmaticus could be compared to asthmatics with exacerbations treated on the ward versus healthy controls. Currently Ireland is ranked fourth highest in the world for asthma and 1 in 5 Irish children have experienced asthma

symptoms. C5L2 polymorphisms may be relevant in people with paroxysmal nocturnal haemoglobinuria who lack the ability to inhibit complement mediated haemolysis. In response to treatment with a humanised monoclonal antibody (Eculizumab) that binds C5 complement protein they had significant reduction in blood transfusions and improved quality of life score. Patients with cystic fibrosis (of which Ireland has a very high incidence), who are colonised early in childhood with *Pseudomonas aeruginosa* and therefore have a poorer prognosis may have different C5L2 polymorphisms to those who are colonised later or never chronically colonised. Autoimmune disorders such as rheumatoid arthritis and psoriasis are worthy of investigation in view of the increased C5aR and C5a des Arg found in these conditions respectively.

Secondly, measuring C5a/C5aR, C3a/C3aR and C5L2 levels in critically ill patients with sepsis versus ward treated gram- negative bacteraemic patients versus healthy controls may give us insight into the role of C5L2, now known to be critical to the biologically activity of both anaphylatoxins important in sepsis. Recently, a distinct cytokine pattern in humans with severe sepsis or septic shock was demonstrated (O'Dwyer *et al.* 2006) using quantitative real time PCR. IL-6 distinguished between survivors and non survivors on day 1 of I.C.U. stay and was related to severity of the disease on day 7, in keeping with previous studies (Goldie *et al.* 1995, Damas *et al.* 1997). Higher IL-10 mRNA levels were detected in the I.C.U. group with severe sepsis/septic shock compared with the control group. Lower IFN-γ mRNA levels were detected in the I.C.U. group versus the bacteraemic group and the control group. Lower levels of both IFN-γ and TNF-α were associated with the presence of shock day 7 while survivors had higher TNF-α mRNA day 7 of I.C.U. stay than non survivors. Furthermore, IL-27 mRNA distinguished between the three groups, with highest levels in the I.C.U. group, intermediate in the bacteraemic group and lowest in the control group. IL-23 distinguished between the groups in an opposite pattern with lowest levels in the I.C.U. group. Greater IL-27: IL-23 ratios in late sepsis were associated with the development of septic shock, higher organ failure scores and an increased risk of death, both early and late. In late sepsis IL-23 and TNF-α mRNA levels were directly related ($p<0.0001$).

This study is particularly interesting because of the complex interaction and positive feedback between IL-6 generation and C5aR up regulation, between C5aR activation (via C5a) and IL-6 production and between C5L2 and IL-6. C5a enhances mediator induced production of cytokines and chemokines. C5a negatively regulates Toll like receptor 4 (TLR4) and CD40 induced synthesis of IL-12 family cytokines (IL-12, IL-23 and IL-27) from inflammatory macrophages. This decreased cytokine response translates into a decreased T helper type 1 (Th1) response *in vitro* and *in vivo*. Knowing that C5L2 modulates the effects of C5a and C3a, a study examining C5L2 polymorphisms, C5a/C5aR, C3a/C3aR and C5L2 levels and cytokine profile as above could prove very interesting.

Viral bronchiolitis is the leading cause in paediatrics for hospitalisations in the developed world and causes an estimated one million deaths per year worldwide (Collins *et al.* 1996, Leader *et al.* 2002). Respiratory syncytial virus (R.S.V.) is associated with the majority of cases

(Phelan *et al.* 1994) Each winter, outbreaks of R.S.V. infection result in over 2% of all infants under 1 year of age requiring hospitalisation (Leader *et al.* 2002).While 70-80% of previously healthy infants are infected with R.S.V. in the first year of life, the majority develop upper respiratory tract infection alone and a small proportion develop bronchiolitis, pneumonia, apnoea and respiratory failure. Infants hospitalised with severe R.S.V. disease have been shown to have reduced IFN-γ levels in nasopharyngeal aspirates and reduced levels of IFN-γ expressed by peripheral blood mononuclear cells (Mobbs *et al.* 2002, Aberle *et al.* 1999). Reduced IFN-γ at the time of bronchiolitis is associated with reduced lung function and increased airway hyper-responsiveness to histamine challenge 5 months after the bronchiolitis and to later development of asthma (Renzi *et al.* 1999). The superfamily of cytokines have been shown to play a central role in the antiviral response to RSV infection in infants (Tripp *et al.* 2003, Welliver *et al.* 2003, Durbin *et al.* 2004). The largest group regulates immune cell proliferation and differentiation and includes IFN-γ, IL-2, IL-12 (T helper type 1 cytokines) and IL-4, IL-5, IL-6 and IL-10 (T helper type 2 cytokines). Infants hospitalised with RSV have an increased risk of developing asthma up to the age of 13 years (Sigurs *et al.* 2005). Examining RSV disease in infants would be interesting because of the known clinical link between hospitalisation with RSV

bronchiolitis and the later development of asthma and the role C5a, C3a and C5L2 plays in asthma development (increased C3a and C5a in asthmatics, not healthy controls after intra-pulmonary deposition of allergen but not saline) (Humbles *et al.* 2000, Krug *et al.* 2001) Secondly, the exaggerated type 2 cytokine profile seen in severe RSV disease and the strong link between IL-6 and C5a and C5L2, and between C5a and IL-12 family via Toll like receptor 4 is interesting. Excluding the known groups of infants who are at high risk of developing severe RSV disease (premature infants, infants with chronic lung disease, immuno-compromised infants and those with congenital heart disease involving haemodynamically significant left to right shunts) no one can predict which infant will develop severe RSV disease. It is enticing to postulate that C5L2 polymorphisms may answer this question and explain why these infants are at higher risk of developing asthma later in life. This sub-group may be amenable to C5L2 treatment.

6.8 C5L2 as a potential drug target

In a mere seven year period C5L2 has gone from being a newly discovered putative receptor to being critical for the biological activities of the important anaphylatoxins C5a and C3a. It can act as a positive regulator critical for C5a and C3a signalling. C5L2 can work cooperatively with signals mediated by C5aR and C3aR (Chen *et al.* 2007). C5L2 has been shown to be involved in controlling both pro-inflammatory (Chen *et al.* 2007) and anti-inflammatory (Gerard *et al.* 2005) responses and in particular certain aspects of LPS signalling. It is tempting to propose that C5L2 may have significant implications for healthcare world wide. Consider that more money was spent worldwide on asthma treatment alone in one year than on HIV/Aids and Tuberculosis combined for the same period (W.H.O. 2000), that the mortality of sepsis worldwide can be up to 60% and that the mean survival of people with cystic fibrosis is ~28 years in Ireland. Extrapolation from the bench to the bedside is always challenging but is one of the next steps in further understanding the role of C5L2, its mechanism of action and its effects on the pathogenesis of certain disease states. Armed with that knowledge, C5L2 may have a bright future as a

potential target for treating asthma, allergy, sepsis, pneumonia, cystic fibrosis and paroxysmal nocturnal haemoglobinuria to mention but a few.

APPENDIX

APPENDIX

Buffers and reagents

Hanks Balanced salt solution

HBSS	500ml
N-(2-Hydroxyethyl)piperazine-N'2-ethanesulfonic acid, (HEPES) (1M)	10ml

10 X Phosphate Buffered Saline (PBS)

Na_2HPO_4 (8 mM)	14.24g
KH_2PO_4 (1.5mM)	2.04g
NaCl (137 mM)	80.0g
KCL (2.7 mM)	2.0g

Make up to 1 litre final volume.
Sterilise by autoclaving. Dilute to
1 X PBS and adjust pH to 7.4

100mM $CaCl_2$

$CaCl_2$	2.7g

Make up to a final volume of 500ml with deionised water and sterilise by autoclaving

100mM $MgCl_2$

$MgCl_2.6\ H_2O$	10.33g

Make up to a final volume of 500ml with deionised water and sterilize by autoclaving

20mM Tris HCL pH 7.5

Tris Base	2.422g

Dissolve in 950ml de-ionised water, adjust pH to 7.5 with HCL and complete to 1L

Northern Blot Reagents

1.1% Agarose Gel

Agarose	0.55 g
ddH_2O	36.5 ml
X 10 MOPS	5.0 ml
Formaldehyde	8.5 ml

Boil solution to dissolve agarose, allow to cool, pour into tray, insert comb, cover with cling film. It sets in ~30minutes.

X10 MOPS solution

0.2 M MOPS	41.9g
0.05M Na Acetate $_3H_2O$	6.8g
0.01M Na_2 EDTA	20.0 ml

Adjust pH with 5M $NaOH_2$ (pH 7.01)

Make up to 1 litre. Sterilise by autoclaving. This is light sensitive.

Sample buffer for Northern blot

Make up 5 mls by adding

ddH_2O	1.2ml
Formamide	2.5ml
X 10 MOPS	0.5ml
Formaldehyde	0.8mls

Running buffer for northern blot

To make 250 mls of running buffer, add

X10 MOPS	25ml
X1 MOPS.	225ml

Blotting Buffer for northern blot

X 10 SSC

Polyacrylamide gel Electrophoresis (PAGE) reagents

10% Ammonium persulphate (APS)

APS	0.1 g
Deionised water	1.0 ml

Make up fresh before use

Water saturated Butanol

Butanol	10 ml
Deionised water	10 ml

Mix well and allow settle. Use top layer

Western Blot Reagents

Composition of 10% Separating/ Resolving Gel

Protogel	13.2 ml
Sterile distilled water (SDW)	16.6 ml
30% APS	100 µl
TEMED	30 µl
Protogel buffer (lower)	10ml

Composition of Stacking gel

Ultrapure Proto-Gel	1.3 ml
Protogel Stacking buffer (upper)	2.8 ml
Sterile distilled water (SDW)(add first)	7.4 ml
30% APS	30 µl
TEMED	10 µl

Blocking buffer

1% BSA TBS

BSA	1g
Tris Buffered Saline	100mls

Make up fresh

Binding buffer

TBST = Tris buffer solution with Tween 20(0.1%)

Tween 20	1ml
TBS	999mls

Running buffer

Tris	7.5g
Glycine	36g
SDS	25g

Make up to 500 ml in sterile distilled water (SDW)

Washing Buffer

Tween TBS -made from X 10 Tween TBS,
(Sigma), dilute 1 in 10.

SDS Sample Treatment Buffer

1M Tris (ph 6.8)	1ml
10% SDS	2ml
0.1% Bromophenol Blue	200µl
Sterile distilled water	5ml
Glycerol	2ml
B-mercaptoethanol	1ml

Primary and Secondary antibody diluent solution

0.1% BSA Tris buffered saline with Tween 20 (TBST)

ECL Plus- enhanced chemiluminesence.

Place 2 ml on blot, allow to develop x 5minutes, expose to film

PBS, pH 7.2-7.4

NaCl	40g
KCl	1.0g
$Na_2HPO_4.2H_2O$	7.2g
KH_2PO_4 (anhydrous)	1.02g

Dissolve in 4.5 L deionised water, adjust pH to 7.2-7.4 and complete to 1L.Sterilise by autoclaving.

Antibiotic selection

Ampicillin	100mg/ml

Prepare in deionised water, sterile filter (0.2µ), aliquot and store at -20°C. Use at 100µg/ml in medium

Agar plates

LB agar	4gm
Distilled water	200ml

Autoclave

Luria-Bertani (LB) Broth

Gibco LB powder	37g
Deionised water	1000ml

Autoclave

Luria-Bertani (LB) Agar Plates

Gibco LB powder	37g
Agar	15g
Deionised water	1000ml

Autoclave

Competition Binding buffer

20nM HEPES, with a pH of 7.4, 125 mM NaCl, 1mM$CaCl_2$, 1mM $MgCl_2$, 5nM KCl, 0.5 mM glucose and 0.2%BSA

Kinetics of binding: binding solution

HEPES 1M	25 mls
Bovine serum albumin (B.S.A.)	0.5g
Sodium azide (NaN_3) 2%	5mls
Calcium chloride ($CaCl_2$) 1M	0.5mls
Magnesium chloride ($MgCl_2$) 1M	2.5mls

Add 467 mls of double distilled water (ddH_20) for a final total volume of 500mls of binding solution.

Kinetics of binding : filtering/wash solution

HEPES 1M	25mls
BSA	0.5g
NaN_3 2%	5mls
$CaCl_2$ 1M	0.5mls
$MgCl_2$ 1M	2.5mls
NaCl 5M	50 mls = total of 83 mls.

To this add 417mls of ddH_20 to make up to a total volume of 500 mls.

And adjust to pH of 7.4

Receptor phosphorylation-lysis buffer

20mM Tris-HCl pH7.5,1% n-dodecyl-β-D-maltoside (Anatrace),1mM EGTA, 50 mM NaF, 50 μM Na_3VO_4, 20nM p-nitrophenyl phosphate, 5 mM benzamide and 1mM PMSF.

Receptor Internalisation experiment

Buffer made up of DPBS and 2 % non immune goat serum and Na N_3 (sodium azide).

MAP kinase activation- lysis buffer

20mMTris-HCL (pH 7.5),1mM EGTA, 50mM NaF, 50μM Na_3VO_4, 20nM p-nitrophenyl phosphate, 5mM benzamide and 1mM PMSF.

Protocols

QIAquick gel extraction kit

The QIAquick Gel Extraction Kit protocol, which purifies DNA, uses a micro centrifuge. This protocol is designed to extract and purify DNA of 70 bp to 10bp from standard or agarose gels in TAE or TBE buffer.

1. Excise the DNA fragment from the agarose gel with a clean sharp scalpel.
2. Weigh the gel slice in a colourless tube (remember to subtract weight of empty tube). Add 3 volumes of buffer QG to 1 volume of gel (100mg~ 100μl).

e.g. (Weight of insert – weight of empty tube) = true weight insert

1.38g - 0.96g = 0.42g = 420mg

Add three volumes of buffer QG to 1 volume of gel (100mg ~ 100μl) so add three times the weight i.e. add 1260 μls of buffer QG

Weight of vector = (1.585g-weight of empty tube 0.96g) = 625 mg so add a volume of 1875 μls of buffer QG.

3. Incubate at 50°C for 10 minutes (or until the gel slice has completely dissolved).

To help dissolve, mix the tube every 2-3 minutes during incubation.

4. After the gel slice has completely dissolved, check that the colour of the mixture is yellow (similar to QG buffer without dissolved agarose).
5. Add 1 gel volume of isopropanol to the sample and mix.
6. Place a QIAquick spin column in a provided 2 ml collection tube.
7. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 minute.
8. Discard the flow through and place QIAquick column back in the same collection tube.
9. (Optional) Add 0.5 ml of buffer QG to QIAquick column and centrifuge for 1 minute.
10. To wash, add 0.75 ml of buffer PE (with ethanol) to QIAquick column and centrifuge for 1 minute.
11. Discard the flow through and centrifuge the QIAquick column for an additional 1 minute at >10,000x g (~13,000 rpm).
12. Place QIAquick column into a clean 1.5 ml microfuge tube.

To elute DNA, add 30µl of buffer EB elution buffer (10nMTris-Cl, pH8.5) or H_2O to the centre of the QIAquick membrane. Let the column stand for 1 minute and centrifuge the column for 1 minute at maximum speed

QIAprep Spin Miniprep Kit Protocol

This protocol is designed for purification of up to 20 µg of high-copy plasmid DNA from 1-5 ml overnight cultures of *E. coli* in LB (Luria-Bertani with antibiotic) medium. We use a microcentrifuge.

Procedure:

1. Re-suspend pellet of bacterial cells in 250 µl of buffer P1 and transfer to a microfuge tube. Ensure that RNase has been added to Buffer P1. No cell clumps should be visible after re-suspension of the pellet.

2 Add 250µl of Buffer P2 and gently invert the tube 4-6 times to mix (5 minutes only). Mix gently by inverting the tube. Do not vortex, as this will result in shortening of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 minutes.

3. Add 350µl of Buffer N3 and invert the tube immediately but gently 4-6 times.
4. To avoid localised precipitation, mix the solution gently but thoroughly, immediately after addition of buffer N3. The solution should become cloudy.
5. Centrifuge for 10 min. A compact white pellet will form. During centrifugation, place a QIAprep spin column in a 2ml collection tube.
6. Apply the supernatants from step 4 to the QIAprep column by decanting or pipetting.
7. Centrifuge for 30-60 sec. Discard the flow-through.
8. (Optional) Wash QIAprep spin column by adding 0.5 ml of buffer PB and centrifuging 30-60 seconds. Discard the flow through.
9. Wash QIAprep spin column by adding 0.75 ml of buffer PE and centrifuging 30-60 seconds.
10. Discard the flow through and centrifuge for an additional 1 min to remove residual wash buffer. Important: residual wash buffer will not be completely removed unless the flow through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.
11. Place QIAprep column in a clean 1.5 ml microfuge tube. To elute DNA add 50 µl of Buffer EB (10nM Tris-Cl ph 8.5) or H_2O to the centre of each QIAprep column, leave to stand for 1minute and centrifuge for 1 minute.

Protocol for high speed plasmid Maxi kits

1. Pick a single colony from a freshly streaked selective plate and incubate a starter culture of 2-5ml LB medium containing the appropriate selective antibiotic e.g. kanamycin. Incubate for ~8 hours at 37°C with vigorous shaking (~300 rpm).Use

a tube or flask with a volume of at least 4 times the volume of the culture.

2. Dilute the starter culture 1/500 to 1/1000 into selective LB medium. For high copy plasmids inoculate 50 ml or 150 ml medium. Grow at 37°C for 12-16 hours with vigorous shaking (~300rpm).
3. Use a flask or vessel with a volume of at least 4 times the volume of the culture. The culture should reach a cell density of approximately 3-4 x 10^9cells/ml, which typically corresponds to a pellet wet weight of approximately 3g/l.
4. Harvest the bacterial cells by centrifugation at 6,000 rpm in Sorvall for 15 minutes at 4°C. Remove all traces of supernatant by inverting the open centrifuge tube until all medium has been drained.
5. Re-suspend the pellet in 20 ml of buffer P1 (ensure RNase has been added to buffer P1) which is kept in the refrigerator. The bacteria should be re-suspended completely by vortexing or pipetting up and down until no cell clumps remain.
6. Add 20 mls of P2 lysis buffer, mix gently but thoroughly by inverting 4-6 times and incubate at room temperature for 5 minutes. Do not vortex, as this will result in searing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5 minutes. Close bottle containing lysis buffer P2 immediately after use to avoid acidification from CO_2 in the air. During the incubation prepare the QIA filter cartridge. Screw the cap into the outlet nozzle of the QIA filter. Place the QIA filter cartridge into a convenient tube or the QIA rack.
7. Add 20mls of chilled buffer P3: neutralisation buffer and mix immediately but gently by inverting 4-6 times. Precipitation is enhanced by using chilled Buffer P3. After addition of Buffer P3, a fluffy white precipitate containing genomic DNA, proteins, cell debris and KDS becomes visible ("cottage cheese like"). The buffers must be mixed completely. If the mixture still appears viscous and brownish, more mixing is required to completely neutralise the solution. It is important to transfer the lysate into the QIA filter cartridge immediately in order to prevent later disruption of the precipitate layer.
8. Pour lysate (mixture from 6) into the barrel of the QIA filter cartridge. Incubate at room temperature for 10 minutes. Do not insert the plunger on top of the solution.

This 10 minute incubation at room temperature is essential for optimal performance of the QIAfilter cartridge. Do not agitate the QIAfilter cartridge during this time. A precipitate containing proteins, genomic DNA and detergent will float and form a layer. This ensures convenient filtration without clogging. If, after the 10 minute incubation, the precipitate has not floated to the top of the solution, carefully run a sterile pipette tip around the walls of the cartridge to dislodge it.

9. Meanwhile equilibrate a Hi Speed maxi tip by applying 10 ml buffer QBT and allow the column to empty by gravity flow. Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Allow Hi-Speed tip to drain completely. Tips can be left unattended, since the flow of buffer will stop when the meniscus reaches the upper point in the column.
10. Remove the cap from the QIAfilter outlet nozzle. Gently insert the plunger into the QIAfilter cartridge and filter the cell lysate into the previously equilibrated hispeed tip. Filter until all of the lysate has passed through the QIAfilter cartridge, but do not apply extreme force. Approximately 25ml of the lysate is generally recovered after filtration.
11. Allow the cleared lysate to enter the resin by gravity flow.
12. Wash the Hi-Speed maxi columns twice with 30mls of wash buffer: buffer QC each time. Allow the buffer to move through the Hi-Speed tip by gravity flow.
13. Elute DNA with 15mls of buffer QF. Collect the elute in a tube with a minimum capacity of 30ml.
14. Precipitate DNA by adding 10.5 mls of room temperature isopropanol to the eluted DNA. Mix and incubate at room temperature for 5 minutes. All solutions should be at room temperature in order to maximise salt precipitation
15. During the incubation remove the plunger from a 30ml syringe and attach the QIAprecipitator maxi module onto the outlet nozzle. Do not use excessive force, bending or twisting to attach the QIAprecipitator. Always remove the QIAprecipitator from the syringe before pulling up the plunger.
16. Place the QIAprecipitator over a waste bottle, transfer the elute/isopropanol

mixture into the 30ml syringe and insert the plunger. Filter the elute/isopropanol mixture through the QIAprecipitator using constant pressure.

17. Remove the QIAprecipitator from the 30ml syringe and pull out the plunger. Attach the QIA precipitator to the 30ml syringe again, insert the plunger and dry the membrane by pressing air though the QIAprecipitator. Repeat this step.
18. Dry the outlet nozzle of the QIAprecipitator with absorbent paper to prevent isopropanol carryover.
19. Remove the plunger from a new 5 ml syringe and attach the QIAprecipitator onto the outlet nozzle. Do not use excessive force, bending or twisting to attach the QIAprecipitator. Hold the outlet of the QIAprecipitator over a 1.5ml collection tube. Add 1 ml of buffer TE to the 5 ml syringe. Inset the plunger and elute the DNA into the collection tube using constant pressure. Ensure that the outlet of the QIAprecipitator is held over the collection tube when buffer TE is poured into the syringe, as elute can drip through the QIA precipitator before the syringe barrel is inserted. Water or buffers commonly used to dissolve DNA may also be used for elution. Note: store DNA at –20°C when eluted with water as DNA may degrade in the absence of buffering and chelating agents.
20. Remove the QIA precipitator from the 5mls syringe, pull out the plunger and reattach the QIA precipitator to the 5 mls syringe.
21. Transfer the elute from step 18 to the 5 mls syringe and elute for a second time into the same 1.5ml tube. This re-elution step ensures that the maximum amount of DNA in the QIA precipitator is solubilised and recovered.

RNA isolation procedure Ambion kit

1. Add 250 µl of 95% ethanol to the cleared lysate and mix by vortexing. A precipitate may be visible after addition of ethanol (clumps of DNA form). The purpose of the ethanol is to promote binding of RNA to filter.

2. Place the nucleospin column in a 2ml collection tube and load the sample into the column. Centrifuge at 7000-8,000 x g for 30-60 seconds. Discard the flow through.
3. Centrifuge at 7000-8,000 x g for 30-60 seconds. Discard the flow through.
4. Prepare the DNase 1 reaction mixture: in a sterile microcentrifuge tube, add 10µl of reconstituted DNase 1 to 90µl of DNase1 reaction buffer per isolation. Therefore if we are performing two preps we add 20µl of reconstituted DNase 1 to 180 µl of DNase 1 reaction buffer. (This cocktail is made up earlier, frozen and stored.) This cocktail destroys any DNA which has bound to the filter because the filter binds mainly RNA and DNA.
5. Add 95µl of DNase 1 reaction mixture to the nucleospin column.
6. Incubate at room temperature for 15 minutes
7. Add 500 µl of buffer RA2 and centrifuge at 7,000-8,000 x g for 30-60 seconds. Discard the flow through and place the Nucleospin column into a new 2 ml collection tube. Buffer RA2 inactivates the DNAse.
8. Add 600 µl of Buffer RA3 to the Nucleospin column. Centrifuge at 7,000-8,000 x g for 30 seconds. Discard the flow through, and place the Nucleospin column into a new 2ml collection tube.
9. Add 250µl of Buffer RA3 and centrifuge at maximum speed for 2 minutes (to dry). Discard the flow through and place the Nucleospin column into a 1.5 ml microcentrifuge tube.
10. Elute the RNA by adding 100µl of nuclease–free water and centrifuging at g/speed for 1 minute. It is recommend to elute in 2 steps using 50µl each time. For concentrated RNA samples, elute with 40µl of Nuclease –free water. Estimate the yield by UV spectrophotometry.

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FOR

C5L2, A NON SIGNALLING C5A RECEPTOR IS UNCOUPLED TO G PROTEINS

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