Dimerization of complement C5a receptors in inflammatory responses

By:

AHMED ALI ABD

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The University of Sheffield
Faculty of Medicine, Dentistry & Health
The Medical School
Department of Department of Infection, Immunity & Cardiovascular Disease

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Abstract

Inflammation is a complex pathophysiologic process that occurs in response to tissue injury induced though various stimuli. It involves cellular and humoral responses in which various cell types and inflammatory mediators are engaged. The inflammatory response must be tightly controlled, otherwise it results in chronic inflammation and perhaps continuous tissue damage. During the activation of the complement cascade, several small fragments, known as anaphylatoxins, are released. One of these anaphylatoxins is produced from the complement protein C5 and is known as C5a. C5a is a multifunctional polypeptide that is involved in cellular immune responses. The receptors for C5a, C5a1 and C5a2, are among the large family known as G protein-coupled receptors. Unlike C5a1, C5a2 is incapable of signalling through G proteins but can induce β-arrestin translocation and recruitment. C5a2 function is still enigmatic and it has been suggested as a decoy receptor for its ligands, as a signalling receptor or as a signalling modifier of C5a1, possibly through the formation of heterodimers.

In the current study, we aimed to study possible interactions between the C5a1 and C5a2 receptors. To achieve these goals, heterologous expression of different C5a receptors was studied in clearly defined settings using transfected RBL cells. The possibility of direct physical interaction between the two receptors was explored using fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) using tagged C5a1 and C5a2 receptors. Possible dimerization was further examined using untagged receptors through co-internalization of the C5a1 and C5a2 receptors. Various signalling assays were used to analyse the possible effect of C5a2 on C5a1 such as degranulation, intracellular Ca^{2+} mobilization, MAPK (ERK1/2, p38 and

JNK) signalling and internalization assays. FRET results showed possible homodimerization of C5a1 receptors but not heterodimerization with C5a2. BRET and receptor co-internalization studies also could not detect clear heterodimerization between C5a1 and C5a2. The possible indirect effect of C5a2 on C5a1 was assessed by comparing the C5a1 signalling upon co-expression with C5a2. Intracellular Ca^{2+} mobilization was similar when C5a1 expressed with or without C5a2. However, the RBL degranulatory response to C5a was lower when C5a2 was co-expressed with C5a1. Phosphorylation of ERK1/2 was also decreased when C5a2 was co-expressed with C5a1. In addition, the presence of C5a2 decreased C5a1 internalization. Taken together, when co-expressed in RBL cells, possible interaction between C5a receptors was observed. This interaction is not necessarily due to direct physical interaction but could be through scavenging effect on the ligand or sequestration of intracellular partners.

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List of Abbreviations

A3R	Adenosine type 3 receptors
aa	Amino acid
ACTH	Adrenocorticotropic hormone
AMD	Age-related macular degeneration
ANOVA	Analysis of variance
ASP	Acylation stimulating protein
BBN	BSA/BSS/Sodium azide
BRET	Bioluminescence resonance energy transfer
BSA	Bovine serum albumin
BBS	Balanced salts solution
C5	Complement protein 5
CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
cDNA	Complementary DNA
CFP	Cyan fluorescent protein
CLP	Caecal ligation and puncture
CXCR	C-X-C chemokine receptor type
DMEM	Dulbecco's modified Eagle's media
DMSO	10% dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNP	2, 4-Dinitrophenol conjugated to human serum albumin.
DOR	Delta opioid receptors
EC	Extracellular
EC50	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELIZA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERKs	Extracellular-signal-regulated kinases
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FP	Fluorescent proteins
FPR	Formyl peptide receptors
FRET	Fluorescence resonance energy transfer
GABA receptors	Gamma-aminobutyric acid receptors
GPCR	G protein-coupled receptors

GFP	Green fluorescent protein
GRKs	G protein-coupled receptor kinases
HBSS	Hank’s balanced salt solution
hC5a1	Human C5a1 receptor
hC5a2	Human C5a2 receptor
HEK	Human embryonic kidney
HIV	Human immunodeficiency virus
HMGB1	High-mobility group box 1
IC	Intracellular
IC50	50% inhibitory concentration
Ig	Immunoglobulin
IgE	Immunoglobulin E
IL	Interleukin
IP3	Inositol triphosphate
JNK	c-Jun N-terminal kinases
kDa	Kilo Daltons
KOR	Kappa opioid receptors
LB	Lysogeny broth
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MAPKs	Mitogen-activated protein kinases
MASPs	Mannose-binding lectin-associated serine proteases
MFI	Median fluorescence intensity
MOR	Mu opioid receptors
OD	Optical density
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PMN	Polymorphonuclear neutrophils
PTX	pertussis toxin
RBL	Rat basophil leukaemia
Rluc8	*Renella* luciferase enzyme 8
TM	Transmembrane
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumour necrosis factor
YFP	Yellow fluorescent protein

Table of contents

List of figures

Chapter One

Introduction

Chapter 1: Introduction

1.1 Overview:

The complement system is composed of proteins and glycoproteins (about 35-40) present either in the liquid phase in the plasma or on cell surfaces. Most of the soluble molecules are produced by the liver, but they can be produced locally in many tissues such as brain and kidney.

The complement system is part of the innate immune system and play an important role in coordinating the innate and adaptive immune responses (Walport, 2001, Carroll, 2004, Ricklin et al., 2010).The fundamental function of the complement system is to identify potentially dangerous nonself cells and macromolecules and facilitate their removal by the phagocytic cells (opsonisation) or direct action through perforation of the lipid bilayer (e.g bacteria). Moreover, it has a significant homeostatic role by removal of abnormal or damaged self-components, including necrotic and apoptotic cells; and abnormal protein aggregations as well as immune complexes. Therefore, there are two important roles of complement: an immune response to pathogens and clearance function to remove the damaged host components. Because of the ability of complement to attack cells by opsonisation and lysis, uncontrolled complement activation may lead to damage to host cells and tissues. The release of the anaphylatoxins such as C5a, C3a and C4a (Hugli, 1986) during complement activation results in inflammation. Many control mechanisms (soluble and on host cell surfaces) are involved in the regulation of complement activation (reviewed in Baines and Brodsky, 2017). Nevertheless, many diseases are associated with failure of these regulatory mechanisms, which may be either the result of excessive complement activation (e.g. rheumatoid arthritis) or insufficient

complement activation (e.g. infections or systemic lupus erythematosus) (reviewed in Carroll and Sim, 2011).

One of the important molecules within the complement cascade is C5, which is the precursor of the effector molecules: C5a and C5b. C5b has a well-known function in triggering the formation of the membrane attack complex (MAC) that is responsible for cell lysis, while C5a through its classical receptor C5a1 is a potent inflammatory mediator and chemoattractant (reviewed in Klos et al., 2013). Consequently, it is considered a crucial factor in the pathogenesis of many diseases such as ischemia-reperfusion injuries, sepsis, asthma, rheumatoid arthritis, and inflammatory bowel disease. Nevertheless, anti-inflammatory effects of C5a have also been reported and thought to be also mediated via its classical receptor C5a1 (Bosmann et al., 2012).

However, a recently discovered second receptor for C5a, C5a-like receptor 2 (C5a2 or C5aL2), does not seem to work like C5a1 creating a controversy among complement researchers (reviewed in Li et al., 2013).

The concept of forming dimers or oligomers (homo/hetero-oligomer) by G protein couple receptors (GPCR) is a recently developed one. There is also increasing consideration that the dimerization of GPCR may be necessary for function. Therefore, studying the dimerization of C5a receptors may clarify some of the controversy of the C5a2 receptor function and may have some implications for the receptor pharmacology.

1.2 History of complement research

The activity of the complement system was first observed for the first time between 1884 and 1894. It was reported in 1888 that the fresh plasma had a bactericidal activity that was lost by heating to 55°C (reviewed in

Carroll and Sim, 2011). In 1891, a heat sensitive substance was identified and named as "alexin" (reviewed in Skarnes and Watson, 1957). Then, it was proposed that the antibacterial activity in blood involved two factors: a heat-sensitive factor (analogous to alexin) and a heat-resistant factor (now known as antibodies) (reviewed in Carroll and Sim, 2011). The name “alexin” was then replaced with “complement” describing its role in complementing the activities of antibodies in inducing bacterial lysis (Kaufmann, 2008).

Four complement components were purified and characterised partially in 1941 (Pillemer et al., 1941). Between the 1950s and 1960s, the phenomenon of immune adherence (attaching complement coated particles to human red blood cells), complement activity assay standardization; the isolation of 9 distinct components from guinea pig; and the second complement activation pathway (called the properdin pathway, now the alternative pathway) were investigated in detail (reviewed in Carroll and Sim, 2011).

In the 1970s and 1980s, several complement protein components were isolated and sequenced; and thereafter genomic cloning and sequencing furthered our understanding of complement biology (Muller-Eberhard, 1988, Müller-Eberhard, 1975, Campbell et al., 1988).

Although research is still progressing, the complement homeostatic role has been described, which involves the clearance and removal of damaged self-particles (Walport et al., 1998).

Many genetic variants of complement proteins have been recognised to have altered functions and probably associated with diseases. For example, a factor H (a regulatory protein) polymorphism was found to be associated with AMD (Day et al., 1988, Hageman et al., 2005, Herbert et

al., 2007). Subsequently, altered complement protein function was found to be related to polymorphisms in other proteins such as factor I and factor B (Clark et al., 2006, Nilsson et al., 2011, Gold et al., 2006).

Many complement proteins' three-dimensional structures were investigated by X-ray crystallography and NMR and this could form the basis for better understanding of their functions and interactions (Arlaud et al., 2007, Gros et al., 2008).

In the 1970s, the anaphylatoxin C5a was purified and characterized from activated sera and its biological functions explored (Vallota and Muller-Eberhard, 1973, Vallota et al., 1973). All the functions of C5a were at first attributed to a single receptor, which was cloned in 1991 (Boulay et al., 1991, Gerard and Gerard, 1991). However, the presence of other receptors for C5a was thought possible because of the observation of some puzzling results. The differences in response of leukocytes to the C5a agonists and antagonists were thought to be the result of either different posttranslational modification, unique gene products (Gerard et al., 1989), or perhaps due to differences in the interaction with intracellular partners (Paczkowski et al., 1999, Eglite et al., 2000). In 2000, a previously described orphan receptor, GPR77, was found to be a second receptor for C5a (C5a receptor-like 2, C5L2; or C5a2) and was cloned by Ohno et al., 2000 (Ohno et al., 2000, Lee et al., 2001, Cain and Monk, 2002).

The momentum for developing reagents that can manipulate complement proteins' activity has increased recently (Qu et al., 2011, Schwaeble et al., 2011, Morgan and Harris, 2003, Morgan and Harris, 2015, Woodruff et al., 2011). All complement pathways activation leads to cleavage of complement protein C5 and results in the formation of the anaphylatoxin C5a in addition to C5b. C5b is required for the formation of

the membrane attack complex (C5-9), which has cytolytic and non-cytolytic immune functions. C5a is a potent inflammatory mediator, in addition to various other functions, including some anti-inflammatory behaviour. Therefore, the C5-C5a receptor axis is gaining increasing pharmacological attention and an anti C5 monoclonal antibody (eclizumab) is one of the products that is approved for clinical use in paroxysmal nocturnal haemoglobinuria and atypical haemolytic uremic syndrome (reviewed in Woodruff et al., 2011, Risitano and Marotta, 2016, Baines and Brodsky, 2017).

1.3 The complement activation pathways:

The complement system involves proteins that are secreted in inactive forms into the serum, functioning in a proteolytic cascade after activation. The results of these enzymatic reactions are often two fragments, a large fragment and a small fragment. The large fragment has an attachment site to the triggering surfaces and enzyme site for the cleavage of the subsequent complement component in the activation sequence (Chapel et al., 2014). Some of the small fragments that are generated by the cleavage have important inflammatory and/or homeostatic functions. For example, the cleavage of the complement component C5 into C5a and C5b by the enzymatic function of the activated complement complex called C5 convertase (Figure1.1).

The activation of some complement proteins trigger their binding to the next proteins in the cascade and arrange themselves into complexes on the cell membrane such as the membrane attack complex (MAC), sometimes called terminal attack complex.

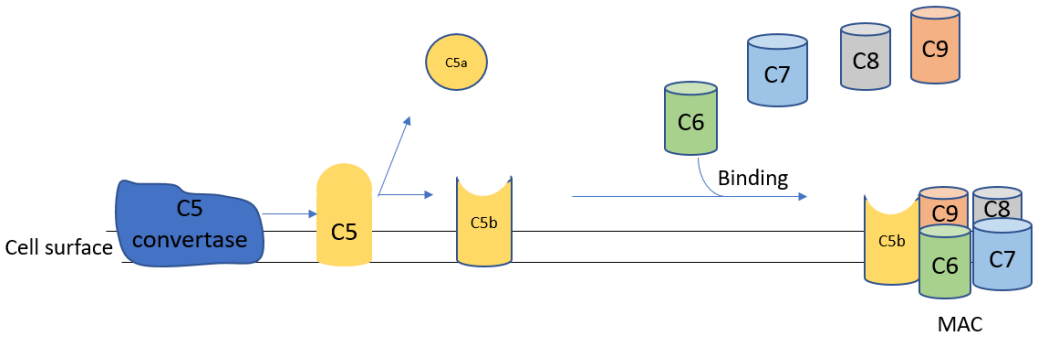


Figure 1.1: Functions of various proteins in the complement cascade. The figure illustrates the various functions of complement proteins such as, enzymatic activity, chemoattraction and formation of membrane attack complex. Some of the complement proteins when they are activated form complexes with enzymatic functions such as C5 convertase. The C5 precursor protein is cleaved into small (C5a) and large (C5b) fragments by the enzymatic function of the C5 convertase. The small fragment C5a exerts several functions related to chemoattraction, inflammation and homeostasis. The large fragment C5b has the potential to activate the next component in the complement cascade. Other complement proteins such as C6, C7, C8 and C9 when they are activated bind to each other and arrange themselves into a complex called the membrane attack complex (MAC). The MAC inserts itself inside the cell membrane and forms pores, which results in cell lysis.

The activation of complement proteins occurs through serial proteolytic processes that lead to cleavage of the inactive molecules to produce the following active protease in the cascade. There are three known pathways of complement activation (reviewed in Sarma and Ward, 2011) (Figure 1.2). First, the classical pathway, which usually involves the recognition of IgM or IgG1 immune complexes with pathogens or nonself antigens, and molecular patterns on pathogen surfaces. These immune complexes activate the multimeric C1 protein, which is composed from C1q, C1r, and C1s. Secondly, the lectin pathway, which is similar to the classical, depends on the pattern recognition receptors for detecting nonself substances. Mannose-binding lectins or ficolins recognise and bind to carbohydrate groups on the surface of pathogens. After this binding, the mannose-binding lectin-associated serine proteases (MASPs) are activated.

In both the classical and lectin pathways, sequential activation and cleavage steps for several complement components (including C2, C3 and C4) results in the formation of C5 convertase, which is C4b2a3b. In addition to the release of the anaphylatoxins C3a and C4a, which accompanies the activation of the classical and lectin pathways.

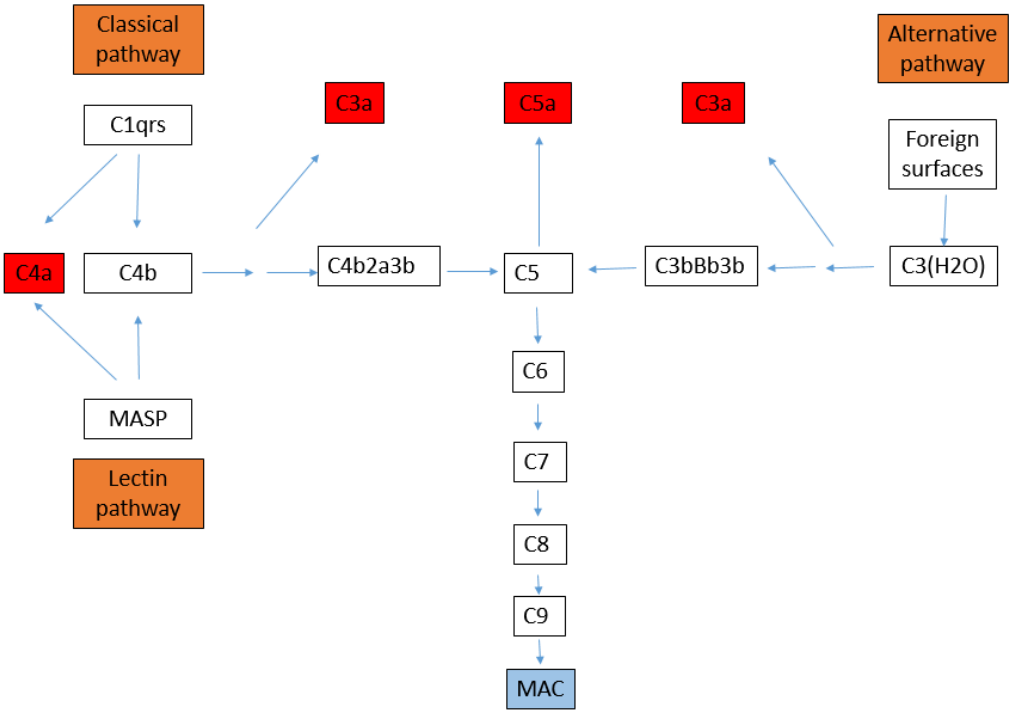


Figure 1.2: Complement activation pathways. The complement activation pathways include the classical, lectin and alternative pathways are described. The classical pathway and lectin pathways are closely related pathways and result in the formation of the same C5 convertase, C4b2a3b. The classical pathway starts with activation of the first complement component C1qrs. The lectin pathway starts with the activation of the mannose-associated lectin-associated serine proteases (MASPs). The alternative pathway starts with the hydrolysis of C3 complement component and results in the formation of C3bBbC3b, which is C5 convertase of the alternative pathway. All the three pathways lead to the cleavage of complement component C5 and formation of the membrane attack complex (MAC) after activation and binding of C6, C7, C8, and C9 components. The activation of the complement system is accompanied by the release of the anaphylatoxins C3a, C4a and C5a.

The third pathway is the alternative pathway that depends on C3 hydrolysis spontaneously with no need for antibodies like the classical

pathway. The activation of the alternative pathway leads also to sequential activation and cleavage that results in the formation of C5 convertase, which is in this case C3bBbC3b. The activation of the alternative pathway is accompanied by the release of C3a anaphylatoxin.

Consequently, all three pathways lead to the cleavage of C5 into C5a and C5b. C5b then triggers the formation of the membrane attack complex. The formation of the membrane attack complex is the final step in this chain, which is a transmembrane channel that leads to cell lysis and death. C5a is another anaphylatoxin that exerts various functions related to inflammation and haemostasis.

There are several inhibitory factors that prevent complement from attacking host tissues when it is activated in proximity to cells or in the fluid phase. These factors include CD59 and the decay-accelerating factor. Cells lacking these factors, for example pathogens or xenografts, will predispose the affected tissue or cells to an amplified complement attack.

Upon detecting threats there will be a potent and rapid reaction due to the presence of the high concentration of complement proteins. However, inflammatory disorders may be developed as a consequence of failure in the controlling mechanisms (Klos et al., 2013).

Activation of the complement system provides the means to remove the target antigens. This purpose could be achieved through the lysis of the whole cell or microorganism by the complete sequence activation and formation of the MAC. However, opsonisation of the invading micro-organisms or the immune complexes is probably the key role. Opsonisation makes the antigens more easily attacked and digested by the immune cells such as macrophages.

1.4 Complement C5a

The large C5 protein (190 kDa) is composed of a beta chain (approximately 75 kDa) and an alpha chain (approximately 120 kDa) from which the C5a is split. Human C5a has a mass of about 15 kDa with 74 aa. It is a globular protein composed of helices and disulfide bridges in the core to stabilize these helices with a flexible tail at the C-terminal end (Huber-Lang et al., 2003).

When the carboxyl-terminal residue (Arg) is cleaved from C5a by carboxypeptidases, the biological function is altered and then it is called C5a desArg (Manthey et al., 2009).

At low concentrations (nanomolar), C5a acts as a potent chemoattractant for different myeloid cell types such as polymorphonuclear neutrophils (PMN), macrophages, monocytes, basophils, and eosinophils. However, C5a can stimulate the generation of superoxide and enzyme release at higher concentrations (mainly in PMNs) (Gerard and Gerard, 1994). A reduction in acute inflammatory response was observed through the genetic absence of C5a receptors or blockade of C5a, which also affected murine neutrophil functions (Czermak et al., 1999, Rittirsch et al., 2008)

Moreover, other functions have been reported for C5a including apoptosis, smooth muscle contraction, and vasodilation (reviewed in Sarma and Ward, 2012). Many of these functions have been reported also be stimulated by C5a desArg but higher concentrations are required to induce the biological responses. Nevertheless, there may be some differences in the activities between C5a and C5a desArg. For example, human basophils, in the presence of interleukin 3 (IL-3), can produce leukotriene C4, IL-4, and IL-13 in response to C5a, while less release of

leukotriene C4 occurs in response to C5a desArg which might act as a super agonist for the release of IL-13 (Eglite et al., 2000). Furthermore, it has been suggested that C5a can enhance the formation of a heterodimer between C5a1-C5a2, while C5a desArg lacks this function. In addition, there were significant differences in the secretion of IL-10 from macrophages derived from human monocytes after exposure to C5a or C5a desArg (Croker et al., 2013).

On the other hand, abnormal production of high levels of C5a was reported to compromise host defences (Riedemann et al., 2003a). Bosmann *et al.* provided evidence for anti-inflammatory effects of C5a and suggested a mechanism for the effect of C5a on the reduction of acute inflammation (Bosmann et al., 2012). This suppression was mediated by C5a1, but not C5a2, receptor. C5a is thought to activate PI3K-Akt and MEK1/2-ERK1/2 pathways, which leads to production of IL-10. IL-10 in turn inhibits IL-17A and IL-23, which appear during endotoxemia. Other studies suggested that production of IL-10 and TGF-β was induced by C5a and favoured generation of Tregs (Vadrevu et al., 2014, Engelke et al., 2014). Recently, immunosuppressive responses has been reported to be promoted by C5a, which was thought to be via C5a1 receptor (An et al., 2016). In addition, a more recent study has reported that C5a1 receptor showed inhibition effect on T helper 1 cell polarization, which allows certain strains of *Mycobacterium tuberculosis* to propagate (Sabio y García et al., 2017). Therefore, there is a growing body of evidence that suggests that inflammatory response can be regulated positively and negatively by C5a production and that the role of C5a in regulation of the innate and adaptive immune response is complex and could be determined by the local microenvironment.

1.5 Complement C5a receptors:

1.5.1 C5a1 receptor (C5a1, C5aR):

The 7-transmembrane receptor, C5a1 is a member of the G protein-coupled receptor superfamily within the rhodopsin family. It binds both C5a and C5a desArg but with a lower affinity for the desarginated form (Boulay et al., 1991, Gerard and Gerard, 1991) The typical signalling pathway involves activation of heterotrimeric G proteins upon binding to C5a.The G proteins are formed from subunits. The guanosine triphosphate (GTP) displaces guanosine diphosphate (GDP), which is attached to α subunit and leads to the dissociation of β and γ subunits of the G protein from the α, allowing the signal to be transmitted further downstream. C5a1 receptor was reported to interact with different G proteins in different cell types. In neutrophils, C5a1 receptor was found to couple to pertussis toxin (PTX) sensitive G α_{i2} (Sheth et al., 1991, Skokowa et al., 2005). However, in monocyte like cells, C5a1 receptor can interact with PTX-insensitive $G\alpha_{16}$ (in human)/G15 (Davignon et al., 2000, Monk and Partridge, 1993, Amatruda et al., 1993). Unlike most other GPCR, C5a1 was found to pre-couple to G proteins (Siciliano et al., 1990).

One downstream signal of C5a1 receptor activation is Ca^{2+} mobilization, which is also cell type dependent. In neutrophils, Ca^{2+} is released mainly from intracellular stores after C5a stimulation, while extracellular influx was observed in the monocyte lineage (Monk and Partridge, 1993). In addition, rapid and transient Ca^{2+} response was reported in mast cells (Hartmann et al., 1997). However, a more prolonged Ca^{2+} response was demonstrated in microglial cells (Moller et al., 1997). The release of Ca^{2+} from intracellular compartments was thought to be dependent on sphingosine-1-phosphate (S-1-P) production in neutrophils and macrophages (Ibrahim et al., 2004). S-1-P, which also plays a role in

lymphocyte trafficking, is produced in response to C5a stimulation in macrophages (Maceyka et al., 2012). Several other signaling pathways were reported to be activated through C5a1 such as the phospholipase C (PLC) (Jiang et al., 1996, Klinker et al., 1996) phospholipase D (Mullmann et al., 1990), phosphoinositide 3-kinase/Akt (Wrann et al., 2007), PKC, and mitogen activated protein kinase (MAPK) (la Sala et al., 2005, Monsinjon et al., 2003, Coffer et al., 1998) pathways.

The PTX-sensitive G proteins induce MAPK signalling mainly through βγ subunits. These subunits were thought to activate PI3K that leads to MAPK signalling (Stephens et al., 1996, Chiou et al., 2004). C5a was demonstrated to differentially activate MAPK pathways. C5a can stimulate ERK1/2 and p38 through independent pathways. ERK1/2 activation was found to be dependent on PLC, PI3K, and Akt activation after activation of the PTX sensitive G proteins. However, p38 was only dependent on the G protein activation and inhibition of the other proteins (PLC, PI3K, and Akt) did not affect its signalling (Chiou et al., 2004).

The termination of signalling is mediated by the GRKs (G protein-coupled receptor kinases) that phosphorylate the C-terminal cytoplasmic tail of the receptors, stimulating β-arrestins recruitment (reviewed in Sarma and Ward, 2012). In the C-terminus of C5a1, the serine residues are the main targets for GRKs. Two pairs of serine residues, Ser332 and Ser334 or Ser334 and 338, are the targets for phosphorylation by GRK2 and GRK3. Subsequently, other serine residues in the C-terminus are phosphorylated and internalization occurs (Boulay et al., 1991).

β-arrestins assist in GPCR internalization through clathrin coated pits after blocking the binding of G protein, causing desensitization of the receptor (Rajagopal et al., 2010).

GPCR signalling is a complex process and not a simple on/off switch. Many factors are involved in the resultant signal. These factors may relate to the agonist, the receptor conformational changes, or the preferred activated pathways (Rajagopal et al., 2010). In addition to G protein dependent signalling pathway, there is G protein independent signalling via the β-arrestin that involves MAP kinase activation (Rosenbaum et al., 2009). It has been suggested that the selection of the pathway through which the signaling takes place namely, the G protein or β-arrestin, depends on the agonist and the subsequent conformational changes of the receptor (Rajagopal et al., 2010). β-arrestin can inhibit the signaling through G protein while permitting an alternative downstream signaling pathway at the same time. β-arrestin undergoes conformational changes and modifications that are responsible for the different functions (Shenoy and Lefkowitz, 2011).

In 1991, two groups independently cloned C5a1 (Gerard and Gerard, 1991, Boulay et al., 1991) . It is composed of 350 aa with a mass of 42 kDa. C5a1 consists of seven transmembrane regions, which are connected by extracellular and intracellular loops (Figure 1.3). The N-terminus segment is located extracellularly, while the intracellular tail contains the C-terminus (Sarma and Ward, 2012).

In human C5a1 receptors, Asn2 is a potential site for N-linked glycosylation. The ligand affinity and receptor expression are slightly affected by removal of glycosylation (Pease and Barker, 1993). At the N-terminus of C5a1, there are three tyrosine residues. Two of them (Tyr11, Tyr14) are sulphated and play an essential role in ligand binding (Scola et al., 2007, Farzan et al., 2001).

At the C-terminus, the main phosphorylation sites are located (Giannini et al., 1995). These sites play an important role in receptor internalization (Naik et al., 1997).

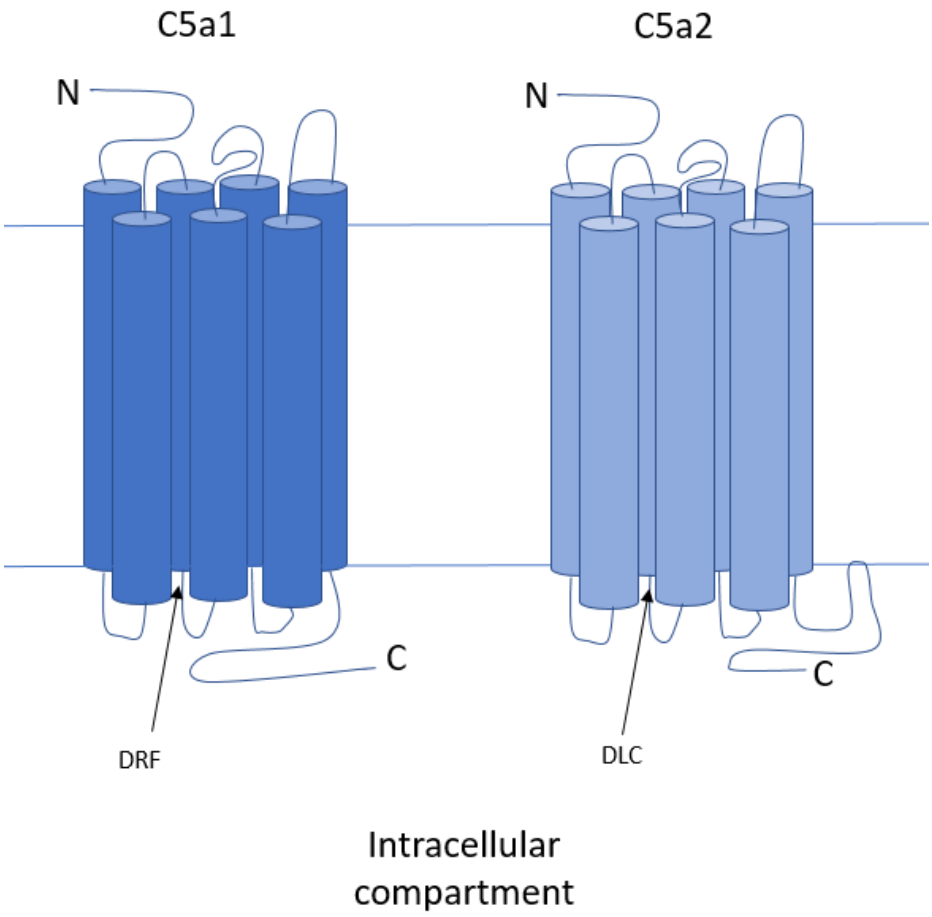


Figure 1.3: Complement C5a receptors. C5a1 and C5a2 receptors structures are shown with seven transmembrane domains connected by intracellular and extracellular loops. The differences in the DRY (Asp-Arg-Tyr) motif following the 3rd TM domain is shown, which in C5a1 is DRF (Asp-Arg-Phe), while in C5a2 is DLC (Asp-Leu-Cys).

1.5.2 C5a2 receptor (C5a2R, C5L2):

An orphan receptor, GPR77, was first reported by Ohno et al. 2000 as expressed on immature (but not mature) dendritic cells. It shares 58% homology with C5a1 receptor and 55% with C3a receptor in the transmembrane domain (Lee et al., 2001). It has been determined that the gene is located on chromosome 19 in the same region as the C5a1 gene.

In 2002, Cain and Monk demonstrated that this orphan receptor GPR77, now termed C5a2, can bind C5a with high affinity and can bind C5a desArg with 10-fold higher affinity than C5a1 receptor (Cain and Monk, 2002). Mouse C5a2 was reported to have 4000-fold higher affinity for C5a desArg than C5a (Scola et al., 2007). It has been suggested that C5a2 binds to C5a and C5a desArg by different mechanisms (Scola et al., 2007). C5a2 shares the same pattern of N-terminal acidic and tyrosine residues with C5a1 that seems to be an important region for the binding of C5a desArg to the extracellular domain (Scola et al., 2007). Other similarities between the two receptors are found in the regions that interact with C-terminus of C5a, which involves a number of hydrophobic and charged residues (Buck et al., 2005, Monk et al., 2007). Moreover, it is also reported that C5a2 can bind C3a and C4a at distinct sites from that of C5a binding (Cain and Monk, 2002, Kalant et al., 2003). It is also supposed that C5a2 can bind C4a desArg and C3a desArg (also known as acylation-stimulating protein or ASP) and acts as a functional receptor for ASP leading to stimulation of triglyceride synthesis and glucose transport in adipose tissue (Kalant et al., 2005, Kalant et al., 2003). However, this is a controversial subject, because Okinaga et al 2003 did not detect interaction of C5a2 with C3a or C4a (Okinaga et al., 2003). In addition, another report has shown that neither C3a nor C3a desArg can interact with C5a2 receptor and it is unrelated to the metabolism of carbohydrate and lipid (Johswich et al., 2006).

The 7-transmembrane C5a2 is considered as a member of the A8 subfamily in the G protein superfamily of receptors with other chemoattractant receptors such as C5a1 and C3a receptors (Joost and Methner, 2002). However, C5a2 is unable to couple to heterotrimeric G proteins (Cain and Monk, 2002, Scola et al., 2009, Kalant et al., 2003).

This is thought to be due to an alteration in the highly conserved DRY motif, which is located following the third transmembrane domain. In C5a1, this DRY motif is aspartate-arginine-phenylalanine (Asp-Arg-Phe). While C5a2 has a leucine instead of arginine at this region (Figure 1.3) (Okinaga et al., 2003). No Ca^{2+} mobilization, ERK1/2 pathway activation or degranulation occur in C5a2-transfected cells in response to C5a or C5a desArg (Cain and Monk, 2002, Okinaga et al., 2003, Johswich et al., 2006, Kalant et al., 2003).

Perhaps in accordance with their location on the same chromosome, C5a1 and C5a2 are generally co-expressed on similar cells and tissues but with overall lower expression for C5a2 receptors (Li et al., 2013, Okinaga et al., 2003, Bamberg et al., 2010). In general, C5a2 is widely expressed on immune cells such as macrophages, neutrophils, monocytes, lymphocytes, and immature dendritic cells (Ohno et al., 2000, Chen et al., 2007). Moreover, as with C5a1, it is also expressed on non-inflammatory cells including vascular smooth muscle, skin fibroblasts, adipocytes, astrocytes (reviewed in Li et al., 2013, Monk et al., 2007).

Although this coexistence may suggest a functional interaction, a recent report that demonstrates distinct renal expression patterns for C5a1 and C5a2 (van Werkhoven et al., 2013) may indicate that these receptors may differ in their key promoters or post translational modification.

Within the cell, although C5a1 is predominantly on the plasma membrane, in agreement with most other GPCR, C5a2 has been suggested to have tendency for more intracellular localization. This has been reported in transfected rat basophil leukaemia (RBL) cells using flow cytometry and ligand binding to demonstrate the more intracellular expression in comparison with C5a1 (reviewed in Li et al., 2013). The same localization

has been displayed in human neutrophils and it is proposed that this predominant intracellular position of C5a2 may affect its interaction with C5a1 (Bamberg et al., 2010). It is still unclear whether C5a2 is intracellular among all cell types and in different disease conditions (Li et al., 2013). It has been reported that there is a wide natural difference in the surface expression of C5a2 receptors among human individuals (Scola et al., 2009). Huber-Lang et al., (2005) demonstrated a positive correlation between survival rate in sepsis and the surface expression of C5a2 in neutrophils (Huber-Lang et al., 2005).

The regulation of C5a2 expression seems to be affected by different factors. C5a itself can influence the expression of C5a2. It has been reported that during sepsis, the lysates of rat polymorphonuclear cells have low levels of C5a2 protein as an effect of exposure to C5a and it is independent from C5a1 expression (Huber-Lang et al., 2005). This is supported by another study that shows that there was no significant differences between C5a1-/- mouse and the wild type neutrophils in terms of expression of C5a2 (Rittirsch et al., 2008). Another factor that affects C5a2 is LPS, which has been shown to decrease C5a2 expression in peripheral blood mononuclear cells, associated with increased pro-inflammatory responses to C5a (Raby et al., 2011). Moreover, it has been identified that some hormones may affect these receptors. For example, oestrogen receptor agonists can upregulate C5a1 expression. However, C5a2 is differentially affected by these hormones, a subject that needs further investigation (Farkas et al., 2012). Many other factors that affect the up-regulation of C5a2 were reported such as interferon γ, insulin and noradrenaline (Li et al., 2013, Gavrilyuk et al., 2005).

Concerning the receptor functions, C5a2 is still enigmatic. Generally, there are three theories to explain the behaviour of this receptor. Two of

them suggest anti-inflammatory roles (Bamberg et al., 2010, Scola et al., 2009), while the other supports a pro-inflammatory function (Rittirsch et al., 2008, Chen et al., 2007):

1. Recycling decoy receptor theory

Due to the lack of coupling with G protein (Cain and Monk, 2002, Okinaga et al., 2003), a "recycling decoy receptor" hypothesis has been described. This model proposes that C5a2 takes C5a and prevents or decreases its binding to, and activation of C5a1 (Scola et al., 2009). After binding of C5a to C5a2, the complex is internalized where the C5a is removed and the C5a2 returns to the surface. Therefore, the C5a is sequestered or degraded and the inflammatory response is prevented.

2. Anti-inflammatory role through β-arrestin signalling

In the second anti-inflammatory model, Bamberg et al have suggested that the inflammatory response to C5a is regulated by C5a2 making complexes with β-arrestin (Bamberg et al., 2010). Upon binding to C5a, C5a1 is internalized and interacts with C5a2 and both receptors are phosphorylated by G protein receptor kinases, which promotes an interaction with β-arrestin. It is thought that ERK1/2 is activated when β-arrestin complexes with C5a1, while inhibition of ERK1/2 occurs if β-arrestin complexes with C5a2. The two pathways compete to give the net signal (Bamberg et al., 2010).

3. Proinflammatory hypothesis

C5a2 was also considered to have a pro-inflammatory function, which is in direct contrast to the previous two models. It has been shown that C5a2 can work as a positive modulator for the signalling of C5a1 and C3a receptors in mouse neutrophils (Chen et al., 2007). Another study

demonstrated that in cecal-ligation and puncture model of sepsis in mice, C5a2 deficiency or blockade provided modest protection in "mid-grade" sepsis, while the combined blockade of both C5a1 and C5a2 receptors was the only protective measure against "high-grade" sepsis according to that study (Rittirsch et al., 2008). This suggests the harmful consequences of sepsis are a result of synergistic contributions of both C5a1 and C5a2 receptors. In addition, C5a2, but not C5a1, signalling was postulated to be a trigger for the release of inflammatory mediator high-mobility group box 1 (HMGB1) from the white blood cells (Rittirsch et al., 2008).

Due to the unclear role of the C5a2 receptor, its therapeutic potential is still unknown. The function of C5a2 in health and disease needs to be well understood before logical attempts can be made to target it for clinical therapy in the future. Activating the receptor or promoting its cellular expression may be a successful potential therapy in case the receptor has an anti-inflammatory role, while the opposite will be in case it has a proinflammatory behaviour. Several laboratories have been trying to target the receptors in different ways. Recently, two selective peptides have been shown to induce β-arrestin recruitments to C5a2 (Croker et al., 2016). Antibodies that target C5a2 have also been developed (Rittirsch et al., 2008, Bamberg et al., 2010). Moreover, the originally described C5a1 inhibitor, the C5a mutant $A8^{\Delta 71\text{-}73}$, also has been reported to inhibit C5a2 (Otto et al., 2004).

Pharmacologically, it is not easy to target C5a2 due to its location inside the cell. Furthermore, other factors challenge the therapeutic effects such as the lack of clear signalling and activation ramifications.

1.6 Pathologies associated with inappropriate complement activation:

The complement system, when it is activated, can attack foreign particles and lyse cells. In addition, it produces anaphylatoxins (such as C5a) that play a role in chemotaxis and inflammation. This capacity if inappropriately controlled can lead to damage to host tissues. These tissue damage effects have been explored since 1970s. Inappropriate complement activation is associated with many disorders. These disorders include sepsis (Huber-Lang et al., 2002b), systemic lupus erythematosus (Pickering and Walport, 2000), drug-induced lupus (Sim et al., 1984), multiple sclerosis (Ingram et al., 2014), amyotrophic lateral sclerosis (Lee et al., 2017), rheumatoid arthritis (Woodruff et al., 2002), gouty arthritis (Khameneh et al., 2017) neurodegeneration (Woodruff et al., 2008, Fonseca et al., 2009, Pavlovski et al., 2012, Ager et al., 2010, Hernandez et al., 2017), ischemia reperfusion injuries (Arumugam et al., 2003, Proctor et al., 2004, Woodruff et al., 2004, Poppelaars et al., 2017), asthma and allergies (Gerard, 2005, Abe et al., 2001, Baelder et al., 2005, Lambrecht, 2006), paroxysmal nocturnal haemoglobinuria (Hill et al., 2005, Hillmen et al., 2004), atypical haemolytic uremic syndrome (Noris et al., 2014), age-related macular degeneration (Zipfel et al., 2010), inflammatory bowel diseases (Woodruff et al., 2003, Johswich et al., 2009, Jain et al., 2013), acute liver failure (Lei et al., 2016) C3 glomerulopathy (Williams et al., 2017) and even in cancer (Rutkowski et al., 2010). Some of these pathologies are associated with diminished complement activity, while the others are associated with over activation and dysregulation of its activity.

Complement deficiencies result either from rare genetic aetiologies (Colten and Rosen, 1992) or from consumption of complement proteins during infections, inflammations, wounds or surgery. Insufficient

complement activity may predispose to increase susceptibility to infections especially bacterial or fungal. In addition, diminished clearance of apoptotic cells could lead to the development of autoimmune disorders such as SLE (Pickering and Walport, 2000) and neurodegenerative disorders such as Alzheimer's disease (Mukherjee and Pasinetti, 2000).

On the other hand, excessive or prolonged activation of the complement system may result from chronic or excessive presence of activators. These activators involve microorganisms such as bacteria and yeasts, immunocomplexes, or damaged tissues. In addition, decreased production of complement down-regulators may also predispose to complement over activation. In many conditions such as rheumatoid arthritis, ischaemia reperfusion (IR), myasthenia gravis and multiple sclerosis, the tissue damage is due to attack on host tissue by complement (Morgan and Harris, 2003). For example, in IR, the tissues are exposed to interruption of blood supply as the case in myocardial infarction or ischemic stroke. Then, when the circulation is returned to the affected tissues, complement activation occurs and attacks the host blood vessels because it considers the anoxic endothelium as altered-surfaces or foreign. This kind of damage is similar to the damage that happens to transplanted organs. Therefore, research interest is growing for the development of complement inhibitors that can protect against such damage.

The complement system was found to have an ambivalent role in cancer development. It was thought that C5a implication in cancer is through promoting angiogenesis, proliferation, invasion, production of growth factors, prevention of apoptosis, suppression of antitumor immunity (reviewed in Rutkowski et al., 2010, Darling et al., 2015, Markiewski et al., 2008). However, at low concentrations, C5a was

suggested to have a tumor inhibition activity by facilitating the infiltration of macrophages and natural killer cells (reviewed in Darling et al., 2015)

Sepsis is another systemic inflammatory response that is associated with uncontrolled activation of the complement system. It involves release of anaphylatoxins such as C3a and C5a excessively and ensuing neutrophils dysfunction (Goya et al., 1994, Hecke et al., 1997, Solomkin et al., 1981, Botha et al., 1995). The role of C5a in sepsis has been described as "too much of a good thing" (Gerard, 2003). Blood polymorphonuclear cells have lower ability to bind C5a in experimental sepsis (Huber-Lang et al., 2001) through down-regulation of C5a1 receptor (Guo et al., 2003a). However, IL6 dependent up-regulation of C5a1 was found in kidneys, lung, heart and liver (Riedemann et al., 2003b). In addition, in animal models of sepsis, animals' survival rate was improved with blockade of C5a or C5a1 (Czermak et al., 1999, Huber-Lang et al., 2002a). Another study showed that in sepsis, the blockade of C5a1 or C5a2 receptors was associated with improved survival from mid-grade cecal ligation and puncture (CLP) sepsis (30-40% survival). The combined blockade was the only protective condition in high-grade sepsis (100% lethality), which suggests C5a1 and C5a2 were possibly acting synergistically (Rittirsch et al., 2008). Moreover, in C5a1- or C5a2 deficient mice with CLP, the G-CSF plasma levels were substantially lower in wild type mice (Bosmann et al., 2013b). On the other hand, a decrease in IL6 levels was reported in sepsis after anti-C5a1 infusion, whereas IL6 was increased up to nearly fourfold after treatment with anti-C5a2. The same study observed similar effects on IL6 release from normal blood neutrophils pretreated with anti-C5a2 after stimulation with LPS and C5a (Gao, 2005). In addition, in CLP rat, C5a2 receptors expression was decreased in PMN cells in a time dependent manner. C5a2 expression was

preserved after blockade of C5a *in vivo* in experimental sepsis. Similarly, C5a2 surface expression was decreased in patients with progressive sepsis, and in patients with multiorgan failure, the expression was virtually abolished. In contrast, retention of C5a2 receptors was observed in sepsis survivors (Huber-Lang et al., 2005).

The role of C5a and its receptors was also explored in experimental models of pulmonary inflammation and allergies. In asthma, C5a1 receptor has been reported to promote the development of asthma (Baelder et al., 2005). C5a2 receptor did not show a clear role in pulmonary diseases. It is thought that C5a2 has a complex and dual role in allergic asthma pathogenesis and this critical role is beyond the function of a decoy receptor for its ligands (Zhang et al., 2010). In C5a dependent immune complex pulmonary injury, an exaggerated inflammatory response was observed in C5a2 deficient mice, which suggests that C5a2 has a role in limiting C5a and C5a desArg effects (Gerard, 2005). In addition, in LPS-induced lung injury in $C5a2^{-/-}$ mice, C5a2 was also suggested to play an important anti-inflammatory role at least by negative modulation of C5a1 receptor (Wang et al., 2016). On the other hand, a decreased build-up of inflammatory cells was described in the lungs of C5a2 deficient mice in an experimental model of asthma (Chen et al., 2007). In acute lung injury, the C5a1- and C5a2- mediated tissue damage and inflammation was suggested to be due to the appearance of extracellular histones (Bosmann et al., 2013a).

C5a2 was also found to play a role in suppressing C5a-C5a1 signalling in allergic contact dermatitis (Wang et al., 2013).

The role of C5a and its receptor in metabolic processes has been explored by many studies. ASP has been proposed as an adipokine that has

metabolic and immune functions. It may play a role in triglyceride synthesis and glucose transport. It was proposed that this ASP is identical to C3a desArg through sequence analysis and purification from human serum (Cianflone et al., 1987). In adipocytes and other cells, C5a2 was reported to act as a receptor for ASP (Kalant et al., 2005, Kalant et al., 2003). C5a2 deficient mice placed on a high-sucrose and high-fat diet demonstrated insulin resistance and increased serum proinflammatory cytokines (Fisette et al., 2013). This could be due to interruption of the interaction between C5a2 and ASP. However, this is a controversial subject because the direct interaction between ASP and C5a2 could not be detected by other groups (Johswich et al., 2006).

The C5a receptors are also thought to have a role in the communication between the immune, nervous, and endocrine systems. This communication could play a pivotal role in controlling inflammation that is primarily mediated by the hypothalamic–pituitary–adrenal axis. Both C5a1 and C5a2 receptors were reported to be expressed in anterior pituitary gland. They were supposed to play a role in inhibiting the release of macrophage migration inhibitory factor (MIF) and triggering adrenocorticotropic hormone (ACTH) release and, therefore, participate in damping down inflammation (Francis et al., 2008). However, it is unclear if this effect is mediated by C5a1 or C5a2. C5a2 expression has also been reported in astrocytes, which is regulated by noradrenaline and exerts anti-inflammatory properties (Gavrilyuk et al., 2005). Another hormone that was found to regulate C5a2 expression is progesterone. It acts to down-regulate C5a2 expression in preadipocytes and mature adipocytes (Wen et al., 2008).

Therefore, the complement system generally, and C5a and its receptors specifically, play important and sometimes vital roles in

development of diseases that could be life threating such as sepsis. Thus, studying this area deserves more attention to possibly identify the factors that are involved in the pathogenesis of such diseases and may results in development of novel therapies that could limit the morbidity and mortality.

1.7 Complement therapeutics:

Inappropriate complement activation clearly plays an important role in the pathogenesis of a multitude of diseases. Therefore, targeting the generation of its components or their functional pathways has become an attractive drug development target. Since all the complement pathways activation results in cleavage of C5 and production of C5a and C5b, which are key perpetuators in the initiation and propagation of inflammatory events in many diseases, targeting the complement system activation at this step is a useful strategy. The C5a production can be inhibited at different levels. It could be targeted at the stage of C5 cleavage to prevent its generation. In addition, C5a can be a target itself after production. Furthermore, the C5a signalling through its receptors could be another attractive target.

1.7.1 Inhibiting generation of C5a:

C5a is produced after cleavage of C5 by C5a convertases, C4b2a3b and C3bBbC3b from the classical/lectin and alternative pathways respectively. These convertases are formed by the association of the C3 byproduct, C3b, with other components of the complement pathways. One option to inhibit C5a production is through the inhibition of C5a convertases. This approach is already found in nature. Several natural complement regulators such as CR1, CD46 and CD55 are working by limiting the C5 convertases activity (Nangaku, 1998). In addition, some

bacteria such as *S. aureus* produce molecules that can inhibit C5a convertases (Jongerius et al., 2007). However, all these molecules are non-selective and target C3 convertases in addition to C5 convertases. Another method to prevent cleavage of C5 is by targeting the protein itself and rendering it not susceptible to enzymatic cleavage by the C5 convertases. In mouse model of C3 glomerulopathy, a recent study has shown that renal failure was prevented by inhibiting C5 (Williams et al., 2017). An example of such strategy is the anti-C5 antibody eculizumab (Solaris^{TM}). It binds to C5 with high affinity and prevent its cleavage (Matis and Rollins, 1995). In paroxysmal nocturnal haemoglubinurea, eculizumab has been used successfully, and promising results have been obtained from patients with atypical hemolytic uremic syndrome (reviewed in Woodruff et al., 2011).

1.7.2 C5a as a target:

Although inhibiting C5 has been shown to be a useful therapeutic strategy in preventing the production of C5a and MAC, in certain cases it may more desirable to develop a more selective method in targeting one of the two cleavage products of C5. This could preserve the immune function of the other one. This has directed the pharmaceutical attention to target C5a solely. Like C5 inhibition, this method is used by certain bacteria to target C5a directly. For example, group A *streptococci* produce the C5a peptidase, ScpA, which cleaves 7 residues from the C-terminus of C5a to inactivate it (Kagawa et al., 2009). Several C5a inhibitors have been developed and they are at various stages of clinical development (Ricklin and Lambris, 2013, Woodruff et al., 2011). An interesting approach was inducing the production of C5a neutralizing antibodies by the host through vaccinating with maltose binding protein with C5a as a recombinant fusion construct (MBP-C5a) (Nandakumar et al., 2010). This immunotherapy resulted in generation of host-specific antibodies to C5a with no significant

alteration to C5/C5b activity. In a mouse model of Alzheimer's disease, C5a-peptide active vaccine was reported as a safe and well-tolerated new therapeutic approach (Landlinger et al., 2015). This could be a useful strategy to exploit host immune response to produce sustained antibodies for its own benefit in addition to avoidance of formation of neutralizing antibodies towards the injected therapeutic antibodies or inhibitors.

1.7.3 C5a1 receptors as a target:

The other methods of inhibiting the C5-C5a1 receptors axis is by targeting C5a1 receptors. The pharmacological preparations that target C5a1 receptors are one of the largest developing groups of compounds, which are attractive to pharmaceutical companies (reviewed in Woodruff et al., 2011). The benefit of such approach is the specificity of targeting an individual complement component and sparing the other components for the immune defensive functions. In addition, C5a1 receptor has various roles in many diseases (Ager et al., 2010).

Targeting C5a2 receptor is still a debatable subject within the literature because its role in health and pathophysiology is yet unclear. Its enigmatic function, in addition to potential reliance on C5a1 receptor for possible activity and its predominant intracellular position make this receptor not easy for targeting and drug development (Cain and Monk, 2002, Scola et al., 2007, Bamberg et al., 2010). Therefore, studies are needed to be carried out to clarify the function of C5a2 receptor and its effect on C5a1 and this could help in development of new strategies to target these receptors.

Inhibition of complement activities for a long time may affect the beneficial aspects of complement function and developing complement targeting therapeutics needs to take this subject in consideration. For

example, meningococcal disease susceptibility may arise after interfering with C5 cleavage. In addition, *Leishmania major* infections could be exacerbated by the C5a inhibition due to diminished Th1 immune response (Hawlisch et al., 2005). A dual role of C5a was reported in an animal model of asthma (Kohl et al., 2006). C5a was also suggested to have a role in protecting neurons from apoptosis induced by glutamate (Mukherjee and Pasinetti, 2001); it is essential in liver regeneration (Strey et al., 2003) and has anti-inflammatory role in pancreatitis (Bhatia et al., 2001). Furthermore, it was considered as a potent inhibitor of angiogenesis (Langer et al., 2010). C5a role in neurodevelopment (Denny et al., 2013) and neurorepair (Beck et al., 2010) was also reported. Therefore, it still needs to be determined whether the long-term of use of complement therapeutics will affect these beneficial functions.

One of the possible approaches to solve the problem of the potential unwanted effects after long-term C5/C5a inhibition, is by improving the specificity of targeting. This could be done by limiting treatment to specific tissues. It has been reported that developing mini-bodies against C5 fused to a peptide was a successful strategy because this construct was selectively delivered to inflamed synovium (Macor et al., 2012). Therefore, this approach leads to local inhibition of complement function without affecting the functions circulating complement components.

1.8 Receptor dimerization and oligomerization:

An increasing body of evidence assumes that the GPCR can interact with other receptors of their family in the form of either homodimers, heterodimers or higher-order oligomers. As most cells are expressing many types of receptors on their surfaces, it may be possible to propose that multiple GPCR may be involved in formation of different oligo/dimers.

The exact factors and conditions that affect the formation of these dimers are not clear. It has been suggested that the relative affinity and the rate of expression of the receptors may influence the ratio of hetero-/homodimer (Ayoub et al., 2004).

The engagement in heterodimers can affect the characteristics and behaviours of the receptors towards the ligand (Jordan and Devi, 1999). It has been described that two-non-functional receptors can interact and form a functional receptor. This is due to the requirement of the presence of both receptors to be delivered to the cell surface (i.e. $GABA_B$ receptors and the vast majority of the olfactory receptors).

Considering that the GPCR have significant pharmacological attention, a clear understanding of the heterodimers' ligand binding features may play an important role in the development of new therapies.

The effect of receptor interactions on receptor pharmacology is one of the intensive areas of research. The formation of receptor dimers or oligomers could result in changes of ligand affinities. This could be due to allosteric interactions between the receptors. In addition, a new binding site could result from transmembrane regions swapping between the interacting receptors. The opioid receptors are prominent examples in this context. These receptors are involved in many physiological conditions such as analgesia and pain perception. The main cloned opioid receptors are delta (DOR), kappa (KOR) and mu (MOR). Heterodimerization between DOR and KOR has distinct pharmacological implications compared to their homodimerization (Levac et al., 2002). The selective DOR and KOR agonists and antagonists have shown decreased binding affinities, while the non-selective antagonists showed increased binding affinity. A novel

binding site is also created between the DOR and MOR receptors, which affects the binding affinities of the synthetic selective ligands (including morphine) and the endogenous opioid peptides. It has been reported that blocking DOR can enhance analgesia induced by MOR. A bivalent ligand that can work as agonist for the MOR and antagonist for the DOR has been developed (Daniels et al., 2005). This new pharmacological strategy of bivalent ligands can help in development of analgesics with less side effects.

Due to the significant functional changes that have been observed in some heteromers, such as binding and signalling, it is proposed that heterodimerization could have a considerable influence on receptor response (Gazi et al., 2002). For example, monomeric expression of the DOR and MOR results in inhibition of adenylyl cyclase through a pertussis toxin-sensitive pathway. This toxin catalyses the ADP ribosylation of Gi/o-proteins with notable exception of G_z. However, co-expression of both DOR and MOR inhibits adenylyl cyclase by a pertussis toxin-insensitive rout, which suggests the involvement of G_z. In similar way, the dopamine D1 and D2 receptors couple to Gs and Gi proteins, respectively, when expressed separately. Nevertheless, the D1-D2 heterodimer couples to Gq/11.

Internalization of receptors after ligand binding is part of the desensitization process of majority of GPCR. It is not clear for homodimers whether both receptors need to bind the ligand to be endocytosed. However, many examples were reported that co-internalization could occur in response to agonist of only one of the receptors (reviewed in Dickenson et al., 2013). For the β2-adrenoceptor-DOR heterodimers, co-internalization of both receptors occurs after agonist stimulation of either receptor (isoprenaline for β2-adrenoceptors or etorphine for opioid receptors).

However, this co-internalization does not occur with β2-adrenoceptor-KOR heterodimers (Jordan et al., 2001).

Internalization in certain cases is part of the signalling process. This is the case for β2-adrenoceptors. Mitogen-activated protein kinase pathway activation occurs after internalization of the receptors by β2-agonists. This signalling is reduced when β2-adrenoceptor are co-expressed with KOR (Jordan et al., 2001). Therefore, heterodimerization could be a method for regulation of receptors function.

It is estimated that about 30% of all pharmaceutical drugs target are GPCR. Therefore, they can be considered a major therapeutic target. The GPCR heterodimerization may provide an additional novel drug discovery path. As mentioned earlier, pain treatment with a bivalent ligand for MOR-DOR heterodimers may prove a useful treatment (Dietis et al., 2009). Another example on the clinical relevance of GPCR dimerization is from the study of chemokine receptors. Two receptors were thought to function as receptors for the entry of HIV into the cells. These are CXCR4 and CCR5. Both receptors heterodimerize with CCR2. A single nucleotide polymorphism of CCR2 was associated with increased heterodimerization with CXCR4 and CCR5 and was linked to a decrease in HIV progression. Since such heteromer formation has clinical significance, promoting its formation by ligands or antibodies could be of use in HIV treatment. Moreover, GPCR heterodimerization was linked to the pathogenesis of certain diseases. The first evidence of such link was appeared in 2001. It was found that in pre-eclampsia cases angiotensin AT1 and bradykinin B2 receptor heterodimer levels increase compared to normotensive pregnancies. This increase results in increase in blood pressure due to increase in response to angiotensin II (reviewed in Dickenson et al., 2013).

Regarding C5a receptors, studies have shown that C5a receptors can form homodimers or higher orders oligomers (Klco et al., 2003, Floyd et al., 2003, Rabiet et al., 2008), while other studies elicited the formation of C5a1 heteromers with C5a2 or with other receptors such as CCR5 (Poursharifi et al., 2013, Huttenrach et al., 2005, Croker et al., 2013). However, the functional consequences of these interactions still need to be more explored.

Dimerization may play a role in receptor interaction with its downstream partners such as β-arrestin and receptor kinases after the activation of the receptor by the agonist, and may be required for internalization of the receptor. If the dimerization occurs between the targeted receptor and other receptor species then both of them might be co-internalized, for example: β2-adrenergic receptor dimer (Sartania et al., 2007); β2-adrenergic receptor heterodimers with V2 vasopressin receptors (Klein et al., 2001), or with α-opioid receptors (Jordan et al., 2001); and of C5a1 and CCR5 chemokine receptor heterodimerization (Huttenrach et al., 2005). In the last example, the inactive unliganded receptor may be cross-phosphorylated when the homo or hetero-dimerization occurs with another receptor, which is phosphorylated by the GRKs. Efficient down regulation of CCR5 chemokine receptor occurs when it forms heterodimers with C5a1, activated with C5a, due to the cross-phosphorylation and co-internalization of this heterodimer.

It has been proposed that the non-signalling receptor CXCR7 (Levoye et al., 2009) forms dimers with CXCR4, which then associates constitutively with β-arrestin and decouples from Gαi that leads to the potentiation of CXCR12-dependent MAP kinase signalling for promoting cell migration (Decaillot et al., 2011). It may be expected for C5a2 to act in the same way with C5a1 depending on β-arrestin downstream signalling

rather than the classical G protein pathway. Association of β-arrestin with C5a2 receptors was reported and it is thought to have a modulatory role on C5a1 receptors and possibly fine tuning the host defence (Bamberg et al., 2010). The ability of C5a2 to recruit β-arrestin2 in macrophages was also reported and it is thought to perhaps exert an inhibitory effect on C5a1 signalling through modulating ERK1/2 signalling (Croker et al., 2014). Some researchers suggest that the underlying mechanism might be due to differential downstream signalling rather than the dimerization (Chen et al., 2007, Bamberg et al., 2010).

1.9 Techniques for measuring the dimerization of receptors:

The dimerization of GPCR is studied using various biochemical, biophysical, and functional techniques (Dickenson et al., 2013). In many cases, the dimerization was explored using heterologous expression of receptors in transfected cell lines. The receptors are either tagged with fluorescent or immunological tags. Issues could be raised about using such approach because receptor overexpression in comparison to the endogenously expressed receptors could affect the results. Dimerization of endogenous GPCR has been reported for rhodopsin in retinal disc membranes (Fotiadis et al., 2003). These membranes are characterised by very high rhodopsin expression levels.

1.9.1 Biochemical techniques:

Many biochemical approaches have been used to detect GPCR dimerization. Using SDS-polyacrylamide gels for measuring the molecular mass of a migrating proteins complexes, which may be expected to show the molecular weight of dimers or oligomers (Nimchinsky et al., 1997). Another approach is by using co-immunoprecipitation (Cvejic and Devi,

1997, Hebert et al., 1996). The first evidence for the possibility of GPCR existing in dimers was provided using this technique for the β2-adrenoceptor homodimerization. The concern regarding such technique is the possibility of the formation of artificial aggregations after solubilisation of hydrophobic membrane receptors before the immunoprecipitation.

1.9.2 Biophysical approaches:

1.9.2.1 Fluorescent resonance energy transfer (FRET):

FRET was discovered 80 years ago, and Theodor Forster, in 1940s, quantitively described and established a basis for it. In biological research, however, only more recently FRET has been harnessed as a tool (reviewed in Dye, 2005).

FRET occurs between two molecules: one (donor) that, after excitation, emits energy to a second molecule (acceptor) (Selvin, 2000, Szollosi et al., 1998). The energy is generated via the vibrational relaxation of the excited donor until it reaches its ground excitation level. If the acceptor is a chromophore, the received energy will eventually be emitted as a fluorescence (sensitized emission).

At a sufficiently small distance (10–100 Å) and appropriate orientation between the donor and acceptor, energy transfer happens (Truong and Ikura, 2001, Heyduk, 2002). FRET is considered to be an attractive method to measure the conformational changes of the receptors due to the very high sensitivity to small modifications (Ayoub et al., 2004). A small increase in the distance between the donor and acceptor leads to a dramatic decrease in FRET because the energy transfer is inversely proportional to the sixth power of the separating distance between them.

To monitor a particular protein by these strategies, two approaches have been used (Figure 1.4). First, to measure intramolecular FRET by making a construct composed of the protein of interest with the donor and acceptor attached to its extremities. This could help in measuring the conformational changes of a protein. Second, measuring the energy transfer between molecules (intermolecular) by attaching the donor to one molecule and the acceptor to the other. GPCR dimerization or oligomerization could occur independent of ligand binding (Uberti et al., 2003). However, other receptors are affected by the ligand and the energy transfer changes depend on the ligand dose, which may suggest the concept that the distance between the donor and acceptor or their orientation can be modified by the conformational changes after ligand binding (Ayoub et al., 2004).

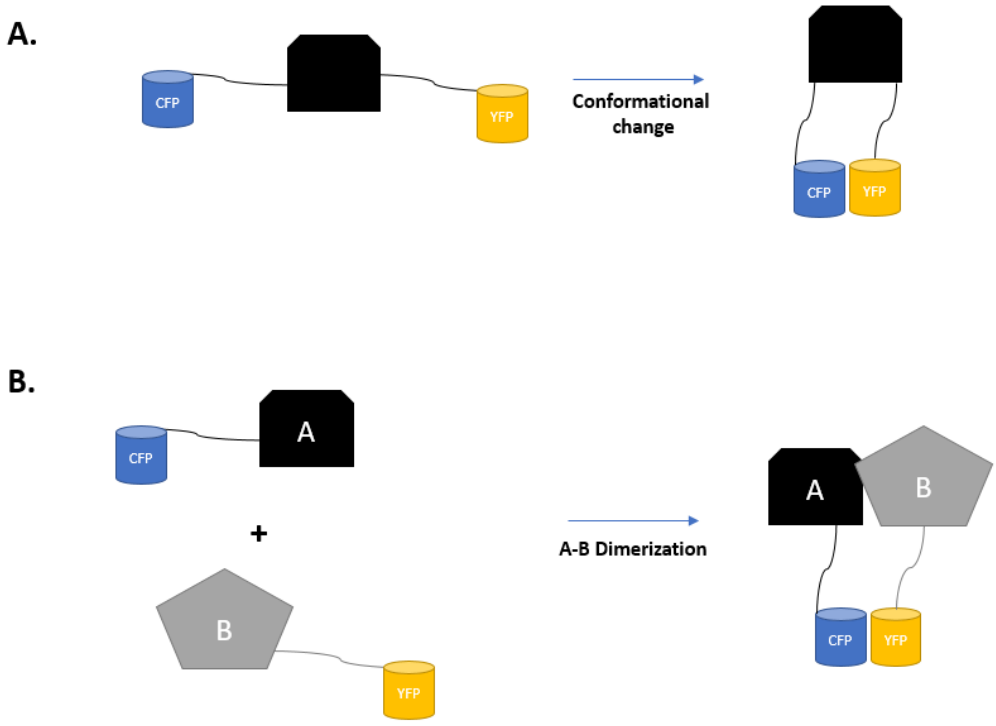


Figure 1.4. Fluorescent resonance energy transfer types. A. intramolecular, the two FRET labels cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) are attached to ends of a protein. If the protein undergoes conformational changes that bring both ends together, the CFP and YFP would be brought together inducing FRET signal. B. Intermolecular, one protein molecule (A) tagged with CFP and the other (B) with YFP. The FRET efficiency increases when the two proteins interact bringing the fluorophores close to each other.

Various receptors have been studied by using this approach (Boute et al., 2001, Overton and Blumer, 2000, Dinger et al., 2003, Couturier and Jockers, 2003, Rocheville et al., 2000a, Kroeger et al., 2001, McVey et al., 2001, Stanasila et al., 2003). Three essential properties must be present in the donor and acceptor for the FRET to occur: (1) distance, they must be close (10-100 Å) to each other, (2) geometric orientation must be suitable, and (3) spectral overlap must be significant between the two chromophores (Dye, 2005).

Cell surface proteins interaction can also be investigated by FRET using fluorescently labelled antibodies, which are specific to the interacting proteins (Horvath et al., 2005, Broudy et al., 1998). Intracellularly, however, it may be difficult to detect protein-protein interaction using antibodies. Therefore, new techniques have been developed to detect intracellular interactions by fluorescent proteins, which can be added to the target proteins and expressed as fusion proteins. The derivatives of green fluorescent protein (GFP), from *Aequorea victoria*, for example cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), or the red fluorescent protein (dsRed), from *Discosoma* sp, are most commonly used to perform FRET intracellularly (Mizuno et al., 2001, Campbell et al., 2002).

1.9.2.2 Bioluminescence Resonance Energy Transfer (BRET):

BRET occurs in some marine creatures as a natural phenomenon. It was used for the first time to detect protein-protein interaction in living cells in 1999 by Xu et al (Xu et al., 1999). The principle of this technique is similar to FRET assay with the advantage of avoiding external excitation of the donor and its problems. In BRET, luciferase is used as a donor, which in the presence of a substrate, produces bioluminescence to excite

the acceptor fluorophore by the same mechanism of energy transfer that occurs in FRET (Milligan and Bouvier, 2005). Many studies have reported the use this technique to measure GPCR oligo/dimerization (Kroeger et al., 2001, Ayoub et al., 2002, Issafras et al., 2002, Babcock et al., 2003, McVey et al., 2001, Angers et al., 2000).

1.9.3 Functional complementation techniques:

The principle of this approach is the regaining of the functionality after co-expression of receptors that are mutant and functionless when expressed individually (Lee et al., 2002, Dickenson et al., 2013, Carrillo et al., 2003). It could show the functional consequences for such interactions.

1.10 Aim of the study and the hypothesis:

We aim to determine:

1- If C5a1 receptor is interacting with C5a2 and whether the interaction is due to formation of heterodimers.

2- If the interaction between C5a1 and C5a2 can affect receptor behaviour.

Hypothesis:

Human C5a2 receptor affects C5a1 receptor signalling directly by dimerization or indirectly by sequestering both ligand and signalling molecules, such as β-arrestin.

Chapter Two

Materials

and

Methods

Chapter 2: Materials and methods

2.1 Materials:

2.1.1 Instruments and equipment:

Equipment	Supplier
Centrifuges	-SIGMA 3K15
Microscopes	-Confocal microscopy (Nikon A1 confocal microscopy) -Olympus CK40 light microscope, Nickon light microscope
Spectrofluorimeter (Varioskan)	-Thermoscientific
Micro plate reader	LabTech LT-4000
Flow cytometers	-LSRII (BD-Bioscience) -Attune autosampler (Life technologies) -FACS Caliber (BD-Bioscience) -FACS Aria (BD-Bioscience)
Electroporator	-Biorad gene pulser II

2.1.2 Prepared solutions and buffers:

Buffers and solutions	**Components**
BBN	Hank's balanced salt solution (HBSS) (with divalents cations), 0.2% BSA (1g/500ml) and 0.1% sodium azide (0.5g/500ml)
Internalization buffer	HBSS+0.2% BSA
Release buffer	HBSS (with divalent cations) with 0.1% BSA (0.5g/500ml)
Lysogeny broth (LB) media	10g tryptone, 10g NaCl and 5g yeast extract Made up to 1L in water (for agar, 15g agar added)
Kanamycin	Stock solution prepared in ultrapure water. Used at 25 µg/ml in LB media
Carbenicillin	Stock solution prepared in ultrapure water. Used at 50 µg/ml in LB media
Freezing mixture for mammalian cells	90% foetal calf serum 10% DMSO
Neomycin (G418, Stock 250mg/ml)	Used at 0.5-1 mg/ml in DMEM
Tris buffer 1M (pH9.0)	121.14g of Tris dissolved in 800ml dH_2O, adjust to 9.0 with HCl and make up to 1Litre
Triton-X buffer	0.5% Triton-X100
Citrate buffer, pH4.5 (0.2M)	250ml (5.85g citric acid and 6.47g sodium citrate)

2.1.3 Standard reagents, chemicals, solutions and kits:

Name	Product no.	Company	composition
Mouse anti-human C5a1	sc-53794	Santa Cruz Biotechnology	
Mouse anti-human C5a2	B147507	Biolegend	
Mouse IgG1 isotype	GR76062-1	Abcam	
Mouse IgG 2a isotype	Cat. 401502	Biolegend	
Alexa fluor@633 rabbit anti-mouse	1270146	Life Technologies	
Foetal calf serum	10846	Biosera	Sterile, filtered, heat inactivated
P/S Pen-Strep	2MB251	Lonza	10000 u penicillin/ml 10000 u streptomycin/ml Sterile filtered
DMEM Dulbecco's modified Eagle's Medium	Cal: BE12-604FMBO13	Lonza	With 4, 5, L-glucose With L-Glutamine
Dimethyl sulfoxide (DMSO)	Lot: RNBC 6511	Sigma	
Fluo 3-AM	F 1241	Invitrogen	
Pluronic F- 127	Lot 1084328	Invitrogen	

(20% solution in DMSO)			
Albumin from bovine serum	Lot SLBC2188V	Sigma	Lyophilyzed powder Essentially fatty acid free
HBSS Hank's Balanced salt solution without divalents (Ca^{2+} and Mg^{2+}) and phenol red	Cat. N: BE10-547F3MBO97	Lonza	
Neomycin	13200	Cayman	
Cell dissociation solution (CDS)	S-044-B	MilliPore	
Hi Speed Plasmid Midikit	12643	Qiagen	
Substrate solution for release assay	N9376	Sigma	P-nitrophenyl N-acetyl β-D-glucosaminide 50 mM stock prepared in DMSO
Trypsin 10X	NE02-007E	Lonza	
Turbofect reagent	00120877	Thermo-scientifific	
Mounting media for fluorescence	2B0324	Vectashield H-1200	With DAPI

Dinitrophenyl-albumin (DNP-A)	A-6661	Sigma	
Zeocin	1621193	Invitrogen	
Coelentrazine h	S201A	Promega	
RHYPYWR peptide	P11001501	GenScript	
Cell-Based Human/Mouse/Rat ERK1/2, JNK, p38 MAPK Phosphorylation ELISA Kit	CBEL-ERK-SK	RayBio®	

2.1.4 plasmids:

Name	**components**	**Source**
pcDNA3-YFP	Yellow fluorescent protein (YFP) in pcDNA3 vector	Addgene (ID13033)
pECFP-EYFP	CFP linked to YFP	LMBP 8242
Rluc8-hC5a1	Human C5a1 receptor attached to *Renella* luciferase enzyme 8	Invitrogen™ Life technology

2.2 Mammalian cell culture methods:

2.2.1 Cell lines:

The rat basophilic leukemia cell line (RBL-2H3) is a widely-used cell line in many applications that involve inflammatory or allergic immunological reactions. It is frequently referred to as a mast cell line, although it originated from basophils (Passante and Frankish, 2009). Its secretory properties and simple growth conditions qualify it to be used in a broad range of studies. It has been used for degranulation assays; studying the interaction between IgE antibodies with their receptor (FcεRI); and intracellular Ca^{2+} mobilization (Maeyama et al., 1986, Gao et al., 2010). Therefore, this cell line has been chosen to express various types of human complement C5a receptors to study the behavior of these receptors in response to C5a. The cell lines used were supplied kindly by Prof. Andreas Klos, MHH, Hannover:

1-RBL-2H3-hC5a1 receptor: these cells are expressing wild type human C5a1 receptor.

2-RBL-2H3-hC5a1-CFP receptor: these cells are expressing human C5a1 receptor tagged with cyan fluorescent protein (CFP).

3- RBL-2H3-hC5a1-YFP receptor: these cells are expressing human C5a1 receptor tagged with yellow fluorescent protein (YFP)

4-RBL-2H3-hC5a2-YFP receptor: these cells are expressing human C5a2 receptor tagged with yellow fluorescent protein (YFP)

5-RBL-2H3-hC5a1-CFP+hC5a2-YFP receptor: these cells are expressing both human C5a1-CFP and human C5a2-YFP receptors.

6- RBL-2H3-hC5a1-CFP+hC5a1-YFP receptor: these cells are expressing human C5a1-CFP and human C5a1-YFP receptors.

7-RBL-2H3-hC5a1+hC5a2 receptor: these cells are expressing both human C5a1 and human C5a2 receptors.

In addition, RBL cell lines were made during this study expressing:

1-pECFP-YFP plasmid.

2-Rluc8-hC5a1 receptor: these cells are expressing human C5a1 receptor tagged with *Renella* luciferase enzyme 8 (Rluc 8).

3-Rluc8-hC5a1+hC5a2-YFP receptor: these cells are expressing both human C5a1-Rluc8 and human C5a2-YFP receptors.

4-hC5a1/YFP: these cells are expressing hC5a1 receptors and YFP separately.

2.2.2 Cryopreservation and thawing of cryopreserved mammalian cell lines:

The cells were usually suspended in freezing solution at a density of about $5x10^6$ cell/ml in a cryovials. The freezing solution is composed from 90% foetal calf serum (FCS) and 10% dimethylsulphoxide (DMSO), which is used to decrease ice crystal formation during freezing to protect the cells. The mixture was cooled slowly in liquid nitrogen vapour to about -80ºC for about 2 hours then transferred to liquid nitrogen for long-term storage (about -196ºC).

Thawing the cryopreserved cells is a stressful procedure for the cells that needs to be done carefully and as quick as possible. The cells need to be placed into a 37°C water bath immediately after removing from the liquid nitrogen. When a small piece of ice remained, the vial was transferred to a laminar flow hood. After sterilizing the vial with 70% ethanol, the lid was opened and cells were transferred into a universal tube containing 9ml pre-warmed complete media Dulbecco's Modified Eagle's media (DMEM) + 10%

FCS + penicillin/streptomycin diluted 1/100). After centrifugation (200xg for 5 minutes at 4°C to remove the DMSO, which is toxic to the cells in solution) the supernatant was discarded and the pellet re-suspended in complete media and transferred into a 100mm petri dishes suitable for tissue cultures. The cells were then incubated at 37°C, 5% CO_2 in humidified incubator. The medium was changed next day to remove the dead cells and cell debris due to cryopreservation.

2.2.3 Maintenance of mammalian cell Lines:

The cell lines were grown in complete medium, which is composed of DMEM, 10% (v/v) foetal calf serum (FCS) and penicillin/streptomycin antibiotics (dilute stock solution 1/100). The FCS was added as a source of vitamins, minerals and growth factors, while the antibacterial agents were used to prevent accidental bacterial contamination. Moreover, selective antibiotics were added to the medium to keep selection of the transfected cells. These selective markers are toxic to mammalian cells, therefore allowing the growth of the cells that retain the plasmids with the corresponding antibiotic resistance.

The cells usually grow until confluent then the cells stop dividing and may start dying. They, therefore, need to be spilt and subcultured continuously in order to be maintained. When the cells were approximately 70%-90% confluent the medium was aspirated off and the cells were washed with sufficient sterile HBSS (Hank's buffered salt solution without Ca^{2+} or Mg^{2+}). Then the cells were incubated with trypsin/EDTA solution (2.5 ml for 100 mm petri dishes) for 7 minutes at 37°C, 5% CO_2 in humidified incubator. The cells were dislodged and equal volume of complete medium was added to the

cell suspension and transferred to a 30ml universal tubes. Subsequently, the cells were centrifuged (400 xg, 5 minutes and at 4°C). After decanting the supernatant, the pellets were re-suspended in new complete medium and subcultured into a suitable vessel.

2.2.4 Transfection of Mammalian Cells:

2.2.4.1 Transfection by electroporation:

Mammalian cells (e.g. RBL-2H3) from nearly confluent flask or 100 mm petri dishes were harvested using 2-3 ml trypsin/EDTA as in section 2.2.3. Then the cell suspension was centrifuged and $5x10^6$ cells were re-suspended in 0.8 ml complete media. Plasmid DNA (20 µg) was added to the cell suspension. The mixture was transferred into 4mm gap electroporation cuvette. The cuvette was incubated on ice for 15 minutes and then electroporated (250V and 960µF) once using electric pulse machine and returned back to ice quickly for another 15 minutes. Subsequently the cells were plated into 4x 100mm petri dishes and incubated at 37°C, 5% CO_2 in humidified incubator in complete medium overnight. Next day, the medium was replaced with complete medium with suitable selective markers. The cells were kept under selective pressure by changing the selective media every 2 days until the cells approach confluence and become ready for sorting using the fluorescence activated cell sorting (FACS) technique.

2.2.4.2 Transfection by a chemical-based method:

RBL-2H3 cells were seeded in 6 well plates at a density of $2x10^5$ cell/well with complete medium (4 ml/well) for 24 hours at 37°C, 5% CO_2 in

humidified incubator. A mixture of 400 µl/well plain DMEM, 4 µg/well plasmid DNA and 6 µl of Turbofect reagent (Thermo Scientific) was prepared, vortexed immediately and incubated for 15-20 minutes at room temperature. The mixture was then added to the wells drop wise when the cells were 70-80% confluent and incubated overnight at 37°C, 5% CO_2 in humidified incubator. Next day, the medium was changed with selective one that contains suitable antibiotic agent. The cells were kept under selection by changing the selective medium every 2 days until become confluent and ready for sorting by FACS method.

2.2.5 Sorting RBL-2H3 cells after transfection:

The transfected RBL cells from two T75 confluent flasks were harvested using cell dissociation solution into two tubes and centrifuged at 400g and 4°C for 5 minutes. The cells were then re-suspended in 10 ml HBSS with divalent ($Ca^{2+}$and Mg^{2+}) and re-centrifuged again as before. Then, primary antibodies (mouse anti-human C5a1 antibodies) was added to one tube and isotype (mouse IgG1) antibody control to the other (both were sterilized by filters and at concentration of 10 µg/ml) and incubated for 1 hour on ice. After incubation, the cells were washed with 10 ml HBSS with divalent and centrifuged as above. The cells were re-suspended in 1/400 diluted secondary antibodies (sterilized by filter) and incubated for further hour on ice in dark. Subsequently, the cells were washed with 10 ml HBSS with divalent cation and centrifuged. After re-suspension in 1ml cold medium the cells were transferred to the flow cytometer. The cells were sorted by the FACS-Aria machine. The cells then were re-plated in new dishes with fresh selective medium.

2.2.6 Antibiotic sensitivity test for mammalian cells:

This test was used to determine the minimum dose of Zeocin™ antibiotic reagents can be used for selection of resistant mammalian cells. Zeocin™ antibiotic sensitivity test was done for RBL-2H3 cells wild type (Non-x) and RBL-2H3 expressing hC5a2-YFP plasmid. These cells were used because they will be transfected with Rluc8-hC5a1 plasmid that carries Zeocin resistance gene.

Zeocin is part of bleomycin/phleomycin family of antibiotics. To determine the minimum dose of Zeocin that can be used in mammalian cell selection, sensitivity test was done for cells that do not carry Zeocin-resistant gene. RBL-2H3 were seeded overnight in 24 well plate at $5*10^4$ cell/well in complete medium. Next day, the medium was removed and replaced with selective medium that contains various concentrations of Zeocin (0, 50, 100, 200, 400, 600, 800, and 1000 µg/ml) to respective well. The selective media was then changed every 2 days and cell survival is observed. The minimum concentration of Zeocin that is needed to kill the majority of the cells was 50 µg/ml for RBL-2H3-Non-x cells and 100 µg/ml for the RBL-2H3-hC5a2-YFP (Figure 2.1).

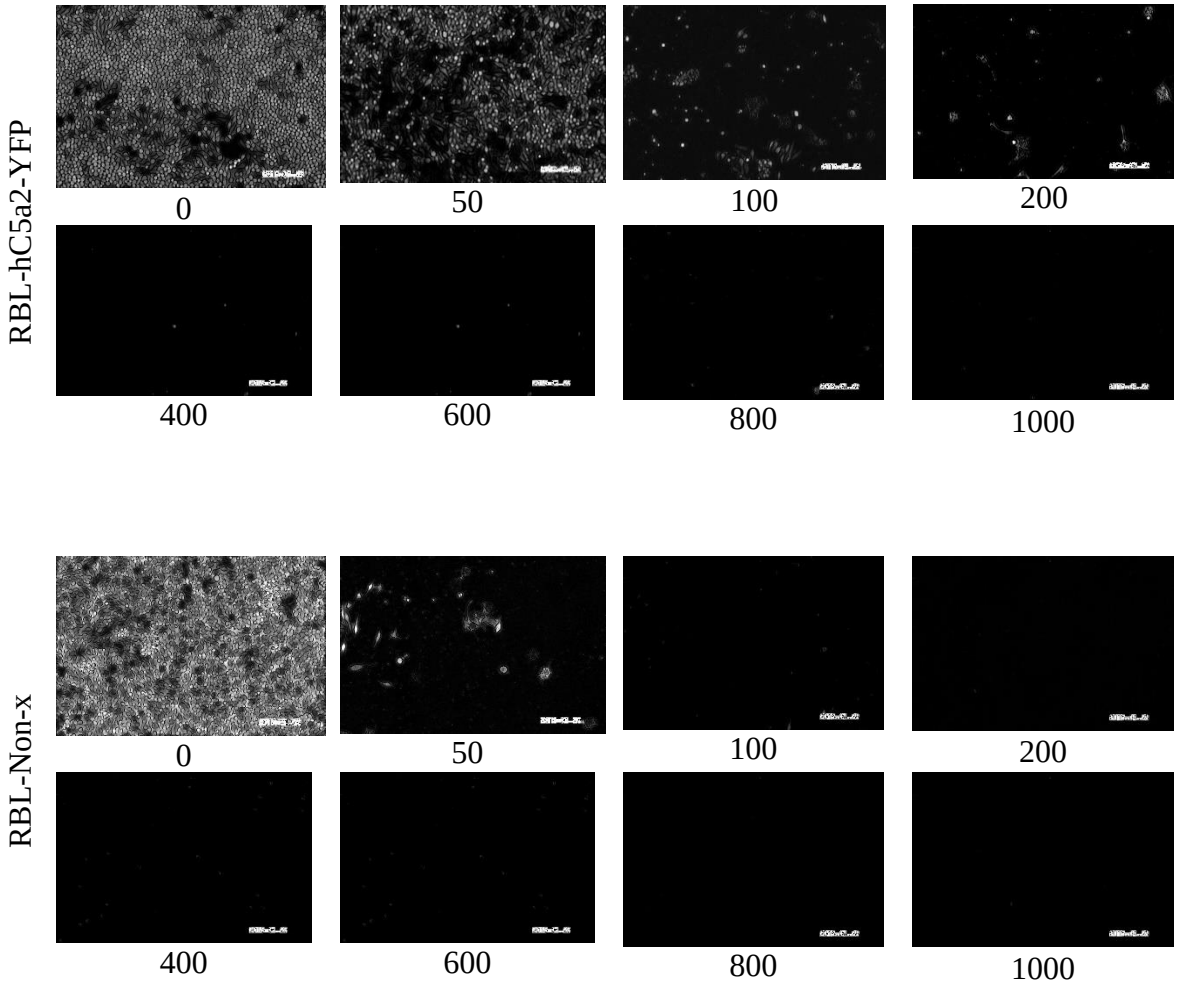


Figure 2.1: Zeocin antibiotic sensitivity test. RBL-2H3 cells wild type (Non-x) or RBL-2H3 expressing hC5a2-YFP plasmid were grown overnight in 24 well plate at $5*10^4$ cell/well. The cells were then treated with different concentration of Zeocin (0, 50, 100, 200, 400, 600, 800, and 1000 µg/ml) for 7days. The background was subtracted from images after converting the images to 8-bit grayscale using image J (FIJI) software (Schindelin et al., 2012) and then optimised for brightness/contrast.

2.3 Proteomics:

2.3.1 Receptor expression assay by flow cytometer:

Staining of the receptors with antibodies was used to confirm cell surface expression of the receptors. Therefore, primary anti-human C5a receptors antibodies were used to target the specified receptors and then fluorescently-tagged secondary antibodies were used to attach to the primary antibodies in order to be detected by the flow cytometer. First, the cells were harvested using cell dissociation solution and re-suspended in BBN at $1x10^5$ cells/well and plated into 96-well U-bottom plate. The cells were then centrifuged (200xg for 5 minutes at 4˚C) and washed with 150 µl/well BBN and centrifuged again as previously. The supernatant was discarded and 50 µl BBN, isotype [for anti-human C5a1: mouse IgG1 isotype (Abcam); for anti-human C5a2: mouse IgG2a isotype (Biolegend)] or primary antibody [mouse anti-human C5a1(Santa Cruz biotechnology) or mouse anti-human C5a2 (Biolegend)] was added to the wells. After incubation on ice for one hour, the cells were washed with BBN as above. Subsequently, secondary antibodies (Alexa fluor@633 rabbit anti-mouse IgG, Life Technology) were added and incubated for further hour on ice in dark. After spinning down, the cells were re-suspended in 300 µl BBN and transferred to flow cytometer.

2.3.2 Ligand binding assay:

C5a labelled with Alexa 488, as described (Bell, 2017), was used to assess the ability of the transfected receptors to bind to their ligand. The binding was detected by measuring the fluorescence using flow cytometer. The cells were harvested using cell dissociation solution; plated ($1x10^5$ cell/well) into 96 U-bottomed plate; and washed in the same way as mentioned

in section 2.3.1. Mixtures of 100 nM Alexa 488 C5a and serial dilutions (1 µM, 300 nM, 100 nM, 30 nM, 10 nM, 1 nM, 0.1 nM and 0 nM) of unlabeled C5a were added to the cells at a volume of 50 µl per well and incubated for 15 minutes on ice in a dark place. The cells were transferred to flow cytometer after adding 150 µl of BBN to each well.

2.3.3 Intracellular Ca^{2+} mobilization assay:

This assay was done to assess the cellular response to C5a through C5a receptors. After harvesting the cells with CDS, the cells re-suspended in HBSS (+0.1% BSA) at a count of 5 x 10^5 cell/ml. The cells washed once with HBSS (+0.1% BSA) and centrifuged at 400xg for 5 minutes at 4˚C. After re-suspension in 10 ml HBSS (+0.1% BSA), 4 µl of 20% pluronic (Invitrogen) and 11 µl Fluo3-AM (Invitrogen) were added to the cells. The cell suspension was incubated for 30 minutes in dark on ice. The cells were then washed with HBSS (+1% BSA), centrifuged as above and re-suspended in HBSS (+1% BSA) at 5x10^5 cell/ml. Subsequently, 0.5 ml was transferred into flow cytometer tubes on ice. At the FACS Caliber flow cytometer, each sample was warmed for 2 minutes before starting the measurements. After loading the samples, the basal fluorescence was measured for 20 second and then after the addition of C5a (0.1, 0.5, 1, 5, 10 nM) for 2 minutes. The fluorescence rise was then calculated by subtracting the basal fluorescence from the peak fluorescence after C5a stimulation.

2.3.4 RBL-2H3.1 β-hexosaminidase release assay:

Degranulation is a known function of mast cells during the innate immune respose. The degranulation process can be stimulated by at least two

types of receptors: 1) the IgE receptors and 2) the GPCR. Therefore, the degranulation assay can be used to examine one functional step in C5a receptors signalling. Mast cells and basophils release a range of mediators upon crosslinking of their IgE-bound FcεRI through several allergens. RBL-2H3 cell line, which is derived from basophils, has been widely used to test degranulation for both IgE and GPCR receptors in allergy and inflammatory research. The RBL-2H3 granules contain many mediators such as histamine, serotonin and β-hexosaminidase enzyme. Beta-hexosaminidase enzyme is an exoglycosidase that is commonly used to monitor degranulation in RBL-2H3 cells as it is released in parallel with histamine (Passante and Frankish, 2009). It can work at pH of about 4.5, which is typical condition during inflammation. The enzyme activity can be measured through the production of yellow colour p-nitrophenol from the colourless hexosaminidase substrate (p-nitrophenyl-N-acetyl-β-D-glucosamide). The resultant colour can be quantified by measuring the absorbance at 405nm.

In this study, this method was performed to check a functional step in the downstream signalling pathway of C5a1 receptors, which is the secretion of intracellular mediators such as β-hexosaminidase in response to C5a stimulation. The RBL-2H3 cells, which are transfected with wild type or tagged hC5a receptor, were plated in a sterile flat bottom 96 well plate at $5x10^4$ cell per well and incubated overnight at 37ºC, 5% CO_2 in humidified incubator. Next day, the cells were washed with 50 µl/well warm (37ºC) release buffer twice. Then, 40 µl/well release buffer was added to the cells and incubated for 10 minutes at the same conditions above. Subsequently, 10 µl C5a (at a serial dilution of 1/3 in release buffer starting with 1 µM was added to the cells. Alternatively, 10 µl release buffer or 0.5% Triton-X100 were added as

negative and positive controls respectively. The plate was incubated for 15 minutes at the same conditions as above. After incubation, 40 µl of the cells supernatant was removed and added to the 40 µl substrate solution (diluted 1/25 with 0.2 M citrate buffer pH 4.5) in new flat-bottom 96 well plate and incubated for 2-3 hours at 37°C, 5% CO_2 in humidified incubator. Then, the reaction was stopped by addition of 120 µl/well of 1 M Tris (pH 9.0). Consequently, the absorbance was measured at 405 nm using a plate reader. Release was calculated as a percentage of total cell associated enzyme activity as in the following equation:

Enzyme release = [(supernatant of C5a treated cells – supernatant of buffer treated cells)/supernatant and lysate of Triton-X100 treated cells] x100

The degranulation assay was also performed to examine enzyme release in response to IgE binding. In this assay, the RBL cells were grown on flat bottom 96 well tissue culture treated plated at a density of $5x10^4$ cell/well in 100 µl. The cells were then incubated at 37°C, 5% CO_2 in humidified incubator for 2 hours to allow the cells to adhere to well bottom. After that, 100 µL of DNP-A-specific IgE antibody solution (diluted 1/500) was added to the wells at final concentration of (1ng/ml) and incubated for 16 hours at 37°C, 5% CO_2 in humidified incubator. This incubation is to allow the IgE antibodies to bind to FcεRI receptors. After the incubation time, the cells were washed twice and incubated with 40 µl release buffer for 10 minutes at 37°C, 5% CO_2 in humidified incubator. Then, 10 µL of 5x DNP-A was added to the cells in 1/10 serial dilutions (starting with 1µg maximum final concentration) and incubated for 15 minutes at 37°C, 5% CO_2 in humidified incubator to allow degranulation to occur. The other steps of detecting the release of β-

hexosaminidase enzyme are the same as in C5a-mediated degranulation mentioned above.

2.3.5 Internalization assay:

This test was used to measure the percentage of surface receptors internalized in response to various treatments. RBL-2H3 cells expressing either hC5a1, hC5a2, hC5a1+hC5a2, hC5a1-CFP, or hC5a1-Rluc8 surface receptors were used. The cells were detached using non-enzymatic cell dissociation solution and resuspended at 2 $x10^6$ cell/ml. The cells were then incubated with appropriate dilutions of anti-hC5a1 or anti-hC5a2 antibodies on ice for 30 minutes. The effect of pre-treatment of the cells with antibodies was checked and did not appear to affect the ligand-induced internalization as illustrated in chapter 6. Subsequently, the cells were washed with ice-cold internalization buffer (HBSS + 0.2% BSA). After that, different treatments 500 nM C5a, 500 nM C5adesArg, selective C5a2 receptor ligand (100 µM peptide RHYPYWR) or control buffer were added to the cells and incubated part on ice (0°C) and part at 37°C, 5% CO_2 in humidified incubator. The cells were removed from the incubator at appropriate time points (5, 15, 30, 45, or 120 minutes) and quenched with ice-cold buffer on ice. Subsequently, the cells were then washed with ice cold buffer and incubated with Alex633 secondary antibodies for 30 minutes on ice in dark. After washing with ice-cold buffer, the fluorescence intensity was measured using flow cytometer. The percentage of internalization was measured as [(MFI of cells incubated on 0°C - MFI of cells incubated at 37°C)/ MFI of cells incubated on 0°C] x 100.

Internalization assay was also used to measure the behaviour of both C5a receptors internalization in the presence of C5a1 receptor inhibitor PMX53 (gift from Associate Professor Trent Woodruff, University of Queensland, Australia), which has no discernible activity on C5a2 receptor (Scola et al., 2007, Klos et al., 2013). This assay may indicate whether C5a2 receptor is working in dimer with C5a1 or not. Therefore, RBL-2H3 cells co-expressing hC5a1 and hC5a2 receptors were treated with 1µM PMX53 for 10 minutes before adding 500 nM C5a or C5a desArg ligands and incubating the cells for another 45 minutes. The cell surface receptors were stained and quantified by indirect immunostaining and flow cytometer as above.

2.3.6 Fluorescence resonance energy transfer (FRET) measurements:

The dimerization between hC5a1 and hC5a2 receptors was explored in different methods, which are confocal microscopy and spectrofluorimetry as described below. In all methods RBL cells expressing C5a receptors tagged to fluorescent proteins CFP or YFP were used. These fluorescent proteins share a significant spectral overlap, which makes them suitable for such experiment. In the ideal situation, excitation of CFP by an external source leads to emission of wavelength that is suitable for YFP excitation. Consequently, YFP emission can be detected, which is called “FRET” emission, when the two fluorophores are in close proximity (Figure 2.2). However, many factors, such as crosstalk, limit this process and make detecting “FRET” a challenging procedure.

2.3.6.1 FRET measurement using confocal fluorescent microscopy:

Confocal microscopy has several advantages over the conventional wide-field fluorescent microscopy. The key element is that it can measure light emission from a very small spot in a focal plane and image can be built up by scanning across the sample. In addition, the out of focus light from above and below the focal plane can be blocked. This results in measuring a very shallow section and several sections can be obtained for thick samples.

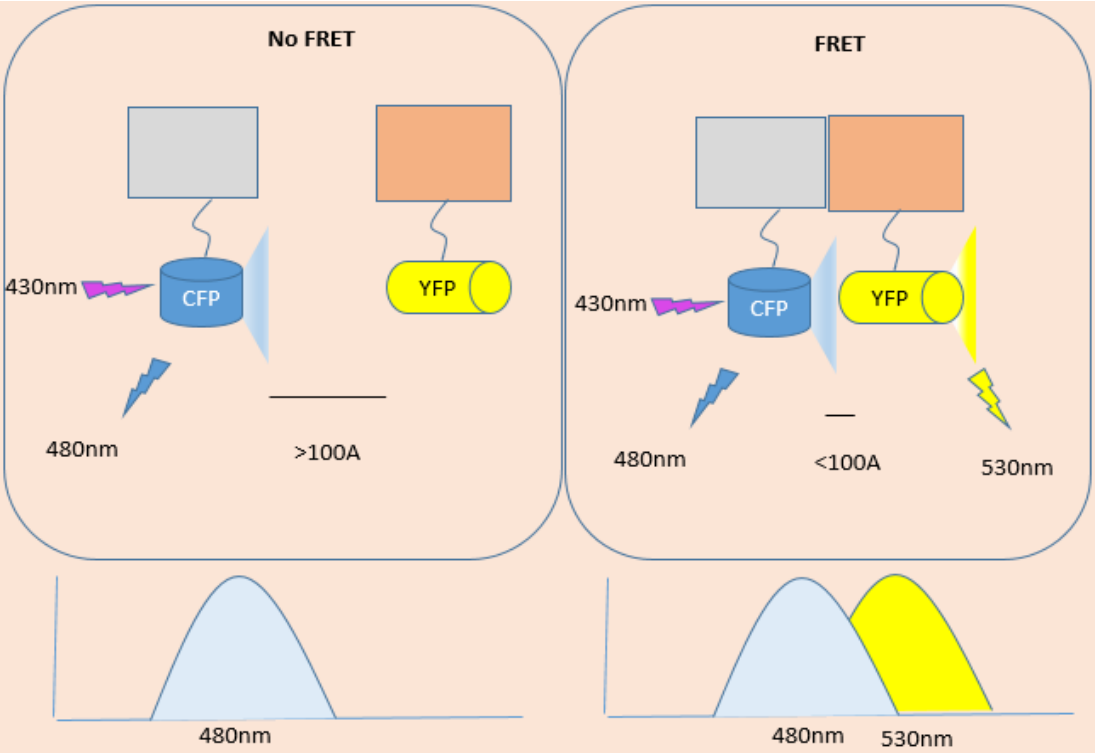


Figure 2.2 Fluorescence resonance energy transfer (FRET) illustration. The figure demonstrates the principle of FRET between CFP and YFP fluorophores. The two fluorophores are attached to two homologous or heterologous proteins. If the two proteins are not interacting, the CFP and YFP will be too far away for FRET to occur (left panel) and only CFP emission (~480 nm) can be detected. When the two proteins approach each other to a distance <100Å, FRET occurs and YFP (~530 nm) emission can be detected (right panel).

In this experiment RBL cells co-expressing hC5a1-CFP and hC5a2-YFP were used. Cells expressing only one of the fluorophores (either CFP or YFP) were also used for correction of the bleed-through between different wavelengths. RBL cells expressing CFP physically linked to YFP were used as a positive FRET control (pECFP-YFP). Cells were seeded in 8 chamber Labtech slides at a density of 10 x 10^4 cell/chamber overnight. Next day, the cells were treated with 500 nM C5a or control buffer and incubated for 10 or 20 minutes at 37°C, 5% CO_2 in humidified incubator. The cells were then washed twice with PBS and fixed using 2% paraformaldehyde for 15 minutes. The chambers were then washed with PBS twice and the plastic chamber was removed as per manufacturer's instructions. Mounting medium for fluorescence with DAPI was added to each well, covered with a cover slip and sealed with nail varnish. The slides stored at 4°C until visualised by Nikon A1 fluorescence confocal microscopy using 60x oil immersion objective.

The CFP (FRET donor) was excited using laser with a wavelength of 457 nm and the emission was detected at 482 nm. The YFP (FRET acceptor) was detected using the 540 nm detector. The normal YFP emission was detected at 540 nm after excitation by the 514 nm laser.

The images obtained from the confocal microscopy were in nd2 format. These images can be opened using image J (Fiji) (Schindelin et al., 2012) or by NIS-Element viewer software. For FRET measurement, an Image J plugin was used, which is "FRET and colocalization analyser" (Hachet-Haas et al., 2006, Schindelin et al., 2015, Schneider et al., 2012). This software can calculate FRET index pixel by pixel. The plugin corrects the donor and acceptor bleed-through and subtracts it from the raw FRET channel image. It requires images taken from cells expressing the donor and acceptor

individually. It can show only the pixels that correspond to co-localization and produce a "colocalized FRET index" image.

2.3.6.2 FRET measurement using spectrofluorimetric method:

Spectrofluorimetry is a useful method to examine the spectrum of fluorescent proteins. It is known that CFP has emission spectrum that peaks around 476 nm and YFP at around 529 nm (Dye, 2005). Therefore, in cells expressing either CFP or YFP, the fluorescence spectrum should be corresponding to the fluorophore present inside the cells if it is excited with its appropriate wavelength. However, in the cells co-expressing CFP and YFP, the detection of YFP emission spectrum upon CFP excitation may indicate FRET happening between CFP and YFP. Therefore, the spectrofluorimetric method was used to detect possible FRET between CFP and YFP in cells co-expressing hC5a1 tagged with CFP and hC5a2 tagged with YFP by measuring the emission spectrum of the resulting fluorescence.

RBL-2H3 expressing either hC5a1 (mock), hC5a1-CFP, hC5a2-YFP, hC5a1-CFP+hC5a2-YFP or hC5a1-CFP+hC5a1-YFP were used in this experiment. Control cells expressing CFP directly linked with YFP were also used. Cells from nearly confluent 100 mm culture petri dishes were detached using non-enzymatic cell dissociation solution and re-suspended in 1 ml HBSS. The cells were washed twice with HBSS and distributed into 96 well black plates at a volume of 200 µl/well. The cells stimulated with either 500 nM C5a final concentration or buffer control for 20 minutes. The fluorescence spectrum was measured using a spectrofluorimeter (Varioskan, Thermoscientific). The emission spectrum of cells expressing untagged receptors was subtracted from the fluorescence spectrum of cells expressing

fluorescent tagged receptors. This was done to eliminate nonspecific fluorescence and light scattering of the cells. In order to do this, the cells were excited by 430 nm wavelength and the emission intensity measured at 450 nm. This emission wavelength was used considering that the CFP and YFP emission is negligible at this wavelength and only the emission of light scattering is detected at such a wave length. The correction factor of the background fluorescence F(BG) was then calculated by dividing the emission at 450 nm of untagged receptors (mock) containing cells by the FP-tagged receptors containing cells (I).

(I) F(BG)=mock cells/FP-cells

After that, the spectrum of mock transfected cells was divided by the correction factor F(BG) and then subtracted from the spectra of cells expressing FP-tagged receptors. A second normalization step was applied to the cells co-expressing CFP and YFP fusion proteins to normalise their CFP and YFP expression. To do so, correction factors F(CFP) and F(YFP) were calculated. The F(CFP) was measured by dividing the CFP emission of cells expressing CFP alone by the CFP of cells expressing both CFP and YFP proteins (II). The F(YFP) was calculated by measuring the ratio between YFP of cells expressing YFP alone and the cells expressing both CFP and YFP (III). Then, the fluorescence spectra of cells expressing either CFP or YFP was divided by the corresponding correction factor (IV) and (V).

(II) F(CFP)= CFP of CFP alone cells/CFP of CFP&YFP cells

(III) F(YFP)= YFP of YFP alone cells/YFP of CFP&YFP cells

(IV) Normalised CFP (NCFP)=CFP spectrum of CFP alone cells/F(CFP)

(V) Normalised YFP (NYFP)=YFP spectrum of YFP alone cells/F(YFP)

After these normalization steps, the FRET was calculated by subtracting normalised CFP and YFP spectra from the spectra of cells co-expressing CFP and YFP after excitation at 430 nm (VI). The resulting spectrum from the cells expressing both donor and acceptor can be considered as exclusively FRET spectrum. FRET ratio was then calculated using the ratio of YFP(FRET) to YFP emission spectrum between 520 nm and 530 nm (VII).

(VI) FRET curve= spectrum of CFP&YFP cells-NCFP-NYFP

(VII) FRET ratio=YFP(FRET)/YFP

2.3.7 Bioluminescence resonance energy transfer (BRET) assay:

BRET is a naturally occurring phenomenon between a luminescent donor and a fluorescent acceptor. The energy is nonradiatively transferred from the donor to the acceptor. The luminescence from the sea pansy *Renilla reniformis* occurrs due to the catalytic degradation of the substrate coelenterazine by the enzyme luciferase.

The emitted light energy is transferred to green fluorescent protein or its derivatives (such as YFP) when the two proteins dimerize (Figure 2.3).

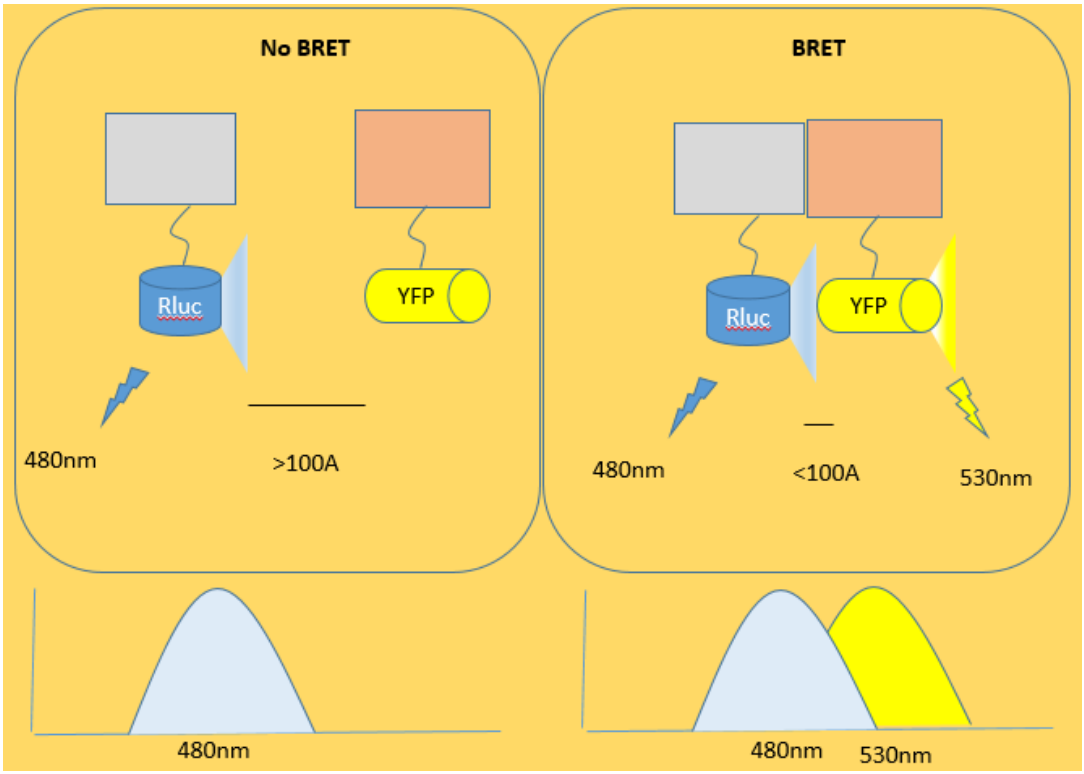


Figure 2.3: Bioluminscence resonance energy transfer (BRET). Two proteins are linked to either *Renella* luciferase (Rluc) or YFP. When the two proteins are far from each other, BRET does not occur and only Rluc emission (~480 nm) can be detected (left panel). When the two proteins are interacting, and are in close proximity (<100Å), BRET can occur and both Rluc and YFP emission (~530) can be detected.

This method, like FRET, needs strict proximity between the donor and acceptor in order for the BRET phenomenon to occur. It was first applied by Xu *et al*.to monitor protein-protein interactions in intact cells (Xu et al., 1999). In this BRET approach, the red-shifted GFP (YFP) was used as acceptor for the luminescence emission of *Renilla* luciferase (*R*luc) to measure the cyanobacteria clock protein KaiB homodimerization in *Escherichia coli*.

In the current study, C5a heterodimerization was assessed using hC5a1 tagged with *Renilla* luciferase 8 (Rluc8), which is an improved version of Rluc enzyme as a donor. In addition, hC5a2-YFP fusion protein was used as BRET acceptor. RBL cells expressing either Rluc8-hC5a1 alone or both Rluc8-

hC5a1+hC5a2-YFP were used in this experiment. Cells were grown at $5x10^4$ cells/well overnight on 96 well white plates. Next day, the cells were washed twice with HBSS and treated with coelentrazine (5 µM final concentration) for 10 minutes at room temperature. The cells were then stimulated with 1 µM C5a and emission was measured at 480 nm and 525 nm at different time points by Varioskan plate reader (Thermoscientific). The ratio of emission signal detected at 525 nm versus emission at 480 nm was calculated for cells expressing donor only (Rluc8-hC5a1) or both donor and acceptor (Rluc8-hC5a1+hC5a2-YFP). Then the ratio of cells expressing only donor were subtracted from cells expressing both donor and acceptor to calculate the BRET ratio.

2.3.8 Cell-based ERK1/2, JNK, p38 MAPK Phosphorylation assay:

This assay was performed to test intracellular protein phosphorylation in response to the signal transmitted after ligand binding. Protein phosphorylation is an essential step in activation or regulation of signal transduction. Many cellular kinases or phosphatases have been explored to test their role in regulating protein functions. Among these proteins are the mitogen activated protein kinases (MAPK). Three main enzymes are involved in signal transduction after being phosphorylated. These are the ERK1/2, JNK, and p38 MAPK. Determining the specific protein phosphorylation suggests pathway activation in the experimental model system.

This assay is a cell-based assay ELISA assay and does not need the preparation of a cell lysate. The RBL cells that express C5a receptors were grown overnight in 96 well plates at 37°C. The cells were then washed with HBSS (with divalent cations and 0.1% BSA) twice. After that, the cells were

incubated with 500 nM C5a or C5a desArg for 0, 5, 10 or 15 minutes at 37˚C. Subsequently, the ERK1/2 (Thr202/Tyr204), JNK (Thr183/Tyr185) and p38 MAPK (Thr180/Tyr182) phosphorylation assay was performed as per manufacturer instructions. Briefly, the cells were fixed after the treatment with C5a/C5a desArg. Then, after blocking, primary anti-phospho-specific protein or anti-pan protein were added. After washing, HRP-conjugated secondary antibody was added. Consequently, TMB enzyme substrate was added, which results in production of colour that can be measured at 450 nm.

2.4 Molecular biology methods:

2.4.1 Bacterial transformation:

This method aims to transform an artificially-competent bacterium with plasmid DNA. M15 [pREP4] *E. coli* (0.25 ml) were thawed on ice for 30 minutes. Then 1 µl of plasmid (about 1 µg) was added to the cells and gently mixed. The mixture was then incubated on ice for 30 minutes. The bacteria were heat shocked using water bath at 42ºC for 30 seconds then immediately returned back to the ice for 2 minutes. Subsequently, LB recovery medium (250 µl) was added to the cells and mixed carefully. The cells were then incubated in a shaking incubator for one hour at 37ºC. After that, the bacteria were plated on agar media containing antibiotics (kanamycin 25 mg/ml and carbenicillin 50 mg/ml) and incubated overnight at 37ºC. Next day, a single colony was transferred into 10 ml LB medium containing the same antibiotic concentrations as above and incubated for 16 hours at 37ºC inside a shaking incubator. Next day, 1 ml of this cell culture was transferred into 100 ml antibiotic containing LB medium (same antibiotics as above) and incubated overnight at 37ºC. The cell culture was checked next day and glycerol stock

was generated (see section 2.4.2) and plasmid purification using Midiprep kits (Qiagen) were performed per manufacturer's instructions.

2.4.2 Glycerol stocks:

This procedure was done for long-term storage of the transformed bacteria. A mixture of 500 µl of 50% glycerol and 500 µl of overnight culture (from 2.4.1) was prepared and then frozen at -80 ˚C.

2.4.3 Designing hC5a1 receptor tagged with luciferase enzyme Rluc8:

The construct of C5a receptor linked with luminescence donor (Rluc8) was needed to measure C5a receptors dimerization using the BRET technique mentioned above (2.3.7). To generate such a fusion protein, the human C5a1 receptor coding region (http://www.cdna.org/files/data/C5R0100000seq.pdf) was cloned in frame with *Renilla* luciferase 8 (Rluc8) (http://www.ncbi.nlm.nih.gov/nuccore/127951035). Rluc8 is a new version of the original *Renilla* luciferase (Rluc) with eight mutations from the Rluc. These mutations result in higher stability and quantum yield (Loening et al., 2006).

The two genes were linked with a PVAT (proline, valine, alanine and threonine) linker sequence. The DNA sequence was synthesized by Invitrogen™ Life Technology and inserted into pcDNA3.1/Zeo (+) vector. This vector carries a Zeocin antibiotic resistant gene for mammalian cells transfection (Figure 2.4). The plasmid was received lyophilised and handled as per manufacturer instructions.

The structure of the fusion protein was predicted using online software RaptorX (Kallberg et al., 2012) (Figure 2.5).

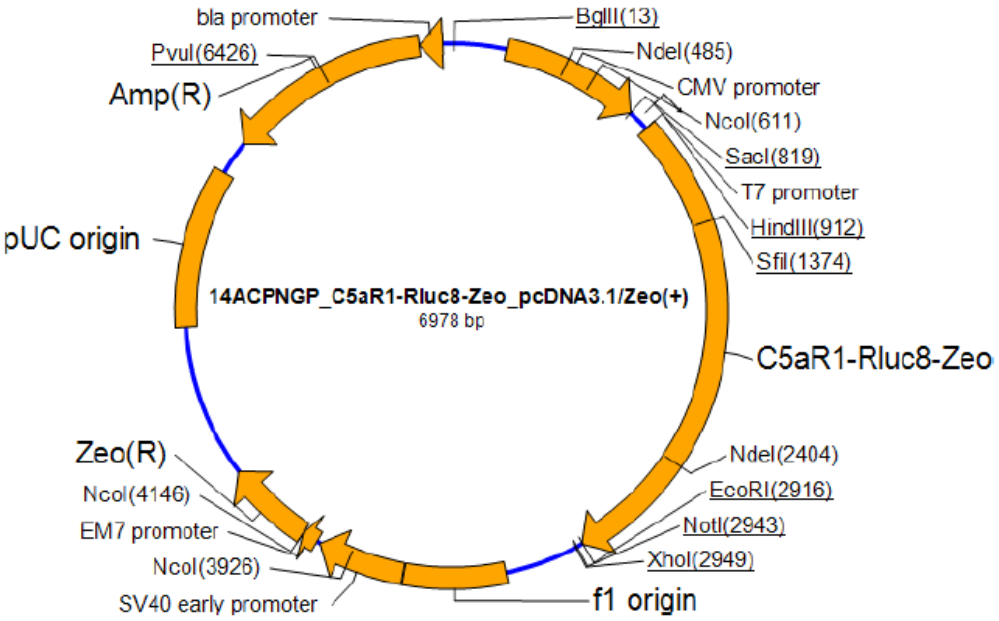


Figure 2.4: The structure of C5aR1-Rluc8-Zeo plasmid. The C5aR1-Rluc8 gene was inserted in pcDNA3.1/Zeo vector. The insert is under the CMV promotor. The plasmid encodes for bacterial antibiotic resistance for ampicillin (AMP) and mammalian antibiotic resistance for Zeocin (Zeo)

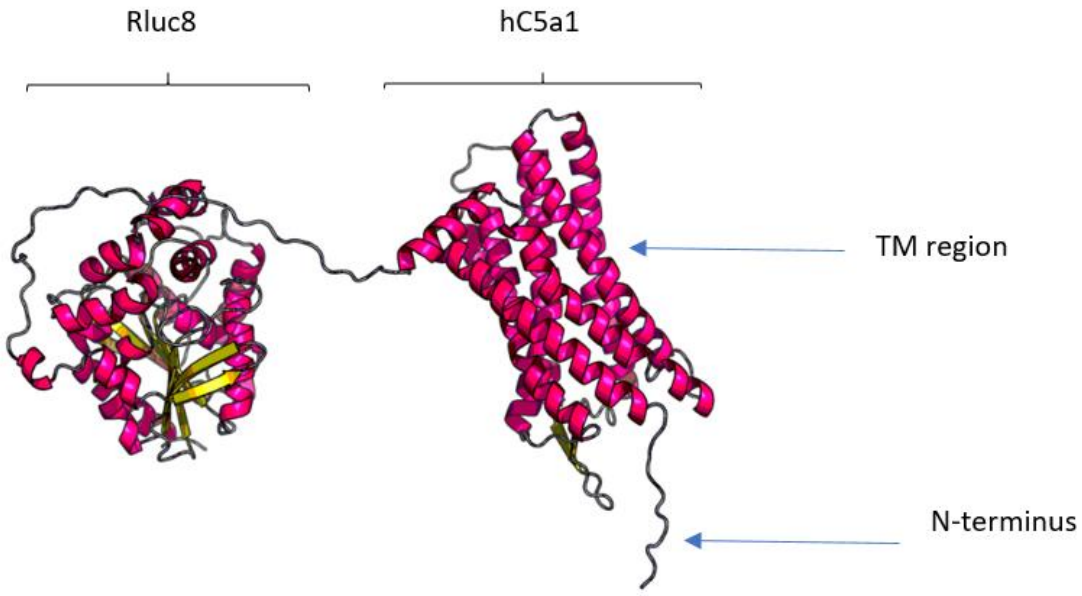


Figure 2.5: Predicted Rluc8-hC5a1 fusion protein structure. The construct structure was predicted using online software RaptorX (Kallberg et al., 2012) and shown in secondary structure (red, helix; yellow, sheets; TM, transmembrane).

2.5 Statistical Analysis:

Statistical analysis and curve fitting were computer assisted using GraphPad Prism 6 for windows. Data are presented as mean ± SEM (standard error of the mean). Unpaired t test, one-way ANOVA or two-way ANOVA were used as indicated in the graphs. P value less than the 0.05 level of significance was considered statistically significant and marked as stars: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; or ****, $p \leq 0.0001$.

Chapter Three

Characterization of Fluorescent Protein-tagged Receptors

Chapter 3: Characterization of fluorescent protein-tagged receptors

3.1 Introduction:

Protein-protein interactions can be measured in several different ways. One of these methods is fluorescence resonance energy transfer (FRET). In order to study the interaction between the C5a1 and C5a2 receptors using this method, human C5a receptors tagged with fluorescent proteins (FP) such as cyan fluorescent protein (CFP) and yellow fluorescent proteins (YFP) were used. The following transfected cell lines were available: RBL2H3-hC5a1-CFP; RBL2H3-hC5a2-YFP; RBL2H3-hC5a1-CFP+hC5a1-YFP; and RBL2H3-hC5a1-CFP+hC5a2-YFP.

Before starting studying the FRET between these FP-receptors, it was necessary to characterize these cell lines. First, confirming the expression of the receptors. Then, testing the tagged-receptor ligand binding capability. Other cellular functions were also explored to examine the potential effects of FP tagging on receptor functions. It was also necessary to develop a control cell line that co-expressed hC5a1 receptors and fluorescent proteins such as YFP separately. Therefore, pcDNA3-YFP plasmid was used to transform competent bacteria (*E. coli*) and then the plasmid was purified and transfected into RBL-2H3-hC5a1 by electroporation. Subsequently, the newly transfected cell line (hC5a1/YFP) was sorted and the expression of the receptor and YFP was examined by flow cytometer. Then the effect of presence of fluorescent proteins on receptors function was explored.

3.2 Detection of receptor expression by flow cytometry:

In order to confirm the presence of hC5a1 and hC5a2 receptors expressed on the surface of RBL-2H3 cells transfected with FP-tagged hC5a1 or 2 receptors, anti-hC5a1 and anti-hC5a2 antibodies were used as described in section 2.3.1. Alexa Fluor@633 antibodies were used as a secondary antibody because the higher emission wavelength does not overlap with the emission of either fluorescent protein (CFP or YFP). A flow cytometer was used to detect the Alexa 633 secondary antibodies (Figure 3.1 and 3.2). The graphs show the expression of hC5a1 or hC5a2 receptors in cells expressing different fusion constructs of tagged hC5a1(Figure 3.1) or hC5a2 (Figure 3.2) receptor.

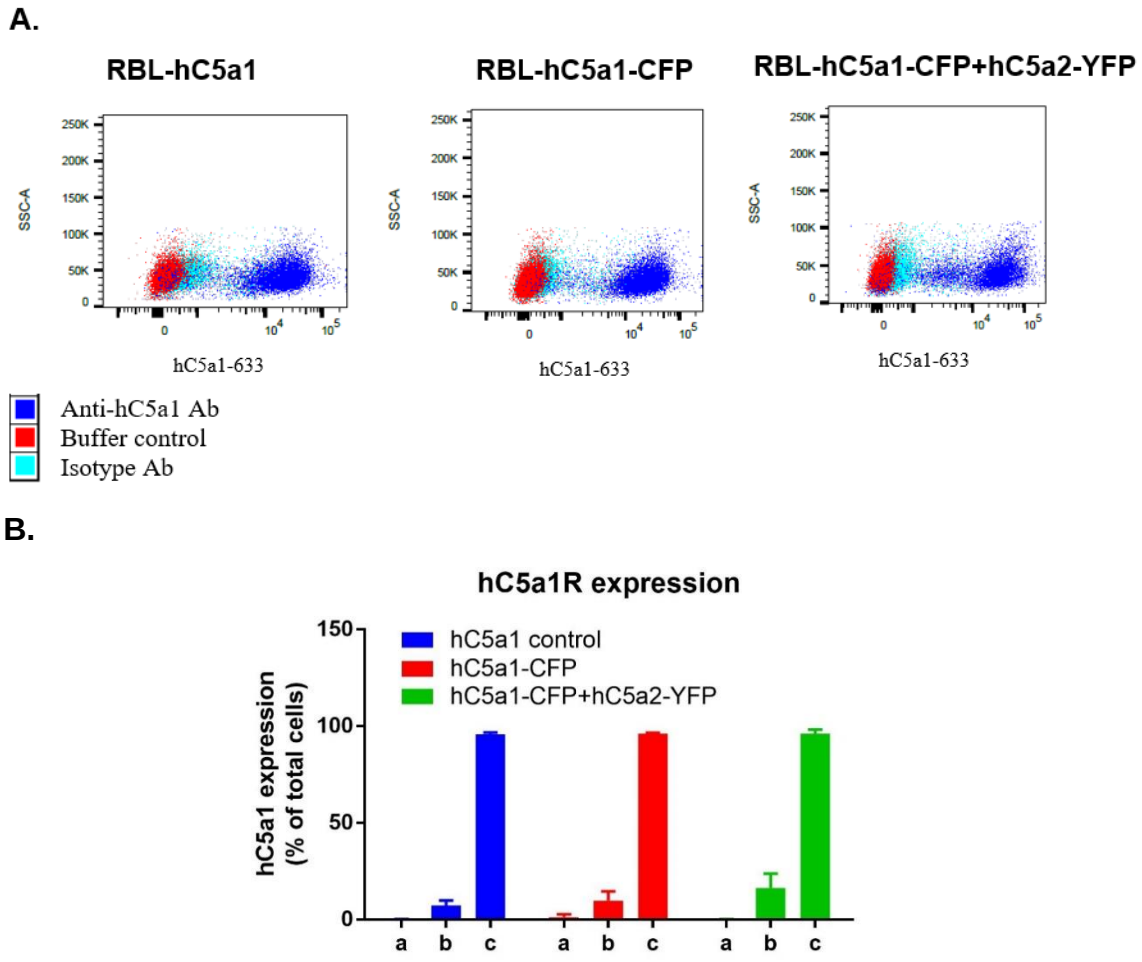


Figure 3.1: Expression of human C5a1 receptor on RBL-2H3 cells by flow cytometry. A. Dot plots show RBL cells expressing either hC5a1, hC5a1-CFP or hC5a1-CFP+hC5a2-YFP treated with antihuman C5a1 antibodies, isotype or buffer control. B. Bar chart for hC5a1 receptor expression shows the percentage of positive cells. a, buffer control. b, isotype control. c, anti-hC5a1 antibodies.

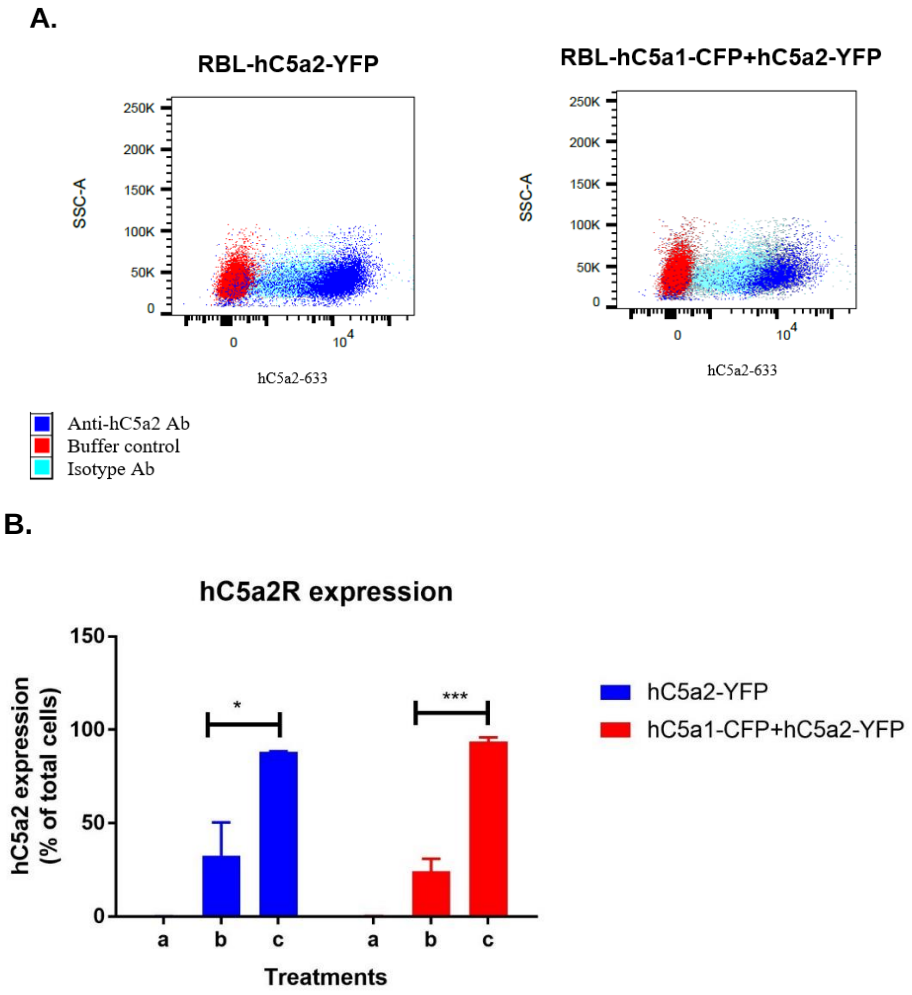


Figure 3.2: Expression of human C5a2 receptor on RBL-2H3 cells by flow cytometry. A. Dot plots show RBL cells expressing either hC5a2-YFP or hC5a1-CFP+hC5a2-YFP treated with antihuman C5a2 antibodies, isotype or buffer control. B. Bar chart for hC5a2 receptor expression shows the percentage of positive cells. t test was used to test statistical significance. *, p ≤0.05 and ***, p ≤0.001. a, buffer control. b, isotype control. c, Anti-hC5a2 antibodies.

3.3 C5a binding assay:

The ligand binding assay was performed as described in section 2.3.2 to compare the capability of the CFP-tagged hC5a1 receptor and the wild type hC5a1 receptor to bind to C5a. Figure 3.3 shows that there is no significant difference between the hC5a1-CFP and the wild type receptor in the binding to ligand.

a. **b.**

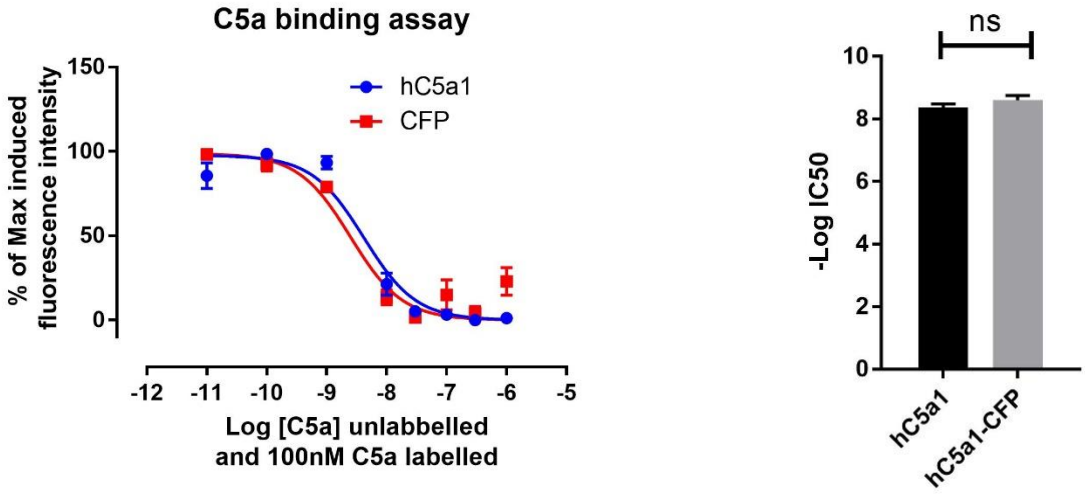


Figure 3.3 C5a binding assay for hC5a1-CFP in RBL-2H3 cell line determined by competitive 488-hC5a binding study. a. Dose response (inhibitory) curve of serial dilutions (starting with 1 µM) of unlabelled hC5a with 100 nM of Alexa488 labelled hC5a. b. Bar chart to compare IC50 of hC5a-CFP with the control. Unpaired t test is used to test statistical significance. ns, non-significant

3.4 Intracellular Ca^{2+} mobilization assay:

One of the steps in some G protein-coupled receptor signalling is the mobilization of Ca^{2+} and it was used to test the effect of the FP tagging to the C5a receptors on their signalling at this level. As mentioned in chapter 2.3.3, Ca^{2+} mobilization was measured after adding C5a at 10, 5, 1, 0.5, 0.1 nM final concentration. The dose response curve is shown in Figure 3.4. There was a significant difference in EC50 between the CFP tagged C5a1 receptors and the wild type.

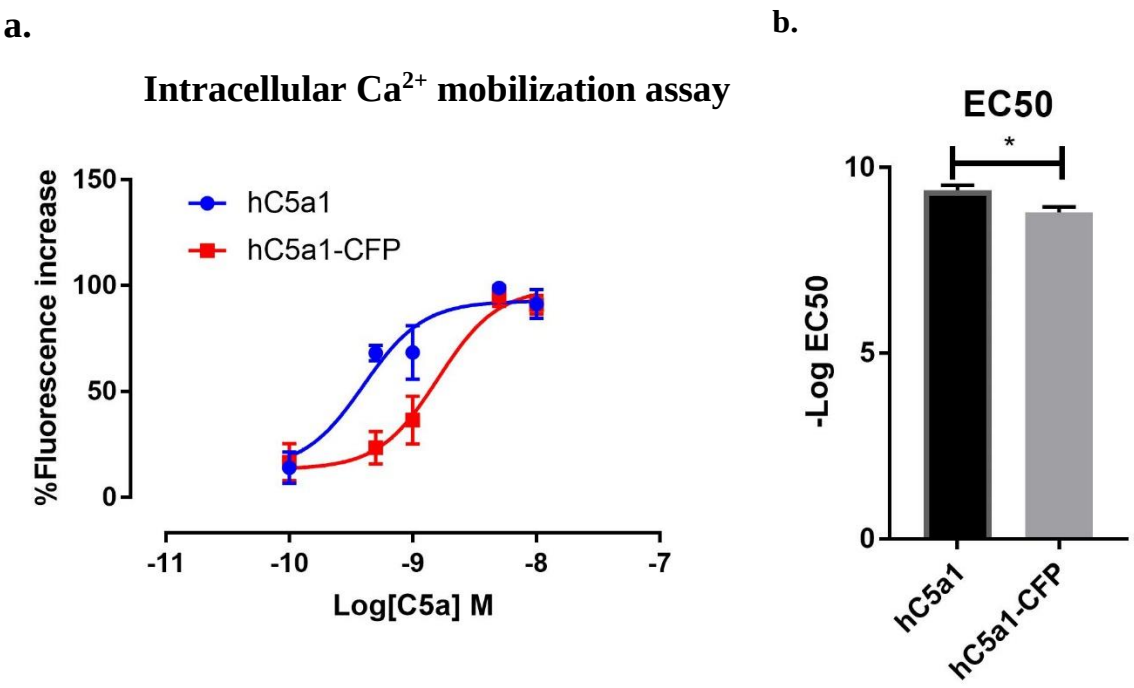


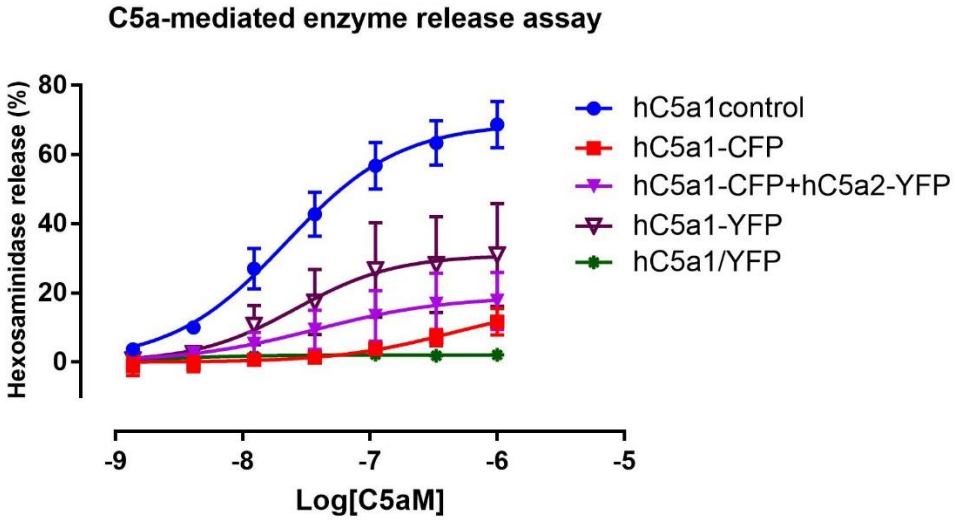
Figure 3.4: Intracellular Ca^{2+} mobilization assay for RBL-2H3 hC5a1-CFP cells. The graph shows the increase in Ca^{2+} after adding C5a to both hC5a1-CFP cells and the wild type receptor cells. C5a was added after 20s to allow recording of the baseline fluorescence. The Ca^{2+} mobilization was measured after addition of agonists. a. Dose response curve for the increase in Ca^{2+} in response to various dilutions of C5a. b. Bar chart for the log EC50 for the Ca^{2+} response. Unpaired t test is used to test statistical significance. *, $p \leq 0.05$.

3.5 β-hexosaminidase release assay:

3.5.1 C5a induced release assay for RBL-2H3 transfected with fluorescently tagged hC5a1 receptors:

This assay was used as a functional output of activation of the C5a receptors in response to stimulation by C5a. The assay is described in section 2.3.4 and different FP-tagged hC5a receptors were used and compared to the hC5a1 control. The maximum responses measured in the presence of hC5a1-CFP (16.22±6.23), hC5a1-YFP (31.73±6.95), hC5a1-CFP+hC5a1-YFP (18.14±4.61), and hC5a1/YFP (2.11±0.21) receptors, were all significantly lower than enzyme release in the presence of the wild type receptor (68.59± 3.94) (Figure 3.5).

a.



b.

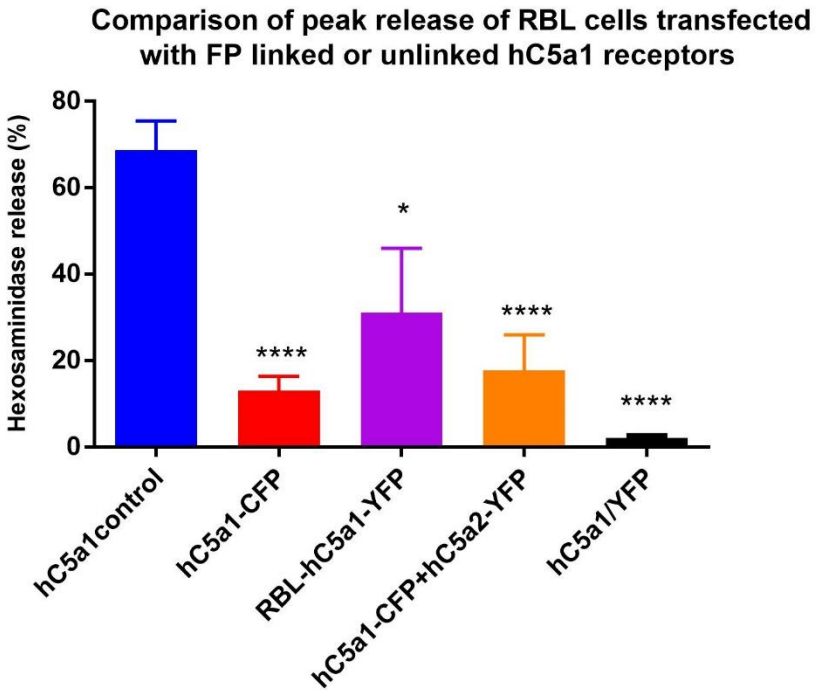


Figure 3.5: C5a-induced enzyme assay for fluorescent tagged/untagged hC5a1 receptors on RBL-2H3 cell line. a. Dose response curve for hC5a1, hC5a1-CFP, hC5a1-YFP, hC5a1-CFP+ hC5a2-YFP, and hC5a1/YFP receptors. Beta-hexosaminidase release was measured in response to 1/3 serial dilutions of hC5a starting with 1 µM. b. Bar chart for the maximum β-hexosaminidase release. One-way ANOVA is used to test statistical significance. *, $p \leq 0.05$ and ****, $p \leq 0.0001$.

3.5.2 IgE-mediated enzyme release assay for RBL-2H3 cells transfected with YFP:

The previous experiments showed the effect of FP on C5a receptor mediated hexosaminidase release. In order to explore the effect of FP on cell function mediated by other pathways, the RBL-2H3 cells expressing YFP were tested for enzyme release by IgE pathway. The cells were incubated with DNP-A-specific IgE overnight and then stimulated with DNP-A. The hexosaminidase enzyme was then measured in the supernatant as described in section 2.3.4. Figure 3.6 shows the effect of fluorescent protein expression (YFP) on the RBL cell degranulation with a substantial decrease in maximum release.

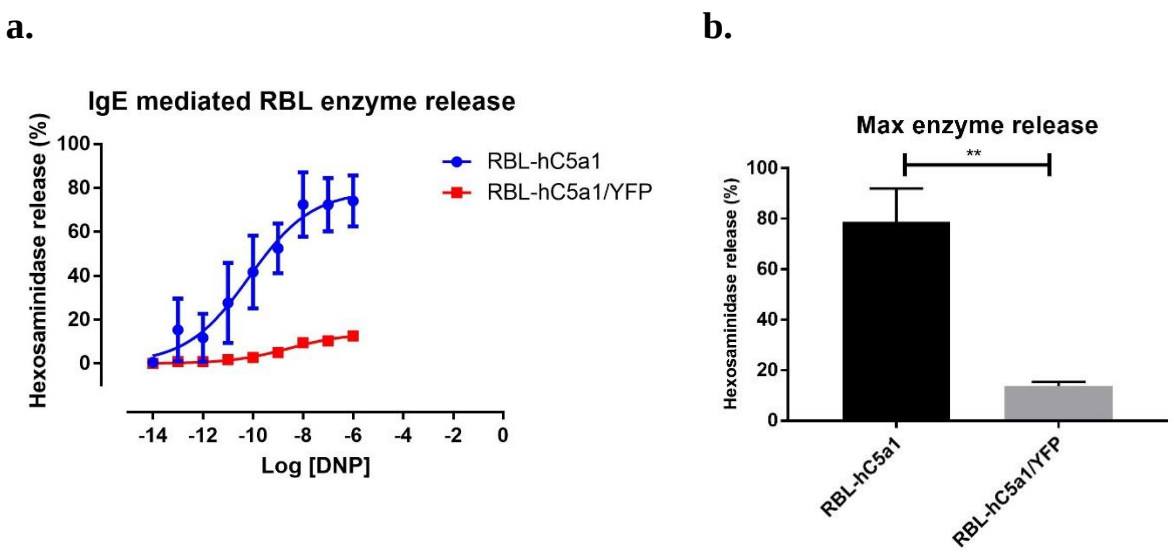


Figure 3.6. IgE-mediated enzyme release assay for RBL-2H3 hC5a1 cell line co-expressing YFP separately. a. Dose response curve for RBL cells with or without expression of YFP. Beta-hexosaminidase release was measured in response to 1/10 serial dilutions of DNP. b. Bar chart for maximum enzyme release. Unpaired t test was used to test statistical significance. **, $p \leq 0.01$.

3.6 The effect of protein tagging on internalization:

The C-terminus of C5a receptor was reported to be involved in receptor internalization (Bock et al., 1997). Therefore, the C-terminus tagged receptors were tested for their internalization capability. Figure 3.7 shows that the tagged receptor endocytosis was less than that of the untagged wild type receptor.

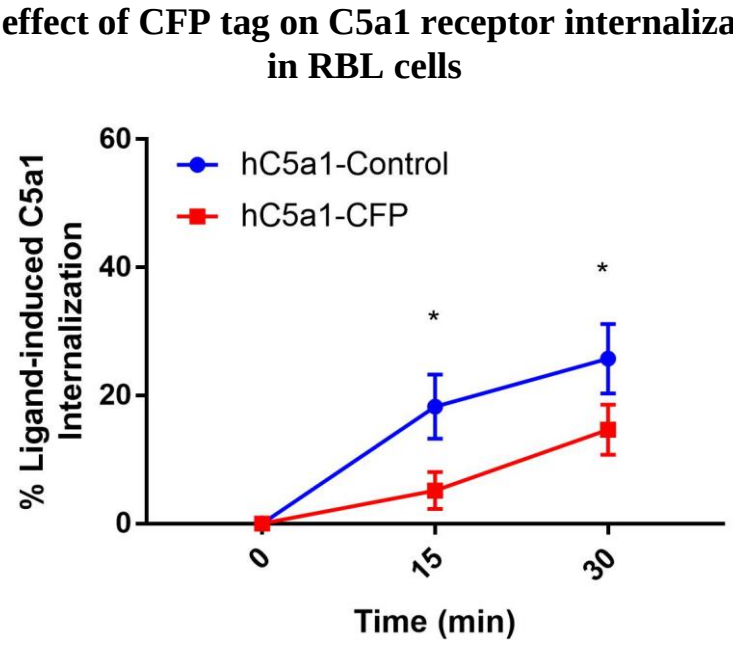


Figure 3.7: C5a-induced internalization of fluorescent protein tagged receptors. RBL-2H3 cells expressing CFP tagged hC5a1 receptors were compared with cells expressing untagged hC5a1 receptors. Cells were stimulated with C5a for 15 and 30 minutes. Two-way ANOVA followed by Dunnett's multiple comparison are used to test statistical significance. *, $p \leq 0.05$.

The internalization of C5a1 receptor was then further analysed in cells co-expressing untagged C5a receptor and YFP separately. The expression of the fluorescent protein appeared to have no effect to the receptor when expressed separately (Figure 3.8).

The effect of separate expression of FP on human C5a1 internalization in RBL cells

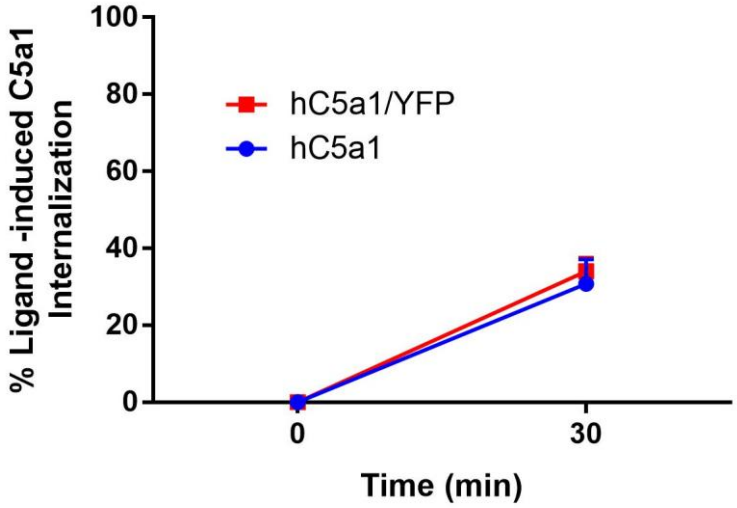


Figure 3.8 C5a-induced internalization for hC5a1 receptors when co-expressed with FP separately. RBL-2H3 cells co-expressing YFP and wild type hC5a1 receptors were compared with cells expressing only the wild type hC5a1 receptors. Cells were stimulated with C5a for 30 minutes.

3.7 Discussion:

Fluorescent proteins (FPs) are one of the most commonly used reporter molecules for observing gene expression, localization of proteins as well as monitoring protein-protein interactions in various cells and organisms such as bacteria, yeast, fish and mammals (Tsien, 1998). The first fluorescent protein to be used was the green fluorescent protein, which was discovered by Shimomura et al.(Shimomura et al., 1962), and its gene was cloned by Prasher et al. (Prasher et al., 1992). Since then, many other fluorescent proteins have been developed to satisfy the evolving need for new tools for biological imaging (Zhang et al., 1996a, Cormack et al., 1996, Shaner et al., 2005, Chudakov et al., 2010). Mutation of the wild type GFP resulted in different colour emitting mutants such as cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). In this part of

the study, the CFP and YFP tags were used to study the effects fluorescent tagging of the C5a receptor on receptor function.

Generally, CFP and YFP have been extensively used to examine protein-protein interactions (Chan et al., 2000, Chan et al., 2001, He et al., 2003, Onuki et al., 2002, Croker et al., 2013, Luo et al., 2001, Ting et al., 2001), considered as innocuous to the cells both *in vivo* and *in vitro*. In fact, GFP was believed to be nontoxic even to transgenic mice that globally express GFP (Okabe et al., 1997). However, it has been reported that these FPs and in particular GFP has various side effects (Liu et al., 1999, Agbulut et al., 2007, Baens et al., 2006, Zhang and Crandall, 2007, Dave et al., 2016, Sokolovski et al., 2015, Swulius and Jensen, 2012, Landgraf et al., 2012, Agbulut et al., 2006, Huang et al., 2000). It has been demonstrated that apoptosis can be induced by GFP although the exact mechanism is still unclear (Liu et al., 1999). In rat adult hepatic stem cells, it was difficult to establish a stable cell line that expressed EGFP (Taghizadeh and Sherley, 2008). In addition, other studies reported an increase in COX-2 expression followed by PGE2 production in endothelial cells due to the introduction of GFP (Zhang et al., 2003). Furthermore, dilated cardiomyopathy was described in transgenic mice (Huang et al., 2000), which was thought to be due to the impairment of actin-myosin interaction in the presence of GFP (Agbulut et al., 2006). There is also concern regarding the natural tendency of the wild type FPs to oligomerize. This is a problem if the protein of interest occurs in a dimeric or oligomeric state because this will result in aberrant aggregations. This problem has been addressed by introducing mutations to produce FP monomers (Shaner et al., 2004). Baens et al. demonstrated that polyubiquitination can be inhibited by EGFP and EGFP fusion proteins (Baens et al., 2006). Ubiquitination, an important post-translational modification for proteins in which ubiquitin is attached to the protein, affects many cellular responses such as kinase signalling, protein degradation, protein interaction or change cellular location of

proteins. Those studies support the finding of this study regarding the defective signal transduction in cells expressing FP in comparison to the cells that do not express FP as shown in the Ca^{2+} and degranulation assays. Therefore, the use of fluorescent proteins should be carefully considered in experiments that involve cellular signalling and protein functions.

The CFP and YFP were attached to the C-terminus of the receptors. This attachment may affect the binding of various intracellular signalling molecules such as the G proteins or β-arrestins or may disturb the phosphorylation of the receptors. The C-terminus also contains the major receptor phosphorylation sites (Giannini et al., 1995). It has been shown that mutations of some of the residues in the C-terminus reduced phosphorylation by 80% (Giannini et al., 1995) in addition to the inhibition of internalization of the receptor (Naik et al., 1997). This might support the result of the current study that shows that less internalisation occurred in the tagged receptors in comparison to the wild type. In addition, the disturbance in the interaction with some intracellular partners such as β-arrestin, dynamin and clathrin may be the reason behind the inhibition of the internalization. It has been suggested that in phosphorylation deficient C5a1 receptors, β-arrestin 1 and 2 still can associate with the receptors albeit more weakly in comparison to the wild type (Braun et al., 2003).

It has been reported that GPCR can activate MAP kinase via β-arrestin independently from G proteins (Rosenbaum et al., 2009). In fact, the G protein signalling pathway may be inhibited by the β-arrestins while transducing downstream signalling. This multifunctional behaviour of β-arrestin may be due to conformational changes in its structure and post-translational modification, (Shenoy and Lefkowitz, 2011) which could be affected by defective ubiquitination as a consequence of the presence of FPs (as mentioned previously). In C3a receptors, it has been demonstrated that β-arrestin have different functions. In human mast cells, the silencing of β-arrestin 2 led to the prevention of

internalization and desensitization, which resulted in prolonged increase in Ca^{2+} (Vibhuti et al., 2011) but no effect on degranulation. However, degranulation can be inhibited by silencing β-arrestin 1. In addition, it was demonstrated that arrestin is necessary for CXCR1-mediated degranulation (reviewed in Vines and Prossnitz, 2004). Therefore, in this study, it could be speculated that the FPs could have an effect on the receptors directly when attached to the C-terminus of the C5a receptors, or indirectly, when FP is expressed separately, that may have affected the interaction with β-arrestins or G proteins and resulted in the observed defect in degranulation.

The rise in intracellular Ca^{2+} is known to induce exocytosis (reviewed in Passante and Frankish, 2009). In this study, the tagging effected the intracellular Ca^{2+} mobilization, which could be the cause for the defect in the enzyme release.

The GPCR was thought to exert their function exclusively from the cell membrane. However, a growing body of evidence suggests that many receptors can continue their signalling process in the endocytic compartment (reviewed in Calebiro et al., 2010). For example, expression of dynamin dominant negative mutant with β2-adrenergic receptors resulted in inhibition of activation of ERK pathway (Daaka et al., 1998). Furthermore, it has been suggested that signalling from internalized receptors is distinct quantitatively and qualitatively from the signalling from the plasma membrane. In thyroid stimulating hormone receptors, the signalling at the cell surface was reported to be rapidly reversible, while the signalling from the internalized receptor continues after removal of the ligand (Calebiro et al., 2009, Calebiro et al., 2010). In the current study, the tagged receptors showed lower internalization rate. This could partly explain the defective signalling, which might suggest signalling possibility during internalization of C5a1 that was decreased by decrease in internalization.

In the current study, the presence of the FP tag does not seem to affect receptor expression. This was also reported in other studies (Zhang and Crandall, 2007), which showed that although the GFP linking to CD36 affected ligand binding, the CD36 expression was normal. Therefore, the FP tagged receptors can still be used to assess the di/oligomerization of the C5a receptors despite the possible limitation due to the presence of FP tag and its potential effect on receptor signalling. This is because receptor signalling and interaction with other intracellular partners are not required for the formation of receptors dimers or oligomers (Floyd et al., 2003, Klco et al., 2003, Rabiet et al., 2008). Floyd et al, (2003) demonstrated the C5a receptor di/oligomerization in the lower eukaryote *Saccharomyces cerevisiae* and this di/oligomerization occurred without the need for ligand, G proteins or other mammalian accessory proteins (Floyd et al., 2003). In addition, Rabiet et al. (2008) showed that both ligand binding deficient and phosphorylation deficient C5a1 receptor mutants can form dimers (Rabiet et al., 2008). Furthermore, it has been reported that C5a receptor di/oligomerization is an early event that occurs at their biosynthesis at the level of endoplasmic reticulum and the receptors are expressed as di/oligomers (Floyd et al., 2003). Therefore, when the receptors are expressed, they are expressed in their final form whether in dimer or oligomers and signalling could not be necessary or responsible for the dimerization or oligomerization (reviewed in Milligan, 2010, Van Craenenbroeck, 2012).

Chapter Four

Fluorescence Resonance Energy Transfer (FRET) Study for Receptor Dimerization

Chapter 4: Fluorescence resonance energy transfer (FRET) study for receptor dimerization

4.1 Introduction

FRET is one of the techniques that can be used to measure protein-protein interaction. This technique requires several conditions to be met before this energy transfer can happen such as spectral overlap, proximity and suitable orientation between the donor and acceptor.

In this chapter, C5a1 receptors tagged with CFP and C5a2 tagged with YFP were used to study possible receptors dimerization using FRET. First, spectra of the donor (CFP) and acceptor (YFP) fluorophores were tested and the localization of the CFP or YFP tagged hC5a1 or hC5a2 receptors within the cells was explored by confocal microscopy. A plasmid encoding CFP directly linked to YFP (pECFP-YFP) was transfected into RBL cells in order to be used as a FRET positive control. Then the possible FRET was examined using different methods, such as spectrofluorimetry and confocal microscopy.

4.2 Emission spectra of chimeric fluorescent hC5a receptor:

The cells that express CFP tagged hC5a1 receptors and YFP tagged hC5a2 receptors were tested for the emission spectra of the fluorophores. The spectrofluorimeter was used to measure the emission of the CFP and YFP (Figure 4.1). The CFP expressing cells were excited at 430 nm and the emission was scanned between 450 nm and 600 nm. The YFP emission was measured between 500 nm and 600 nm after excitation at 480 nm.

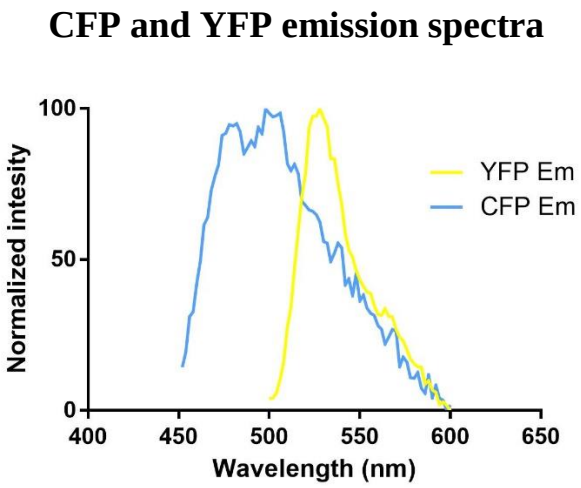
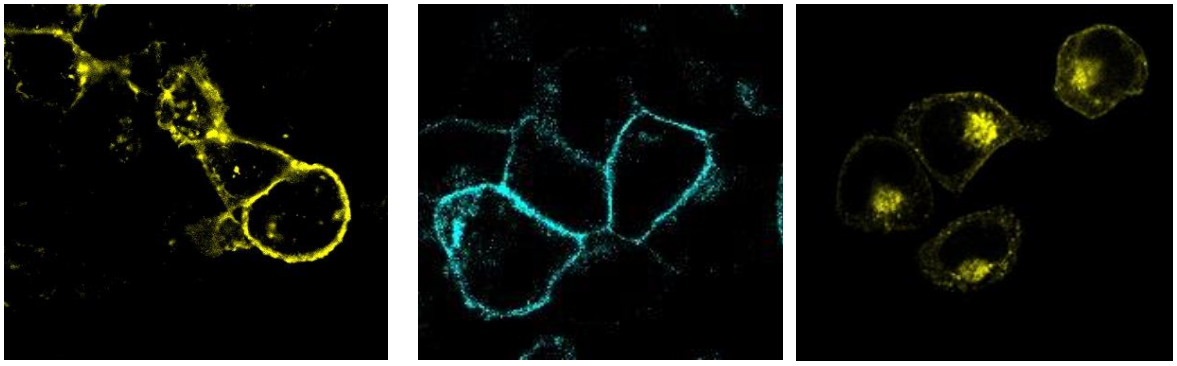


Figure 4.1: Fluorescence spectra of fluorescently tagged receptors. Cyan, spectrum from cells expressing hC5a1-CFP after excitation by 430 nm; Yellow, spectrum from cells expressing hC5a2-YFP after excitation with 480 nm.

4.3 Localization of hC5a1 and hC5a2 receptors within the cells:

The localization of C5a receptors was determined using cells expressing different FP tagged receptors. RBL-2H3 expressing hC5a1-CFP, hC5a1-YFP, and hC5a2-YFP were used. The fluorescence was measured using confocal microscopy. Figure 4.2 demonstrates the expression of C5a1 receptors mainly on the cell surface, while the C5a2 receptors were mainly intracellular.



a. b. c.

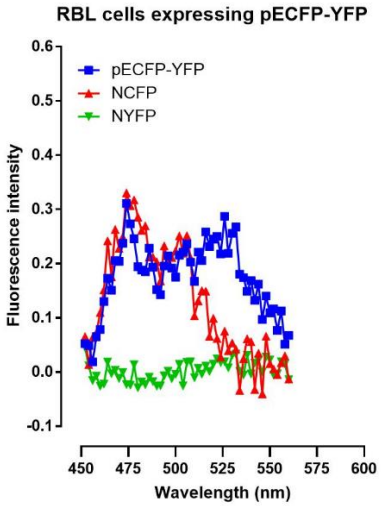
Figure 4.2: Localization of YFP tagged C5a receptors. a. cells expressing hC5a1 tagged with YFP. b. cells expressing hC5a1 tagged with CFP. c. cells expressing hC5a2 tagged with YFP. Images were obtained using Nikon A1 confocal fluorescence microscope at 60x oil immersion objective.

4.4 FRET measurement using spectrofluorimetric technique:

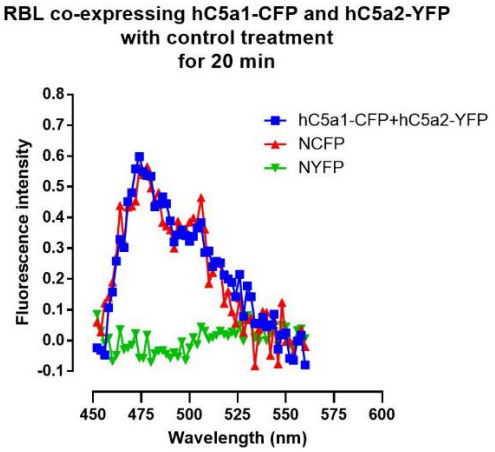
4.4.1 RBL-2H3 co-expressing hC5a1-CFP and hC5a2-YFP:

The spectrofluorimetric method was used to measure the possible resonance energy transfer between CFP and YFP tagged receptors. Cells expressing untagged hC5a1, hC5a1-CFP, hC5a2-YFP, hC5a1-CFP+hC5a2-YFP, or linked CFP-YFP (pECFP-YFP) were used for this experiment. The cells were treated with 500 nM C5a or buffer control for 20 minutes. The emission spectra were measured upon excitation at 430 nm. The potential bleed-through and background artefacts were corrected as described in section 2.3.6.2. The resultant spectrum of pECFP-YFP expressing cells shows two peaks, the first one around 475nm (CFP region) and the other around 530 nm (YFP region). The appearance of the second peak at the YFP region with the use of CFP excitation at 430 nm suggests that this YFP emission was induced emission because of FRET (Figure 4.3a). However, cells co-expressing hC5a1-CFP and hC5a2-YFP did not show a peak emission at YFP spectrum peak region with or without C5a treatment (Figure 4.3b & c). This might suggest FRET has not occurred between the CFP and YFP because the two receptors are not close to each other. The data were further analysed to measure the FRET curves and FRET ratio (Figure 4.4). The FRET curves were calculated after subtracting the CFP (red) and YFP (green) spectra from the sensitized YFP emission spectrum (blue). The FRET curve of the pECFP-YFP positive control was higher than FP-tagged receptors co-expressing cells (Figure 4.4a) and the FRET ratio was significantly different (Figure 4.4b).

a.



b.



c.

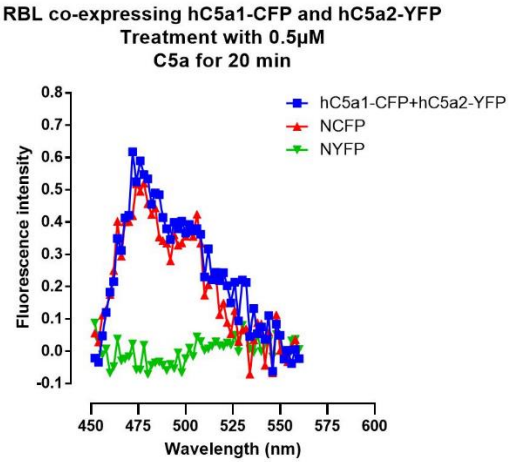
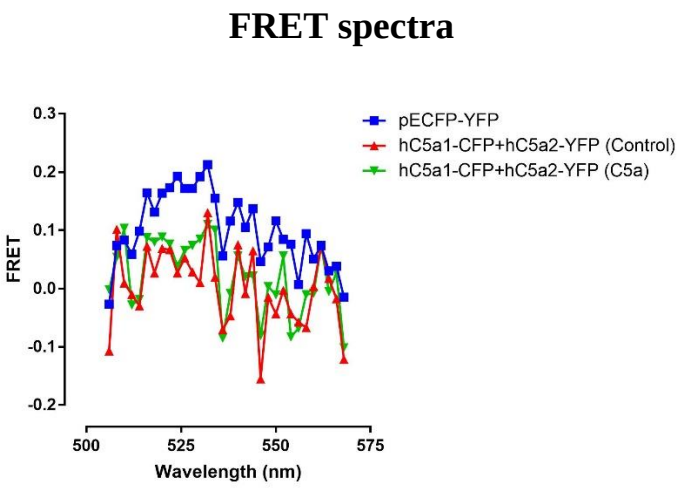


Figure 4.3: Spectral study of cells co-expressing hC5a1-CFP and hC5a2-YFP by spectrofluorimeter. RBL-2H3 expressing either pECFP-YFP linked plasmid (A); hC5a1-CFP+hC5a2-YFP with control treatment for 20 minutes (B); or hC5a1-CFP+hC5a2-YFP with 0.5 μM C5a treatment for 20 minutes (C). Blue, spectrum from cells co-expressing both hC5a1-CFP and hC5a2-YFP or linked pECFP-EYFP plasmid; RED, cells expressing hC5a1-CFP (Normalized as indicated in method chapter); Green, cells expressing hC5a1-YFP (Normalized as indicated in method chapter).

a.



b.

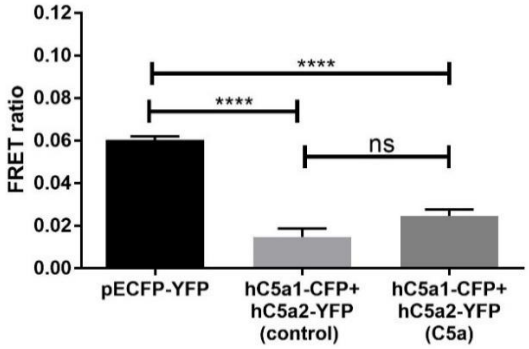


Figure 4.4 Fluorescence resonance energy transfer measurements. (a) FRET curves of the RBL cells co-expressing hC5a1-CFP+hC5a2-YFP treated with C5a or control buffer were compared with FRET curve of cells expressing the FRET positive control pECFP-YFP. (b) FRET ratio of cells co-expressing FP tagged receptors treated with C5a or control buffer in comparison to the pECFP-YFP positive control. The results are from 3 independent experiments. One-way ANOVA was used to test statistical significance. ns, non-significant and ****, $p \leq 0.0001$.

4.4.2 RBL-2H3 co-expressing hC5a1-CFP and hC5a1-YFP:

The FRET method was used to measure the possible homodimerization of C5a1 receptors. The same procedure was used as for the measurement of heterodimerization in the previous section, but with RBL cells co-expressing hC5a1-CFP and hC5a1-YFP. The fluorescence spectra were measured by spectrofluorimetry after treatment with 0.5 µM C5a or control buffer for 20 minutes. The spectra were detected after excitation at 430 nm and corrected for bleed-through and background as in 2.3.6.2. The positive control cells that express linked CFP and YFP (pECFP-YFP) showed one peak for CFP (around 475nm) and one peak for FRET (around 530 nm) (Figure 4.5 a). The cells that co-express hC5a1-CFP and hC5a1-YFP demonstrated the same pattern (Figure 4.5 b and c). The appearance of the peak at the YFP region after excitation with 430 nm strongly suggests FRET emission (Figure 4.5 b). This is because 430 nm is the excitation wavelength for CFP and the appearance of YFP emission means it is a sensitized emission (FRET). Interestingly, the treatment with C5a or buffer control did not affect the amount of FRET. This may suggest a constitutive FRET, which is not ligand induced. The FRET curves of the hC5a1-CFP+hC5a1-YFP were similar to the positive control and the FRET ratio was not significantly different (Figure 4.6).

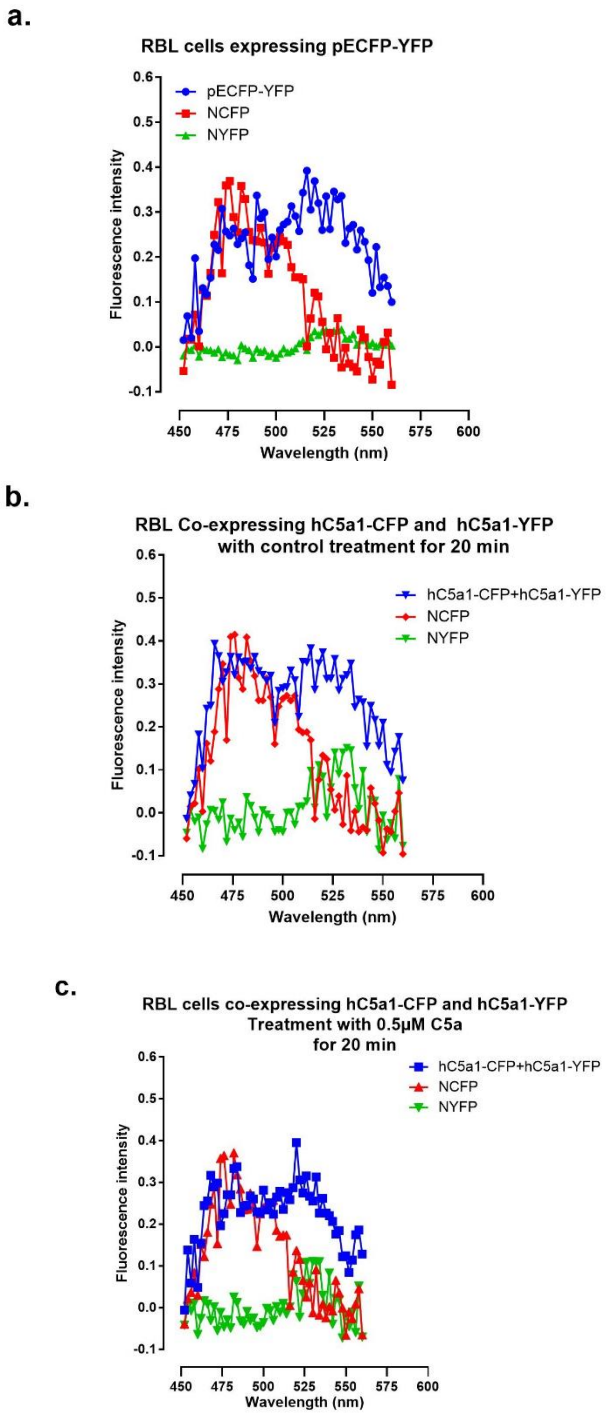
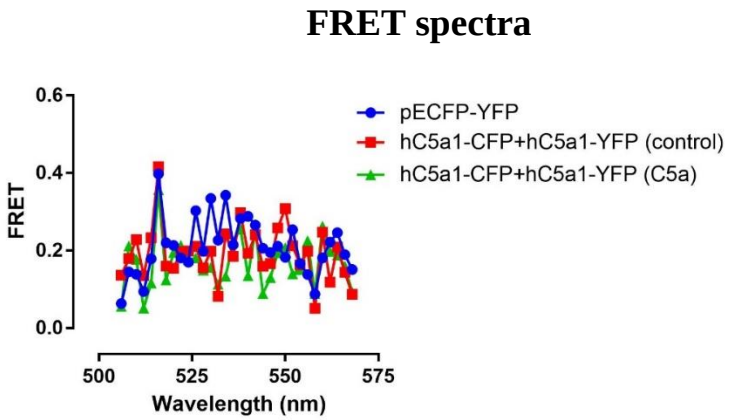


Figure 4.5 Spectral study of cells co-expressing hC5a1-CFP and hC5a1-YFP by spectro-fluorimeter. RBL-2H3 expressing either pECFP-YFP linked plasmid (A); hC5a1-CFP+hC5a1-YFP with control treatment for 20 minutes (B); or hC5a1-CFP+hC5a1-YFP with 0.5 μM C5a treatment for 20 minutes (C). Blue, spectrum from cells co-expressing both hC5a1-CFP and hC5a2-YFP or linked pECFP-EYFP plasmid; RED, cells expressing hC5a1-CFP (Normalized as indicated in method chapter); Green, cells expressing hC5a1-YFP (Normalized as indicated in method chapter)

a.



b.

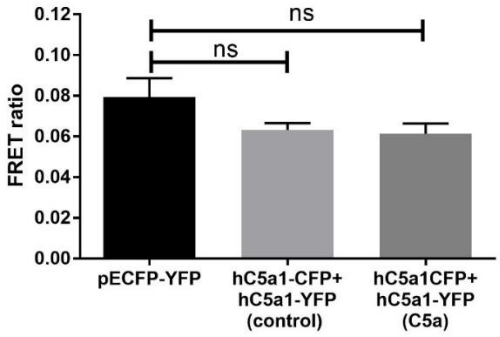


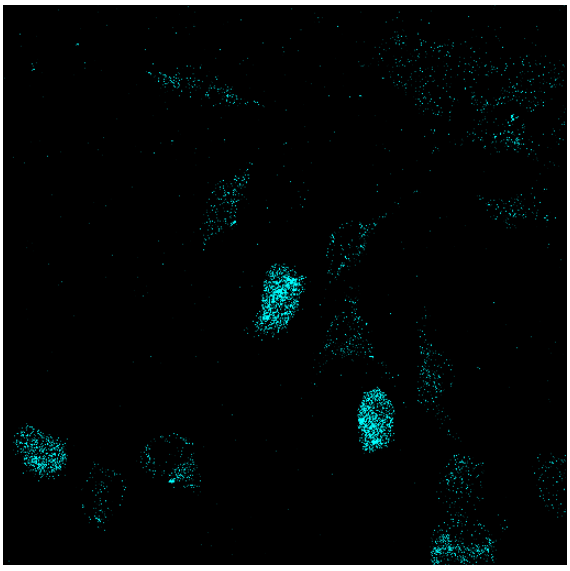
Figure 4.6 Fluorescence resonance energy transfer measurements. (a) FRET curves of the RBL cells co-expressing hC5a1-CFP+hC5a1-YFP treated with C5a or control buffer were compared with FRET curve of cells expressing the FRET positive control pECFP-YFP. (b) FRET ratio of cells co-expressing FP tagged receptors treated with C5a or control buffer in comparison to the pECFP-YFP positive control. The results are from 3 independent experiments. One-way ANOVA was used to test statistical significance. ns, non-significant.

4.5 Localization of the possible receptor dimerization using confocal microscopy:

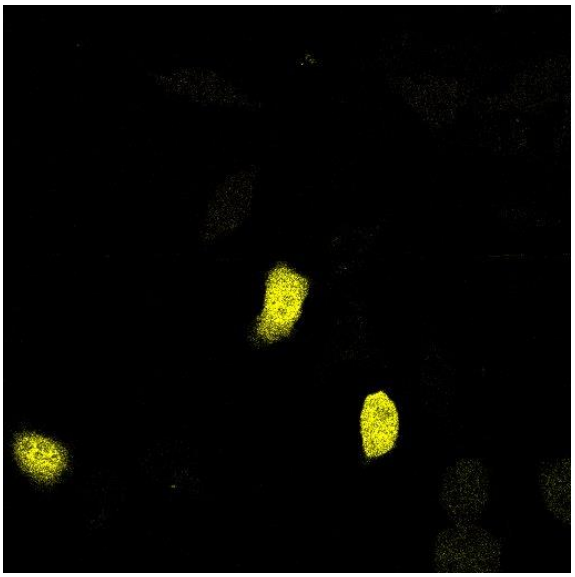
4.5.1 RBL-2H3 expressing pECFP-YFP:

Confocal microscopy was used to visualise the possible sites of FRET signals within the cells. The cells expressing the positive control for FRET were first examined. The cells were grown on chamber slides overnight as in section 2.3.6.1. The fluorescence was measured using two lasers (one for CFP and one for YFP excitation) and three detectors (CFP, YFP, FRET). The FRET image was

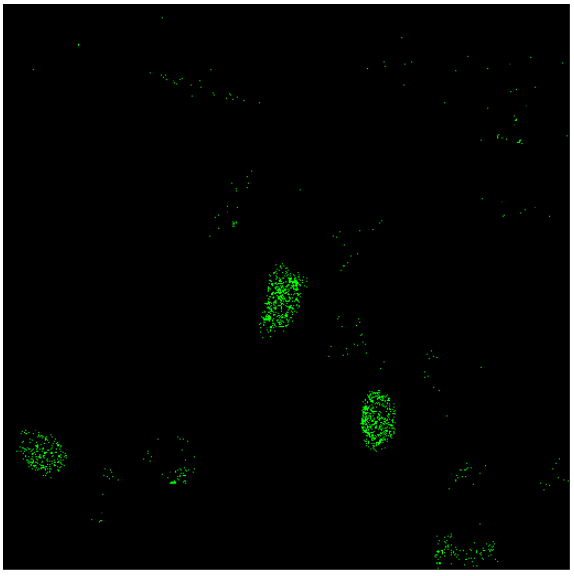
corrected for bleed-through using Image J plugin software and co-localised FRET index (Figure 4.7). The figure shows the FRET in three cells, which is diffused all over the cells, possibly due the distribution of the protein (pECFP-YFP) all over the cells.



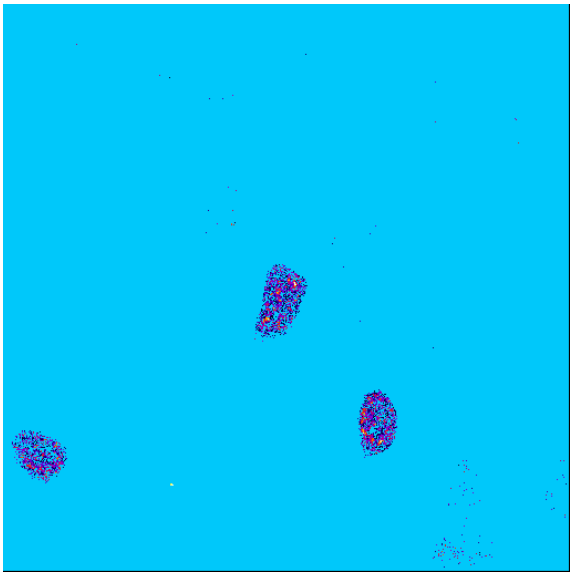
a. CFP



b. YFP



c. FRET

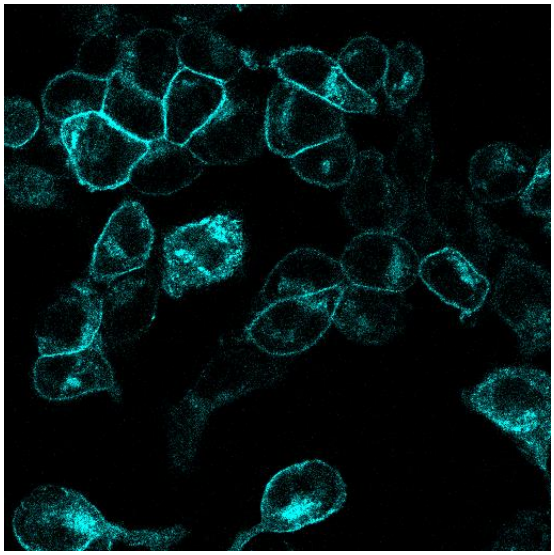


d. Co-localised FRET

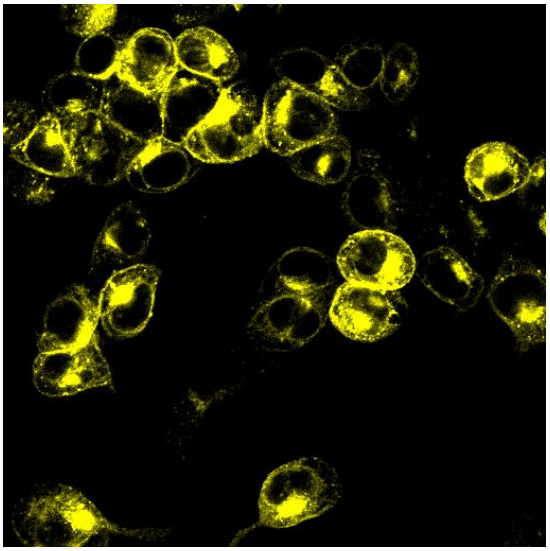
Figure 4.7 FRET measurement for RBL cells expressing pECFP-YFP plasmid. The fluorescence was detected through three channels, CFP (a), YFP (b), and FRET channel (c) by confocal microscopy. The co-localised FRET (d) was analysed using FRET and co-localization analyser plugin of Image J (Hachet-Haas et al., 2006). The image shows possible FRET in three cells and is distributed homogenously within the cells.

4.5.2 RBL-2H3 co-expressing hC5a1-CFP and hC5a2-YFP without C5a stimulation:

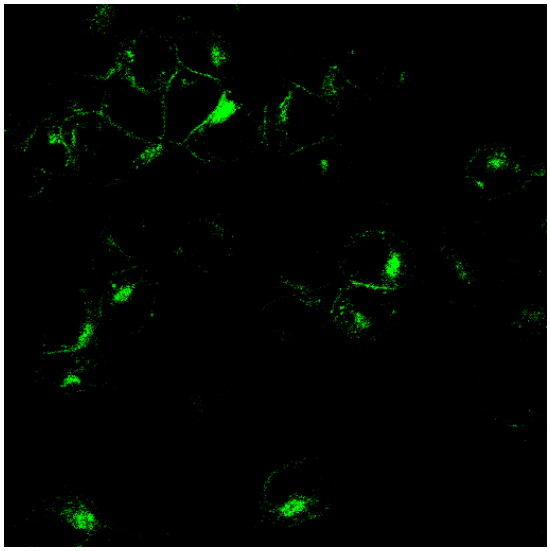
The heterodimerization of hC5a1 with hC5a2 receptors was examined by confocal microscopy. RBL cells co-expressing hC5a1-CFP and hC5a2-YFP were used. Figure 4.8 shows the CFP, YFP and FRET channels and the co-localised FRET image after correction. The CFP channel shows that hC5a1 is distributed mainly on the cell surface, while the hC5a2 in the YFP channel appeared mainly intracellular (perinuclear). Although the FRET channel showed a few areas of FRET but it seems that it was cross talk or a bleed-through, which did not appear after correction in the co-localised FRET image. These data suggest that without C5a treatment no dimerization occurred between hC5a1-CFP and hC5a2-YFP receptors.



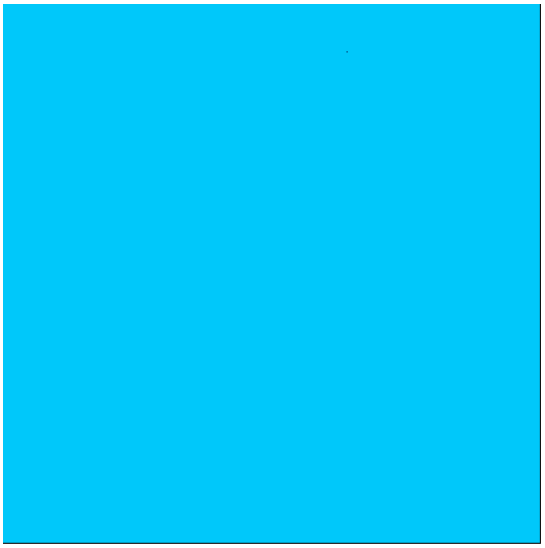
a. CFP



b. YFP



c. FRET



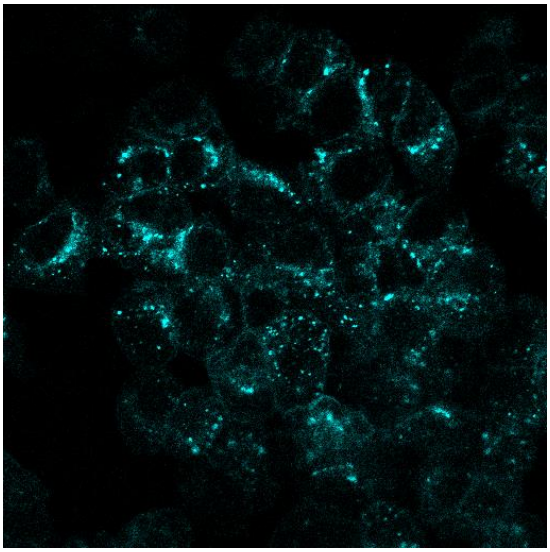
d. Co-localised FRET

Figure 4.8: FRET measurement for RBL cells co-expressing hC5a1-CFP and hC5a2-YFP receptors without C5a treatment. The fluorescence was detected through three channels, CFP (a), YFP (b), and FRET channel (c) by confocal microscopy. The co-localised FRET (d) was analysed using FRET and co-localization analyser plugin of Image J (Hachet-Haas et al., 2006). The graph does not show resonance energy transfer in any of the cells after correction.

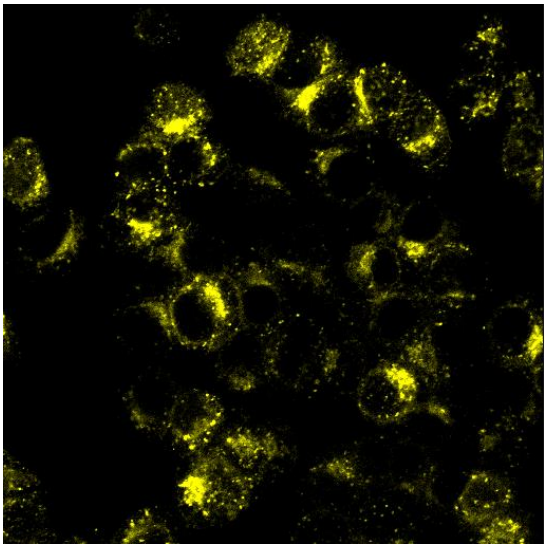
4.5.3 RBL-2H3 co-expressing hC5a1-CFP and hC5a2-YFP with C5a stimulation:

Heterodimerization was also examined after treatment with 500 nM C5a for 10 minutes (Figure 4.9) and 20 minutes (Figure 4.10). Confirming the

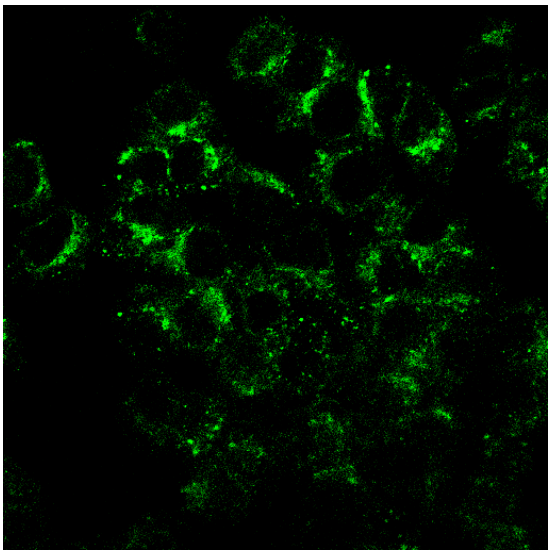
previous results of the specrtrofluorimeter, C5a treatment had no effect on the FRET signal.



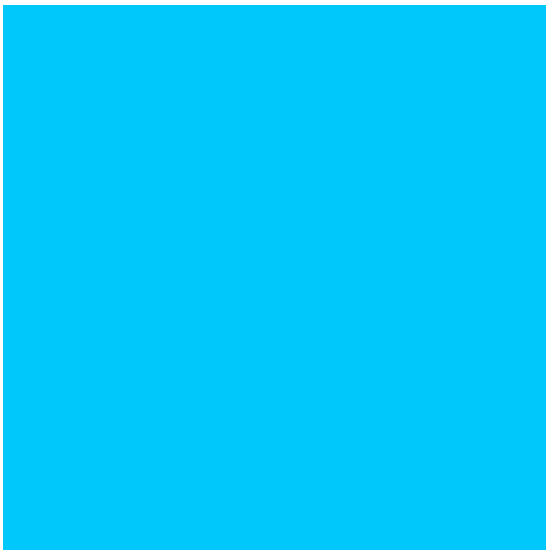
a. CFP



b. YFP

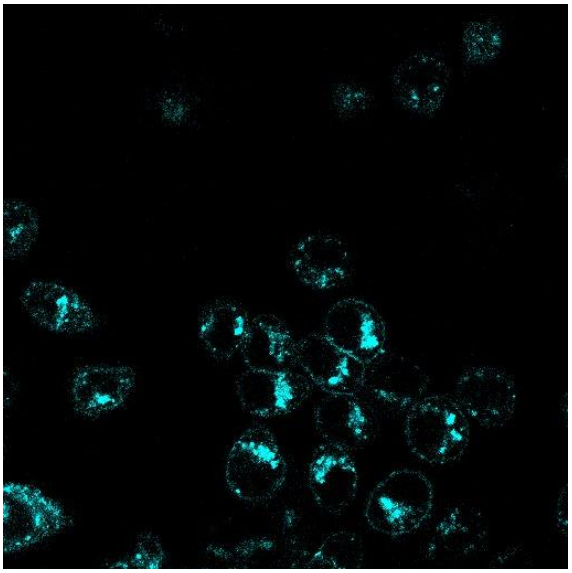


c. FRET

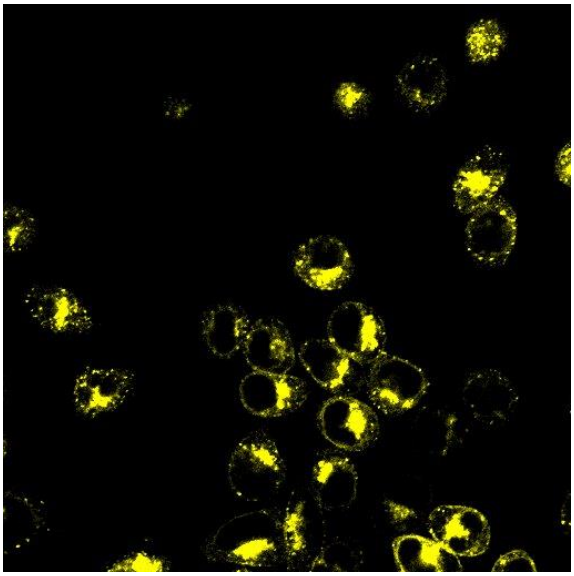


d. Co-localised FRET

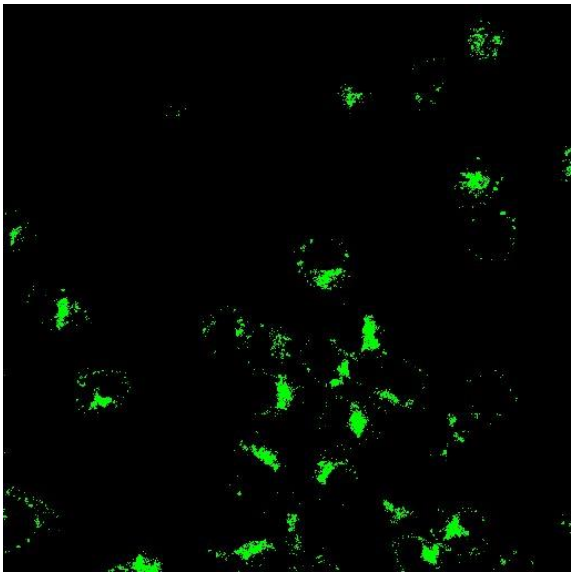
Figure 4.9 FRET measurement for RBL cells co-expressing hC5a1-CFP and hC5a2-YFP receptors after C5a treatment for 10 minutes. The fluorescence was detected through three channels, CFP (a), YFP (b), and FRET channel (c) by confocal microscopy. The co-localised FRET (d) was analysed using FRET and co-localization analyser plugin of Image J (Hachet-Haas et al., 2006). The co-localised FRET image does not show resonance energy transfer in any of the cells after correction.



a. CFP



b. YFP



c. FRET

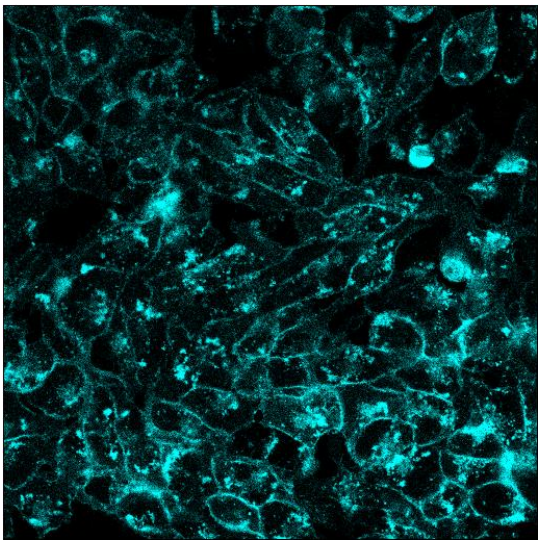
d. Co-localised FRET

Figure 4.10 FRET measurement for RBL cells co-expressing hC5a1-CFP and hC5a2-YFP receptors after C5a treatment for 20 minutes. The fluorescence was detected through three channels, CFP (a), YFP (b), and FRET channel (c) by confocal microscopy. The co-localised FRET (d) was analysed using FRET and co-localization analyser plugin of Image J (Hachet-Haas et al., 2006).

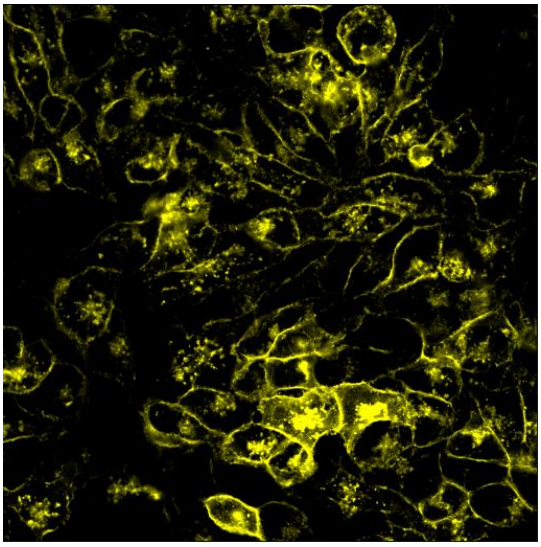
4.5.4 RBL-2H3 co-expressing hC5a1-CFP and hC5a1-YFP without C5a stimulation:

The FRET assay was used to explore the possibility of homodimer formation by hC5a1 receptors. The assay was performed in RBL cells co expressing hC5a1-CFP and hC5a1-YFP. The FRET signal was first analysed for

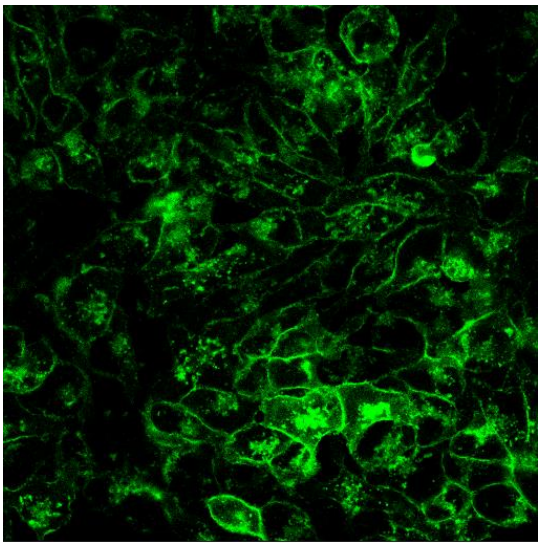
cells without C5a treatment. Figure 4.11 shows FRET signals that are mostly at the boundaries of the cells. This may suggest that the hC5a1 receptors are possibly expressed as homo-di/oligodimers on the cell surface.



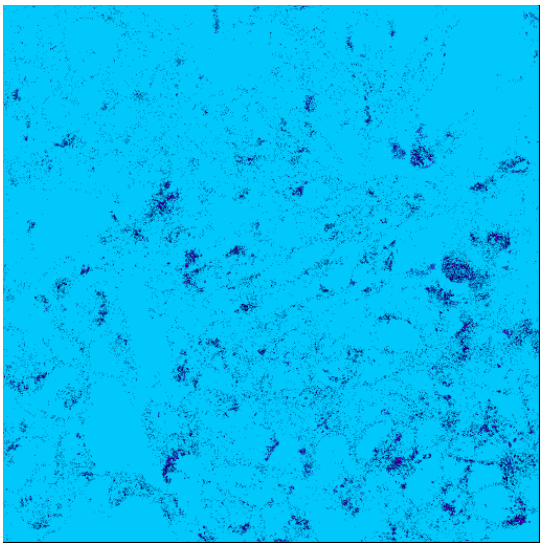
a. CFP



b. YFP



c. FRET

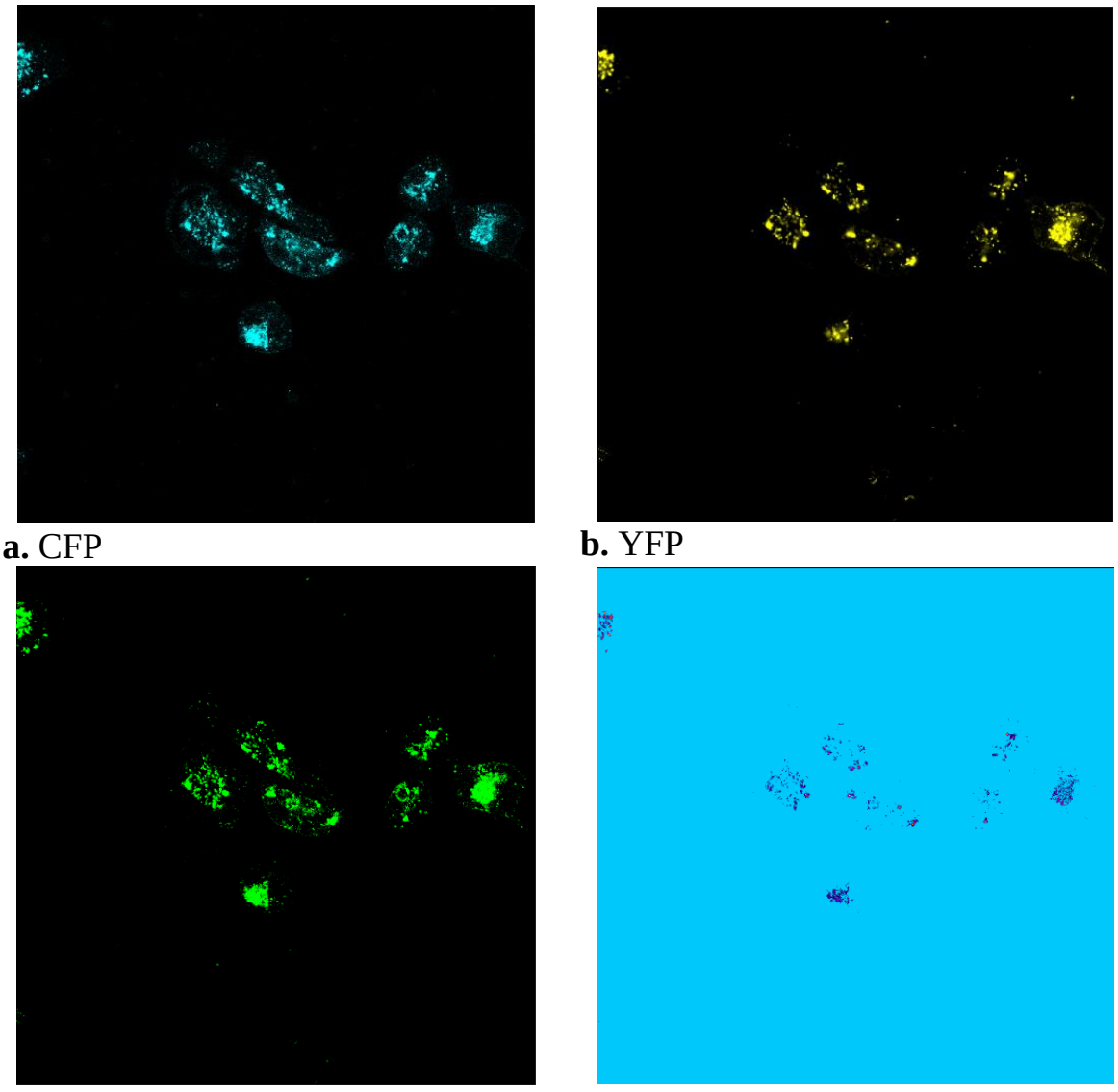


d. Co-localised FRET

Figure 4.11 FRET measurement for RBL cells co-expressing hC5a1-CFP and hC5a1-YFP receptors without C5a treatment. FRET measurement for RBL cells co-expressing hC5a1-CFP and hC5a1-YFP receptors without C5a treatment. The fluorescence was detected through three channels, CFP (a), YFP (b), and FRET channel (c) by confocal microscopy. The co-localised FRET (d) was analysed using FRET and co-localization analyser plugin of Image J (Hachet-Haas et al., 2006). The co-localised FRET image shows FRET in many cells after correction.

4.5.5 RBL-2H3 co-expressing hC5a1-CFP and hC5a1-YFP with C5a stimulation:

The hC5a1 receptor homodimerization was further examined for ligand effect on dimerization. After C5a treatment, the receptors were probably internalised as shown in CFP and YFP channel and the FRET signal was detected at the region of internalization (Figure 4.12).



a. CFP **b.** YFP

c. FRET **d.** Co-localised FRET

Figure 4.12 FRET measurement for RBL cells co-expressing hC5a1-CFP and hC5a1-YFP receptors after C5a treatment for 20 minutes. FRET measurement for RBL cells co-expressing hC5a1-CFP and hC5a1-YFP receptors after C5a treatment for 20 minutes. The fluorescence was detected through three channels, CFP (a), YFP (b), and FRET channel (c) by confocal microscopy. The co-localised FRET (d) was analysed using FRET and co-localization analyser plugin of Image J (Hachet-Haas et al., 2006). The co-localised FRET image shows FRET signal in all cells.

4.6 Discussion:

Protein-protein interaction is thought to play an important role in regulating cellular functions. There are many methods that can be used to detect this interaction. The interaction could be weak and transient and might occur only in natural cellular milieu of molecules. In this case, it is not easily measured by the traditional biochemical methods. Co-localization by fluorescent microscopy historically was the common method. Nevertheless, the resolution of fluorescent microscopy is about several hundred nanometres although the interacting proteins might be few nanometres in size. In addition, the presence of one molecule in the vicinity of another does not mean that they are interacting. It has been thought that "typical fluorescence imaging experiment yields information equivalent to knowing that two students are present in a large lecture hall: merely localizing the two students to the same classroom yields no information about whether the students know each other or not" (Piston and Kremers 2007).

The FRET technique has been developed to overcome the limitations of other methods such as the lack of labelling or appropriate resolution. FRET can occur only when the distance between the interacting molecule is ~10 Å to100 Å (Clegg, 1996) when they are appropriately labelled. The ease of use and the variety of fluorescent proteins have led to the increased popularity of this technique. The fluorescent proteins are now genetically designed to be expressed at one or both ends of a protein. Therefore, FRET can be used to measure intermolecular or intramolecular interactions. In this chapter, CFP and YFP linked to either hC5a1 or hC5a2 were used to explore the interaction between the two receptors. CFP and YFP have been used for many years as FRET donor and acceptor. There are many factors that contribute to their suitability for FRET experiments. The most important is the spectral overlap that they share. This makes the emission maximum of the CFP near the excitation maximum of the YFP, which is necessary for FRET to occur. The second characteristic is that their

excitation maxima are well separated. Therefore, there should be minimal excitation of one of the fluorophore with the wavelength intended for excitation of the other. In addition, their emission maxima are also well separated. This makes the FRET emission easily distinguished from the donor emission (Pollok and Heim, 1999, Sekar and Periasamy, 2003). These properties allow CFP and YFP to be used using excitation wavelengths and filter sets that are commonly available in most fluorescent measuring machines such as microscopes, spectrofluorimeters and flow cytometers. Moreover, the fusion of CFP and YFP to proteins do not affect the native localization of these proteins within the cells (van Rheenen et al., 2004). This might be due to their high stability and compact structure.

CFP and YFP tagged hC5a1 and hC5a2 receptors were used in the current study to show the localization of the receptors within cells. The hC5a1 receptor was expressed mainly on the cell surface. However, C5a2 receptors were mainly intracellular. This result is in support of other studies that reported predominant peripheral positioning of the C5a1 receptors, similar to the majority of GPCR, on the cell surface, while the C5a2 tend to keep more intracellular location (Li et al., 2013, Bamberg et al., 2010, Scola et al., 2009, Croker et al., 2013). This has been reported in primary cell lines and transfected cells. The staining was by either using antibodies or, as in our study, by receptor-reporter fusion proteins. Moreover, it was reported in animal and human native cells (Li et al., 2013). This different localization may not support the possibility of the heterodimerization between the two receptors. Most of the GPCR, which have been reported to form dimers, are expressed on the cell surface and they attach to each other through their transmembrane (TM) regions (Gomes et al., 2001, Angers et al., 2002). For example, the contact surfaces for D2 receptors are in the TM4-5; histamine H1 receptors at TM5-6; and β2-adrenoceptors contact domains are mainly at the TM1-4 (reviewed in Dickenson et al., 2013).

The site of the formation of receptor dimers is still a controversial subject. There are two theories in this context. The first, is that the receptors form the dimers at their destination where they are expressed at the cell surface. This theory also suggests that this dimerization process could be a dynamic process that can be induced, increased or even decreased upon ligand stimulation (Rocheville et al., 2000b, Patel et al., 2002, Cvejic and Devi, 1997, AbdAlla et al., 1999). On the other hand, the second theory suggests that the receptor dimerization is an early event that happens during the biogenesis and maturation of the receptors. It is probably occurring at the level of endoplasmic reticulum and the receptors are delivered as dimers to the cells surface. In this theory, the receptors may form constitutive dimers and their formation is perhaps ligand-independent (reviewed in Milligan, 2004, Drinovec et al., 2012, Floyd et al., 2003). Following heterologous expression, many GPCR are not delivered to the cell membrane in model cell lines. For example, the $GABA_B$ receptor when first cloned and then expressed in heterologous systems, failed to reach surface expression and failed to signal (Couve et al., 1998). These receptors were retained in the endoplasmic reticulum. After one year, a second $GABA_{B2}$ receptor was discovered. This receptor did not bind to GABA ligand, but it was able to physically interact with first receptor $GABA_{B1}$ and both receptors were able to reach cell surface and transduce signal (Marshall, 2001). This was because the $GABA_{B1}$ has an endoplasmic retention signal at its C-terminus that prevent its cell surface expression. However, when co-expressed with $GABA_{B2}$, the retention signal is masked by coil-coil interactions allowing both receptors to release from the ER and be expressed as heterodimer on the cell surface.

In the present study, C5a receptors appeared to have a tendency toward the formation of homodimers rather than heterodimers. This was explored by two different methods, specrofluorimetry and confocal microscopy. Several studies reported that hC5a1 receptors form homodimers (Klco et al., 2003, Floyd et al., 2003, Rabiet et al., 2008). In addition, C5a homodimerization was reported to be

constitutive and not dependent on intracellular proteins, which is in agreement with the result of the current study and with the second theory mentioned above. C5a1 receptor heterodimerization with C5a2 was recently reported to be upregulated by C5a, but not C5a desArg (Croker et al., 2013). This was performed using a BRET method in HEK cells. However, the C5a receptor interaction could not be detected by another study (Chen et al., 2007). Since C5a1/C5a2 heterodimerization was not clear using FRET method in this section, it was decided to construct a C5a receptor BRET system, which is explained in the next section. This was done to develop a tool to explore the nature of this dimerization and testing some pharmacological preparations, such as novel peptides that could work as C5a2 specific ligands (Croker et al., 2016).

Chapter Five

Investigating the Dimerization of C5a Receptors Using Bioluminescence Resonance Energy Transfer (BRET)

Chapter 5: Investigating the dimerization of C5a receptors using bioluminescence resonance energy transfer (BRET)

5.1 Introduction:

BRET is another method for measurement of protein-protein interaction with several advantages over the previously described FRET method. As with any fluorescence technique, many of the drawbacks of FRET techniques results from the use of external source of light for excitation of the donor. This may result in auto-fluorescence, photobleaching or even damage to the tissue. In addition, in FRET, the acceptor may be excited directly by the wavelength used for donor excitation. All these factors may limit the usefulness of the FRET technique and require correction steps to produce the final results. Therefore, BRET, which is a naturally occurring phenomenon, has been developed to be used to measure *in vivo* and *in vitro* proteins interactions and possibly avoid the consequences of fluorescence excitation with external light, by replacing the donor fluorophore with an enzyme. This enzyme emits light upon interaction with its substrate.

The BRET method has been widely used to monitor protein-protein interactions including GPCR dimerization and oligomerization (Hebert et al., 1996, Mercier et al., 2002, Calebiro et al., 2013, Cussac et al., 2012, Croker et al., 2013, Harikumar et al., 2016, Angers et al., 2000). In this study, the BRET method was used to confirm the results from the FRET study in the previous chapter. In order to develop hC5a1 receptor tagged with *Renella* luciferase enzyme (Rluc8, as a luminescent donor), the construct was designed as described in section (2.4.3). RBL-2H3 cell line was transfected using Turbofect method as described in section (2.2.4). The cells were then sorted by a FACS Aria cell sorter. Then the sorted cells were regrown and tested for the functionality of the Rluc8 tagged hC5a1 receptor. The construct was tested for receptor expression, C5a binding, and C5a induced degranulation, and compared with IgE-mediated

degranulation. The hC5a receptors interactions were then measured in cells expressing both Rluc8 tagged hC5a1 and YFP tagged hC5a2 receptors.

5.2 The expression of hC5a1-Rluc8 receptors in transfected cells:

The new plasmid construct that contains the Rluc-8 tagged hC5a1 receptor was first transfected into RBL-2H3 cells. Consequently, the expression was tested after transfection and selection using immunostaining. The fluorescence was detected using the red laser and RL1-A filter set of an Attune flow cytometer (Figure 5.1). This result demonstrated that the transfection was successful and the tagging of the hC5a1 receptor with luciferase enzyme did not affect its expression at the cell surface.

The expression of hC5a1 receptor tagged with luciferase (Rluc8) transfected into RBL-2H3 cells

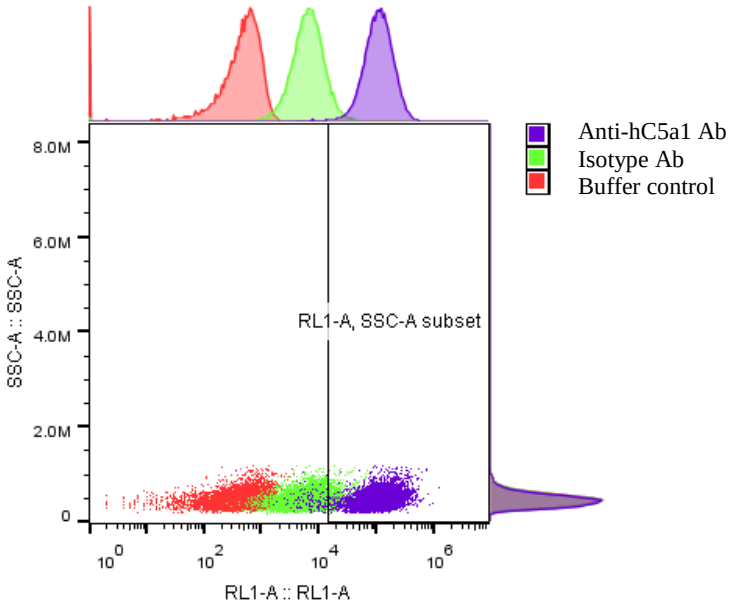


Figure 5.1: The expression of hC5a1 receptor in RBL-2H3 cells transfected with Rluc8-hC5a1. Red, cells treated with buffer. Green, cells were treated with isotype antibody. Purple, cells were treated with mouse anti-hC5a1 primary antibodies. The graph shows combined dot plot and histograms for each treatment. Alexa fluor 633 rabbit antimouse secondary antibody was used to stain the cells, which is shown as RL1-A. SSC, side scatter.

In order to develop the BRET assay to explore the possible heterodimerization of hC5a1-hC5a2 receptors, the Rluc8-hC5a1 plasmid was transfected into RBL-2H3 expressing hC5a2-YFP. The co-expression was then analysed by flow cytometry after immunostaining of the surface receptors. The receptors were treated with either mouse anti-hC5a1 antibodies, isotype antibodies control, or buffer control. Subsequently, the receptors stained using anti-mouse secondary antibodies. Figure 5.2 shows the shift in hC5a1 receptors fluorescence when treated with primary anti-hC5a1 antibodies (green) in comparison to the isotype (red) or buffer (cyan) control treatment. All these cells co-express hC5a2-YFP as represented by the histogram shift in the BL1-A axis in comparison to control cells (purple), which do not express YFP. The flow cytometric analysis revealed receptor expressions of >90% of the transfected cells after the cell sorting (Figure 5.3).

Co-expression of Rluc8-hC5a1 and hC5a2-YFP in RBL-2H3 cells

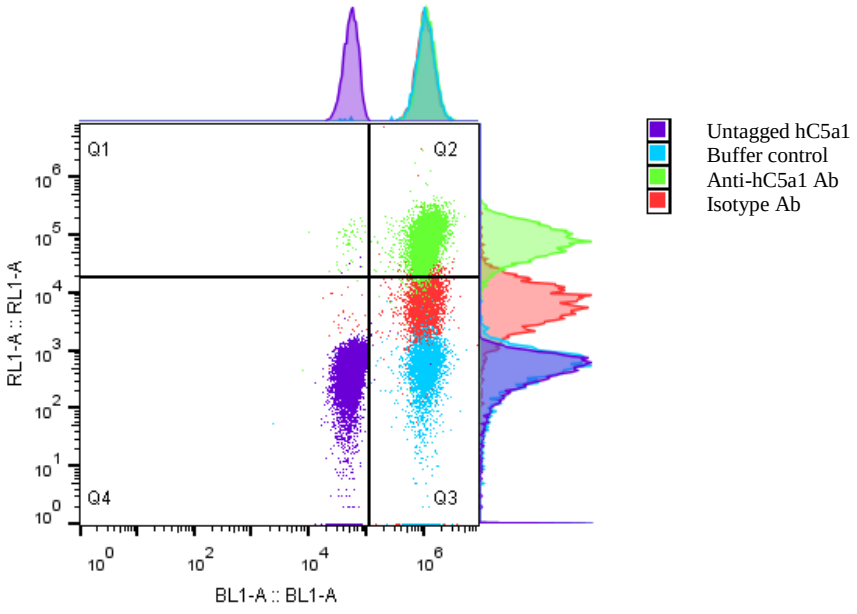


Figure 5.2: The co-expression of C5a receptors in RBL-2H3 cells. Dot plot with corresponding histograms to show Rluc8-hC5a1 expression (RL1-A) and hC5a2-YFP expression (BL1-A). Control cells expressing wild type (untagged) hC5a1 receptor (Purple), RBL-2H3-Rluc8-hC5a1+ hC5a2-YFP were treated with either buffer (cyan); isotype antibody (red); or mouse anti-hC5a1 primary antibodies (green).

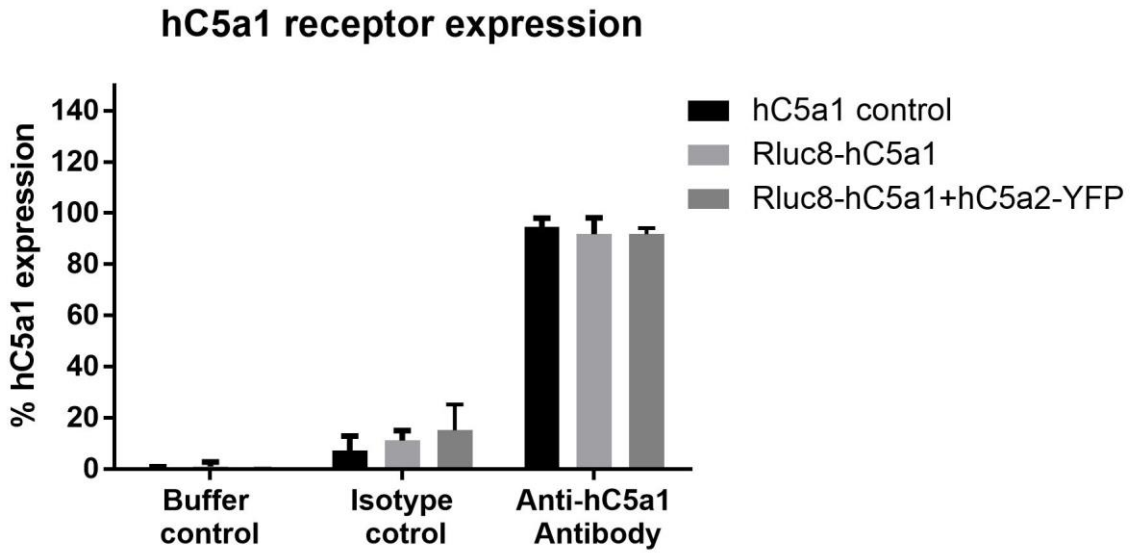


Figure 5.3: The expression of hC5a1 in RBL-2H3 cell lines. Cells expressing either wild type hC5a1 receptors (control); Rluc8-hC5a1; or both Rluc8-hC5a1+hC5a2-YFP were used. Cells were treated either with buffer control, isotype antibody control or anti-hC5a1 antibodies. Alexa633 secondary antibodies were used to stain the receptors to be detected by flow cytometer. Data are mean±SEM of 3 independent experiments in duplicate.

The luminescence of hC5a1-Rluc8 in the transfected cells was also measured. RBL cells that express either hC5a1-Rluc8 or hC5a1-Rluc8+hC5a2-YFP were tested. The treatment with coelenterazine substrate resulted in luminescence in both cell lines (Figure 5.4).

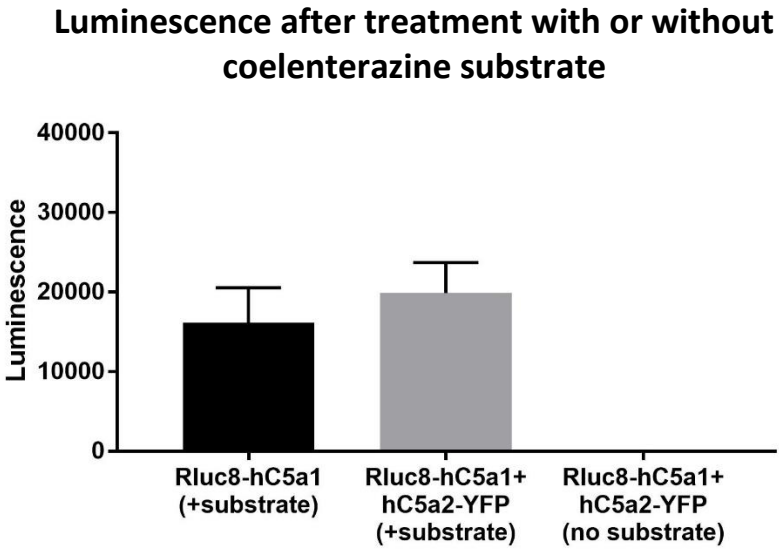


Figure 5.4: Luminescence of Rluc8 tagged hC5a1 receptor. RBL-hC5a1 and RBL-hC5a1-Rluc8+hC5a2 were tested. Luminescence was measured after treatment with or without coelenterazine substrate.

5.3 C5a binding assay by the Rluc8-hC5a1 receptor:

The ligand binding assay was performed to check whether the hC5a1 receptor retains the ability to bind C5a after tagging with luciferase enzyme (Rluc8). Competitive ligand binding assay was done as described in section (2.3.2) and the result shows that IC50 for the tagged receptor was significantly lower than the wild type hC5a1 control (Figure 5.5).

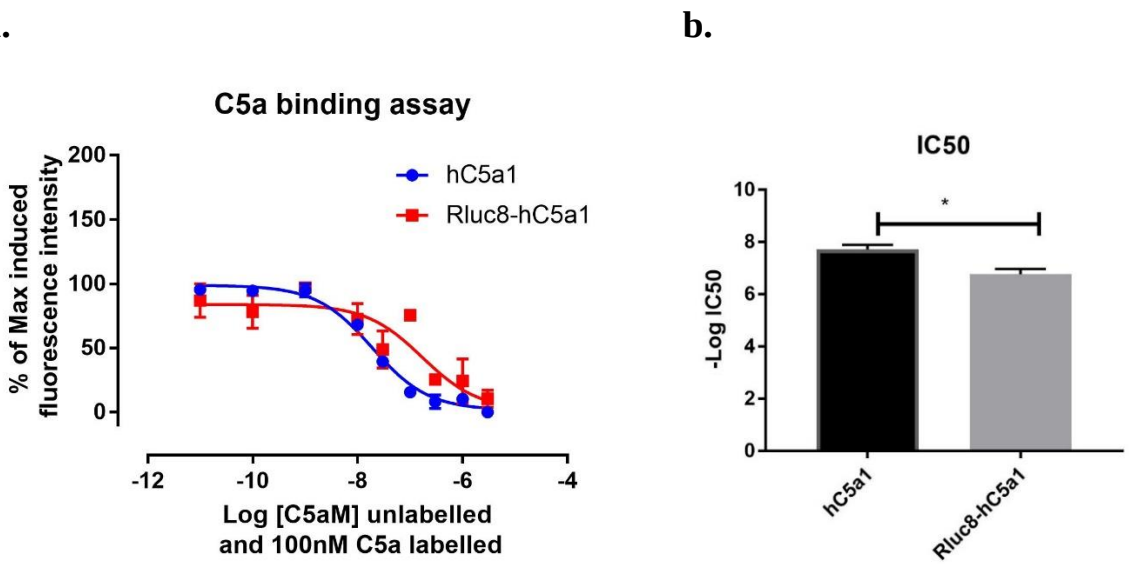


Figure 5.5: C5a binding assay for Rluc8 tagged hC5a1 receptor determined by competitive 488-hC5a binding study. a. dose response (inhibitory) curve of serial dilutions (starting with 3 µM) of unlabelled hC5a with 100 nM of Alexa488 labelled hC5a. b. bar charts to compare the IC50 of Rluc8-hC5a1 with the wild type hC5a1 control. Data are mean±SEM of 3 independent experiments in duplicate. Unpaired t-test is used to test statistical significance. *, P≤0.05; ns, non-significant.

5.4 RBL-2H3 cell degranulation assay:

The degranulation assay was performed to test the function of the Rluc8-hC5a1 receptor. C5a-mediated enzyme release was used to test the Rluc8-tagged C5a1 receptor signalling. In addition, IgE-mediated enzyme release was also examined:

5.4.1 C5a-mediated degranulation assay for Rluc8-hC5a1 receptor transfected cells:

As previously described in section (2.3.4), the enzyme release in the supernatant of RBL-2H3 cells was measured after incubation with C5a for 15 minutes at 37˚C. Figure 6 demonstrates the response to the stimulation in cells expressing either wild type hC5a1 or Rluc8-tagged hC5a1 receptors. Both the EC50 and maximum enzyme release were significantly lower in the tagged receptors.

a. **b.**

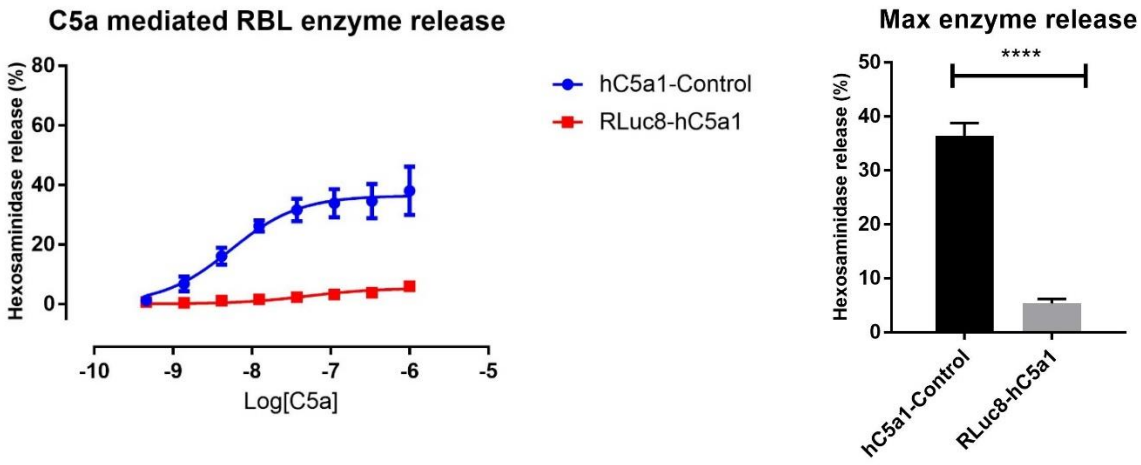
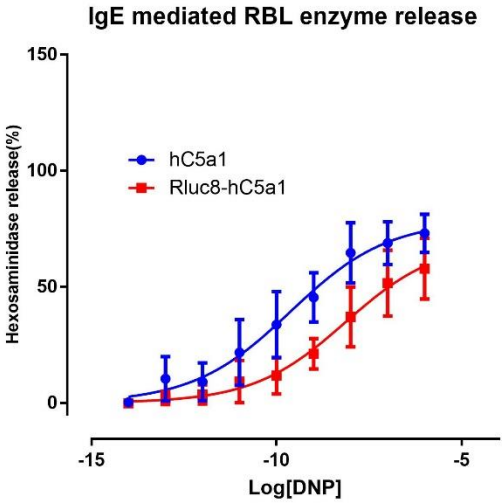


Figure 5.6: C5a-mediated degranulation assay for RLuc8-tagged hC5a1 receptors on RBL-2H3 cells. a. dose response curve for expressing either the wild type or RLuc8 tagged hC5a1 receptor. Beta-hexosaminidase enzyme release was measured in response to 1/3 serial dilutions of C5a starting with 1 µM. b. Bar charts for maximum enzyme release. Data are mean±SEM of 3 independent experiments in duplicate. Unpaired t-test is used to test statistical significance. ****, P≤0.0001.

5.4.2 IgE-mediated degranulation assay:

The hexosaminidase enzyme release was performed in response to IgE stimulation as a comparison to the C5a-mediated response of the tagged hC5a receptor. The current data shows that the EC50 and maximum enzyme release were not significantly different in the Rluc8-hC5a1 transfected cells from the wild type hC5a1 receptors (Figure 5.7). This might suggest that Rluc8 tagging resulted in receptor specific rather than general cellular side effects.

a.



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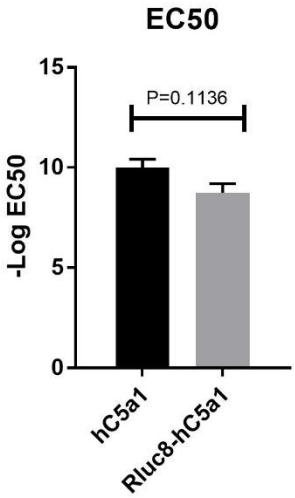


c.

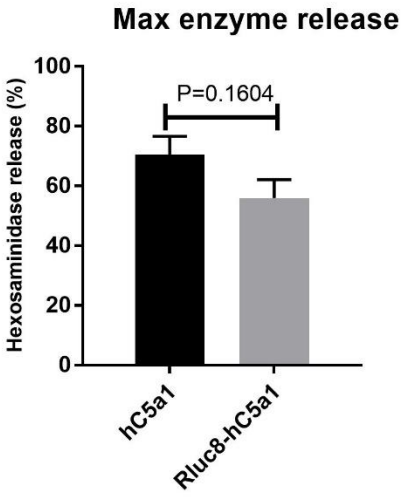


Figure 5.7: IgE-mediated degranulation assay for Rluc8-tagged hC5a1 receptors on RBL-2H3 cells. a. dose response curve for cells expressing either the wild type hC5a1 or Rluc8 tagged hC5a1 receptor. Enzyme release was measured in response to 1/10 serial dilutions of DNP starting with 1 µg maximum. b. and c. are the bar charts for EC50 and maximum enzyme release, respectively. Unpaired t-test is used to test statistical significance. Data are mean±SEM of 3 independent experiments in duplicate

5.5 The effect of luminescent protein tagging on hC5a1 receptors internalization:

In this experiment, the effect of the protein tagging on internalization of the receptor was examined. Human C5a receptors are rapidly internalised after ligand binding. However, the presence of protein tag on the C-terminus of hC5a receptors in the Rluc8-hC5a1 chimera results in significant decrease in receptor endocytosis (Figure 5.8).

C5a-induced hC5a1 receptor internalization for luminescent protein (Rluc8) tagged and wild type hC5a1 receptors

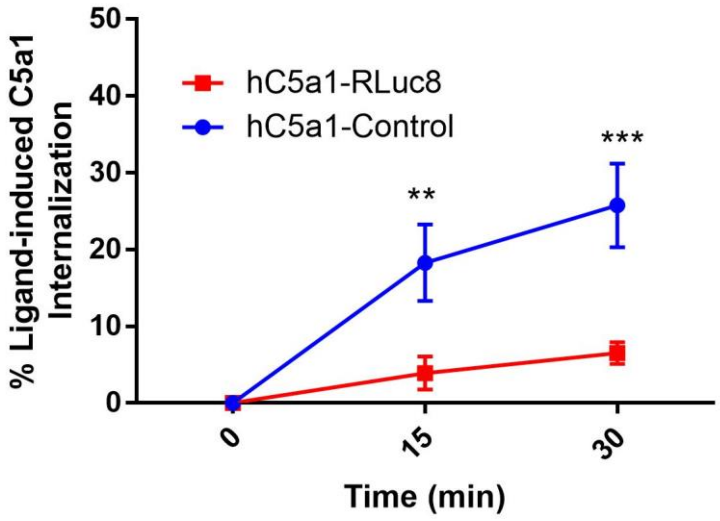


Figure 5.8: C5a-induced internalization of luminescent-tagged receptors. RBL-2H3 cells expressing Rluc8 tagged hC5a1 receptors were compared with cells expressing wild type hC5a1 (control) receptors. Cells were stimulated with C5a for 15 and 30 minutes. Data are mean±SEM of 3 independent experiments in duplicate Two way ANVOVA followed by Dunnett's multiple comparison are used to test statistical significance. **, P≤0.01, ***, P≤0.001.

5.6 BRET assay for hC5a receptors dimerization:

In this experiment, the dimerization between hC5a1 and hC5a2 was measured using the BRET assay. The C5a receptors that were used in this assay were hC5a1 and hC5a2 tagged to luminescent donor (Rluc8) and YFP acceptor, respectively. The assay was performed as a time course before and after adding C5a or buffer control to the cells that co-expressing the tagged receptors. There was no obvious difference between the two treatments over time (Figure 5.9). This result confirms the previous FRET results regarding the lack of tendency of C5a receptors to exist or work in heterodimers.

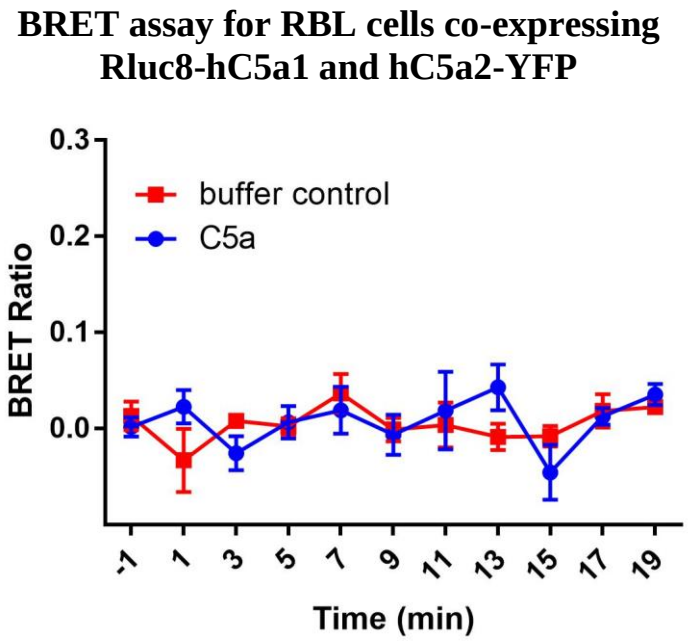


Figure 5.9: BRET assay in RBL-2H3 expressing Rluc8-hC5a1+hC5a2-YFP. Time course of the BRET ratio after adding 1 µM of C5a or buffer control. Luminescence was measured after incubating the cells with 5 µM coelenterazine substrate. Data are mean±SEM of 3 independent experiments in duplicate

5.7 Discussion:

The starting point of this chapter was to generate cells with stable co-expression of both luminescent-tagged hC5a1 and fluorescent-tagged hC5a2 receptors. This cell line could be used to measure C5a receptor heterodimerization using BRET technique. The luminescent tag used to generate the hC5a1 construct BRET donor is *Renella* luciferase enzyme (Rluc8). The addition of such protein, which is about 314 aa, to the C-terminus of C5a receptors may have an influence on receptor pharmacology, G protein coupling or interaction with other intracellular partners. Therefore, in this study, this fusion construct Rluc8-hC5a1 was tested for different pharmacological and signalling properties.

Luciferase has been used in biological research in many applications as a reporter molecule for gene expression when its gene is inserted into the gene of interest and transfected into cells (reviewed in Fan and Wood, 2007). It is used as a high throughput screening for drug discovery as it can be used to monitor cellular events and gene transcription regulation. Numerous major drug targets such as GPCR and nuclear receptors have been assessed using luciferase assays (Katso et al., 2005, Hill et al., 2001, Dinger et al., 2003, Grover et al., 2003). It has been even used for studying cell populations in live animals (Greer & Szalay 2002). However, in this study it appeared that the luciferase tag on hC5a1 receptors had effects on receptor function.

Several effects have been reported for many of the commonly used protein tags (Zhu et al., 2013, Ledent et al., 1997, Zhang and Crandall, 2007). It has been reported that tagging of CD36 with GFP did not affect protein expression. However, the tagging influenced the binding of its ligand (oxLDL) (Zhang and Crandall, 2007). These findings are compatible with this study, which showed that the intracellular tagging of the hC5a1 receptor (C-terminal tagging) did not affect the fusion protein expression, whereas the ligand binding was

disturbed. In addition, ligand binding could be affected when the receptor interaction with its intracellular partners is affected. It has been suggested that G proteins could have an allosteric effect on the ligand binding affinity of some receptors (reviewed in Chabre et al., 2009). Moreover, β-arrestins have also been shown to influence ligand binding to the receptors (Gurevich et al., 1997). This might suggest that the tagging of hC5a1 receptors at the C-terminus may have a direct or indirect influence on receptor physiological and pharmacological function.

The cytosolic C-terminus is essential for many receptor activities such as receptor internalization for majority of G protein-coupled receptors (Benya et al., 1993, Chabry et al., 1995, Huang et al., 1995, Negishi et al., 1993, Nussenzveig et al., 1993, Parker et al., 1995, Thomas et al., 1995a, Thomas et al., 1995b, Tseng et al., 1995). Therefore, it could be speculated that the decreased internalization of the tagged receptor is due to the effect of the tag on the receptor C-terminus. In addition, it could be due to the disturbance in ligand binding as shown in the ligand binding assay. In addition, the C-terminus contains the predominant phosphorylation sites shared with third intracellular loop. These sites are important sites for protein kinase activities (PKC and PKA). In addition, they are the targets for phosphorylation by G protein-coupled receptor kinase (GRK). Therefore, the C-terminus plays an essential role in regulating receptor desensitization in addition to probable role in G protein coupling. Moreover, a splice variant of a GPCR receptor (neuropeptide Y receptor) which lacks part of the transmembrane region and the C-terminal tail failed to couple to second messenger systems such as Ca^{2+} release or MAP kinase stimulation (Dickenson et al., 2013). This might explain the results of the current study regarding the abnormality in signal transduction of the C-terminal tagged C5a1 receptors in terms of defective enzyme release from RBL cells.

The concept of class A GPCR dimerization/oligomerization is a controversial one. This could be partially related to the lack of complete understanding of the methodology used to address the subject. For example, the existence of β_2 adrenergic receptor dimer/oligmerization was both supported (Hebert et al., 1996, Mercier et al., 2002, Dorsch et al., 2009, Calebiro et al., 2013, Angers et al., 2000) and failed to be supported (James et al., 2006, Kawano et al., 2013, Lan et al., 2011, Gavalas et al., 2013, Felce et al., 2014). The C5a receptor heterodimerization is no exception from this type of controversy. Human C5a receptor heterodimerization was reported to be upregulated by C5a ligand (Croker et al., 2013). However, it was shown by Poursharifi *et al*. that C5a heteromer formation was not affected by ligand treatment (Poursharifi et al., 2013). In addition, this form of C5a receptor interaction could not be detected in another study and indirect interactions or parallel functions were suggested (Chen et al., 2007). This discrepancy in reporting interaction between integral membrane proteins may arise from the methods used to study this kind of interaction.

The temporal and spatial factors for the formation of receptors dimerization has been explored in many studies. GPCR dimerization could start at early stages of protein formation and processing at endoplasmic reticulum (ER) and before trafficking to the cell surface (reviewed in Milligan, 2010, Van Craenenbroeck, 2012). For example, as a model for class A GPCR, the β2 adrenergic receptors dimerization is considered as prerequisite for cell surface delivery (Salahpour et al., 2004). The lack of endoplasmic reticulum export motif inhibited trafficking of wild type receptors to the cell membrane. In addition, inhibition of dimerization or disruption of the putative dimerization motifs resulted in inhibition of receptors trafficking to the cell surface. Another example, from class C GPCR, is the metabotropic glutamate receptor-like $GABA_B$ receptors. It was the first GPCR recognised that its delivery to the cell surface require quaternary structure (Pin et al., 2005). $GABA_B$ R1 is retained in ER when expressed alone.

However, cell surface delivery occurs when co-expressed with $GABA_B$ R2 (Milligan, 2010). These findings suggest that once the receptors are expressed on cell surface, they are in their final forms whether they are monomers, dimers or oligomers and the signal transduction capability of the receptors probably has no role in the di/oligomerization. In the current study, the luminescent protein tagging of the receptors does not seem to affect the expression of the receptor. This was also the case with FP tagging as discussed in chapter 3. Other studies also reported similar findings. The tagging of CD36 with GFP did not affect CD36 expression, although it affected ligand binding (Zhang and Crandall, 2007). Therefore, although the presence of luminescent or fluorescent tag could be a limiting factor due to the effect on receptor signal transduction, the receptors can still be used to assess di/oligomerization. This is because the formation of receptor di/oligomers does not depend on the signalling capability of the receptors (Floyd et al., 2003, Klco et al., 2003). C5a receptor dimerization was detected using C5a receptor mutants that lack ligand binding or phosphorylation capability (Rabiet et al., 2008). This can be supported further by the reports that demonstrated that receptor self-association is not required for signalling through G proteins and arrestins (Hanson et al., 2007, Kuszak et al., 2009, Whorton et al., 2007, Whorton et al., 2008).

Using luminescent and fluorescent tagged receptors, the BRET assay could not detect hC5a1 and hC5a2 receptor dimerization. This could be supported by the previous FRET results from chapter 4, which showed a tendency of hC5a1 receptors to form homodimers or oligomers rather than heterodimers with hC5a2. In addition, the discrepancy of the localization of the two receptors within the cells (hC5a1 mainly expressed on the cell surface, while hC5a2 is mainly intracellular in unstimulated cells) does not support the heterodimerization theory. To further confirm this finding, and to rule out the effect of tags on the receptors, in the next chapter, untagged C5a receptors are used to explore the dimerization hypothesis.

Chapter Six

Receptor Interaction During Internalization in RBL-2H3 Cells Co-Expressing Untagged hC5a1 and hC5a2 Receptors

Chapter 6: Receptor interaction during internalization in RBL-2H3 cells co-expressing untagged hC5a1 and hC5a2 receptors

6.1 Introduction:

GPCR are known to internalize upon stimulation with their ligands similar to other receptors. This internalization could be through various pathways that involve interaction with intracellular proteins such as β-arrestins. The internalization could be part of a desensitization process of the receptors, although other functions were reported for internalization such as receptor re-sensitization. In addition, receptors can interact and influence the internalization and trafficking of each other. In this chapter, possible hC5a receptors interactions were tested by observing receptor internalization after treatment with different ligands. These ligands involve C5a, C5a desArg, RHYPYWR peptide, a selective hC5a2 ligand that is thought to stimulate β-arrestin2 (Croker et al., 2016). The internalization was measured in RBL cells that expressed either one of the hC5a receptors (RBL-hC5a1 or RBL-hC5a2) or both (RBL-hC5a1+hC5a2).

The internalization assay was also used to test whether the receptors internalize as dimers or as monomers. Many studies reported that GPCR heterodimerization can be expected if co-internalization of two receptors occurs upon stimulation of either of them (Pfeiffer et al., 2003, Stanasila et al., 2003, Jordan et al., 2001). In this study, the selective hC5a1 inhibitor (PMX53) was used to allow hC5a2 receptor to be activated more selectively by C5a and C5a desArg in RBL cells co-expressing both hC5a1 and hC5a2 receptors.

6.2 Ligand-induced hC5a1 receptor internalization when co-expressed with hC5a2:

Ligand-induced internalization was performed as described in section 2.3.5 at different time points. The RBL cells that express either hC5a1 or both hC5a1+hC5a2 receptors were treated with various ligands. At 5 minutes, there was no significant difference in hC5a1 receptor internalization between either hC5a1 alone or hC5a1+hC5a2 expressing cells. In addition, the different ligand treatments did not result in significant change (Figure 6.1a). After 15 minutes, the cells showed significant hC5a1 internalization in both cell lines (hC5a1 alone or hC5a1+hC5a2) in response to either C5a or C5a desArg, but not to the peptide (Figure 6.1 b). The cells expressing both receptors (hC5a1 and hC5a2) displayed less hC5a1 internalization than cells that express only hC5a1 (Figure 6.1 b). The difference in the hC5a1 internalization between RBL-hC5a1 and RBL-hC5a1+2 was statistically significant in response to ligand treatment compared to buffer treatment (Figure 6.1c). This may indicate that hC5a1 is less internalized when co-expressed with hC5a2 and hC5a2 may have a role in this process. With time, hC5a1 receptor internalization rate appeared to be slowing down and it was slower when hC5a2 was co-expressed with hC5a1. In case of C5a treatment, although RBL-hC5a1 showed significantly more hC5a1 internalization compared to buffer treatment, RBL-hC5a1+2 showed less hC5a1 internalization than RBL-hC5a1 at 45 minutes, which was not significant (Figure 6.1 d). The difference in the hC5a1 internalization between RBL-hC5a1 and RBL-hC5a1+2 became statistically insignificant in response to ligand treatment compared to buffer treatment at 45 minutes (Figure 6.1 e). In case of C5a desArg treatment, the increase in hC5a1 internalization in response to ligand treatment was absent in both cell lines at 45 minutes. This might be due to the lower potency of C5a desArg in comparison to C5a (Figure 6.1 d).

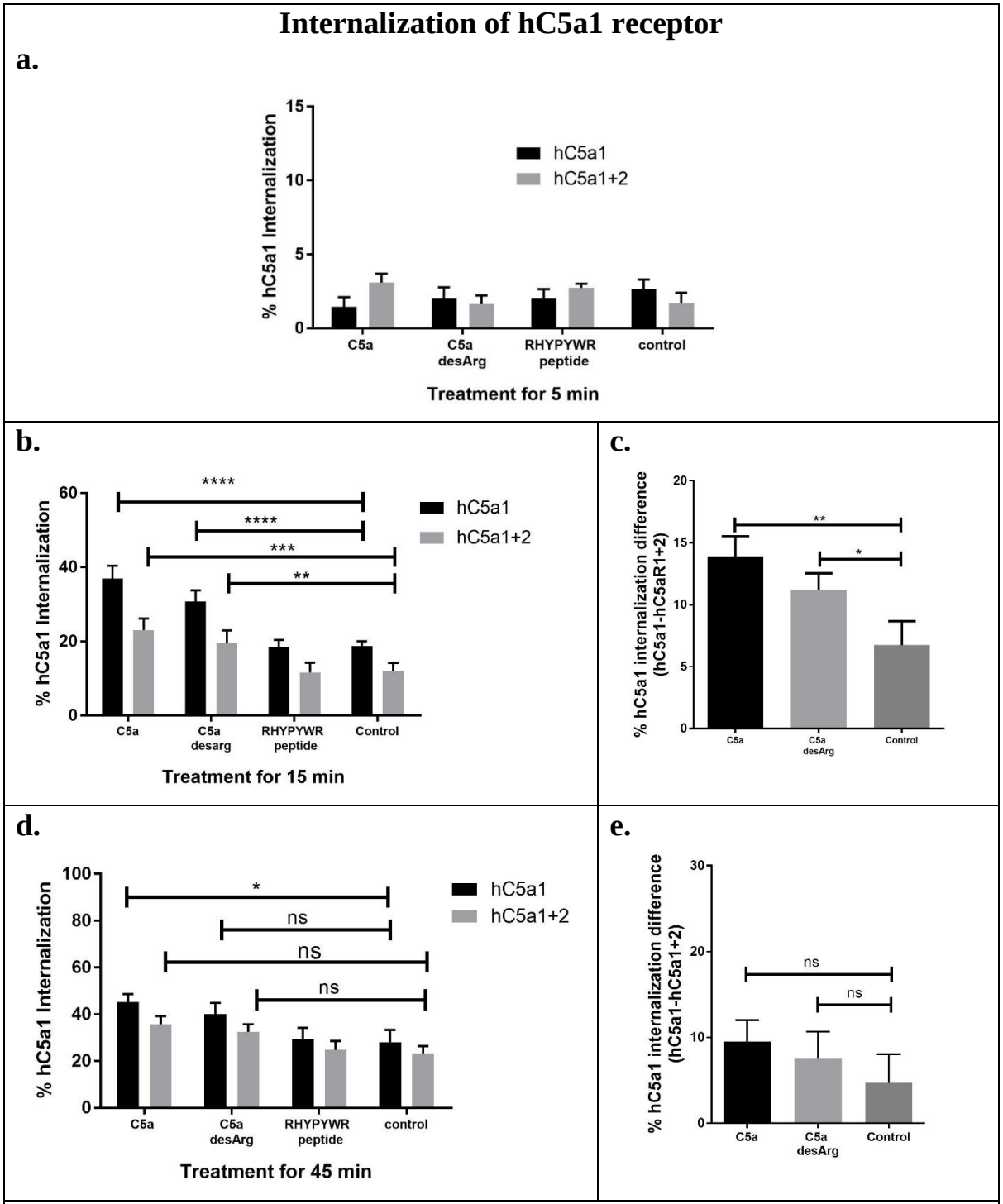


Figure 6.1: hC5a1 receptor internalization. ligand induced internalization in response to either C5a, C5a desArg or RHYPYWR peptide for 5 min (a) 15 min (b) and 45 min (d). Cell expressing either hC5a1 alone or hC5a1+2 were used. Two-way ANOVA test with Sidak's multiple comparisons test were performed to test statistical significance. c. and e. are the hC5a1 receptor internalization difference between RBL-hC5a1 alone or RBL-hC5a1+2 at 15 min or 45 min, respectively. Unpaired t test was used to test statistical significance. Data are mean±SEM of 3-4 independent experiments in duplicate. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, ns, non-significant.

In summary, in case of C5a stimulation, hC5a1 receptor internalization increase rapidly between 5 to 15 minutes in both cell lines and was significantly higher than control treatment at 15 minutes. However, the internalization rate appeared to slow down between 15 to 45 minutes, which still significantly higher than the control treatment in cells expressing only hC5a1 receptors. However, the co-expression of hC5a2 affected hC5a1 internalization at 45 minutes and rendered it insignificantly different from the control treatment. In case of C5a desArg treatment, hC5a1 receptor internalization follows nearly similar pattern. However, at 45 minutes it becomes not significantly different from buffer control in both cell lines. This could be due the lower potency of C5a desArg in comparison to C5a.

6.3 Ligand-induced hC5a2 receptor internalization when co-expressed with hC5a1:

hC5a2 receptor internalization was explored in RBL expressing either hC5a2 alone or co-expressing both hC5a1+hC5a2**.** hC5a2 internalization was measured at different time points after treatment with different ligands. The ligands used in this assay were: C5a, C5a desArg or peptide RHYPYWR. At early time point (5 minutes) of the internalization course of the receptor, the hC5a2 receptor did not internalize at significant percentage upon treatment with any of the ligands (Figure 6.2 a). Although hC5a2 receptors showed higher percentages of internalization at 15 minutes than in 5 minutes, it did not reach statistical significance. This could be because of the higher percentage of constitutive hC5a2 internalization (internalization without ligand stimulation) at this stage (Figure 6.2 b). After 45 minutes, it appeared that hC5a2 underwent high levels of ligand-induced internalization by C5a and C5a desArg, but not for the peptide alone (Figure 6.2 c). This internalization was similar in cells expressing both receptors hC5a1+hC5a2 or cells with only hC5a2. C5a and C5a desArg showed

similar effects on hC5a2 internalization and this is because hC5a2 binds both ligands with similar affinities (Scola et al, 2007). In addition, hC5a2 recruitment of β-arrestin2 was reported to be similar for C5a or C5a desArg treatment (Croker et al., 2014)

In summary, at early time points, 5 and 15 minutes, the C5a/C5a desArg-induced internalization was not significant. However, hC5a2 continued to internalize upon C5a/C5a desArg stimulation and became significant for the difference between ligand and control at 45 minutes incubation with C5a or C5a desArg. In addition, the hC5a2 internalization was similar for C5a or C5a desArg stimulation.

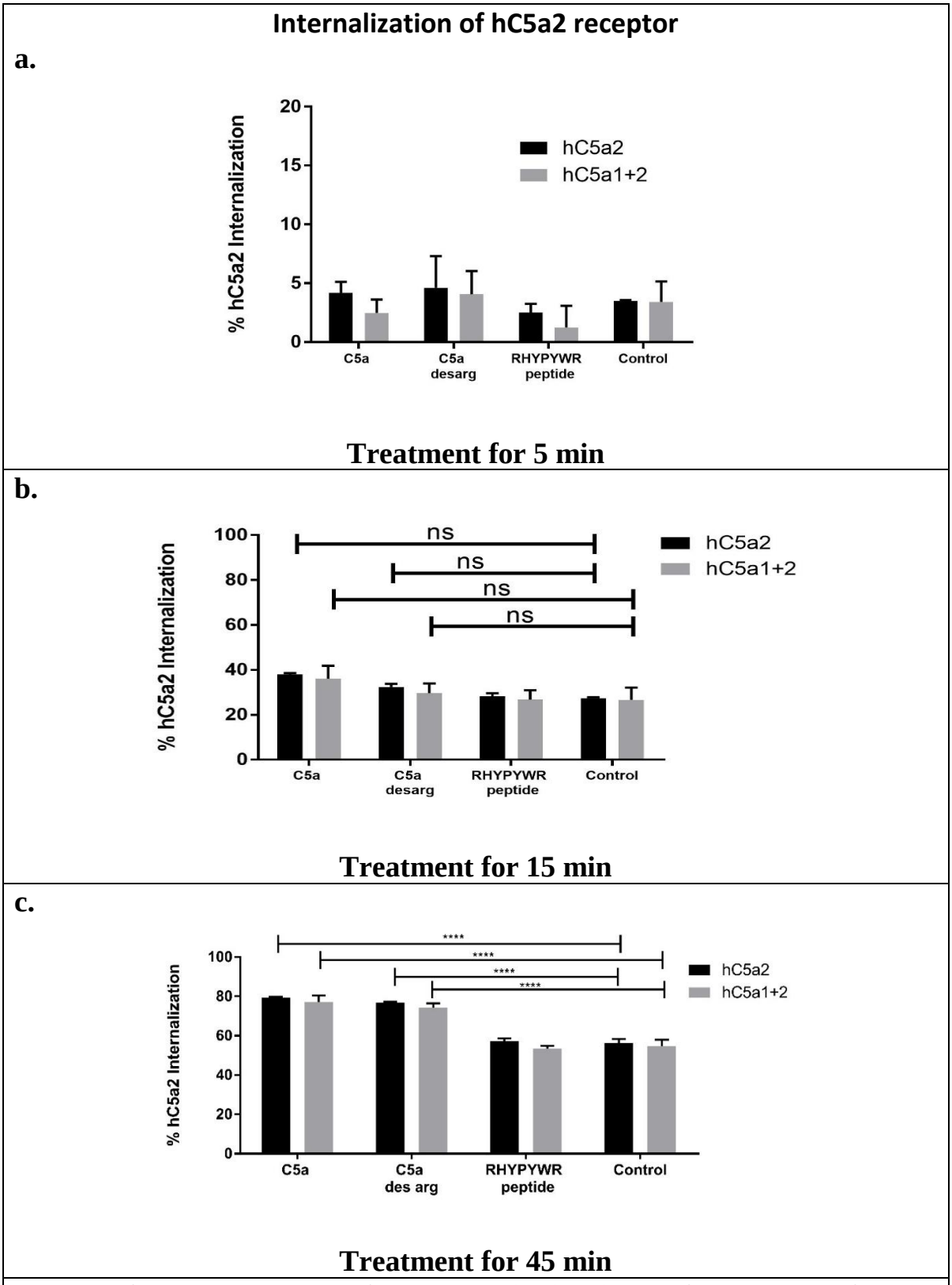
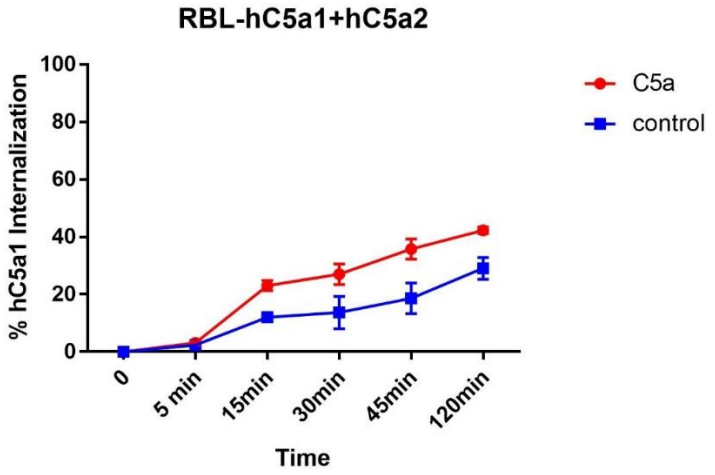


Figure 6.2: hC5a2 receptor internalization. Ligand induced internalization using either C5a, C5a desArg or RHYPYWR peptide for 5 min (a), 15 min (b) and 45 min (c). Cell expressing either hC5a2 alone or hC5a1+2 were used. Data are mean±SEM of 3 independent experiments in duplicate. Two-way ANOVA test with Sidak's multiple comparisons test were performed to test statistical significance. ****, P≤0.0001, ns, non-significant.

6.4 Comparison between hC5a1 and hC5a2 receptor internalization when co-expressed in RBL cells:

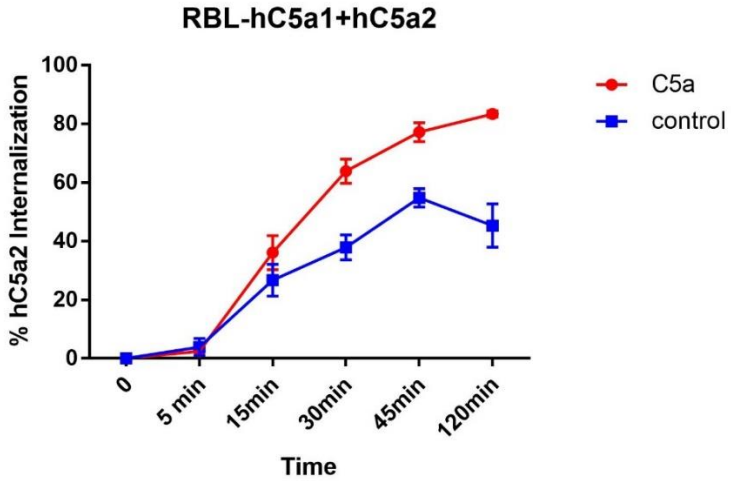
The behaviour of hC5a1 and hC5a2 receptors internalization over time was examined and compared when the two receptors are co-expressed in RBL cells. In case of hC5a1 receptor, it appeared that C5a stimulation induced higher receptor internalization than control treatment over time (Figure 6.3 a). On the other hand, hC5a2 behaves differently in response to different treatment. When there is no C5a, hC5a2 internalizes for about 45 minutes and then probably recycles back to the cell surface (constitutive internalization and recycling; Scola et al., 2009) (Figure 6.3 b). However, when C5a is present in the extracellular (EC) compartment, hC5a2 receptor continues to internalize for longer time points. This probably suggests scavenging role of hC5a2 for removing C5a from the EC compartment to prevent long-term stimulation for hC5a1 receptor as discussed in the discussion section below (Figure 6.3 b).

a.



Source of Variation	P value summary
Interaction	ns
time	****
treatment	****

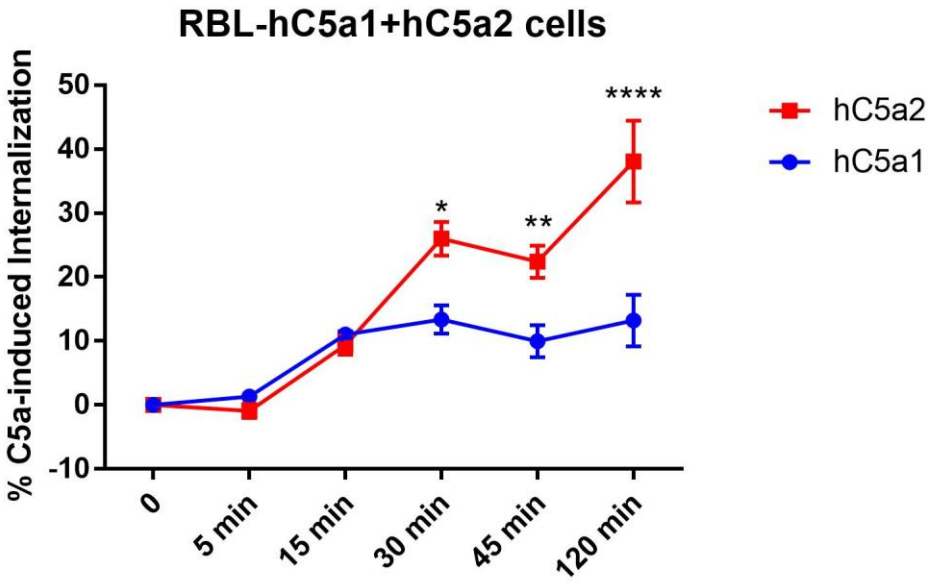
b.



Source of Variation	P value summary
Interaction	***
time	****
treatment	****

Figure 6.3: Receptors internalization time course for hC5a1 or hC5a2 co-expressed on RBL cells. The graphs describe the different patterns of hC5a1 (a) or hC5a2 (b) receptors internalization when co-expressed on RBL. Two-way ANOVA test was performed to test statistical significance. The table shows: interaction, the systematic changes (pattern) of receptor internalization over time in response to different treatment; time, receptor internalization over time; and treatment, receptor internalization upon C5a or control treatment. Data are the mean±SEM for 3 independent experiment in duplicate. ns, non-significant, ***, P≤0.001 and ****, P≤0.0001.

To further illustrate the internalization patterns of hC5a receptors after C5a stimulation, the C5a-induced hC5a1 and hC5a2 receptor internalization was compared after subtracting constitutive (control treatment) internalization (Figure 6.4). The receptors behaviour was different per the receptor type and at various time points. At early time points, both hC5a1 and hC52 displayed ligand dependent internalization. However, hC5a1 receptor internalization slows down after 15 minutes and probably reaches plateau phase. In contrast, hC5a2 continues to internalize after 15 minutes (Figure 6.4). This could explain why hC5a1 internalization slows down after 15 minutes: possibly, hC5a2 receptor uptakes the C5a from the EC compartment and internalizes it for degradation (Scola et al., 2009). Therefore, hC5a1 is not exposed to C5a at late time points and stops activation and subsequent internalization. Therefore, this could be part of the desensitization process of hC5a1 receptor. This role of hC5a2 could be supported more by the previous result that showed when hC5a1 expressed alone, its C5a-mediated internalization continued to be significant after 15 minutes to reach 45 minutes (Figure 6.1 c). However, when hC5a2 co-expressed with hC5a1, the C5a-mediated hC5a1 internalization became insignificant at 45 minutes in comparison to control treatment (Figure 6.1.c).



Source of Variation	P value summary
Interaction	****
Time	****
Receptor type	****

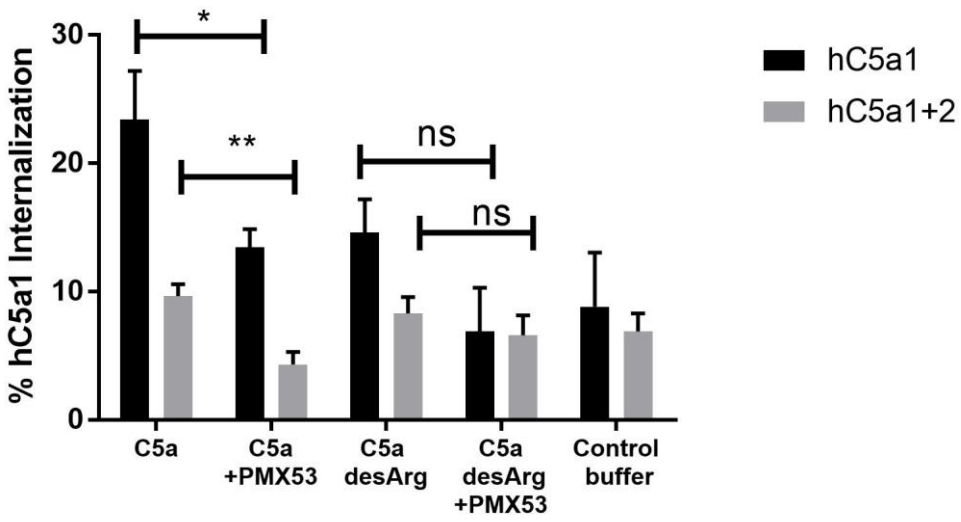
Figure 6.4: C5a-induced hC5a1 and hC5a2 receptors internalization. RBL-2H3 co-expressing hC5a1 and hC5a2 receptors treated with C5a or control buffer at 5,15,30,45 and 120 minutes. The C5a-induced receptor internalization was calculated by subtracting the buffer treated from C5a treated internalization. hC5a1 and hC5a2 show different patterns of internalization upon ligand treatment. hC5a1 internalize rapidly within 15 minutes and reaches probable plateau phase. However, hC5a2 internalization continues after 15 minutes with C5a stimulation. Data are the mean±SEM for 3 independent experiment in duplicate. Two-way ANOVA test with Sidak's multiple comparisons test were performed to test statistical significance. The table shows: interaction, the systematic changes (pattern) of C5a-induced receptor internalization in hC5a1 and hC5a2 over time; time, receptor internalization over time; and receptor type, hC5a1 or hC5a2 receptor internalization. * $P \leq 0.05$, ** $P \leq 0.01$, **** $P \leq 0.0001$.

6.5 Exploring C5a receptors heterodimerization using receptors internalization assay:

This test was performed to examine the internalization forms in which hC5a receptors internalize i.e. whether in monomers or heterodimers. Internalization was measured at 15 and 45 minutes in cells expressing either hC5a1 alone or both hC5a1+2. C5a1 receptor inhibitor PMX53 was used to allow C5a to work selectively on the C5a2 receptor. Figure 6.5 shows the effect of hC5a1 receptor inhibition on receptor endocytosis. C5a induced hC5a1 receptor internalization was inhibited significantly at both time points and in both cell lines in the presence of C5a1 receptor inhibitor PMX53. C5a desArg induced hC5a1 internalization was also inhibited but the difference did not reach the statistical significance at 15 minutes (Figure 6.5 a), probably because C5a desArg is a partial agonist for hC5a1 and the internalization is not as high as the internalization induced by C5a at this time point. However, it became significant in RBL-hC5a1 cells at 45 minutes because the difference in internalization between the PMX53-treated and non-treated at this time point was high (Figure 6.5 b).

The internalization of hC5a1 receptor in the presence of PMX53 and C5a stimulation

a.



b.

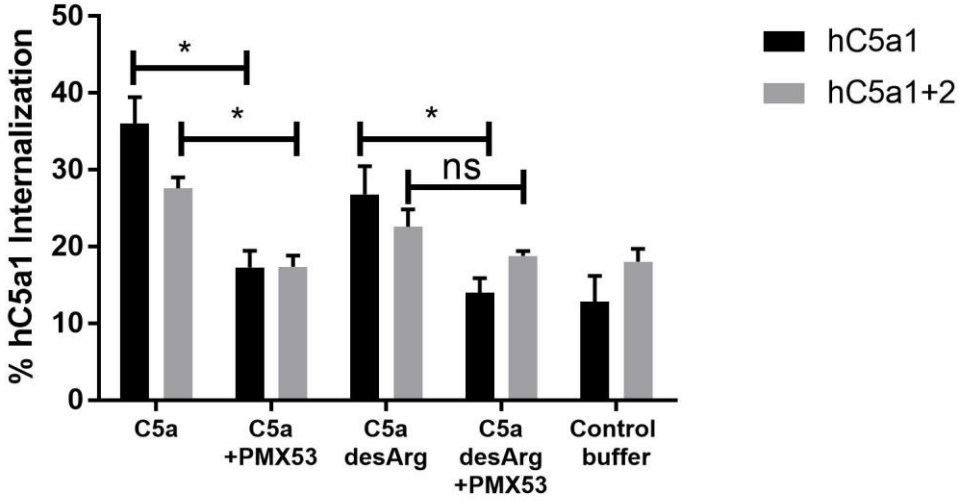


Figure 6.5: hC5a1 receptor internalization in the presence of hC5a1 receptor inhibitor PMX53. The graph shows the hC5a1 receptor internalization at 15 minutes (a) and 45 minutes (b) in RBL expressing hC5a1 alone or co-expressing hC5a1+hC5a2. Cells were pre-incubated with 1 µM PMX53 for 10 minutes before adding C5a or C5a desArg. Data are the mean±SEM for 3 independent experiment in duplicate. Two-way ANOVA was performed with Tukey's multiple comparison to test statistical significance. *, P=≤0.05, **, P=≤0.001, and ns, non-significant.

hC5a2 receptor internalization was also tested in the presence of the hC5a1 inhibitor PMX53. Cells co-expressing both receptors had been pre-incubated with 1 µM PMX53 for 10 minutes and then C5a or C5a desArg were added. Internalization was measured at 45 minutes because hC5a2 receptor internalization is significant at 45minutes (based on previous work, Figure 6.2 c). There was no significant difference in hC5a2 receptor internalization in the presence or absence of the hC5a1 inhibitor PMX53 (Figure 6.6).

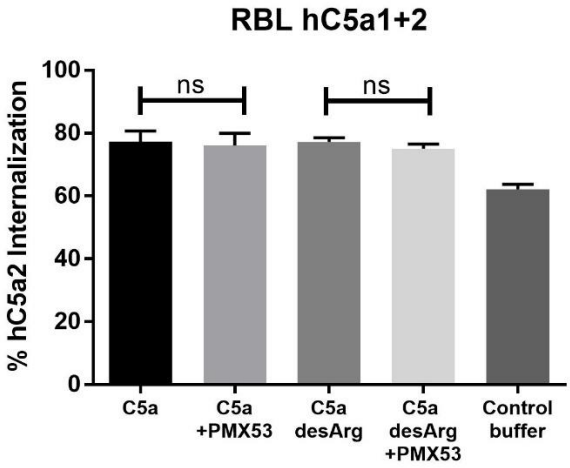


Figure 6.6: hC5a2 receptor internalization in the presence of hC5a1 receptor inhibitor PMX53. The graph shows hC5a2 internalization in cells co-expressing both hC5a1 and hC5a2 at 45 minutes in response to C5a or C5a desArg with or without pre-treatment with PMX53 for 10 minutes. Data are the mean±SEM for 4 independent experiment in duplicate. One way ANOVA was used with Tukey's multiple comparison to test statistical significance. ns, non-significant.

Taken together, it appeared that inhibition of hC5a1 receptor hindered its internalization, but it did not affect hC5a2 internalization in cells co-expressing both hC5a receptors. Therefore, it could be speculated that the two receptors are not in direct physical contact, at least when they are internalizing. This is because it would be expected (if the receptors work in heterodimers) that either both receptors are inhibited from internalization when hC5a1 is inhibited, or both are internalized when the internalization of hC5a2 occurs.

6.6 Discussion:

Many GPCR undergo internalization, which is manifested by decrease in the receptor expression on the cell surface. This could be induced by ligand or could be constitutive (without ligand binding). In this chapter, the internalization of hC5a receptors was studied during a time course with or without ligand treatments. It seems that C5a1 and C5a2 receptors behave differently in terms of their constitutive and ligand-induced internalization. First, the constitutive internalization of C5a2 was higher than C5a1. This could be due to constitutive complexing with β-arrestin2 (Croker et al., 2013). Second, C5a-induced internalization of hC5a1 reached a maximum and then plateaued at earlier stages, while hC5a2 internalization became significant at later time points, in comparison to hC5a1, and continued for a longer time. All these findings may suggest a possible scavenger function of hC5a2. It could be speculated that after exposure to C5a, hC5a1 receptor responds to C5a and internalizes rapidly. hC5a2 endocytosis is less significant directly after exposure to C5a, allowing, hC5a1 to function. However, after long exposure to C5a, hC5a2 starts to uptake C5a and internalizes at a higher rate to decrease hC5a1 exposure to the agonist. In addition, the higher rate of hC5a2 internalization could affect the availability of the intracellular internalization machinery for hC5a1. It was reported that GPCR internalization via clathrin-coated pits was inhibited by expression of a dominant suppressor mutant of β-arrestin1. This mutant β-arrestin1 resulted in 50% reduction in receptor internalization. On the other hand, ligand-induced internalization of β_2-adrenergic receptors was modestly increased when wild type β-arrestin1 was overexpressed. In addition, expression of dynamin mutant resulted in reduction of receptors sequestration by about 70% (Daaka et al., 1998, Ferguson et al., 1996, Zhang et al., 1996b). Thus, hC5a2 may compete for the internalization machinery proteins, such as β-arrestin1, dynamin, and so affect hC5a1 endocytosis. Therefore, hC5a1 did not continue to internalize and reached

plateau phase. In this case, hC5a2 is playing the role of a decoy receptor to prevent further inflammation that could lead to tissue damage. These findings are in agreement with the finding of Scola et al who demonstrated that C5a2 is a recycling decoy receptor (Scola et al., 2009). This has been suggested by several observations. First, C5a2 was completely unable to couple to G proteins even after inserting C5a1 motifs at the critical G protein activation sites. Second, C5a2 could take up C5a and C5a desArg from the extracellular fluid and accumulated them within the cell through clathrin-dependent internalization. Then, these ligands were retained and degraded intracellularly. Nevertheless, C5a internalization by C5a1 was slower and it was released back to the extracellular environment without apparent degradation (Scola et al., 2009). Therefore, C5a2 seems to play a role in removal of the active receptor agonist from the surrounding environment to probably prevent chronic inflammatory response.

One of the difficulties in exploring and clarifying the controversy around C5a2 function is the lack of selective ligands. Recently, peptides have been reported to selectively target C5a2 and thought to induce recruitment of β-arrestin2 to C5a2 receptor (Croker et al., 2016). One of these peptides was used in the current study to explore its effect on C5a-mediated receptors internalization. This peptide did not show discernible effects in this test. This could be due to different β-arrestins subtypes being involved in receptor endocytosis. The expression of β-arrestin1 mutant resulted in inhibition of β2 adrenergic receptors internalization (Daaka et al., 1998). Therefore, internalization function of the receptors is mediated by different β-arrestin subtypes (β-arrestin1) from the one that is the peptides probably induce C5a2 receptor to work on (β-arrestin2).

The internalization of the receptors can also be used to assess potential physical interactions such as heterodimerization. This could be assessed by examining the possibility of co-internalization of the two receptors upon ligand

binding of either receptors (Figure 6.7). This concept was used to examine heterodimerization for many receptors (reviewed in Milligan, 2004).

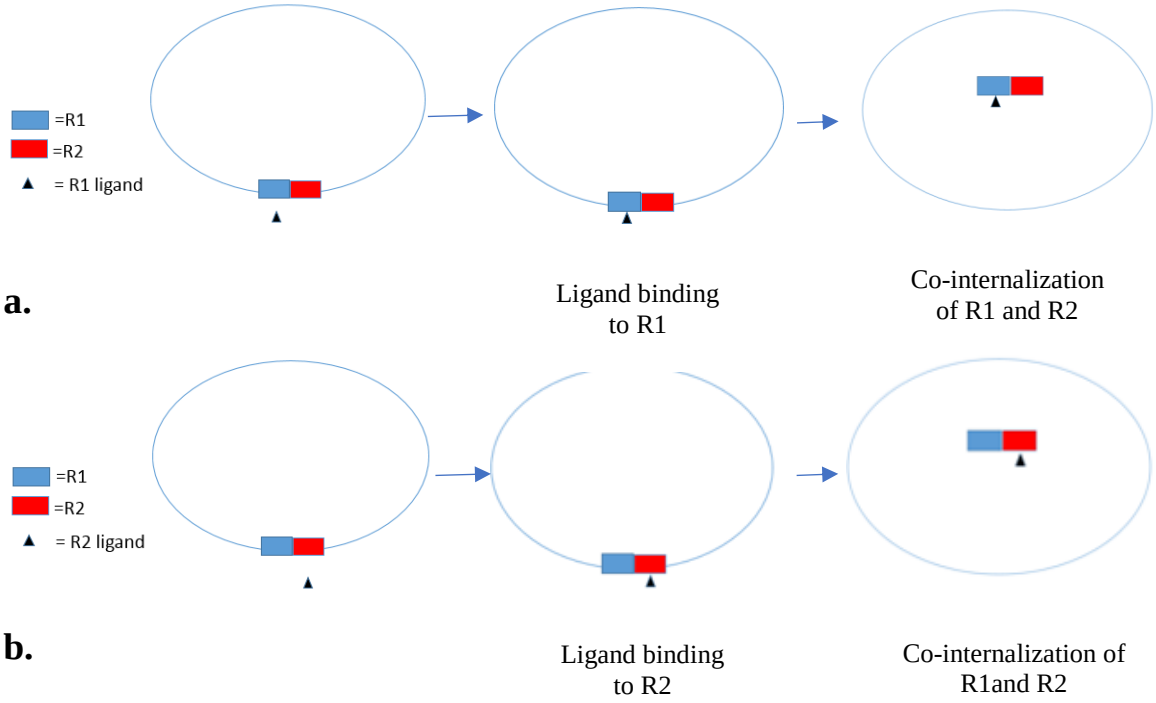


Figure 6.7: The concept of co-internalization of heterodimers upon stimulation of one receptor. If two receptors are working in dimers, internalization of both receptors should occur upon stimulation of either of them. The figure shows co-internalization of receptor 1 (R1) and receptor 2 (R2) after using R1 ligand (a) or R2 ligand (b).

For example, internalization of δ-opioid receptors (DOP) was reported after stimulation of β-adrenergic receptors with isoproterenol when co-expressed together. This co-internalization was specific to DOP receptors because isoproterenol did not cause internalization of κ-opioid receptors (KOP) when co-expressed with β-adrenergic receptors (Jordan et al., 2001). Similarly, co-internalization of NK1 receptors and μ-opioid receptors (MOP) was detected when stimulating either receptors (Pfeiffer et al., 2003). To use this concept to explore hC5a receptors heterodimerization, hC5a1 receptor inhibitor PMX53 was used to allow C5a to work selectively on hC5a2. There are three possibilities to

the outcome of such treatment (Figure 6.8). First, if the two receptors did not internalize this means that they are in dimer form and internalization of hC5a2 was inhibited because it is in contact with hC5a1. Second, if both receptors internalized this may mean again that the receptors are in dimer form and hC5a1 was internalized indirectly by hC5a2 internalization. Third, if hC5a1 receptor was inhibited and only hC5a2 internalized, this might suggest that they are not in dimer form or direct physical contact during the internalization process at least. What happened after treatment with C5a1 inhibitor PMX53 is that hC5a1 internalization was inhibited, while hC5a2 was not, which supports the third possibility. However, this could be due to dissociation of surface receptor dimers that occurs after internalization. A similar scenario was suggested for the β-adrenergic receptors (Lan et al., 2011). Nevertheless, previous chapters' results suggested that the C5a receptors were not delivered to the cells surface as heterodimers.

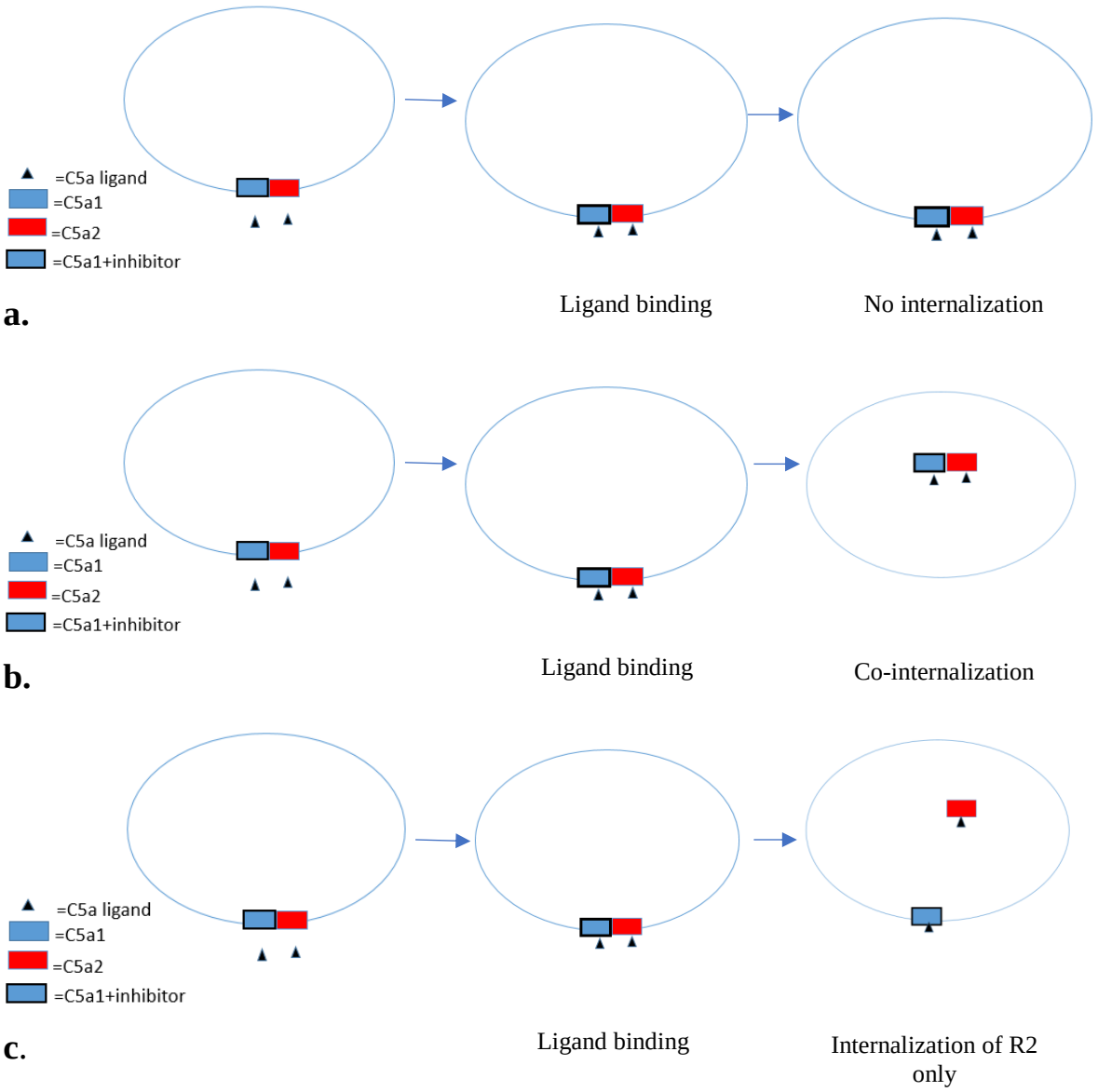


Figure 6.8: The possibilities of C5a-induced C5a receptors internalization after pre-treatment with PMX53 C5a1 inhibitor. RBL cells co-expressing C5a1 and C5a2 receptors were pretreated with PMX53 for 10 minutes before adding C5a. If the two receptors did not co-internalize and both of them remained on the cell surface after inhibition C5a1 with PMX53, this means they are working in dimers and hindering one of the receptors prevented the interaliztion of the other one (a). If the two receptors co-internalized, it means they are also working in dimers and internaliztion of C5a1 happened due to internaliztion of C5a2 (b). However, if C5a2 internalized, but C5a1 not, this suggests the receptors are not working as dimers (c).

Chapter seven

The Effect of C5a Receptor Interaction on Signal Transduction

Chapter 7: The effect of C5a receptor interaction on signal transduction

7.1 Introduction:

In this chapter, the possible effect on signal transduction of co-expressing hC5a2 receptor with hC5a1 receptor on RBL-2H3 cells was explored using untagged wild type receptors. The possible receptor interaction was tested by examining various cellular responses. These involve ligand binding and the signal transduction of the receptors upon ligand stimulation such as intracellular Ca^{2+} mobilization and enzyme release from the RBL cells. The enzyme secretion was examined in RBL cells expressing either hC5a1, hC5a2 or both hC5a1+hC5a2 in response to C5a and compared to stimulation by IgE. The release assay was also used to test the possible effect of peptide that selectively target hC5a2 receptors (RHYPYWR), which is thought to be work through recruitment of β-arrestin 2 (Croker et al., 2016). The effect of hC5a2 on hC5a1 was also examined using MAPK pathways assays. MAPK pathways involve three well-characterized pathways that regulate many physiological processes in response to various stimuli such as inflammatory mediators and cytokines. These pathways include: the extracellular signal-regulated kinases (ERKs); the c-jun amino terminal kinases (JNKs); and the p38 pathway. These MAPKs are regulated by phosphorylation. The phosphorylated MAPKs can be measured using various techniques. In this chapter, the total and phosphorylated MAPKs were measured in response to C5a and C5a desArg treatment and compared in cells expressing either hC5a1 or both hC5a1+hC5a2 to examine the potential influence of hC5a2 on hC5a1.

7.2 hC5a1 receptor expression assay:

The assay was done to compare the expression level of hC5a1 receptor on the RBL transfected with hC5a1 with or without hC5a2 receptor. The cells were treated with primary mouse anti-hC5a1 antibody for 1 hour and then stained with secondary antibody. The median fluorescent intensity was measures by flow cytometer and did not show difference between the two cell lines (Figure 7.1). The data show that hC5a1 receptor expression was similar in cells that express hC5a1 alone or hC5a1+hC5a2.

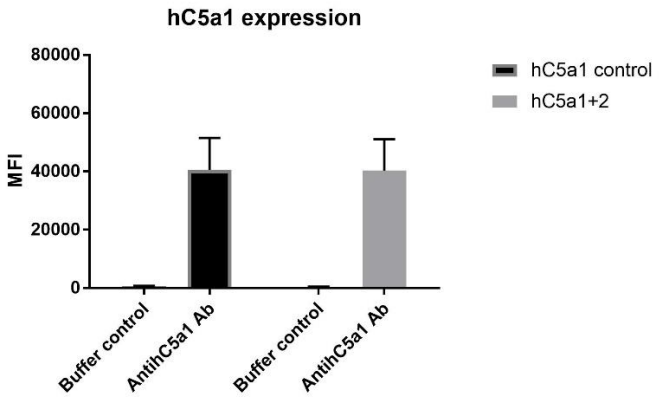


Figure 7.1: Human C5a1 receptor expression. The graph shows the flow cytometric results of hC5a1 receptor stained with Alexa-633 anti-mouse antibody after treatment with primary mouse antihC5a1 antibody. Data are the mean±SEM of 3 independent experiments in duplicate. MFI, median fluorescence intensity.

7.3 Ligand binding assay in RBL co-expressing hC5a1 and hC5a2 receptors:

The ligand binding assay was performed as described in section (2.3.2) to investigate the ligand binding affinity of cells that co-express both hC5a receptors in comparison to cells expressing hC5a1 alone. The result shows that the ligand binding affinity of RBL-RBL-hC5a1 and RBL-hC5a1+2 were nearly similar, IC50= 17.2 nM (-logIC50=7.763) and 13.1 nM (-logIC50=7.883), respectively

(Figure 7.2). This could be due the more intracellular localization of hC5a2 or that both receptors bind C5a with similar affinities.

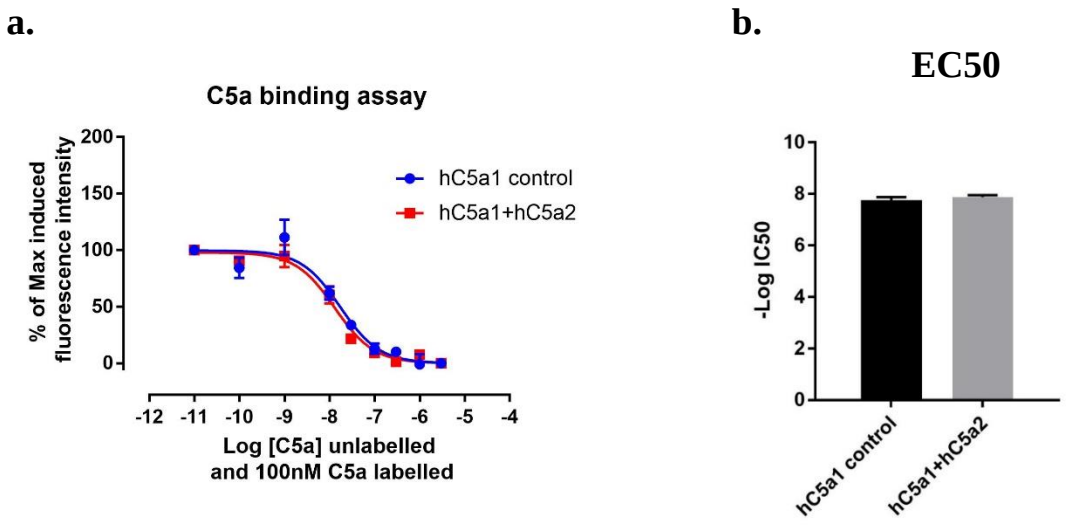


Figure 7.2: C5a binding assay for RBL cells co-expressing hC5a1 and hC5a2 receptors using competitive fluorescent-labelled C5a binding assay. a. The graph shows the inhibitory dose response curves for the cells treated with mixtures of serial dilutions of unlabelled C5a with 100 nM Alexa-488 labelled C5a. b. Bar chart for the IC50. Data are the mean±SEM of 3 independent experiments in duplicate.

7.4 Intracellular Ca^{2+} mobilization assay:

This test was used to test the signalling of the hC5a1 receptor at the level of intracellular Ca^{2+} release in the presence or absence of hC5a2 receptor**.** The test was performed as described in section (2.3.3). There was no obvious difference in response in ligand stimulation in the presence or absence of hC5a2 with approximately similar EC50 1.67 nM and 1.40 nM for hC5a1 and hC5a1+2 cells, respectively (Figure 7.3).

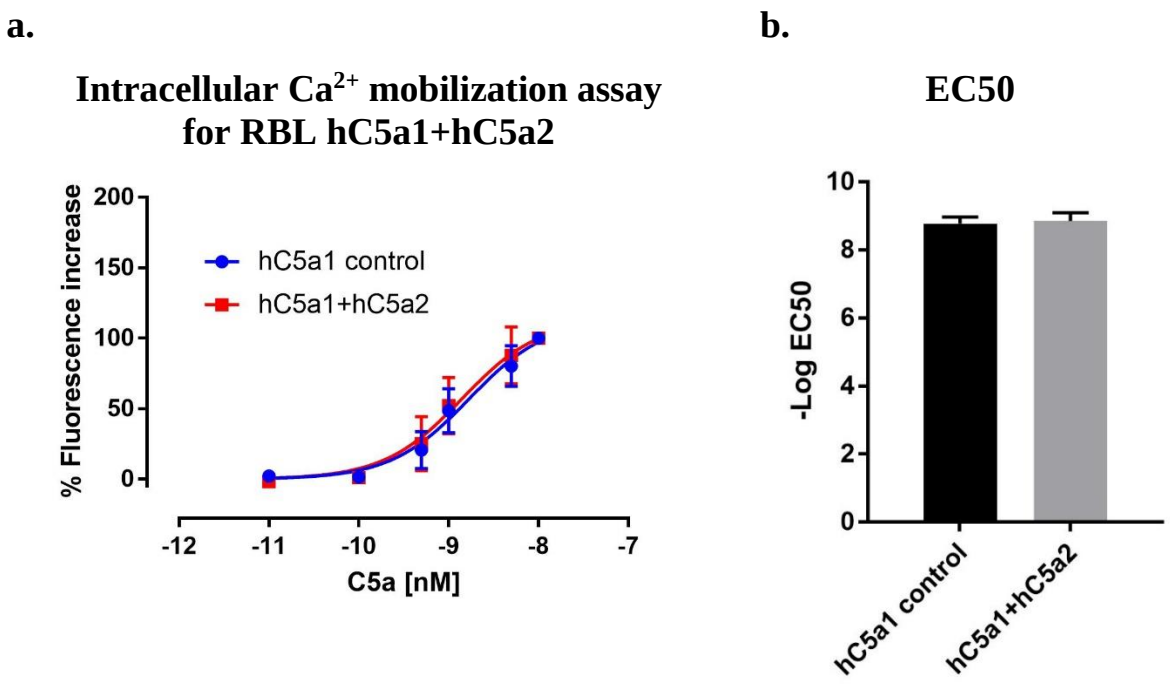


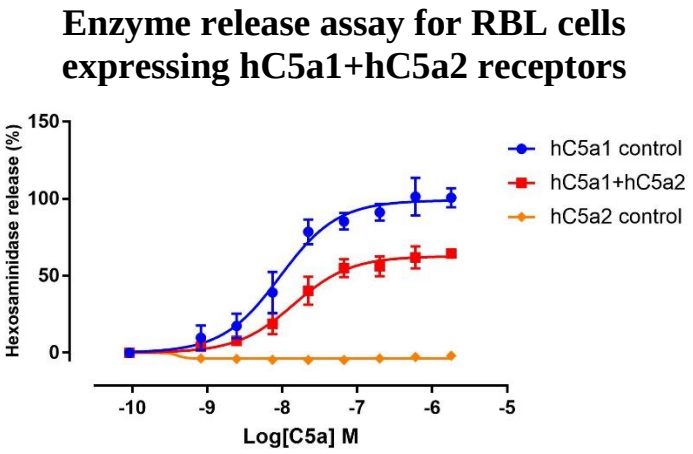
Figure 7.3: Intracellular Ca^{2+} mobilization assay for RBL cells co-expressing hC5a1 and hC5a2 receptors. a. The stimulatory dose response curves show the intracellular Ca^{2+} response to serial dilutions of C5a. C5a was added after 20 second of measuring basal fluorescence. b. the EC50 bar chart. Data are the mean±SEM of 3 independent experiments in duplicate.

7.5 Enzyme release assay for RBL-hC5a1+hC5a2:

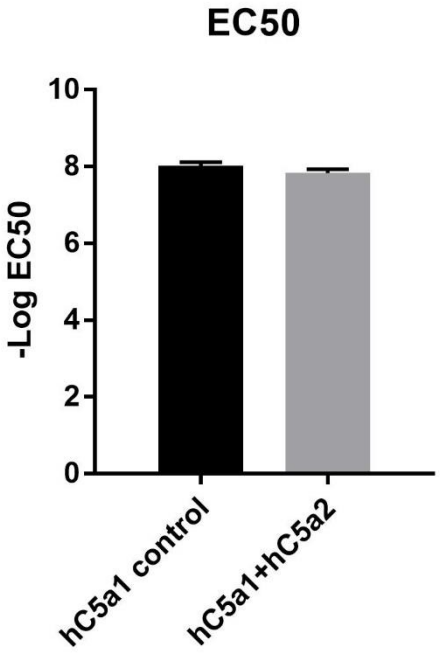
The effect of hC5a2 receptor co-expression with hC5a1 was further explored by testing another functional response, the ligand-induced degranulation. RBL cells, which express both hC5a1 and hC5a2, were tested for β-hexosaminidase enzyme release after stimulation with C5a for 15 minutes. Two control cell lines were used, RBL cells expressing either hC5a1 or hC5a2 receptors. There was a significant difference in maximum enzyme release between cells expressing both hC5a1+hC5a2 receptors from those who expressing only hC5a1, while cells expressing only hC5a2 receptor showed no response to C5a in terms of enzyme release (Figure 7.4).

The enzyme release property of RBL cells in response to C5a was then compared to enzyme release in response to IgE sensitization. This was performed to check if the difference in enzyme release was specific to the C5a stimulation and whether hC5a1 expression renders the cells more sensitive to all degranulation stimuli. The same cell lines, RBL-hC5a1 and RBL-hC5a1+hC5a2, were tested for IgE-mediated enzyme release. The pattern of enzyme release was similar to the C5a-mediated response i.e. the hC5a1 alone expressing cells showed higher enzyme release in comparison to cells co-expressing both receptors (Figure 7.5 a). Furthermore, the IgE-mediated enzyme release was explored for RBL cells expressing either no receptors (non-x) or other receptors such as hC5a2, hC3a and hC5a1. The results demonstrated that hC5a1 receptor expressing cells always have the highest percentage of enzyme release in comparison to other cell lines (Figure 7.5 c). However, the co-expression of hC5a2 with hC5a1 decreased the enzyme release to the levels of RBL-non-x cells. These data suggest that hC5a1 receptor expression could increase the sensitivity of RBL cells for degranulation and it could exert a constitutive activity that is increased upon stimulation and that the co-expression of hC5a2 with hC5a1 decreases this sensitivity to the basal level. The constitutive activity of hC5a1 could be expected because hC5a1 was reported to be pre-coupled to G proteins (Siciliano et al., 1990), which is unusual among other GPCR.

a.



b.



c.

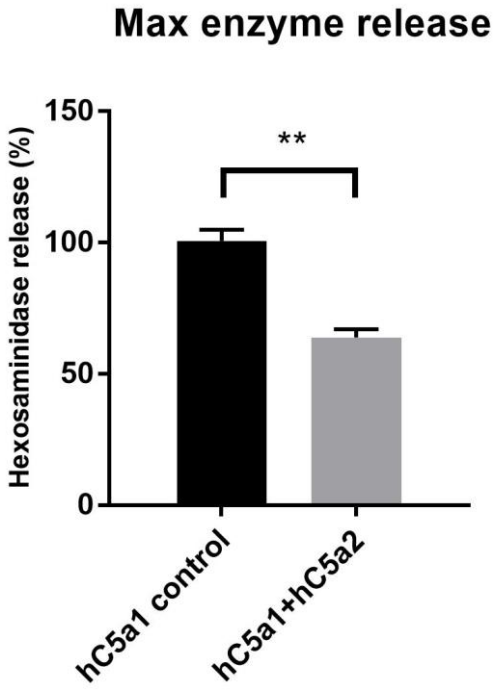
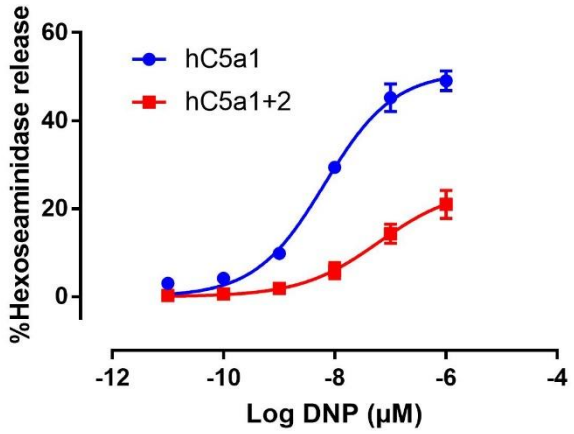


Figure 7.4: C5a-mediated enzyme release assay for RBL cells express both hC5a1 and hC5a2. a. Dose response curve for hC5a receptors. Enzyme release was measured in response to 1/3 serial dilutions of hC5a starting with 2 µM. b. and c. bar charts for the EC50 and maximum enzyme release, respectively. Data are mean±SEM from 3 independent experiments in duplicate triplicate. Unpaired t test was used to test statistical significance. **, $P \leq 0.01$.

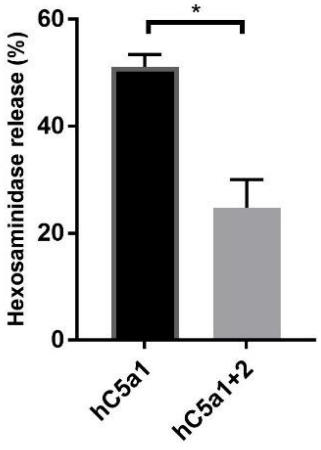
a.

IgE-mediated enzyme release for RBL-hC5a1 and RBL-hC5a1+hC5a2



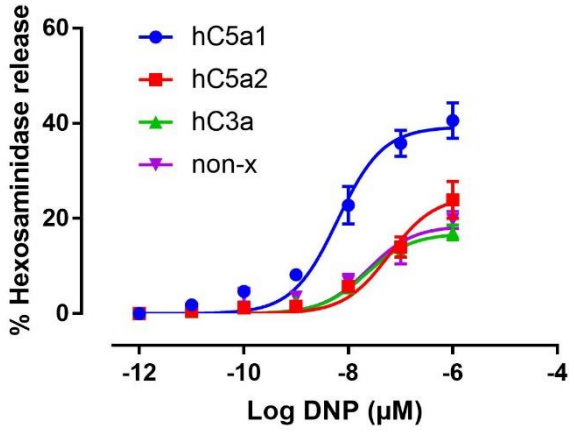
b.

Max enzyme release



c.

Comparison of IgE-mediated enzyme release for RBL expressing hC5a1 with RBL cells expressing other receptors or no receptor



d.

Max enzyme release

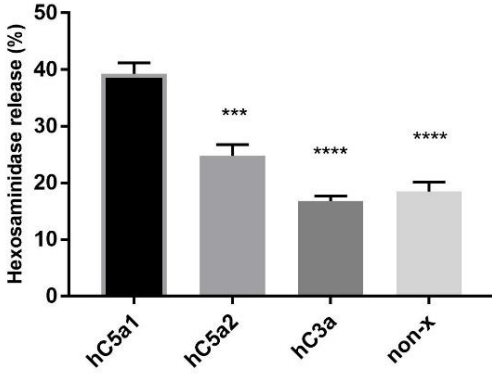


Figure 7.5: IgE-mediated degranulation assay for RBL cells expressing different receptors. a. comparison between hC5a1 alone or hC5a1+2 transfected RBL cells. b. Bar chart for maximum enzyme release from the cells in graph (a). t test was used. c. Comparison between untransfected RBL (non-X), hC5a1, hC5a2, and hC3a receptors. d. Bar chart for maximum enzyme release from the cells in graph (c). One way ANOVA was used to test statistical significance. Data are mean±SEM from 3 independent experiments in duplicate.

7.6 C5a-induced enzyme release assay in presence of peptide RHYPYWR:

The same enzyme release assay was performed but in this case the cells were pre-incubated with 100 µM peptide RHYPYWR (a selective agonist for hC5a2 receptor) for 10 minutes before treatment with C5a. The idea is to see if there is any effect of this peptide on cells expressing hC5a2 receptor in addition to hC5a1. There is no obvious difference in enzyme release between RBL cells that express both hC5a1+hC5a2 or only hC5a1, when treated with either peptide or control buffers (Figure 7.6).

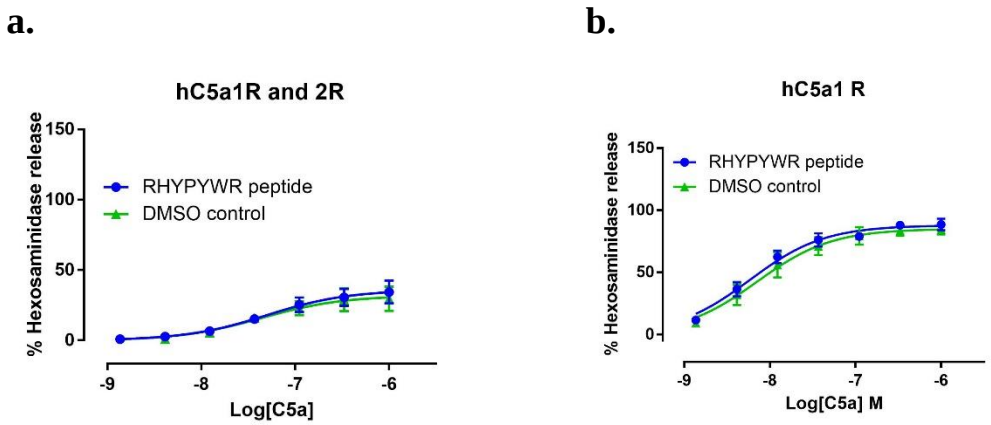


Figure 7.6: Enzyme release assay for RBL cells express both hC5a1 and hC5a2 (a) or hC5a1 (b) in the presence of peptide RHYPYWR. Cells were pre-incubated with 100 µM peptide RHYPYWR for 10 minutes before adding C5a. Enzyme release was measured in response to 1/3 serial dilutions of hC5a starting with 1 µM. Data are mean±SEM from 3 independent experiments in duplicate.

7.7 MAPK signalling:

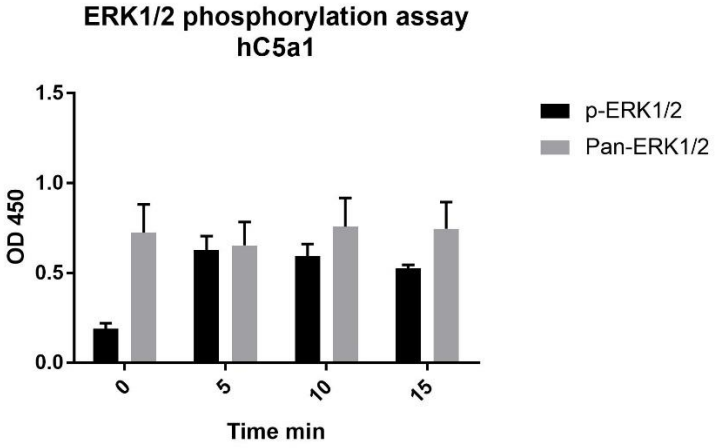
The effect of hC5a2 expression on hC5a1 signalling was further analysed using MAPK signalling pathways. The three major MAPK signalling pathways (ERK1/2, P38 and JNK) were tested after treatment with C5a or C5a desArg. The results were as follows:

7.7.1 C5a-mediated MAPK signalling:

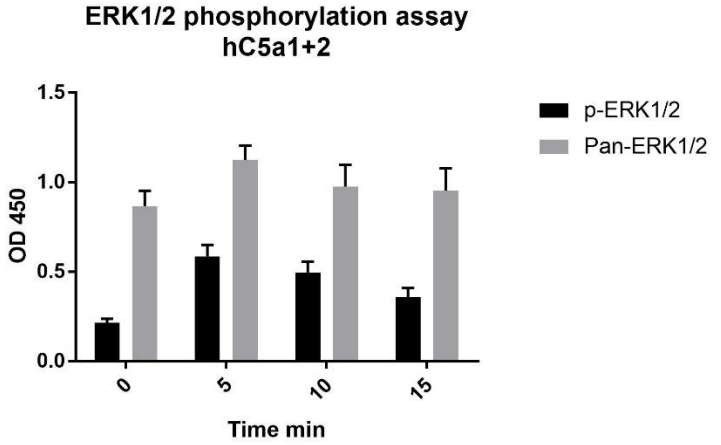
7.7.1.1 ERK1/2 phosphorylation assay:

The C5a-mediated ERK1/2 activation was measured in RBL cells expressing hC5a1 alone or co-expressing both hC5a1+hC5a2 receptors. The cells were stimulated with C5a at 0.5 µM for 5, 10, 15 minutes. The phosphorylation of ERK1/2 increased from 5 minutes in both cell lines (Figure 7.7 a and b). However, the phosphorylated ERK1/2 was lower in cells co-expressing hC5a1+hC5a2 receptors (Figure 7.7 c).

a.



b.



c.

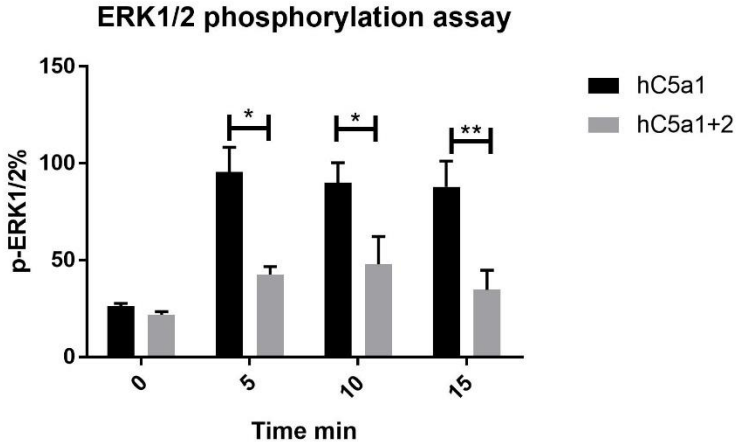
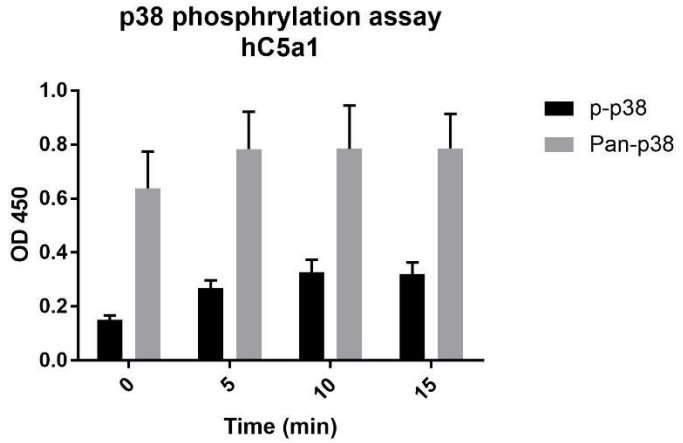


Figure 7.7: C5a-mediated ERK1/2 phosphorylation assay. RBL cells expressing either hC5a1 (a.) or both hC5a1+2 receptors (b.) were used. Cells were incubated with 0.5 µM C5a for different time points. Phosphorylated (p-ERK1/2) and total ERK1/2 (pan ERK1/2) were measured using cell-based ELISA assay. c. The percentage of ERK1/2 phosphorylation was compared between hC5a1 alone or hC5a1+2 cell lines. Data are mean±SEM from 3 independent experiments in duplicate. Two-way ANOVA with Sidak's multiple comparison test were used to test statistical significance. *, P≤0.05 and **, P≤0.01.

7.7.1.2 p38 phosphorylation assay:

The p38 phosphorylation assay was performed using hC5a1 alone or hC5a1+2 RBL cell lines after incubation with 0.5 µM C5a. The phosphorylated and total p38 were measured at different time points. The activation of p38 pathway in RBL-hC5a1+2 was lower than RBL cells expressing hC5a1 alone after 10 and 15 minutes treatment (Figure 7.8).

a.



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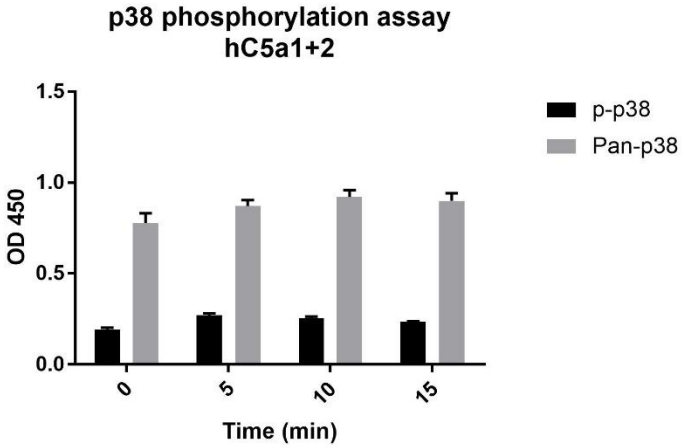


c.

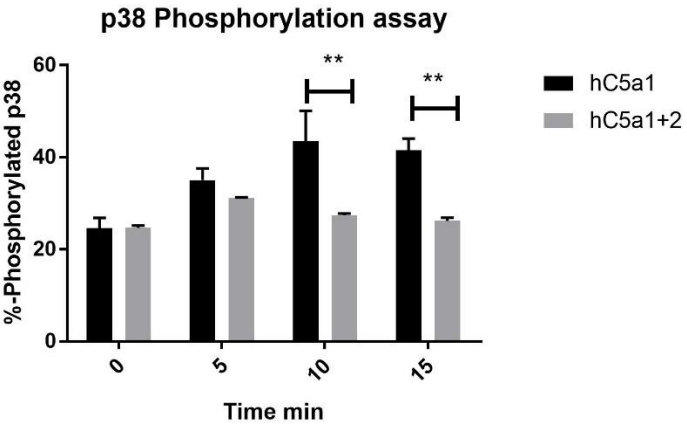
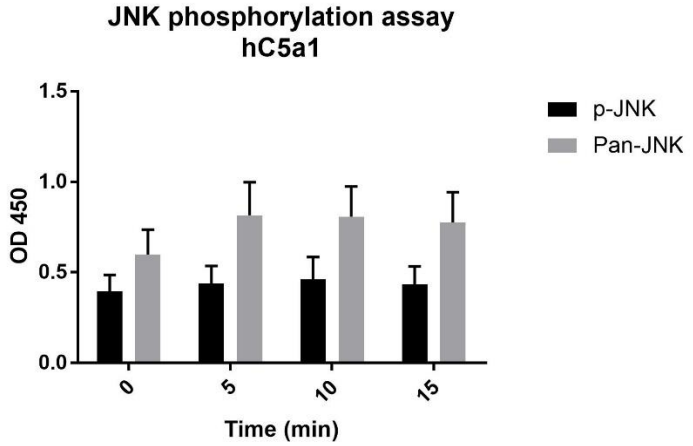


Figure 7.8: C5a-mediated p38 phosphorylation assay. RBL cells expressing either hC5a1 (a.) or both hC5a1+2 receptors (b.) were used. Cells were incubated with 0.5 µM C5a for different time points. Phosphorylated (p-p38) and total p38 (pan-p38) were measured using cell-based ELISA assay. c. The percentage of p38 phosphorylation was compared between hC5a1 alone or hC5a1+2 cell lines. Data are mean±SEM from 3 independent experiments in duplicate. Two-way ANOVA with Sidak's multiple comparison test were used to test statistical significance. **, P≤0.01.

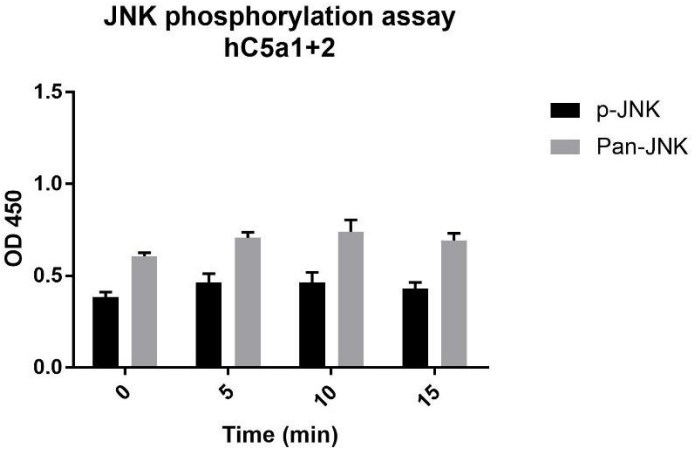
7.7.1.3 JNK phosphorylation assay:

The phosphorylation of JNK was examined in hC5a receptors expressing RBL cells after stimulation with C5a 0.5 µM. The phosphorylated and total JNK were measured at several time points. There were no significant changes in JNK phosphorylation on both cell lines at all time course (Figure 7.9).

a.



b.



c.

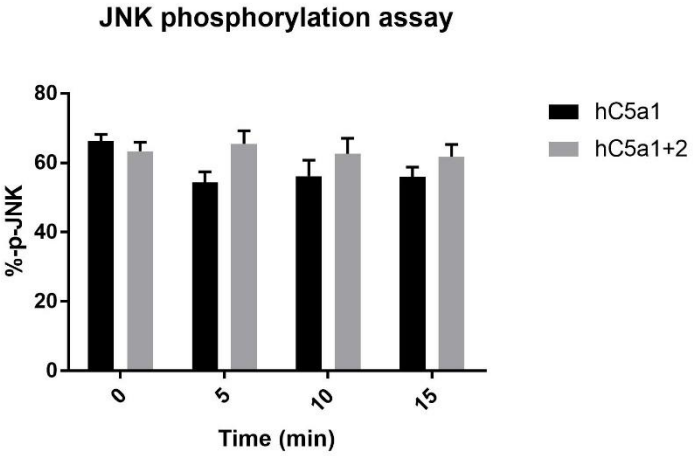


Figure 7.9: C5a-mediated JNK phosphorylation assay. RBL cells expressing either hC5a1 (a.) or both hC5a1+2 receptors (b.) were used. Cells were incubated with 0.5 µM C5a for different time points. Phosphorylated (p-JNK) and total JNK (pan JNK) were measured using cell-based ELISA assay. c. The percentage of JNK phosphorylation was compared between hC5a1 alone or hC5a1+2 cell lines. Data are mean±SEM from 3 independent experiments in duplicate. Two-way ANOVA with Sidak's multiple comparison test were used to test statistical significance.

7.7.2 C5a desArg-mediated MAPK signalling:

7.7.2.1 ERK1/2 phosphorylation assay:

The ERK1/2 phosphorylation assay was performed as described in section 2.3.8. The cells were stimulated with 0.5 µM C5a desArg for 15 minutes. The effect of hC5a2 receptors expression was observed using RBL cells co-expressing both hC5a1 and hC5a2 receptors and compared with cells expressing only hC5a1 receptors. The cells that co-express both receptors showed less ERK1/2 phosphorylation (Figure 7.10).

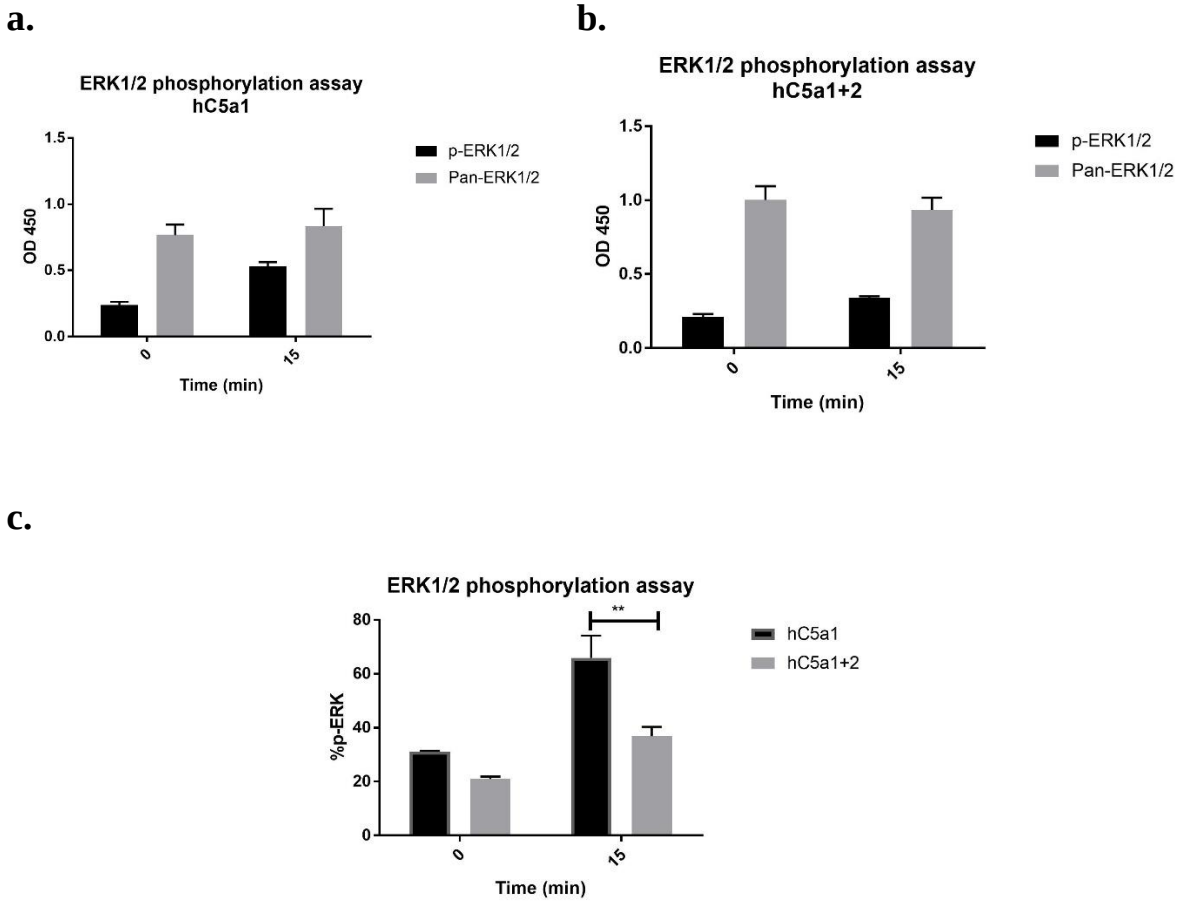


Figure 7.10: C5a desArg-mediated ERK1/2 phosphorylation assay. RBL cells expressing either hC5a1 (a.) or both hC5a1+2 receptors (b.) were used. Cells were incubated with 0.5 µM C5a desArg for 15 minutes. Phosphorylated (p-ERK1/2) and total ERK1/2 (pan ERK1/2) were measured using cell-based ELISA assay. c. The percentage of ERK1/2 phosphorylation was compared between hC5a1 alone or hC5a1+2 cell lines. Data are mean±SEM from 3 independent experiments in duplicate. Two-way ANOVA with Sidak's multiple comparison test were used to test statistical significance. **, P≤0.01.

7.7.2.2 p38 phosphorylation assay:

C5a desArg was used to measure p38 signalling pathway in cells co-expressing hC5a1+hC5a2 and compared with RBL-hC5a1. At 15 minutes stimulation with 0.5 µM C5a desArg, the difference in phosphorylated p38 between the two cell lines was not statistically significant (Figure 7.11).

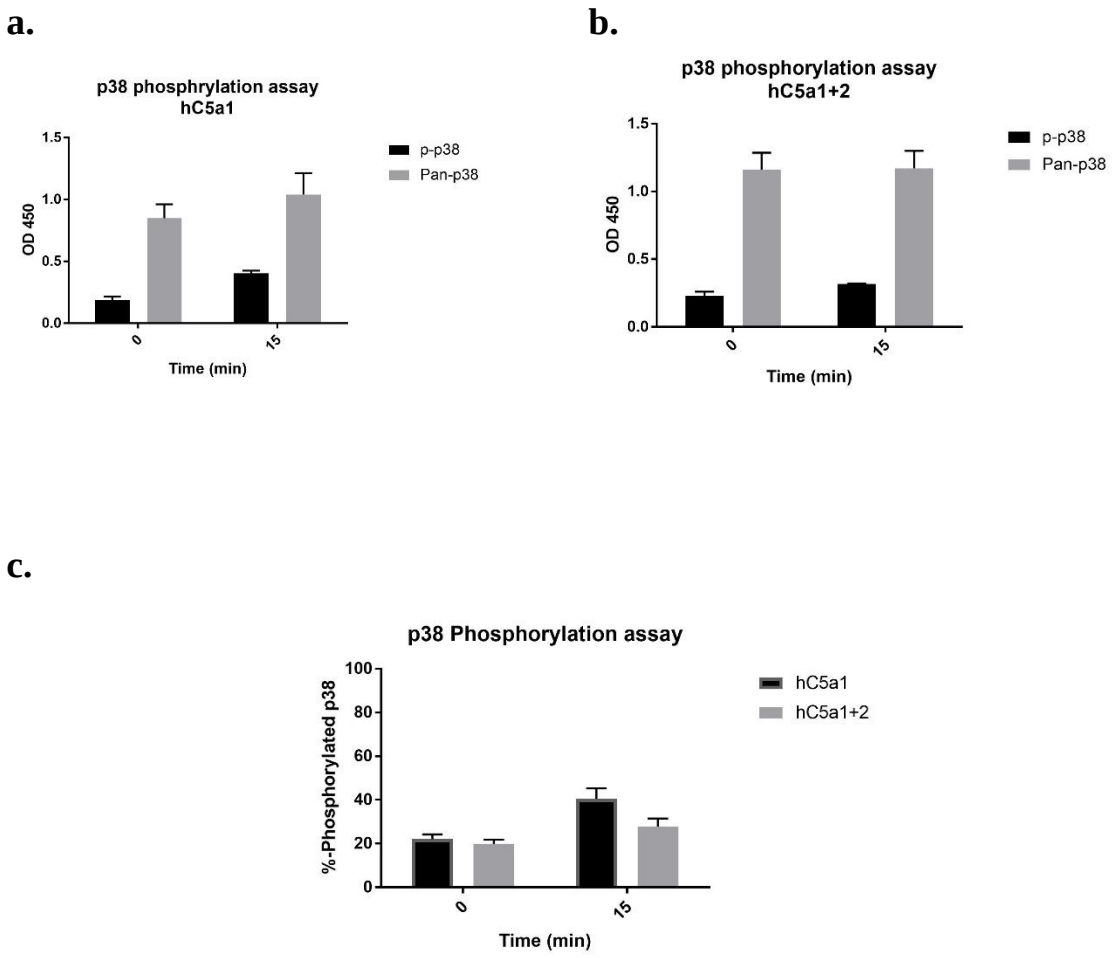


Figure 7.11: C5a desArg-mediated p38 phosphorylation assay. RBL cells expressing either hC5a1 (a.) or both hC5a1+2 receptors (b.) were used. Cells were incubated with 0.5 µM C5a for 15 minutes. Phosphorylated (p-p38) and total P38 (pan-p38) were measured using cell-based ELISA assay. c. The percentage of p38 phosphorylation was compared between hC5a1 alone or hC5a1+2 cell lines. Data are mean±SEM from 3 independent experiments in duplicate. Two-way ANOVA with Sidak's multiple comparison test were used to test statistical significance.

7.7.2.3 JNK phosphorylation assay:

The JNK activation pathway was also tested in response to C5a desArg stimulation. Like C5a treatment, C5a desArg treatment did not induce phosphorylation in JNK after 15 minutes incubation (Figure 7.12).

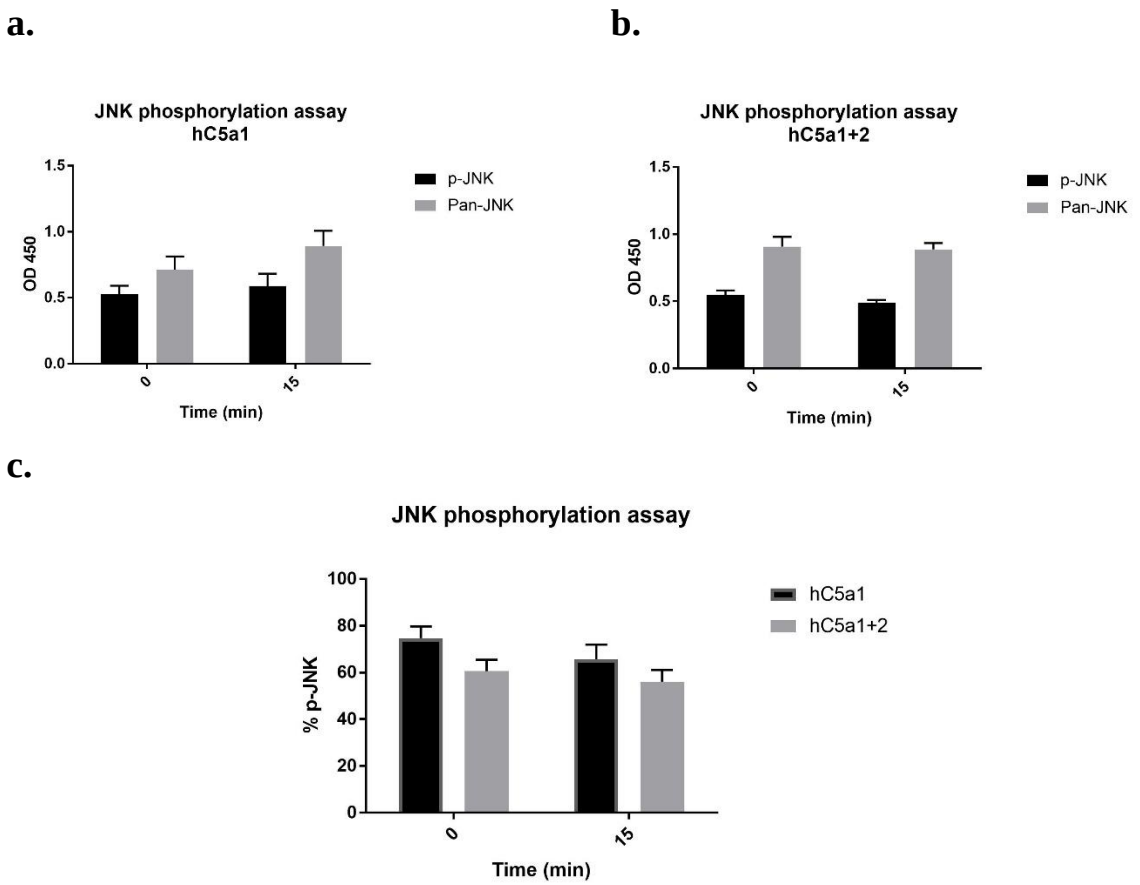


Figure 7.12: C5a desArg-mediated JNK phosphorylation assay. RBL cells expressing either hC5a1 (a.) or both hC5a1+2 receptors (b.) were used. Cells were incubated with 0.5 µM C5a for different time points 15 minutes. Phosphorylated (p-JNK) and total JNK (pan JNK) were measured using cell-based ELISA assay. c. The percentage of JNK phosphorylation was compared between hC5a1 alone or hC5a1+2 cell lines. Data are mean±SEM from 3 independent experiments in duplicate.

7.7.3 Comparison between C5a and C5a desArg effect on MAPK phosphorylation assay:

To show the effect of different ligand treatments on the MAPK pathways activation, the C5a-mediated MAPK signalling was plotted with C5a desArg-mediated MAPK signalling. These treatments were compared between the two cell lines, RBL-hC5a1 alone and RBL-hC5a1+2 (Figure 7.13). Both C5a and C5a desArg stimulated ERK1/2 phosphorylation in RBL cells expressing hC5a1 alone, which was abolished when hC5a2 co-expressed with hC5a1 receptor (Figure 7.13 a and b).

Although it did not reach statistical significant at 15 minutes stimulation, ERK1/2 phosphorylation after C5a desArg treatment was less than C5a treatment in RBL-hC5a1 cells (Figure 7.13 a). However, RBL hC5a1+2 did not show any observed difference (Figure 7.13 b). This is because C5a1 receptor has lower affinity for C5a desArg than C5a, while C5a2 binds C5a and C5a desArg in nearly similar affinity (Scola et al., 2007).

All other MAPK pathways did not show differences in response to different treatments (Figure 7.13 c, d, e and f).

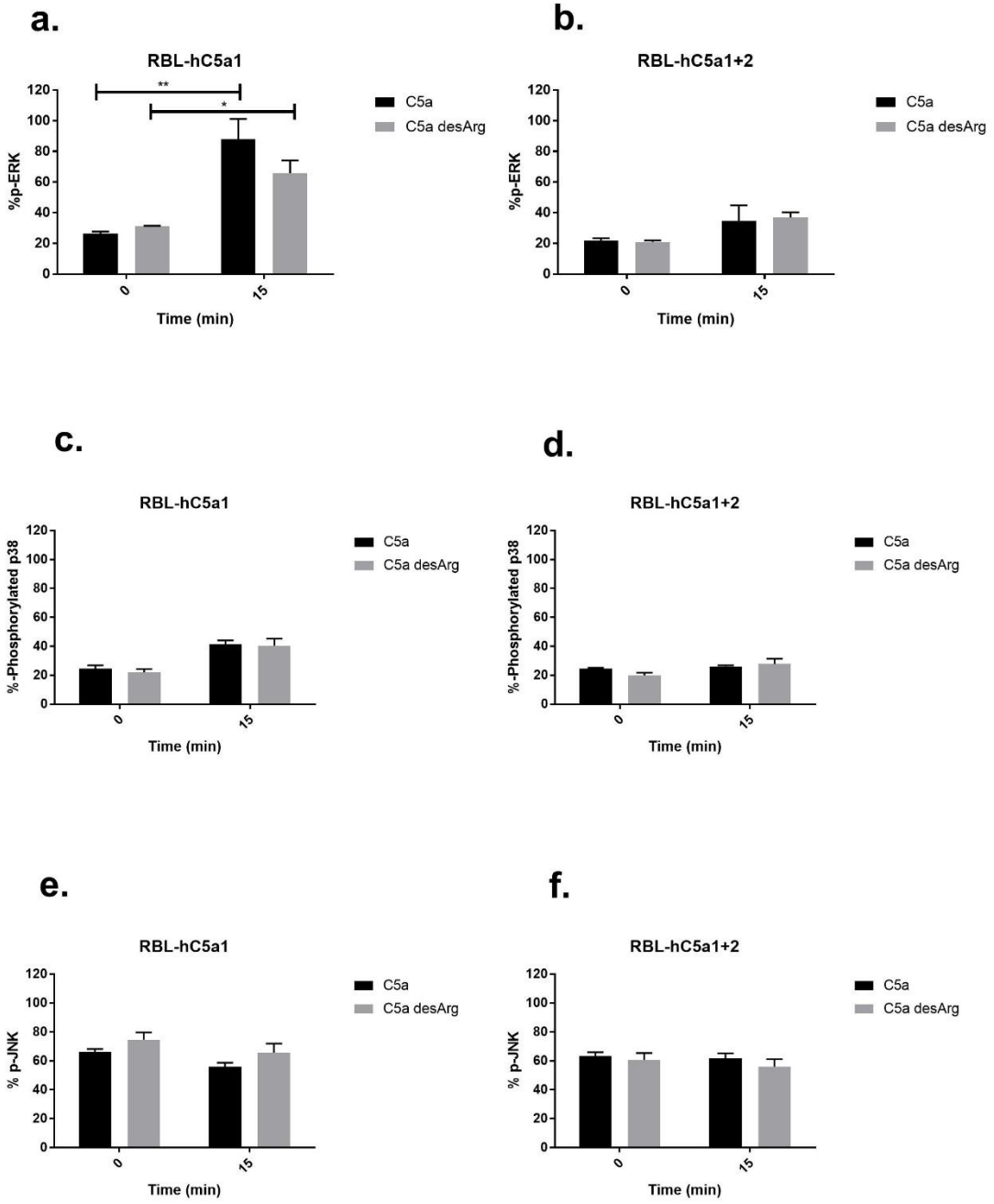


Figure 7.13: Ligand-induced MAPK phosphorylation assay. RBL cells expressing either only hC5a1 or both hC5a1 and hC5a2 were used. The ERK1/2 (a, b); p38 (c, d); and JNK (e, f) signalling was measured after 15 minutes C5a or C5a desArg stimulation. Data are mean±SEM from 3 independent experiments in duplicate. Two-way ANOVA with Sidak's multiple comparison test were used to test statistical significance. *, P≤0.05 and **, P≤0.01.

7.8 Discussion:

In this chapter, untagged receptors were used to explore the possible signalling interactions between hC5a1 and hC5a2. Although both receptors have the seven-transmembrane domains of GPCR, C5a1 can productively couple with G proteins, while C5a2 cannot (Cain and Monk, 2002, Okinaga et al., 2003). This is confirmed in this study by showing that hC5a2 receptor failed to induce degranulation, which is a known function of some G protein-coupled receptors (GPCR) found predominantly in mast cells, basophils, neutrophils and macrophages (Vines and Prossnitz, 2004). This was thought to be due to the lack of the DRY and NPXXY motifs, which are the highly-conserved motifs among GPCR. In C5a2 receptor, a DLC motif replaces the DRY in the third transmembrane domain. The arginine residue appeared to have important role in interaction with G proteins. In several receptors, such as histamine H2, CCR5, FPR, the mutation of the arginine residue greatly affects their G proteins binding capabilities (Prossnitz et al., 1999, Alewijnse et al., 2000, Rovati et al., 2007, Lagane et al., 2005). When the leucine is mutated to arginine in C5a2, the receptor partially regains the ability to bind to Gα16 when co-expressed in HEK cells and low level of Ca^{2+} mobilization was induced (Okinaga et al., 2003). However, when C5a2 mutant at this motif transfected into RBL, other study reported that G protein coupling potential is completely absent in C5a2 even when the C5a1 G protein coupling motifs were inserted and no Ca^{2+} signalling was observed in response to C5a or C5a desArg (Scola et al., 2009). Therefore, the present study, in addition to others mentioned above, suggest a comprehensive inability of the C5a2 receptor to induce G protein signalling.

The expression of hC5a1 receptor in RBL caused a high percentage of enzyme secretion upon stimulation with C5a. In addition, hC5a1 receptor increased the sensitivity of RBL cells for degranulation in response to other stimuli such as IgE. However, when hC5a2 was co-expressed in RBL cells with

hC5a1, the hC5a1-mediated enzyme release was decreased significantly. This may suggest that hC5a2 receptors can possibly modulate the signalling of hC5a1 receptor. This effect could be through signalling pathway that does not involve G protein coupling because C5a2 lacks the ability to activate G proteins. This result is in agreement with the reports that demonstrated that C5a2 can modulate C5a1 signalling (reviewed in Li et al., 2013, Bamberg et al., 2010). Bamberg and colleagues suggested a model in which C5a2 affects the C5a1 signalling through β-arrestin. Translocation of β-arrestin in response to C5a2 was reported in HEK cells transfected with C5a2 (Kalant et al., 2005, Croker et al., 2014, Croker et al., 2016) and in polymorphonuclear cells (Bamberg et al., 2010). It has been postulated that both C5a1 and C5a2 receptors are phosphorylated by G protein receptor kinases (GRKs) after ligand binding of C5a1 receptors. This triggers β-arrestin association. The binding of β-arrestin to C5a1 activates ERK1/2, while C5a2- β-arrestin association inhibits ERK1/2 signalling. The final signal depends on the net signal of the two pathways (Bamberg et al., 2010).

The possible role of hC5a2 on hC5a1 mediated degranulation was further examined by using the hC5a2 selective peptide. However, this peptide did not show effect on C5a-mediated degranulation in RBL cells that co-express hC5a2 and hC5a1 receptors. This could be due to the β-arrestin subtype that is involved in enzyme release signalling. It was reported that silencing β-arrestin1 inhibited degranulation in mast cell in response to C3a (Vibhuti et al., 2011). The C5a2 selective peptide was reported to cause translocation of β-arrestin2 towards C5a2. Therefore, the difference in the target protein (β-arrestin) subtypes could explain the results of the present study.

The present study illustrated that although the cells that co-expressing both C5a1 and C5a2 showed less enzyme release, the intracellular Ca^{2+} mobilization response was similar. This might indicate that the effect of C5a2 intervention is occurring at a later event after Ca^{2+} mobilization or through alternative pathway

and that Ca^{2+} mobilization is not the only trigger for degranulation. It has been shown that some GPCR respond differently after Ca^{2+} mobilization. For example, after stimulation of formyl peptide receptors (FPR) and adenosine type 3 receptors (A3R), inositol triphosphate (IP3) was produced through a pertussis toxin sensitive Gαi-mediated pathway. This led to Ca^{2+} signalling in response to both stimulated receptors. However, degranulation was different in response to activation of each receptor. The FPR stimulation induced degranulation in RBL cells, while A3R did not (reviewed in Vines and Prossnitz, 2004) (Figure 7.14). Therefore, intracellular Ca^{2+} mobilization could be necessary but not sufficient to stimulate enzyme release in GPCR.

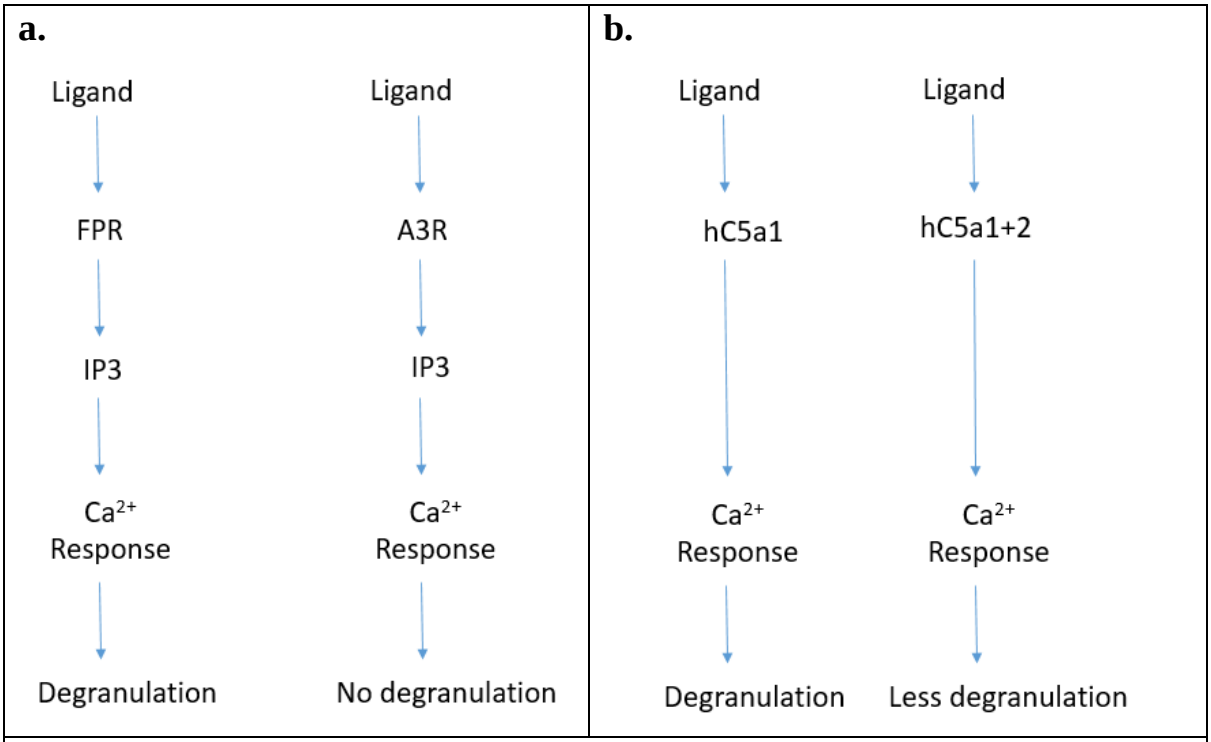


Figure 7.14: The relation between Ca^{2+} response and degranulation. a. Upon ligand binding, both FPR and A3R stimulated IP3 production and then Ca^{2+} response. However, FPR induced degranulation, while A3R did not. b. Similarly, RBL cells that expressing hC5a1 only or co-expressing hC5a1+2 induced Ca^{2+} response. However, the cells that co-expressing both hC5a1+2 showed impaired degranulation. FPR, formyl peptide receptors. A3R, adenosine type 3 receptors. IP3, inositol triphosphate.

C5a is known to induce MAPK activation (Riedemann et al., 2004, Maeda et al., 2015). Three distinct intracellular signaling pathways are involved in MAPK activation. These involve ERK1/2, p38 and JNK (Widmann et al., 1999). They are a family of kinases that are activated by a series of protein kinase reactions (Widmann et al., 1999). MAPK activation was found to be related to increase IL6 and TNF-α production (Riedemann et al., 2004, Clemente et al., 2015, Choi et al., 2015, Fehr et al., 2015, Yang et al., 2015). In addition, they are involved in chemotactic migration of macrophages after stimulation with C5a (Chiou et al., 2004). These effects were thought to be predominantly through the ERK1/2 and p38 pathways, although JNK phosphorylation was found to be nonessential (Chiou et al., 2004). Another study has demonstrated that C5a induced the phosphorylation of MAPK but not p38 (Francis et al., 2008). Taken together, these findings agree with findings of the current study, which demonstrated that C5a and C5a desArg stimulated the phosphorylation of ERK1/2, but not p38 or JNK.

C5a-mediated ERK1/2 phosphorylation was reported to be decreased in bone marrow-derived macrophages from $C5a1^{-/-}$ and $C5a2^{-/-}$ mice and the depletion of C5a2 was thought to ameliorate the acute inflammation in mice with induced colitis (Hsu et al., 2014). However, inhibiting MAPK signaling pathways could also have an anti-inflammatory effect by inhibiting cell migration (Tsai et al., 2004). In the present study, the co-expression of hC5a2 with hC5a1 resulted in a decrease in MAPK signalling and enzyme release, which may support the idea of a modulatory effect of hC5a2 on hC5a1 signalling. The results of the current study are supported by the findings of two other groups. The blockade of C5a2 was found to be associated with a dramatic increase in C5a-mediated ERK1/2 phosphorylation in human PMNs (Bamberg et al., 2010). In addition, in human monocyte-derived macrophages, C5a2 was suggested to modulate

ERK1/2 signalling at C5a concentrations that could induce C5a1 and C5a2 interactions (Croker et al., 2014). In both publications, this modulatory effect was thought to involve β-arrestins. Therefore, C5a2 could play distinct roles in different environments or disease conditions, which may explain the diverse and conflicting reports regarding its function.

In the current study, the ERK1/2 signalling was affected by the co-expression of hC5a2 with hC5a1, whereas the Ca^{2+} signalling was not. This could suggest that the signalling via ERK1/2 and Ca^{2+} is through separate pathways. The C5a2 effect might be through β-arrestin rather than G proteins. Other studies also reported similar findings. C5a2 blockade was demonstrated to increase in ERK1/2 phosphorylation, while Ca^{2+} mobilization was not affected (Bamberg et al., 2010).

To sum up, the co-expression of hC5a2 with hC5a1 seems to affect the functions of hC5a1 differentially. The effect was mainly on the ERK1/2 signalling and the degranulation, while Ca^{2+} response was spared. Therefore, it could be speculated that the MAPK signalling is one of the factors that could be involved in degranulation in RBL cells. In addition, the MAPK pathway may be independent from the Ca^{2+} pathway (Figure 7.15).

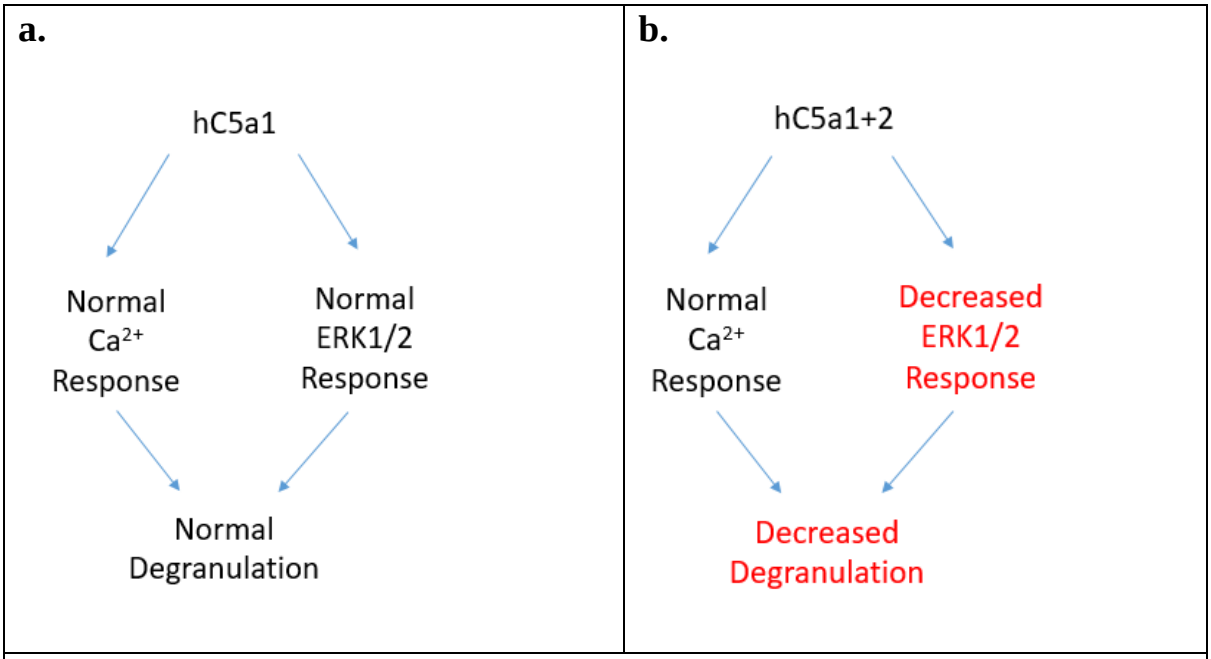


Figure 7.15: The possible hC5a1 signalling pathways that are affected by the hC5a2. In RBL cells that express only hCa1, the Ca^{2+}, ERK1/2 and degranulation response were normal (a). However, in RBL-hC5a1+2, the ERK1/2 and degranulation responses were decreased, while the Ca^{2+} signalling was not affected (b).

Chapter eight

General discussion

Chapter 8: General discussion

During the inflammatory process, many inflammatory mediators are released such as the anaphylatoxins, C3a and C5a. In normal conditions, the effects of these mediators are controlled after the removal of the danger that triggers the inflammatory reaction. However, in some cases the inflammatory process continues and results in a chronic destructive process.

The complement system plays important roles in many inflammatory diseases. Complement involvement, including C5a, has been reported in many diseases (see section 1.6). Therefore, studying and understanding factors that are involved in the inflammatory process may be of great help to decrease the morbidity and mortality of affected patients. One of the central factors is the release of anaphylatoxins both locally in inflamed tissues and systemically. This study deals with the paramount anaphylatoxin, complement fragment C5a. This protein is produced during the activation of the complement cascade in inflammatory conditions and is known to have a proinflammatory function through its classical receptor, C5a1. Nevertheless, anti-inflammatory effects of C5a has been also reported (Bosmann et al., 2012, An et al., 2016). A second receptor for C5a, C5a2, was then discovered, but its function is still complex and controversial. The C5a receptors are among the large GPCR family that is characterised by heptahelical transmembrane domains connected by intracellular and extracellular loops. These receptors have significant physiological functions and are major pharmacological targets.

The RBL cell line was used as a model to study C5a receptors functions and interaction. In this study, a cell culture model represented by rat basophilic leukaemia cell line stably expressing human complement C5a receptors was used. This cell line has been widely used for studying GPCR signalling and functions. It was used to test the response of expression of wild type or mutant human C5a1

receptors by measuring several cellular responses such as intracellular Ca^{+2} mobilization, actin polymerization (Monk et al., 1994b, Cain and Monk, 2002), and granule release such as serotonin (Monk et al., 1994a), hydroxytryptamine (Monk et al., 1994b) and hexosaminidase (Cain and Monk, 2002). Therefore, in the current study, this cell line was used to measure various C5a receptors responses using wild type and tagged receptors. In addition, the behaviour of hC5a1 receptor when co-expressed with hC5a2 was also explored, which could reflect the *in vivo* situation where the two receptors are expressed concomitantly.

The C5a1-mediated degranulation is probably a multifactorial signalling pathway, Ca^{2+} and MAPK signalling could be among these factors. Ligand binding to GPCR results in G proteins and/or β-arrestin activation that leads to a cascade of signalling steps, which result in various functions for the receptors. These signalling steps involve Ca^{2+} mobilization, MAPK activation and degranulation in some immune cells. Many reports investigated the link between these signalling pathways or the dependence of these signalling events on each other. In the current study, the signalling of human C5a receptors was explored in RBL cells expressing various types of these receptors. This study found that when the Ca^{2+} signalling was disturbed for various reasons, the degranulation was disturbed. This was the case for the FP-tagged hC5a1 receptors in chapter 3. However, the degranulation was disturbed even when the Ca^{2+} signalling was normal as in the case of cells co-expressing untagged hC5a1 and hC5a2 receptors. This may suggest that Ca^{2+} signalling is necessary for degranulation from RBL cells but not sufficient and that the degranulation is stimulated through complex and multifactorial pathways (Figure 8.1). It has been shown that inhibition of Ca^{2+} influx resulted in inhibition of degranulation in RBL cells (Tanifuji et al., 2010, Ito et al., 2002). In addition, many antiallergic drugs exert their inhibitory effect on degranulation via the inhibition of Ca^{2+} influx (Kim et al., 2008, Matsubara et al., 2004, Paulussen et al., 1998, Fischer et al., 1998, Spataro and Bosmann,

1976). Furthermore, IgE-mediated Ca^{2+} mobilization and degranulation was almost completely inhibited after chelating Ca^{2+} with EGTA (Ozawa et al., 1993, Nishida et al., 2005). Therefore, impairment of degranulation could result from defects in Ca^{2+} mobilization, albeit degranulation could be affected by other factors when the Ca^{2+} response is normal.

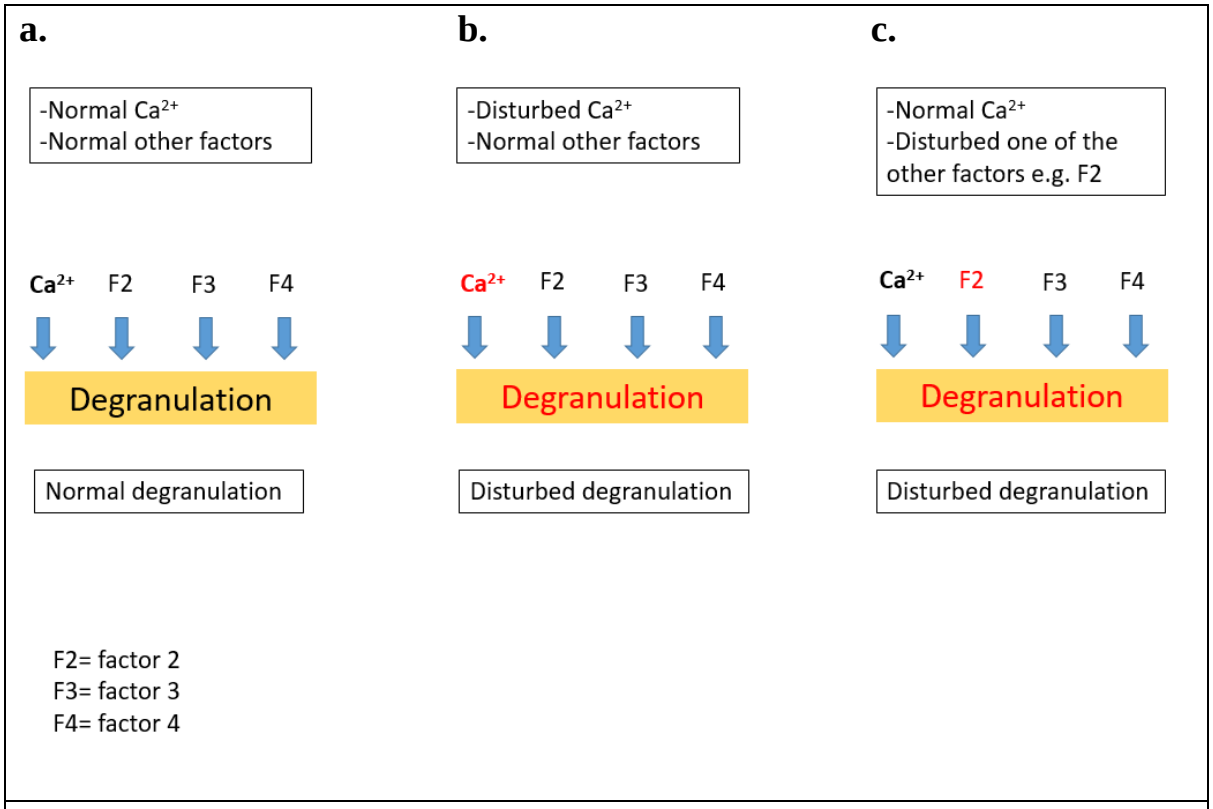


Figure 8.1. The multifactorial nature of degranulation signalling pathway. When the Ca^{2+} and other factors are normal, the degranulation is normal (a). If the Ca^{2+} response is disturbed (red), the degranulation is disturbed (red) (b). If the Ca^{2+} response is normal but one of the other factors is disturbed, for example F2 (red), the degranulation is disturbed (red) (c).

Other factors that may affect degranulation could be the MAPK signalling. The role of MAPK signalling in eicosanoids release and cytokine production was reported (Gilfillan and Tkaczyk, 2006) but its role in degranulation is a controversial subject. The release of histamine from mast cells or human basophils was reported to be not regulated by MAP kinases (Stempelj and Ferjan,

2005, Miura et al., 1999). On the other hand, correlation between ERK phosphorylation and granule release was demonstrated in RBL cells (Hanson and Ziegler, 2002), and mast cells (Choi and Kim, 2004, Koo et al., 2006). This could be in support of the current study as the co-expression hC5a2 with hC5a1 resulted in lower ERK1/2 signalling in addition to decrease in enzyme release.

The GPCR internalization plays several roles in many biological processes. It has been suggested that receptors internalization has implications for β2 adrenergic receptors re-sensitization after their desensitization (Lefkowitz, 1996, Lefkowitz, 1998). In addition, it is an important step in MAPK activation by the receptors. When β2 adrenergic receptors co-expressed with κ opioid receptors, isoproterenol-mediated β2 trafficking and MAPK activation were lost. Furthermore, when internalization inhibited by the expression of β-arrestin1 and dynamin mutants, β2 adrenergic receptors-mediated MAPK signaling was attenuated without affecting the early plasma membrane-delimited signaling events (Daaka et al., 1998). On the same manner, in the current study, the hC5a1 receptor internalization was decreased when co-expressed with hC5a2. This may explain the decrease in MAPK activation and enzyme release when both hC5a1 and hC5a2 are co-expressed on RBL cells.

Human C5a1 receptors possibly exist at the cell surface in homodimers or oligomers. GPCR were first believed to work as monomeric entities, which after ligand binding can activate G proteins. However, a growing body of evidence suggests that they may exist or function as dimers or oligomers (Devi, 2001, Milligan, 2004, Prinster et al., 2005, Pin et al., 2007, Dalrymple et al., 2008, Milligan, 2009, Vischer et al., 2011, Maurice et al., 2011). In the present study, the possibility of hC5a receptor homo- and heterodimerization was investigated. Using the FRET technique, hC5a1 receptors showed possible homodimerization, which is in agreement with other reports that used co-immunoprecipitation to

detect C5a1 homodimers in HEK cells (Rabiet et al., 2008). It is also possible that the FRET signal that was detected in the present study resulted from oligomer formation by hC5a1 and not only dimers. This can be supported by the findings, of Klco et al., 2003, which proposed the formation of hC5a1 high-order oligomers using disulphide-trapping (Klco et al., 2003). In addition, using hC5a1 receptors expressed on *Saccharomyces cerevisiae*, Floyd et al., 2003 showed that hC5a1 receptor oligomerization did not require mammalian accessory proteins (Floyd et al., 2003). That is because the lower eukaryote organism used in that study does not express potential scaffolding proteins such as arrestins and caveolins that might play a role in GPCR oligomerization. Furthermore, hC5a1 receptor oligomerization was found to be constitutive and was not significantly affected by G proteins. Moreover, these hC5a1 oligomers were believed to be formed early in their biosynthesis, probably at the level of endoplasmic reticulum, because the oligomerization signal was detected in yeast endoplasmic membranes similar to the levels detected on the plasma membranes (Floyd et al., 2003). This may suggest early formation of oligomers that are delivered to the cell surface as oligomers and do not need interaction with other intracellular proteins. Therefore, the tagged receptors that were used in this study, which perhaps exerted poor interaction with intracellular proteins as appeared from the disturbance in some of their signalling properties, could still be used to explore receptor dimerization or oligomerization. In addition, Floyd *et al.* reported that the level of receptors self-association was not affected by ligand stimulation. The same result was observed in the current study, which suggests constitutive dimerization/oligomerization.

Human C5a receptor heterodimerization could not be detected in this study. Although the FRET technique confirmed the homo-di/oligomerization of hC5a1, it did not indicate heteromerization with C5a2. The BRET technique was also used to explore possible hC5a1 and hC5a2 association. This technique has the

advantage of avoidance of excitation of the donor because the donor in this method is an enzyme that can emit light directly after interaction with its substrate. Therefore, the side effects of using an external light source such as direct excitation of the acceptor will have no role in such experiments. Although this technique has been used previously to detect C5a receptor heterodimerization (Poursharifi et al., 2013, Croker et al., 2013), in the current study this heterodimerization could not be detected. This could be due to different experimental conditions such as the cell lines. The use of different cell lines, 3T3-L1 adipocytes and J774 macrophages, showed different effects in one study (Poursharifi et al., 2013). The ligand-dependent colocalization of C5a1 and C5a2 was observed in 3T3-L1 adipocytes. However, in J774 macrophages, little and transient C5a-dependent colocalization was observed. Furthermore, the C5a1 and C5a2 colocalization in macrophages was increased by the use adipocyte-conditioned medium (Poursharifi et al., 2013).

The potential dimerization interaction between hC5a1 and hC5a2 receptors was then explored with a different technique that uses untagged C5a receptor co-internalization. There are many examples of receptor dimerization measured using such a technique (reviewed in Milligan, 2004). In this study, C5a1 receptor inhibitor (PMX53) was used to make hC5a2 receptor selectively bind C5a. If hC5a1 is in direct physical contact with hC5a2 (heterodimers), it is expected that either both receptors internalize upon internalization of hC5a2 or both of them are inhibited from internalization due to the effect of PMX53 on hC5a1. The result was that hC5a1 did not co-internalize with hC5a2 after treatment with PMX53, which suggests no physical contact between the two receptors. This confirms the FRET and BRET results of this study, which suggest that hC5a1 and hC5a2 heterodimerization does not occur in RBL cells. In addition, other studies could not detect interaction between C5a2 and the classical anaphylatoxin receptor C5a1 (Chen et al., 2007). Therefore, other possible mechanisms may

explain the effect of C5a2 on C5a1 signalling, which could be through contribution to signalling in parallel with the classical receptor. For example, in rat astrocytes, C5a2 was shown to be regulated with noradrenaline and was found to have anti-inflammatory function (Gavrilyuk et al., 2005). In addition, the co-expression of β2 adrenergic receptors with κ opioid receptors resulted in loss of MAPK activation and β2 adrenergic receptor internalization. However, the cells were still able to activate adenylyl cyclase. This is in support of the results of the current study, which showed a decrease in MAPK activation and hC5a1 internalization when hC5a2 was co-expressed with C5a1, although the Ca^{2+} signalling was not affected. This variability in the effects of hC5a2 on hC5a1 could explain some of the variability of the function of C5a2 in the literature.

C5a1 receptor self-association could prevent or compete with association with hC5a2. The GPCR are thought to form dimers or oligomers through specialized structures. These could be disulphide bonding (Romano et al., 1996, Kunishima et al., 2000) or interaction of the carboxy-terminal (coil-coil) (Margeta-Mitrovic et al., 2000). The dopamine D2 receptors were shown to crosslink through disulphide-trapping experiments (Guo et al., 2003b). This crosslinking was abolished by a single cysteine substitution on TM4. Using the same method, it was proposed that C5a1 receptors crosslinking is mediated by a cysteine in the IC2 loop (klco et al., 2003). The disulphide bond is the strongest bond that plays an essential role in protein stability and folding. If the C5a1 receptor forms these bonds before its delivery to the cell membrane, then it could be speculated that ligand addition cannot change the level of receptor interaction as found in this study and others (Floyd et al., 2003, Rabiet et al., 2008). In addition, this could suggest that the C5a1 receptor self-association using their IC2 cysteine residues in disulphide bonding, which occurs early in their biosynthesis (at the level of ER), might render this IC2 cysteine residue not available for other interactions to form heteromers with C5a2. This could explain tendency of the

C5a1 receptors to homo-di/oligomerize rather than heterodimerize in the present study.

Receptor interaction is not necessarily through the formation of heterodimers. The theory of the possibility of cooperation of GPCR is thought to be not necessarily due to the dimerization. In fact, the relevance of such observations has been questioned and alternative interpretations have been suggested (Chabre et al., 2003, Chabre and le Maire, 2005, James et al., 2006, Gurevich and Gurevich, 2008, Chabre et al., 2009). Although it was suggested that many GPCR, especially group A, are capable of dimerization, the monomeric forms were shown to bind G proteins efficiently (Bayburt et al., 2007, Whorton et al., 2007, Ernst et al., 2007). Using pharmacological studies, when two receptors are co-expressed, the ligand binding of one receptor can affect the affinity of the other receptor for its ligand, both positively and negatively. This was originally thought to be due the formation of heterodimers with the ligand of the first receptor having an allosteric effect on the second ligand-receptor interaction. However, this has been argued that this interaction could be interpreted in different way (Chabre et al., 2009). It was thought that this apparent interaction could be due to interaction of the receptors with a shared G protein pool and not due to direct cross talk between the two receptors. In another word, it is due to simultaneous talk to the same G protein (Chabre et al., 2009). In addition, G proteins are not the sole mechanism that can affect receptor behaviour and arrestins could also play a role. It has been reported that arrestins can affect the ligand binding affinity of some GPCR (Gurevich et al., 1997). In addition, some GPCR have been shown to compete for arrestins (Schmidlin et al., 2002). In the current study, C5a2 could affect C5a1 indirectly through interaction with and/or competing for a β-arrestin pool. The association of β-arrestin 1 and 2 with C5a2 was reported using co-immunoprecipitation and BRET techniques (Bamberg et al., 2010, Croker et al., 2014). In addition, C5a2 was found to co-localize with β-arrestin and C5a1

intracellularly. Moreover, C5a-mediated ERK1/2 and chemotaxis signalling was increased upon C5a2 blockade, whereas Ca^{2+} was not (Bamberg et al., 2010). This could suggest a C5a2 role in the modulation of C5a1 signalling through the β-arrestin pathway.

The C5a2 receptor could work as a scavenging decoy receptor. The anaphylatoxin C5a is thought to be removed from the circulation rapidly because its half-life is only 2-3 minutes, mainly due to C5a desArg formation (Webster et al., 1982, Weisdorf et al., 1981). This prompt removal mechanism may involve the participation of classical C5a receptor, C5a1 (Oppermann and Gotze, 1994). However, the lately discovered C5a2 receptor could play a role in this clearance mechanism. This could be due to the high affinity of C5a2 for C5a desArg and the lack of signal transduction capability. C5a2 could behave in similar way to that of the chemokine scavenging decoy receptor D6. Although it binds to several chemokines, D6 receptor cannot transduce G protein signals. However, it can carry ligands from the extracellular to the intracellular compartment through constitutive internalization. The ligand is then dissociated and degraded. Later, the receptor recycles back to the cell surface. The recycling mechanism is similar to that used by conventional G protein-coupled receptors that involves clathrin and arrestin (Galliera et al., 2004, Weber et al., 2004). The data of the current study suggests a comparable role of C5a2 to the scavenging decoy receptor D6. This is because C5a2 did not show evidence of G protein signalling. In addition, C5a2 has a predominant intracellular localization similar to D6, while the classical receptors C5a1 and CCR5 are mainly expressed on the cell surface (Galliera et al., 2004, Weber et al., 2004). Furthermore, C5a2 showed higher constitutive and ligand-induced internalization, which continues for a longer time than that of C5a1. This result is supported by the finding of Scola et al. 2009, which also showed higher constitutive internalization of C5a2 compared to C5a1 and, as for D6, this internalization led to degradation of the ligands especially

C5a desArg. The relatively low constitutive internalization of C5a1 did not lead to degradation of the ligand (Scola et al., 2009). All these findings suggest a scavenging role of C5a2 by eliminating the extracellular ligands C5a and C5a desArg (Bokisch and Muller-Eberhard, 1970, Budzko et al., 1971). Therefore, it could be speculated that C5a2 has a scavenging behaviour by decreasing the exposure of the classical C5a1 receptor to its ligand. This is supported by other reports that suggest a scavenging decoy function of C5a2 in different models such as mouse model of pulmonary inflammation (Gerard et al., 2005) and also in rat astrocytes (Gavrilyuk et al., 2005). Nevertheless, a pro-inflammatory function of C5a2 was also reported by other groups but it was not evident if it involved G proteins activation (Chen et al., 2007, Rittirsch et al., 2008, Poppelaars et al., 2017). It should be noted that this study cannot rule out the potential multi-functionality of C5a2 receptor in different cells, tissues, organs or even between species.

Future work. The heterodimerization between C5a1 and C5a2 receptors can be further researched using primary cells that endogenously express the C5a receptors. A new approach could be used to examine the possible receptor dimerization in cells or tissues, namely the proximity ligation assay (PLA) (Weibrecht et al., 2010). This technique can detect protein-protein interactions at a distance similar to the FRET distance. In addition, it does not need the receptors to be extracted from the membranes or genetically engineered to be attached to fluorophores. In this technique, two probes, composed of antibody conjugated with oligonucleotides, are used to bind the target the proteins of interest. These antibody-conjugated oligonucleotides can hybridize, if they are in proximity close to FRET-distance, to connector oligonucleotides to form a circular DNA molecule. This circular DNA molecule can serve as an “endless” template for rolling circle amplification. The resultant single-stranded DNA molecule can be visualized by hybridization with fluorescently labelled detection oligonucleotides.

The potential heterodimerization should be compared in health and diseases states to explore the possible factors that affect its formation. If this form of interaction is confirmed between the C5a receptors, it would be interesting to investigate the possible dimerization interfaces to possibly test the effect of disturbance of such interaction on the receptors functions.

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