Study of the capacity of Toll-like receptors to modulate pro-inflammatory responses mediated by receptors for the complement anaphylatoxin C5a.

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For Emma

DECLARATION

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

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PUBLICATIONS

Oral Presentations:

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ABSTRACT

Toll-like receptors (TLRs) and the complement system play a crucial role in the innate immune response by mediating the initial recognition of, and prompt response to a variety of microorganisms. The concerted activation of TLRs and complement ensures efficient clearance of infection. Previous studies have documented synergism between TLRs and the receptor for the pro-inflammatory peptide C5a (C5aR/CD88), and regulation of TLR-induced pro-inflammatory responses by C5aR, suggesting crosstalk between TLRs and C5aR. However, it is unclear whether and how TLRs modulate C5a-induced pro-inflammatory responses. This study tested the hypothesis that a genuine, bi-directional signalling crosstalk between TLRs and C5a receptors exists, involving not only modulation of TLR-mediated responses by C5a receptor activation, but also modulation by TLR activation of the extent and/or quality of cellular responses to C5a. The experiments described in this thesis confirmed this hypothesis by demonstrating a marked positive modulatory effect of TLR activation on cell sensitivity to C5a *in vitro* and *ex vivo* and identifying underlying mechanistic targets. Pre-exposure of peripheral blood mononuclear cells and whole blood to diverse TLR ligands or bacteria enhanced C5a-induced pro-inflammatory responses. This effect was not observed in TLR4-signalling-deficient mice. TLR-induced hypersensitivity to C5a did not result from C5aR up-regulation or modulation of C5a-induced calcium mobilization. Rather, TLRs targeted the second C5a receptor, C5L2 (acting as a negative modulator of C5aR) by reducing C5L2 expression and activity. TLR-induced hypersensitivity to C5a was mimicked by blocking C5L2 and was not observed in C5L2KO mice. Furthermore, TLR activation inhibited C5L2 expression upon C5a stimulation. Expression of the key adaptor molecule β-arrestin 1, which mediates the inhibitory effects of C5L2 on C5aR, was also found to be negatively regulated by TLR activation. These findings identify a novel pathway of crosstalk within the innate immune system that amplifies innate host defence at the TLR-complement interface. Unravelling the mutually regulated activities of TLRs and complement may reveal new therapeutic avenues to control inflammation.

ABBREVIATIONS

Ab	Antibody
BMDC	Bone marrow-derived dendritic cell
C3aR	C3a receptor
C5aR	C5a Receptor 1, CD88
C5L2	C5aR-like receptor 2, gpr47
CLP	Caecal ligation and puncture
CLR	C-type lectin receptor
CMV	Cytomegalovirus
CpG	Hypomethylated cytosine-phosphate-guanine repeats
CR	Complement receptor
CRD	Carbohydrate recognition domain
CVF	Cobra venom factor
DAF	Decay-accelerating factor, CD55
DAMP	Danger-associated molecular patterns
DC	Dendritic cell
DD	Death domain
dsRNA	Double-stranded RNA
ERK	Extracellular signal-related kinase
FH	Complement factor H
fMLPR	f-leu-met-phe receptor
GPCR	G protein-coupled receptor
GRK	G-receptor kinase
HMGB1	High mobility group box protein 1
HSV	Herpes simplex virus
IFN	Interferon
Ig	Immunoglobulin

IKK	IκB kinase
IL	Interleukin
IL-8R	Interleukin-8 receptor
IRAK	Interleukin receptor-associated kinase
IRF	Interferon response factor
IκB	Inhibitor of NF-κB
JNK	c-Jun N-terminal kinase
KC	Keratinocyte-derived chemokine
LAM	Lipoarabinomannan
LBP	Lipopolysaccharide-binding protein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTA	Lipoteichoic acid
mAb	Monoclonal antibody
MAC	Membrane attack complex
MAL	MyD88-like; also known as TIRAP
MAPK	Mitogen-activated protein kinase
MASP	MBL-associated serine protease
MBL	Mannan-binding lectin
MCP-1	Monocyte chemoattractant protein
MIP	Macrophage inflammatory protein
mRNA	messenger RNA
MyD88	Myeloid differentiation gene primary product 88
NF-κB	Nuclear factor-kappa B
NK cell	Natural killer cell
NLR	Nucleotide binding domain leucine-rich repeat receptor

NOS	nitric oxide synthase
Pam3Cys	Tripalmitoyl-cysteinyl-seryl-(lysyl)4-lysine
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PG	Peptidoglycan
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear leukocytes
poly I:C	Polyinosinic:cytidylic acid
PRR	Pattern recognition receptor
RLR	Retinoic acid-inducible gene-I-like receptor
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SARM	Sterile-alpha and HEAT-Armadillo motifs-containing protein
SIGIRR	Single immunoglobulin IL-1R-related molecule
siRNA	Small interfering RNA
SOCS-1	Suppressor of cytokine signalling-1
SR	Scavenger receptor
ssDNA	Single-stranded DNA
ssRNA	Single-stranded RNA
TAB	TAK1-binding protein
TAK	TGF-β-activated kinase
TGF-β	Transforming growth factor-β
TIR	Toll/IL-1R homology domain
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-alpha
TOLLIP	Toll-interacting protein

TRADD   TNF receptor-associating via death domain

TRAF   TNF-α-receptor-associated factor

TRIF   TIR domain-containing inducing interferon β; also known as TICAM1

WT   Wild-type

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1. Chapter 1: INTRODUCTION

1.1 The inflammatory response to infection

The invasion of a normally sterile tissue by pathogenic microbes induces a prompt and robust response aimed at clearance of the infecting organisms. A wide range of cellular and soluble defence mechanisms are activated upon recognition of pathogens, with the common effect of inducing localised changes in the infected tissue [Medzhitov 2008]. These changes involve activation of the vascular endothelium, with consequent vasodilation, hyperaemia and increased vascular permeability. Their overall effect is to promote recruitment and extravasation both of migratory phagocytic cells and soluble mediators into the infected tissue, a process known as the inflammatory response. The inflammatory response is largely a consequence of the release of pro-inflammatory cytokines and chemokines such as tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, IL-8, and monocyte chemoattractant protein 1 (MCP-1).

Whilst this response is beneficial to the host, it is also potentially harmful, since the inflammatory response to infection may itself result in injury to tissues as a consequence of their infiltration by migratory phagocytic cells which release toxic reactive oxygen and nitrogen species. Hence, it is apparent that as well as being sufficiently robust, the response to infection must be proportionate; an excessive response may be as bad as an inadequate one. An example of this is the clinical syndrome of sepsis, in which an exaggerated, systemic inflammatory response to infection leads to the systemic release of massive quantities of pro-inflammatory cytokines and chemokines, which induce inflammation in healthy tissues remote from the site of the infection, with serious consequences for the host [Takeuchi and Akira 2010]. For this reason, the mechanisms that regulate the magnitude of inflammatory cytokine release in response to infection are of critical importance, and have been the focus of much attention during the last few decades, not only in the context of sepsis research, but also in

sterile inflammatory conditions such as trauma, major haemorrhage and burns, as well as the autoimmune diseases.

The mechanisms that trigger and regulate the early pro-inflammatory cytokine response to infection are generally considered to fall within the purview of the innate immune system. An overview of the components of the innate immune system will be undertaken before a more detailed consideration of the specific components of innate immunity that are the primary focus of this study, namely, the Toll-like family of receptors and their ligands, and the complement component C5a and its receptors.

1.2 The components of the innate immune system

The innate immune system is comprised of a range of nonspecific and broadly specific defence mechanisms whose shared characteristic is that they do not require the clonal expansion of antigen-specific lymphocytes [Murphy 2012]. These include the epithelial barrier, soluble components (including the complement system) and pattern recognition receptors (PRRs) (summarised in Figure 1).
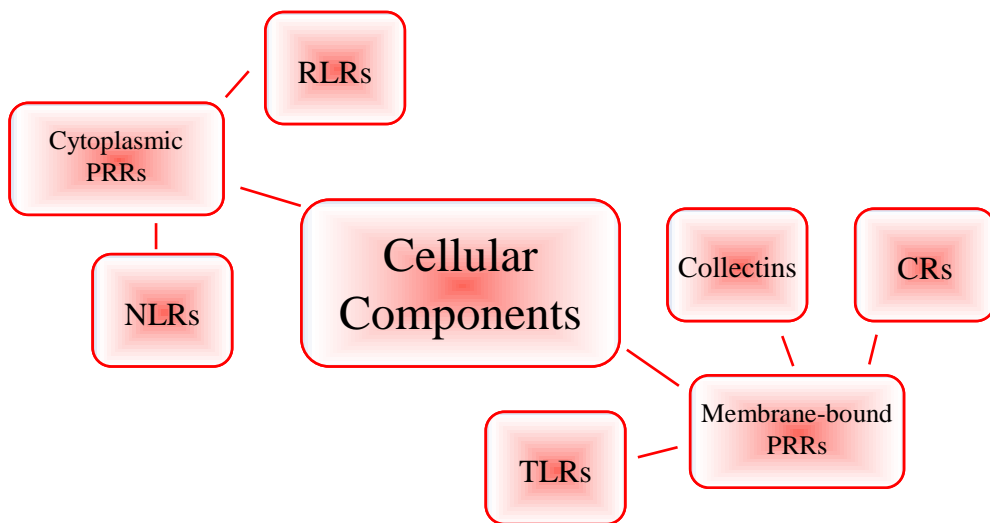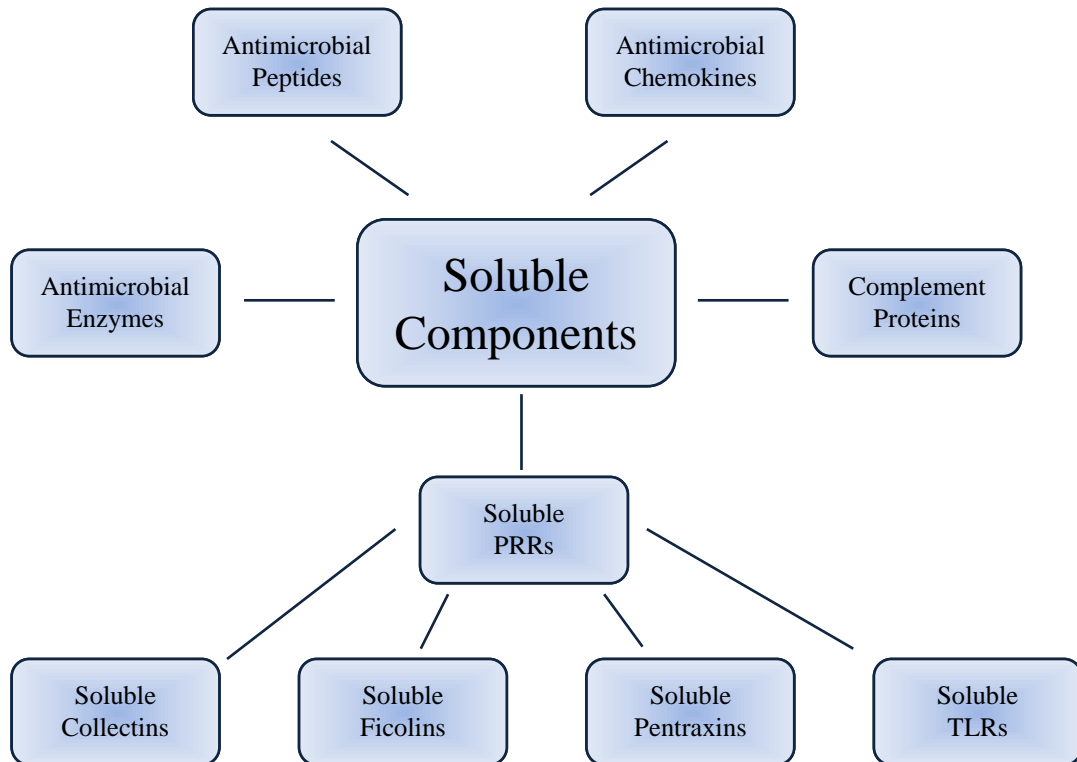
Figure 1. Pathogen recognition by components of the innate immune system. CRs, Complement Receptors; NLRs, Nucleotide Binding Domain Leucine-Rich Repeat Receptors; PRRs, Pattern Recognition Receptors; RLRs, Retinoic Acid-Inducible Gene-I-like Receptors; TLRs, Toll-like Receptors. Adapted from [Murphy 2012].

1.2.1 The epithelial barrier

The first defence against pathogens is a mechanical barrier: the epithelia that cover all of the body surfaces that come into regular contact with the external environment. Bulk flow of body fluids over these surfaces may provide an additional defence against their colonisation by pathogens, for example, the mucociliary escalator in the tracheobronchial tree. Alternatively, the maintenance of a chemical environment hostile to pathogens (by gastric acid, for example) may also serve to protect the endothelial barrier [Murphy 2012].

1.2.2 Soluble components

The epithelial barrier is reinforced by a number of secreted antimicrobial proteins, including antimicrobial enzymes, peptides and chemokines, as well as soluble pattern-recognition receptors that activate other components of the innate immune system.

1.2.2.1 Antimicrobial enzymes

Lysozyme, described by Fleming in 1922 [Fleming 1922], is a glycosidase that cleaves bacterial cell wall peptidoglycans. It is secreted in tears, saliva and by specialised accessory epithelial cells in the gut such as Paneth cells, and is an important defence against Gram-positive organisms [Harder 2007]. Another example is secretory phospholipase A_2, a basic enzyme that is able to penetrate bacterial cell walls and hydrolyse membrane phospholipids [Boyanovsky and Webb 2009].

1.2.2.2 Antimicrobial Peptides

Antimicrobial peptides are conserved, phylogenetically primitive defence mechanisms that have been extensively studied in insects. Human antimicrobial peptides include defensins, histatins and cathelicidins, and are widely expressed in cells such as epithelia that are

commonly exposed to microbes [Wiesner and Vilcinskas 2010]. Defensins are ubiquitous small cationic peptides able to disrupt bacterial and fungal cell membranes as well as the envelope proteins of certain viruses [Lehrer and Lu 2012]. Histatins are predominantly secreted by salivary epithelia, and are important in protecting the oral mucosa against commensal yeasts such as *Candida albicans* [Peters 2010]. Cathelicidins are produced constitutively by phagocytic cells and inducibly by keratinocytes and gut and lung epithelial cells in response to infection; they are known to play an important role in wound healing [Steinstraesser 2011].

1.2.2.3 Antimicrobial chemokines

Chemokines are a crucial component of the innate immune response to infection, as their principal role in immune defence is the recruitment of phagocytic cells to sites of infection. However, a number of chemokines are also reported to exert a direct antimicrobial effect. CCL-28 has been reported to show broad microbicidal activity and to play an important role in mucosal defence [Hieshima 2003]. The neutrophil chemokines CXCL-7 [Durr and Peschel 2002], CXCL-9, CXCL-10 and CXCL-11 [Cole 2001], the platelet-derived chemokines CXCL-4, CATP-3 and CCL-5 [Tang 2002] have also been reported to have direct antimicrobial activity.

1.2.2.4 Soluble pattern recognition receptors

The distinctive characteristic of the receptors of the adaptive immune system is high specificity for a particular non-self antigen, through receptor affinity maturation achieved by VDJ gene segment rearrangement during leucocyte maturation and selection [Murphy 2012]. In contrast, pattern recognition receptors (PRRs) of the innate immune system are "ready-made", germ line-encoded, and thus display broad specificity for classes of conserved microbial component molecules not found in the host [Janeway and Medzhitov 2002]. These are also known as pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs).

Endogenous molecules such as DNA, whose presence in the extracellular milieu is indicative of host injury, are known as damage-associated molecular patterns or DAMPs, and may also be ligands for the PRRs of the innate immune system. PRRs are expressed either on or inside cells, or as soluble molecules and in certain cases, as both. The cellular PRRs will be considered in more detail below; the soluble PRRs include collectins, ficolins and pentraxins.

a. Collectins

Collectins are calcium-dependent members of the lectin family of proteins, and are found both as membrane-bound receptors such as dectin-1 and as soluble proteins. Soluble collectins include mannan-binding lectin (MBL), surfactant proteins A and D, conglutinin, and hepatic and placental collectins. Soluble collectins are secreted by myeloid cells as well as epithelia, and typically bind to carbohydrate residues on the surface of microbes, where they induce cell lysis by disrupting the integrity of the membrane and cause agglutination and opsonisation of pathogens. MBL also has a vital role in the innate immune response as the initiator of the lectin pathway of complement activation; bound MBL interacts with MBL-associated serum proteases (MASPs), resulting in cleavage and activation of C4 and C2 [Gupta and Surolia 2007] – discussed in more detail in section 1.2.3 below.

b. Ficolins

Ficolins are structurally related to MBL and can also cause complement activation via the lectin pathway as well as opsonising pathogens for phagocytosis. They differ in their specificity for different carbohydrate moieties [Endo 2007].

c. Pentraxins

Pentraxins include C-reactive protein, serum amyloid P component (short pentraxins) and pentraxin 3 and its family members (long pentraxins). Short pentraxins are synthesized in the

liver and secreted into the blood in response to inflammatory stimuli, while long pentraxins are secreted by myeloid cells and epithelia. Their typical ligands vary widely, but include a range of molecules associated with pathogens or damage to host tissues. They can activate complement via the classical pathway and also mediate opsonophagocytosis via F_c receptors. They have been likened to the innate immune system's analogue for antibodies [Bottazzi 2010].

1.2.3 The complement system

Another major component of the innate immune system is the complement system, which comprises a series of more than 35 proteins and glycoproteins found in plasma and other bodily fluids, and on cell surfaces [Carroll and Sim 2011].

1.2.3.1 History

The complement system was first recognised late in the 19th century when the leading microbiologists Bordet, Ehrlich and Nuttall discovered a bactericidal function of blood on anthrax bacilli [Bordet 1895 and 1898]. In 1888 Nuttall demonstrated that fresh plasma possessed a bactericidal activity that was abolished when heated to 56°C [Nuttall 1888]. Bordet later showed that the bactericidal activity of heat-treated immune serum could be restored with fresh non-immune serum, which alone had no activity. On the basis of these observations, Ehrlich and Morgenroth [Ehrlich and Morgenroth 1899] proposed that there were two soluble bactericidal components in blood: heat-stable 'amboreceptors' (later identified as antibodies) that were found in immune serum and a heat-labile component found in non-immune serum which 'complemented' the microbicidal properties of the amboreceptors.

In the 1920's and 30's substantial progress was made with the identification of 4 complement components and the recognition that a lytic mechanism was responsible for their activation [Whitehead 1925, Gordon 1926, Gordon and Wormall 1929]. By 1941 four protein components had been identified and at least partly purified [Pillemer and Ecker 1941]. In 1958 Louis Pillemer reported the discovery of a second, antibody-independent pathway of complement activation, which he named the properdin pathway (and which we know now as the Alternative Pathway) [Wedgewood and Pillemer 1958]; sadly the scientific community of the day was not ready for Pillemer's breakthrough, and the professional ridicule that he endured as a consequence of his discovery contributed to his suicide in 1957.

Developments that occurred during the 1960's included a detailed investigation of 'immune adherence', the phenomenon by which complement-coated particles adhere to red blood cells (part of the process of opsonisation), standardisation of complement function assays by measuring the capacity to lyse red blood cells, and the separation of 9 distinct complement proteins from guinea-pig serum [Nelson and Nelson 1959 and Nelson 1966]. In the 1970's and 1980's, protein components were identified and sequenced, and cDNA and genomic DNA clones were also obtained and sequenced [reviewed in Müller-Eberhard 1975, Müller-Eberhard 1988 and Campbell 1988]. Moreover, cell-surface complement receptors were identified and characterised, as were both cell-surface and soluble complement regulatory proteins [reviewed in Wilson 1987 and Hourcade 1989]. Another substantial development of the 1980's was the beginning of an appreciation of the role that complement activation plays in driving the adaptive immune response, as a result of ligation of complement receptors on B-lymphocytes and dendritic cells (DC) [Pepys 1974, reviewed in Fearon 1998]. A third activation pathway, the lectin Pathway, was discovered by the Yamashina group in 1987 [Ikeda 1987].

In the last 20 years, complement research has explored the role of the complement cascade in the clearance of damaged-self material [Walport 2001a and b], the function of complement regulatory proteins and the part played by their dysregulation in chronic inflammatory diseases such as age-related macular degeneration [reviewed in Rodriguez de Cordoba and de Jorge 2008]. The discovery of new proteases (mannan-associated serum proteases or MASPS) [Schwaeble 2002] and new recognition molecules (ficolins and collectin-11) [Matsushita 2009 and Hansen 2010] has led to a new appreciation of the complexity of the lectin pathway. X-ray crystallography and nuclear magnetic resonance studies have revealed the three-dimensional structures of many complement proteins [Arlaud 2007 and Gros 2008], and the development of agents aimed at therapeutic manipulation of complement activation [Qu 2011] has culminated in the clinical use of the anti-C5a monoclonal antibody eculizumab.

1.2.3.2 Pathways of complement activation

Soluble complement proteins circulate in plasma and other bodily fluids as inactive precursors. Specialised sensor molecules detect non-self signals such as characteristic patterns of microbial carbohydrates and respond by inducing conformational changes in their structure that trigger an auto-amplifying catalytic cascade which results in rapid generation of activated complement products. These activated complement products have a wide range of pro-inflammatory and immunomodulatory effects, including direct lysis and opsonisation of pathogens as well as inducing chemotaxis, granule enzyme release and the generation of reactive oxygen and nitrogen species by phagocytic cells. They may also activate the coagulation cascade and have profound effects on vascular smooth muscle tone. Complement activation can occur by any or all of four pathways: the classical, alternative, lectin and extrinsic protease pathways (summarised in Figure 2). The classical, alternative and lectin pathways converge in the activation of C3, the most abundant complement protein, which has

a number of effects: first, it acts as an auto-amplification mechanism, catalysing the activation of more C3; second, it generates opsonic protein fragments that coat non-self surfaces and label them for phagocytosis by migratory leucocytes; third, it results in the generation of chemotactic anaphylatoxins that recruit migratory phagocytes to the site of inflammation; and lastly it results in the assembly of a C5 convertase complex that triggers activation of the terminal pathway, resulting in the assembly of the lytic membrane attack complex (MAC) and the release of the most potent anaphylatoxin, C5a [Carroll and Sim 2011]. In the extrinsic protease pathway, C5 may be directly activated by serine proteases of the coagulation cascade and by a cellular protease expressed by differentiated phagocytes [Huber-Lang 2006, Amara 2008].

Each of these pathways is initiated by different recognition proteins; for the classical pathway, this is the C1q multimer and for the lectin pathway, MBL or other structurally similar molecules. The alternative pathway is more complex, since the existence of a specific sensor molecule is somewhat controversial; also there is a constant low-level 'tick-over' activation of the pathway due to spontaneous hydrolysis of C3. The catalytic activity of this spontaneously-generated $C3(H_2O)$ is strongly inhibited by complement regulatory proteins on host tissues, and it is the absence of these inhibitors on exogenous surfaces (such as pathogens or synthetic materials) that is critical for amplifying complement activation via the alternative pathway [Carroll and Sim 2011, Ehrnthaller 2011].
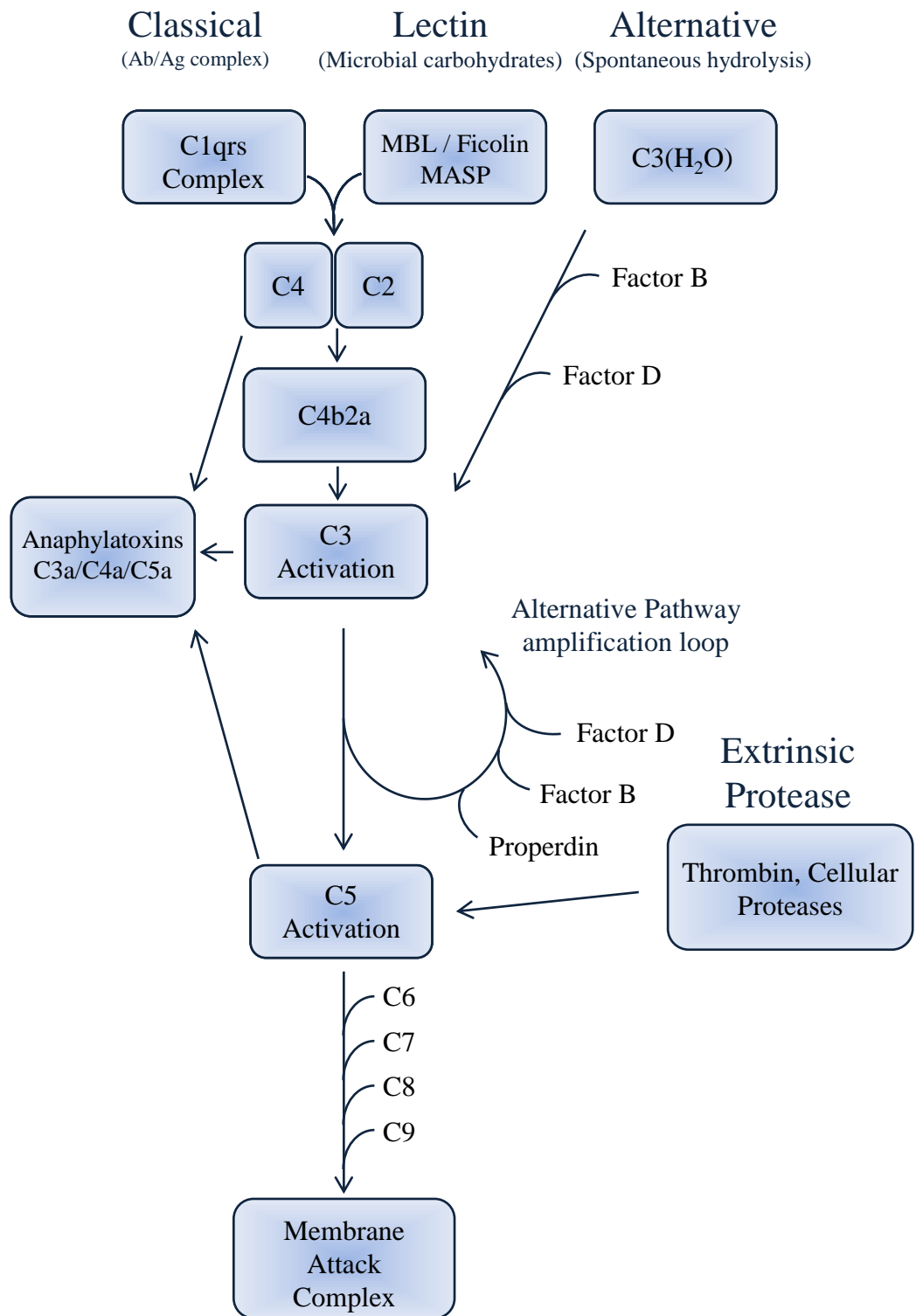
Figure 2. Overview of complement activation pathways. MASP, mannan-associated serine protease; MBL, mannan-binding lectin. Adapted from [Amara 2008], [Carroll and Sim 2011] and [Huber-Lang 2002].

The different activation pathways have a number of components that are functionally, and to some extent structurally analogous. The sensor components of the classical and lectin pathways are the C1 complex and MBL (L-ficolins or collectin-11), respectively; these molecules are structurally related and play a similar role in both pathways, namely that of target recognition and activation of the C3 convertase complex. The large opsonin/convertase proteins C3b (alternative) and C4b (classical/lectin) are structurally similar, and serve to anchor catalytically active convertases to non-self surfaces, as well as linking complement-coated pathogens to cell-surface complement receptors. Component C2a of the classical/lectin pathways and factor Bb of the alternative pathway are also structurally similar and functionally analogous, mediating the C3 convertase activity of the active complex.

a. The classical pathway

The classical pathway sensor is the C1 complex, which is composed of a C1q hexamer and two molecules of each of two serine proteases C1r and C1s. As befits its role, soluble C1 is found abundantly in blood, with a mean plasma concentration of 115 µg/ml [Tan 2010]. Each C1q monomer is comprised of a three intertwined chains (A, B and C chains) with a collagen-like region and a globular head. Disulphide bridges stabilise adjacent C1q monomers, giving the hexamer the overall appearance of a bunch of tulips. The catalytic elements, the two C1s/C1r dimers, bind in a Ca^{2+}-dependent manner to the 'stems' (Figure 3) [Pflieger 2010].

Figure 3. Structural organisation of the C1 complex. (A) Structure of the C1q hexamer and its association with C1r/C1s tetramer. **(B)** Representation of the association between the A (blue), B (green) and C (light blue) chains. Inset in B highlights the positions of lysine residues in the A (position 59), B (position 61 and 65) and C (position 58) chains likely to be involved in the binding site of the C1r/C1s tetramer. CLR, collagen-like region. Adapted from [Pflieger 2010].

The classical pathway is activated by C1 binding to a target molecule. The F_c region of antigen-bound IgM and IgG is the primary target of C1.It is recognised however that C1q can also bind to a wide range of non-immunoglobulin activators including endogenous DAMPs and acute phase reactants such as free nucleic acids or C-reactive protein, microbial antigens such as Group B streptococcal capsular proteins and synthetic materials such as carbon nanotubules [reviewed in Kang 2009]. Binding of the globular heads of C1q induces a conformational change in the C1q hexamer that results in cleavage and activation of the C1r subunits, which in turn cleave the C1s molecules to form an active serine protease [Dodds 1978]. The activated C1r/s tetramer cleaves C4 and C2 to generate the classical/lectin pathway C3 convertase, C4b2a, with release of the anaphylatoxin C4a. C4b2a activates C3, yielding the small peptide anaphylatoxin C3a and a large opsonic and catalytic fragment C3b. C3b can either bind covalently to the target surface or to C4b in the C4b2a complex. The former results in activation of the alternative pathway via the formation of C3b-factor B complex (the alternative pathway C3 convertase), and the latter in formation of the C5 convertase C4b3b2a, which initiates the terminal pathway [Ehrnthaller 2011, Carroll and Sim 2011].

The classical pathway is regulated by the C1 inhibitor (C1-INH). C1-INH binds the C1 complex and initiates the dissociation of the fragments C1r and s, resulting in an irreversible inactivation of the serine protease.

b. The alternative pathway

The alternative pathway can be activated directly by contact with many types of complex carbohydrate structures present on the surfaces of pathogens [Ehrnthaller 2011]. As mentioned above, however, an important characteristic of the alternative pathway is its 'tick over' generation of C3b and thus large-scale complement activation via the alternative

pathway may be more a consequence of the *absence* of complement inhibitors on the surface of pathogens or synthetic materials, than of the *presence* of a specific trigger.

C3 is a member of the C3/α2-macroglobulin protein family, which are large proteins of 1400-1800 amino acids in length; complement components C4 and C5 are structurally similar members of this family and share about 30% sequence homology with C3. C3 and C4 are characterised by a reactive thioester moiety necessary for covalent attachment to molecular or cellular targets, and undergo profound conformational changes upon activation [Blandin and Levashina 2004]. The crystal structure of unactivated human C3 was determined in 2005 [Jansen 2005] (Figure 4) and comprises a β-chain consisting of residues 1-645 and an α-chain consisting of residues 650-1641. Together these chains form 13 domains [Fredslund 2006]: eight homologous macroglobulin domains which comprise the core of the molecule and the linker, anaphylatoxin, CUB (C1r/s, Uegf, Bmp1), thioester and C345c domains [Gros 2008].

Figure 4. Complement component C3. (A) Ribbon diagram of C3. Thioester group (red spheres), anchor region (grey) and α chain N-terminus (black) are shown. Domains are colour-coded: Macroglobulin (MG) domains 1-8 (1, blue; 2, orange; 3, magenta; 4, gray; 5, green; 6, pink; 7, cyan and 8, yellow), anaphylatoxin (ANA, red), C1r/s, Uegf, Bmp1 (CUB, dark blue), thioster (TED, green), linker (LNK, gold), and C345C (russet) domains. **(B)** Domain sequence and arrangements in C3. Colour scheme matches that in (A). Sequential removal of peptide segments is shown, representing proteolytic activation and inactivation. Black lines represent disulphide bridges. [Adapted from Janssen 2005].

The alternative pathway of complement activation is summarised in Figure 5. Spontaneous slow hydrolysis of C3 (half-life ~ 230 h) generates a fluid-phase C3b-like molecule $C3(H_2O)$, which is capable of associating with complement factor B, and is in turn cleaved by the soluble factor D, yielding a soluble complex $C3(H_2O)Bb$ which has C3 convertase activity [Pangburn 1992]. Further molecules of C3 are activated by clipping off the anaphylatoxin domain
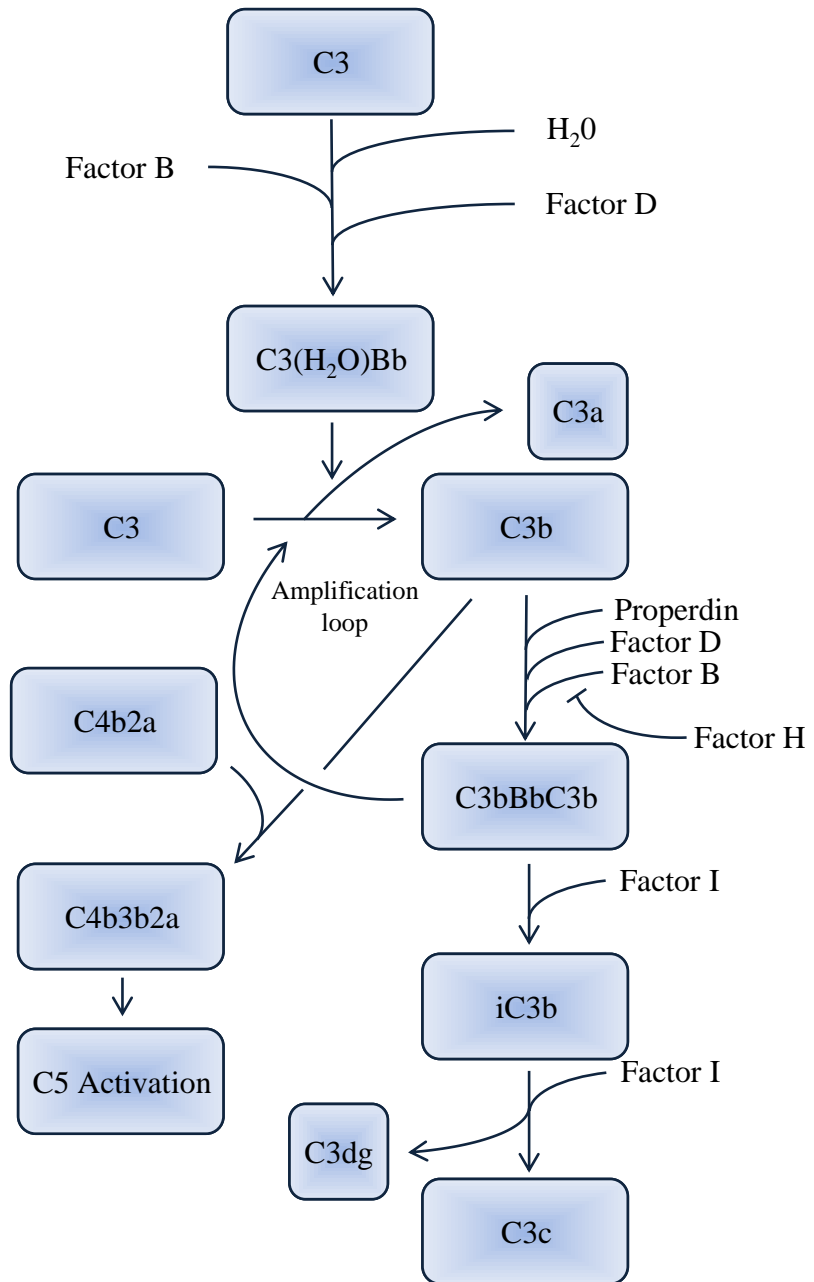
Figure 5. Overview of the alternative pathway. Spontaneous hydrolysis of C3 yields C3(H_2O) which binds factor B. Bound Factor B is activated by Factor D to yield a C3 convertase C3(H_2O)Bb. This activates C3 by cleaving off the anaphylatoxin domain to release the anaphylatoxin C3a and C3b which rapidly binds to pathogen surfaces. Properdin stabilises C3b and facilitates the recruitment of the pro-enzyme factor B; recruitment of factor B is inhibited by factor H. Binding of a second molecule of C3b yields the alternative pathway C3 convertase (C3bBbC3b). Alternatively, bound C3b may associate with C4b2a (the classical pathway C3 convertase) to yield the C5 convertase (C4b3b2a). This activates C5 and initiates the terminal pathway. C3b is sequentially degraded by the soluble protease Factor I. Adapted from [Carroll and Sim 2011] and [Ehrnthaller 2011].

between Arg726 and Ser727, resulting in a conformational change that exposes the highly reactive thioester group, which rapidly (half-life < 100 µs) bonds covalently to any accessible nucleophile molecules (such as sugar residues on microbial surfaces). Surface-bound C3b forms the base for the alternative pathway C3 convertase: the plasma globulin protein properdin binds to C3b and facilitates the recruitment of the pro-enzyme factor B as well as greatly increasing the half-life of the intrinsically unstable C3bB complex. The bound factor B is then activated by the soluble protease factor D to yield factor Bb. Binding of another molecule of C3b yields the alternative pathway C3 convertase C3bBbC3b, which contributes to a positive feedback loop, activating more molecules of C3. Bound C3b may alternatively associate with C4b and C2a, yielding the C5 convertase C4b3b2a, which activates the terminal complement pathway by cleaving C5 to C5a and C5b. C3 may also be activated by the classical pathway convertase (C4b2a) or by MASPs of the lectin pathway [Gros 2008].

Regulation of the alternative pathway is achieved by a number of soluble and membrane-bound complement regulatory proteins, as well as by the intrinsic instability of C3bBb, which decays spontaneously with a half-life of 60 seconds [Kerr 1980]. Complement regulatory proteins inactivate C3bBb in two ways: by enhancing the spontaneous decay of C3bBb (decay-accelerating activity), and/or by enhancing the proteolytic cleavage of C3b into iC3b by the soluble protease factor I (cofactor activity) (Figure 5).

Factor I breaks down surface-bound C3b, yielding the fragments iC3b, C3c and C3dg. Membrane-bound regulators such as decay-accelerating factor (DAF, CD55) and membrane cofactor protein (MCP, CD46), and complement receptor 1 (CR1, CD35) are cofactors for factor I as well as having decay-accelerator activity.

The soluble complement regulatory factor H (FH) is a 155-kDa elongated glycoprotein consisting of 20 complement control protein (CCP) domains, each comprising 60 amino acids

[Sim and Di Scipio 1982]. Binding sites for C3b are found at CCP1-4, 12-14 and 19-20. FH is found in plasma at concentrations between 150-750 µg/ml [Tan 2010]. FH binding to C3b prevents formation of C3bB and so inhibits assembly of the convertase complex; it also possesses decay-accelerating activity and cofactor activity, enhancing the proteolytic breakdown of C3b. FH is reported to bind preferentially to C3b on endogenous cell surfaces due to the presence of negatively charged residues such as scialic acids that are not found on pathogens. FH can also bind to negatively charged residues on endogenous molecules such as heparin. Certain pathogenic bacteria, such as *Neisseria meningitidis* also express negatively charged residues on their cell surfaces which are capable of binding FH. Since FH is a complement inhibitor, this represents microbial 'hijacking' of a host defence strategy and helps the bacteria to evade complement-mediated lysis and phagocytosis [Meri and Pangburn 1990].

c. The lectin pathway

The lectin pathway is in many respects similar to the classical. The lectin pathway analogues of the C1 complex are MBL, L-ficolin and collectin 11 [Hansen 2010]; the MASPs are structurally similar and functionally analogous to the C1r/s tetramer and similarly catalyse the breakdown of soluble C4 and C2 to the classical pathway C3 convertase C4b2a.

MBL, L-ficolin and collectin 11 are somewhat less abundant in plasma than is C1q, with a plasma concentration ranging from less than 1 µg/ml to about 25 µg/ml [Hansen 2010]. MBL and collectin-11 are members of the collectin family [Holmskov 2003], which are proteins composed of collagen-like and carbohydrate recognition domains (CRD). Ficolins are structurally and functionally related, but their CRD is a fibrinogen-like domain.

MBL consists of 18 identical 32 kDa subunits typically arranged as a hexamer of trimers (Figure 6A), although tetramers or dimers may also form [Jensenius 2009]. Each monomer is

comprised of an N-terminal region, a collagen-like domain, an α-helical coiled-coil 'neck' region and a CRD [Drickamer 1986] (Figure 6B). The N-terminal domain contains 21 amino acids with cysteines in positions 6, 13 and 18. Cys^6 is critical for the formation of a disulphide bridge that links monomers within the trimer; Cys^{13} and Cys^{18} are involved in disulphide bridges that stabilise the overall hexamer. The collagen-like region is comprised of 12 repeats of a Gly-X-Y motif where Y is typically proline or hydroxyproline. The monomer spirals in a left-handed helix, each of which is coiled with two others in a right-handed helix to form the trimer. The trimers are stabilised by interactions between the NH group of the glycines and CO groups of the prolines. This 'stem' region forms the binding site for the MASPs. Each monomer terminates in a globular carbohydrate recognition domain. The close approximation of 6 groups of 3 CRDs is indispensable for the high affinity of the molecule for microbial sugar residues [Jensenius 2009].

Figure 6. Structure of human mannan-binding lectin (MBL). (A) Schematic of human MBL structure showing the conformation of hexamer of trimers. **(B)** Diagram of MBL trimer showing N-terminal region (amino acids 1-21), collagen-like domain (amino acids 22-81), neck region (α-helix coiled-coil, amino acids 82-111) and carbohydrate recognition domain (amino acids 112-230). Adapted from [Holmskov 2003] [Sheriff 1994] and [Drickamer 1988].

MBL binds terminal mannose, glucosamine and fucose sugars on the surface of bacteria and fungi, resulting in the activation of MASPs which catalyse the activation of C4 and C2 to generate the classical/lectin pathway C3 convertase C4b2a. C3 activation results in an auto-amplification loop as described above. As well as its role in triggering complement activation, MBL and the other detector molecules of the lectin pathway have been shown to function as opsonins, able to bind to certain microbes in serum-free conditions and mediate enhanced uptake by phagocytic cells without requiring complement activation [Kuhlmann 1989]. However, this plays a proportionally lesser role in opsonisation of pathogens, since relatively few molecules of C1q, MBL or ficolin are found on the foreign surface, whereas brisk complement activation will rapidly cover a foreign particle in C3b.

d. The extrinsic protease pathway

The three canonical pathways of complement activation described above converge at the level of C3 activation. The classical pathway C3 convertase C4b2a is reported to have C5 catalytic activity, but has very low catalytic efficiency [Rawal and Pangburn 2003] and thus C3 activation is the primary mechanism for generation of an efficient C5 convertase and subsequent initiation of the terminal pathway. Thus, in the total absence of C3, activation of the terminal pathway would be predicted to be at the least greatly inhibited. Ward and co-workers used a murine model of immune complex-mediated lung injury to demonstrate that complement-dependent lung injury occurred at an equivalent intensity in spite of the genetic deletion of C3, suggesting the presence of an alternative C5 convertase in C3-deficient mice [Huber-Lang 2006]. The observation that C5a levels in bronchoalveolar lavage fluid and pulmonary injury scores in C3-deficient but not wild-type mice were greatly reduced by inhibitors of the coagulation cascade indicated that the extrinsic C5 convertase might be a serine protease of the coagulation cascade.

Thrombin (factor IIa) is a serine protease that catalyses the conversion of fibrinogen to fibrin, the final common pathway of the coagulation cascade. Ward and colleagues found that in the mouse model of complement-mediated lung injury, thrombin activity in the C3-deficient mice was up-regulated compared with that of wild-type mice, and that purified human thrombin was capable of activating purified or recombinant human C5 in a dose-dependent manner. Hence it appears that thrombin is capable of activating C5 independently of other components of the coagulation cascade. The investigators also found evidence that factors Xa, and XIa and plasmin (but not factor VII or tissue factor) were able to directly activate both C3 and C5 [Amara 2008]. The physiological significance of this pathway (in the presence of functional C3) is not established, but conditions such as sepsis which cause massive complement activation

are typically accompanied by dysregulated activation of the coagulation cascade (for example in disseminated intravascular coagulation), and thus it is plausible that thrombin might contribute to pathological systemic complement activation in sepsis. It has also been shown that terminally differentiated phagocytic cells are able to activate C5 directly, presumably through the action of a membrane-bound or secreted serine protease [Huber–Lang 2002].

e. The terminal pathway

The pathways of complement activation converge with the activation of C5. C5 is a large (196 kDa) protein that is structurally similar to C3 and C4. It is composed of 8 macroglobulin domains, a CUB (C1r/s, Uegf, Bmp1) domain, the C5d domain (analogous to the thioester domain in C3 and C4) and an extended linker region between macroglobulin domains 1-2 and 4-6 [Fredslund 2008]. Activation of C5 results from cleavage at Arg^{751}-Leu^{752}, which releases the potent chemotactic and vasoactive peptide C5a, (discussed in more detail below) and the larger fragment C5b which forms the basis of non-enzymatic assembly of the membrane attack complex. The C5b fragment sequentially combines with C6 and C7 and integrates into the phospholipid membrane [Podack 1980]. The addition of one C8 molecule creates the complex of C5b678, which forms a small transmembrane pore which is enlarged by the polymerisation of between 10 and 18 molecules of C9, creating a hole of about 100 Å in diameter [Podack 1984]. With loss of the membrane barrier function, unrestricted movement of water and small molecules along their concentration gradients may result in lysis of the target cell.

1.2.3.3 Effects of complement activation

a. Opsonisation of pathogens

All of the three canonical pathways result in the activation and degradation of C3b. C3b degradation products are potent opsonins that greatly facilitate the clearance of pathogens. Surface-bound C3b is a ligand for complement receptor 1 (CR1), which is strongly expressed on red blood cells [Fearon 1981]. Thus, complement-coated particles are transported to the spleen bound to red blood cells. As the particle circulates, its affinity for CR1 decreases as C3b is degraded to iC3b by Factor I; CR1 has cofactor activity for Factor I. iC3b, on the other hand, has a high affinity for CR3 and CR4, which are expressed by phagocytic cells. When the red blood cell passes through the spleen, where there are abundant macrophages, the opsonised particle - now mainly coated with iC3b - is transferred to resident macrophages, which ingest and destroy it [Carroll and Sim 2011]. Bound iC3b is itself gradually degraded to the smaller fragment C3dg, which is a ligand for CR2. CR2 is not found on phagocytic cells, but is a component of the co-stimulatory B-cell receptor complex. It is predominantly through CR2 that the complement system acts as an adjuvant to the B cell response, promoting proliferation of antigen-specific B-cell clones that have bound a specific antigen via their immunoglobulin-like B-cell receptor [Carroll 2000].

b. Cell lysis

Although apparently dramatic in its effects, the capacity to form MAC seems to be largely dispensable in host defence; the only adverse effect of deficiency of C5-9 appears to be an increased susceptibility to meningococcal infections [Nagata 1989]. Extracellular lysis of these organisms appears to contribute substantially to clearance of infections, since they are capable of intracellular survival [Walport 2001a]. Paradoxically, deficiency of terminal pathway components may actually convey a survival advantage in *Neisserial* infections, as witnessed by

the high prevalence of inherited C6 deficiency in areas in which they are endemic. It has been suggested that C6 deficiency is protective against *Neisserial* endotoxaemia in infantile gastroenteritis [Orren 1987].

c. Formation of the anaphylatoxins C3a, C4a and C5a

The small peptide fragments C3a, C4a and C5a play a vital role in the host response to infection. They are chemotactic for neutrophils, potentiate phagocytosis and the generation of reactive oxygen species in phagocytic cells, induce complex effects on vascular smooth muscle tone, cause degranulation of mast cells and have a varying capacity to induce inflammatory cytokine release [Guo and Ward 2005]. C5a, the primary focus of this study, is discussed in more detail in section 1.3 below.

d. Adverse effects of complement activation

The complement system plays an important role in host defence and homeostasis, but like many other components of host immunity, it appears to be a two-edged sword; as well as having a vital role in the clearance of pathogens, inappropriate or dysregulated complement activation may result in 'friendly fire' injury to endogenous cells and tissues. Complement activation contributes to the initiation and maintenance of chronic sterile inflammatory conditions such as rheumatoid arthritis [Ballanti 2011], atypical haemolytic uraemic syndrome [Norris and Remuzzi 2009], age-related macular degeneration [Khandhadia 2012], systemic lupus erythematosus and many others [reviewed in Chen 2010].

The potent anaphylatoxin C5a in particular is strongly implicated in the pathophysiology of sepsis. It is thought to contribute to the development of septic cardiomyopathy (a form of sepsis-induced cardiac failure that contributes to septic shock). It has been implicated in the pathological apoptosis of adrenal medullary cells during sepsis, with a resulting deficiency of

the adrenergic amine hormones that are critical for maintaining vascular tone and blood pressure. Excessive intravascular C5a generation in models of sepsis also contributes to neutrophil dysfunction, resulting in failure of neutrophil chemotaxis and impaired microbial killing; this is probably a key factor in the immunosuppression and susceptibility to 'second-hit' infections by opportunistic pathogens that is commonly seen in sepsis patients who survive the initial inflammatory insult [reviewed in Ward 2010a and b]. However, C5a is also a potent inducer of inflammatory cytokine and chemokine release from leucocytes and vascular endothelial cells. The contribution of C5a to the 'cytokine storm' that triggers systemic inflammation and widespread tissue injury and multi-organ failure is perhaps its most significant contribution to the pathophysiology of sepsis [Ward and Gao 2009]; this is the particular focus of this study.

1.3 The anaphylatoxin C5a and its receptors

The discovery of C5a began with the observation that incubation of an antigen with its corresponding antiserum resulted in the production of a heat-stable substance that was chemotactic for neutrophils [Boyden 1962]. Although itself heat-stable at 56˚, this substance was not produced if the serum was heat-treated (to 56˚ for 30min) prior to incubation, suggesting the existence of a heat-labile chemotaxin-generating factor, probably an enzyme, in serum containing antibody-antigen complexes. The observation by Müller-Eberhard that perivascular neutrophil infiltration in experimental immune complex-induced vasculitis was abolished by complement depletion raised the possibility that the chemotaxin was a complement protein [Ward 1965]. Mayer demonstrated in 1968 that sheep erythrocytes pre-treated with guinea-pig IgG and exposed to the first four complement components were capable of cleaving C5 with the production of a high MW complex of C5, 6 and 7 and release of a peptide fragment with a molecular mass of ~ 15000 Da [Shin 1965]. This peptide fragment

was chemotactic for neutrophils, and induced smooth muscle contraction in isolated guinea-pig ileum strips; it was also shown to induce degranulation of basophils and mast cells [Johnson 1975]. Subsequent studies have demonstrated a wide range of effects of C5a, including induction of oxidative burst and generation of reactive oxygen species in phagocytic cells, expression of activation markers and increased leucocyte adhesion and diapedesis, regulation of tissue regeneration and fibrosis and neurodevelopment, and induction of pro-inflammatory cytokines and chemokines release by leucocytes [Strey 2003 and Guo and Ward 2005], the latter being the primary focus of this study (discussed in more detail below).

A   TLQKKIEEIA AKYKHSVVKK CCYDGACVNN DETCEQRAAR
          ISLGPRCIKA FTECCVVASQ LRANISHKDM QLGR

B

This image has been removed by the author for copyright reasons.

Figure 7. Structure of human C5a. Amino acid sequence **(A)** and secondary and tertiary structure of human C5a **(B)**. Four α-helices are shown in grey and inter-α helical loops 1-4 are shown in red (D1, amino acids 12-20), green (D2, amino acids 28-33), and blue (D3, amino acids 38-46). Disulphide bridges are shown in yellow. Adapted from [Guo and Ward 2005].

1.3.1 Structure

The amino acid sequence of C5a was determined between 1976 and 1978 by Fernandez and Hugli [Fernandez and Hugli 1976 and 1978] and is shown in Figure 7. It is a 74-amino acid peptide, arranged in an antiparallel four-helix bundle stabilised by three disulphide bridges between Cys^{21}-Cys^{47}, Cys^{22}-Cys^{54} and Cys^{34}-Cys^{55} [Zuiderweg 1989, Zuiderweg and Fesik 1989, and Zhang 1997]. The helix bundle is highly cationic and confers high affinity for the negatively charged cell surface. The N-terminal segment (residues 1-69) is responsible for receptor binding, while the C-terminal pentapeptide (residues 69-74) form a bulky 'tail', which is responsible for receptor activation. In the absence of the C-terminal pentapeptide, the N-terminal helix (residues 4-12) will bind to the receptor but without agonist activity. Neutralizing antibodies to C5a have implicated residues Lys^{20}-Arg^{37} as critical for the molecule's affinity for its main receptor, C5aR, while its potency is determined by the composition of the terminal pentapeptide (especially Arg^{74}), and by the composition of loop 1 (especially the lysines at positions 12, 14, 19, and 20) and loop 3.

The predicted mass of the 74-amino acid peptide is 10.4 kDa. Approximately 25% of the observed mass (~12.9 kDa) of the native peptide is contributed by a carbohydrate moiety of ~2.5 kDa attached to Asn^{64}, the composition of which shows some variability. The carbohydrate side chain appears to contribute to the chemotactic activity of the native peptide, since the absence of glycosylation results in a reduction of C5a-induced chemotaxis in human neutrophils [Perez 1981]. Interestingly, the chemotactic effect of C5a for human neutrophils is augmented by an anionic polypeptide factor present in human serum, proposed to bind to a sialic acid residue in C5a's carbohydrate side chain [Perez 1986]. This 'co-chemotaxin' was identified as vitamin D-binding protein [Kew and Webster 1987], and was shown to enhance neutrophil chemotaxis to both C5a and its degradation product, C5adesArg

[Binder 1999]. This finding may have implications (largely uncommented-on) for the interpretation of subsequent C5a research, since most studies of the activity of C5a have been conducted using recombinant C5a expressed in bacteria, which typically results in a polypeptide that altogether lacks the carbohydrate side-chain.

1.3.2 Clearance

In vivo, C5a is an ephemeral peptide, due to the rapid kinetics both of its enzymatic deactivation and receptor-mediated uptake by leucocytes. Ubiquitous serum and cell membrane-bound carboxypeptidases rapidly (within seconds) convert it to the more stable C5adesArg by clipping off the N-terminal arginine, which is reported to decrease its biological activity ~10-100 fold [Bokisch and Muller-Eberhard 1970]. It is likely that this also occurs in *in vitro* models of C5a stimulation of myeloid cells, which express both membrane-bound and secreted carboxypeptidases [Krause 1998].

As well as being enzymatically deactivated, C5a and C5adesArg are rapidly cleared from plasma by receptor-mediated uptake. I^{125}-labelled C5a administered by intravascular injection to rabbits was found to have a plasma half-life of 2 min, and C5adesArg only slightly longer [Webster 1982]. Radiolabelled C5a and C5adesArg rapidly redistributed from the plasma to highly vascular organs, where they accumulated. This presumably reflected massive neutrophil activation and extravasation. Consistent with this, it was found that a neutropenia followed intravascular C5a activation, and numerous *in vivo* models have found neutrophil infiltration of the lungs and other highly vascular tissues to be a rapid consequence of intravascular complement activation [reviewed in Ward 2010a].

1.3.3 Biological activity

C5a is chemotactic for migratory phagocytic cells, and induces contraction of smooth muscle and degranulation of mast cells. It induces oxidative burst in neutrophils and macrophages and potentiates oxidative burst in response to other stimuli. It is a weak inducer of inflammatory cytokines in its own right, but interacts synergistically with other inflammatory stimuli to regulate release of inflammatory mediators, and thus is a vital component of the cytokine response to infection and sterile inflammatory stimuli (discussed in more detail below). As well as being a potent mediator of innate immune responses, it also has a role in the regulation of the adaptive immune system, promoting survival and proliferation of both CD4$^+$ and CD8$^+$ T cells [Fang 2007 and Lalli 2008]. It has also been shown to have roles outside the immediate scope of the immune system, mediating bone remodelling and directional migration of neural tissue cells, and it has also been proposed to mediate *in utero* implantation and placentation [Salmon 2011].

It has been observed that C5a and C5adesArg appear to elicit qualitatively different effects in a manner that is difficult to explain simply on the basis of the reduced potency of the stable metabolite. For instance, basophils stimulated with C5a release both pro-inflammatory cytokines and lipid mediators of inflammation, whereas stimulation with equipotent concentrations of C5adesArg results in cytokine release alone [Eglite 2000]. These findings are suggestive of the action of more than one receptor for the anaphylatoxins C5a and C5adesArg.

1.3.4 Receptors for C5a[1]

1.3.4.1 C5aR (CD88)

The existence of a specific cell-surface receptor for C5a was demonstrated by Chenoweth in 1978 [Chenoweth and Hugli 1978] using I^{125}-labelled C5a competition binding studies in human neutrophils. The gene for the receptor was cloned and sequenced in 1991 [Boulay 1991], [Gerard and Gerard 1991] and encodes a 42-kDa protein consisting of 350 amino acids, with a carbohydrate moiety attached at Asn^5. Additional post-translational modifications include sulphations at Tyr^{11} and Tyr^{14}. The gene is localised to chromosome 19q13.33 and consists of two exons, the first of which contains the start codon and the 5' UTR, and the second contains the entire translated sequence, stop codon and 3' UTR. This gene structure is characteristic of the family of 7 transmembrane region G protein-coupled receptors (GPCRs), which contains both of the C5a receptors as well as other chemokine receptors. The gene is known to have two non-synonymous single nucleotide polymorphisms resulting in Asp^2Asn and $Asn^{278}Lys$ substitutions; neither is known to be associated with disease [Birney 2006].

a. Structure

The C5aR protein has an overall structure typical of G protein-coupled receptors (see Figure 8). C5aR ligation involves two distinct events: the aspartate-rich acidic N-terminus of the receptor interacts with the basic core of C5a, and the C-terminus of C5a interacts with a pocket formed by hydrophobic residues in the transmembrane domains and charged residues at the base of the extracellular loops [Monk 2007]. The first event is responsible for ligand binding (affinity) and the second for receptor activation (agonist activity). The ligand-binding domain of C5aR

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[1] Here and subsequently in this thesis, the term **C5a receptor** is used generically to refer to either or both of the receptors for C5a. **C5aR** is used to indicate CD88 or C5a Receptor 1, and **C5L2** to indicate gpr77 or C5a Receptor 2.

Figure 8. Structure of human C5aR. Structure of human C5aR (CD88) showing possible interaction sites with C5a. Transmembrane regions of C5aR are shown as mauve cylinders. Possible interaction sites with C5a in the second extracellular loop and N-terminus are shown in red. Inter-α helices of C5a are shown in red (D1), green (D2) and blue(D3). From [Guo and Ward 2005].

has been identified in a number of ways: antibodies raised against the N-terminal peptide sequence exhibit C5aR antagonist activity [Morgan 1993], and deletion of the N-terminus prevents ligand binding [Mery and Boulay 1993]. A C3aR-C5aR chimera, having the N-terminal region of C3aR also fails to bind C5a, but all three retain the ability to respond to peptide analogues of the C-terminal region of C5a [Crass 1999]. Mutational analysis of C5aR using a yeast selection system suggested that Arg^{175}, Glu^{199}, Arg^{206} and Asp^{282} are critical residues in the juxta-membrane region of the extracellular loops for the activation site.

b. Tissue distribution

Initially shown to be expressed on myeloid leucocytes (which show the strongest expression), C5aR is known to be widely expressed as a cell-surface receptor on a range of cell types including lymphocytes (B and T-cells), endothelial cells and a range of cells in the central nervous system, skin, heart, connective tissue, kidney, liver and lung [reviewed in Monk 2007]. Its tissue expression is up-regulated in non-myeloid cells by inflammatory stimuli such as bacterial lipopolysaccharide (LPS), IL-6 and TNF-α, and in models of sepsis [Hunt 2005, Riedemann 2003 and Stahel 2000], but downregulated in myeloid cells (especially neutrophils) in response to sepsis [Huber-Lang 2005]. Stimulation of neutrophils with C5a leads to rapid receptor internalisation followed by slower recycling to the cell surface, possibly with re-release of functional C5a back into the extracellular medium [Scola 2009].

c. Ligand specificity

Radiolabelled C5a competition binding studies showed that C5aR binds C5a avidly, and C5adesArg with 20 to 200x less affinity. Reported IC_{50} values of C5a for C5aR are in the range of 2-20 nM and of C5adesArg for C5aR 400-700 nM [Cain and Monk 2002, Kalant 2003, Okinaga 2003]. It has also been shown that C5aR is capable of binding a homodimer of the ribosomal protein S19, resulting in activation of downstream signal transduction pathways [Nishiura 2010].

d. Signal transduction

GPCRs like C5aR are the largest protein receptor superfamily in the body. GPCRs transduce extracellular signals through a cascade of protein phosphorylation events and second messenger intermediates (Figure 9). GPCRs undergo a conformational change upon ligand binding that promotes activation (phosphorylation) of the heterotrimeric GTPase (G protein).
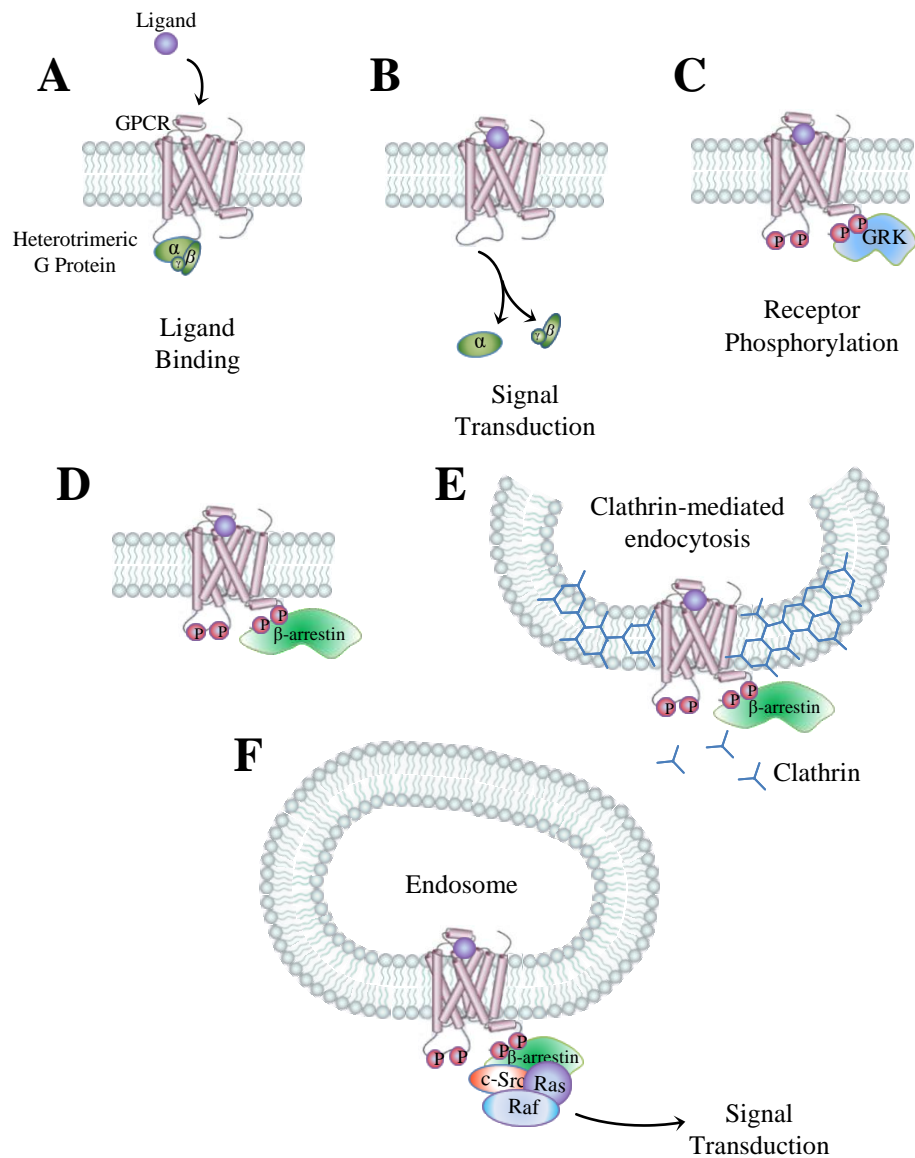
Figure 9. Model of G protein-coupled receptor signal transduction. (A) Heterotrimeric G protein bound to the intracellular loops of a G protein-coupled receptor (GPCR) in its 'resting' state. **(B)** Ligand binding induces a conformational change in the GPCR, causing dissociation of G protein monomers and subsequent activation of signal transduction cascades. **(C)** Phosphorylation of intracellular loops of the GPCR by G receptor kinases (GRK) prevents re-association of G proteins and promotes **(D)** association of β-arrestins with the phosphorylated receptor. **(E)** β-arrestin promotes clathrin-mediated internalisation of the receptor-ligand complex, leading to **(F)** β-arrestin-dependent recruitment of signal transduction intermediates (such as non-receptor tyrosine kinase Src and the rho-family GTPases Ras and Raf), resulting in G protein-independent signal transduction. [Adapted from Luttrell 1997, Lefkowitz 2004 and Shukla 2011].

Activation of the G protein results in its dissociation into α and βγ subunits, which initiate G protein-dependent signalling [Audet and Bouvier 2012]. After dissociation of the G-protein subunits, G-receptor kinases (GRKs) phosphorylate serine/threonine residues primarily in the C-terminal intracellular 'tail' of the receptor. Receptor phosphorylation promotes binding of the multifunctional adaptor proteins, β-arrestins, which sterically hinder further G-protein coupling with the activated receptor and lead to clathrin-mediated internalisation of the receptor [Lefkowitz 1998].

The role of β-arrestins is wider than just termination of GPCR signalling: as well as mediating clathrin-dependent receptor internalisation, β-arrestins may recruit a range of other signalling intermediates to the activated receptor and serve as a scaffold for a signalling complex that engages G protein-independent signalling pathways. Importantly in the context of this study, β-arrestins have been shown to be able to scaffold upstream components of many of the signalling pathways critical for inflammatory responses (such as cytokine production). In particular, β-arrestin 1 has been shown to recruit non-receptor tyrosine kinases of the Src family which form a catalytic complex with small rho-family GTPases and are known to activate the mitogen-activated protein kinases (MAPKs) p38 (MAPK14), extracellular signal-related kinase 1/2 (ERK1/2, MAPK3/1) and c-Jun N-terminal kinase 1/2 (JNK1/2, MAPK8/9) as well as phosphoinositol-3-kinase (PI3K) and Akt [Luttrell 1997, McDonald 2000, Povsic 2003, Gong 2008].

Furthermore, it appears that GPCR signalling is more complex than a 'two-state model' in which the G protein is switched 'on' by ligand binding or 'off' by GRK phosphorylation. First, GPCRs may adopt multiple 'active states', engaging different pathways with different G-protein subunits, one or other of which may be favoured according to different circumstances. For example, the β_2-adrenergic receptor may sequentially engage $G\alpha_s$ and $G\alpha_i$ subunits as well

as signalling via β-arrestin 2, depending on the degree of phosphorylation of its intracellular loops [Lefkowitz 1998]. Second, GPCRs may form homo- or heterodimers, which appear to exhibit distinct behaviour in regards of internalisation/deactivation and signalling; C5aR has been reported to homodimerise [Floyd 2003] and heterodimerise with the chemokine receptor CCR5 [Huttenrauch 2005]. Third, receptor clustering in membrane microdomains such as lipid rafts may affect G-protein coupling and trafficking [DeFea 2008].

C5aR G protein-dependent signal transduction proceeds through $G\alpha_{i2}$ [Sheth 1991, Skokowa 2005], a pertussis toxin - sensitive G protein. $G\alpha_{16}$ coupling has been shown in cultured cell lines but its occurrence in primary cells is controversial [Monk and Partridge 1993]. The Gβγ protein subunits have also been shown to be critical for C5aR signalling [Hwang 2004 and 2005]. Consistent with this, it has been reported that the Gβγ subunits (rather than Gα subunits) are principally involved in activation of MAP kinase cascades in other GPCRs [Lefkowitz 1998]. C5aR is known to bind to β-arrestins 1 and 2 [Braun 2003] and an effect of β-arrestin 1 binding on signal transduction has been documented [Bamberg 2010].

C5aR ligation may elicit a number of different cellular responses, including the cytoskeletal rearrangements involved in chemotaxis, induction of oxidative burst metabolism and the release of pro-inflammatory cytokines (the particular focus of this study). C5aR signal transduction (Figure 10) has been studied predominantly in the context of chemotaxis and the induction of oxidative burst in professional phagocytic cells, and consequently the pathways involved specifically in the induction of cytokine synthesis have not been completely documented. However, many of the signal transduction pathways reported to mediate chemotaxis and oxidative burst are also known to be involved in the induction of cytokine secretion. This is discussed further in Section 1.5 and Figure 18 (below).
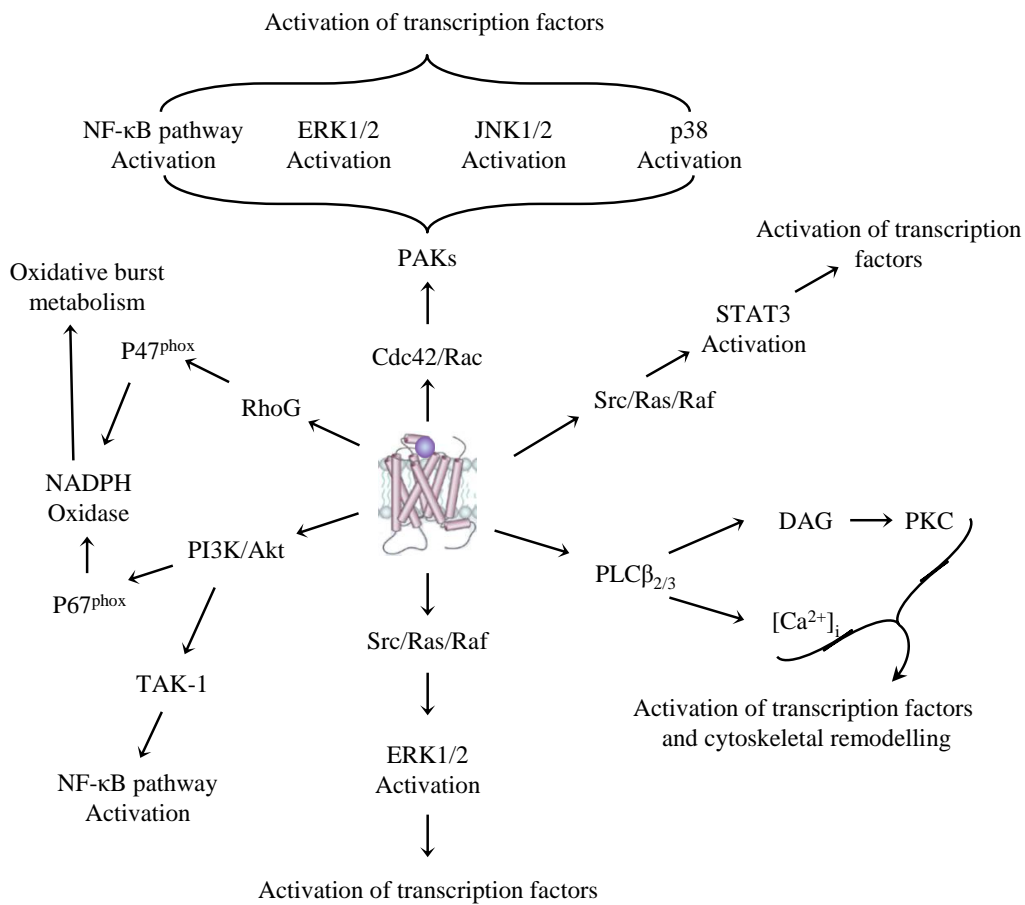
Figure 10. Overview of C5a receptor signal transduction. C5aR interacts with protein kinases, GTP-binding proteins, non-receptor tyrosine kinases and other signalling enzymes, which in turn activate downstream signal transduction intermediates, leading to: actin remodelling and cytoskeletal rearrangement, activation of transcription factors leading to changes in gene expression, and initiation of oxidative burst metabolism leading to generation of reactive oxygen species. DAG, diacyl glycerol; ERK, extracellular signal-related kinase; PAKs, p21-activated kinases; JNK, c-Jun N-terminal kinase; NADPH, nicotinamide adenine dinucleotide phoshate; NF-κB, nuclear factor κB. Adapted from [Monk 2007].

1.3.4.2 C5L2 (*gpr*77)

a. Structure

The existence of a second receptor for C5a was first reported by the Takahashi group in 2000 [Ohno 2000]. The group amplified a 700-bp fragment of DNA from a cDNA library isolated from human immature dendritic cells using degenerate primers based on known conserved regions in the second and seventh transmembrane regions of non-chemokine chemoattractant receptors (including IL-8R, C5aR, C3aR and f-met-leu-phe receptor {fMLPR}). An expression plasmid was constructed and stably transfected into HEK-293 cells. Sequence homology with human C5aR (38%), C3aR (33%) and fMLPR (29%) suggested that the new receptor might be a GPCR. Analysis of the amino acid sequence suggested that the protein possessed a serpentine structure with several common characteristics of the GPCRs including several Ser/Thr residues that provide the phosphorylation site for binding of β-arrestins to C5aR in the intracellular C-terminus.

b. Tissue distribution and subcellular localisation

Expression of mRNA for C5L2 is reported in a wide range of human tissues; its expression parallels that of C5aR mRNA, but is generally of lesser abundance. Early reports [Okinaga 2003] suggested that C5L2 might be expressed in greater abundance than C5aR in certain tissues (especially skeletal and cardiac muscle), whereas Gerard et al found C5L2 consistently less abundant than C5aR [Bamberg 2010]; this might reflect intersubject variability in the relative expression levels of the mRNA for the two receptors. Expression of C5L2 at the protein level has been reported in a range of leucocyte subtypes including monocytes, macrophages and neutrophils, as well as in astrocytes, glial cells, fibroblasts and adipocytes [reviewed in Monk 2007].

The subcellular localisation of C5L2 is still a subject of some controversy. Many of the early data concerning the subcellular localisation of C5L2 pertain to transfection systems in cultured cell lines and may not reflect the cellular localisation of the receptor in cells that naturally express it. A number of techniques have been used to assess the presence of C5L2 either at the cell-surface or in intracellular compartments, with apparently conflicting results.

The Takahashi group [Ohno 2000] detected cell-surface expression of C5L2 on immature but not mature human monocyte-derived dendritic cells by flow cytometry. Gerard *et al*. reported that cell surface expression of C5L2 was detectable by flow cytometry on human neutrophils and monocytes [Okinaga 2003]. Köhl and co-workers found strong surface expression of C5L2 on purified human monocytes and the mast cell line HMC-1, but none on human neutrophils [Otto 2004]. In this study, the investigators found that mean fluorescent intensity of C5L2 staining on monocytes varied by a factor of 3 between four donors, raising the possibility that cell-surface expression of C5L2 may vary substantially between different donors of the same species. Ward *et al.* used Western blot of a whole-cell lysate and flow cytometry to demonstrate C5L2 expression in human and rat neutrophils [Huber-Lang 2005]. The Ward group also used confocal microscopy to investigate the sub-cellular localisation of C5L2 in rat neutrophils [Gao 2005], which showed a uniform peripheral staining for both C5L2 and C5aR, indicating a cell-surface expression pattern for both receptors. However, neutrophils isolated from blood samples taken 24 h after caecal ligation and puncture (CLP) showed a cortical redistribution of C5aR (suggesting receptor internalisation) but unchanged peripheral C5L2 distribution.

Monk and colleagues used I^{125}-labelled C5a to assess cell-surface expression of C5L2 on human neutrophils [Scola 2009]. Briefly, neutrophils were loaded with I^{125}-labelled C5a in the presence and absence of a specific C5aR antagonist. C5a uptake in the presence of an excess

of C5aR antagonist was attributed to C5a binding to C5L2, and the difference between this and C5a uptake in the absence of C5aR antagonist attributed to C5a binding to C5aR. The investigators found that C5L2 expression on neutrophils was weak compared with that of C5aR (4,617 ±1,373 C5L2 molecules per cell compared with 97,306 ± 42,658 of C5aR, mean ±SD). These findings were consistent with their observation that C5L2 expression in stably transfected RBL cells was predominantly intracellular, with >60% of the receptor localised within the cell, compared with <20% of C5aR. Notably, the ratio of cell-surface C5L2 to C5aR expression exhibited significant variation between the 4 donors tested (range 2.4% to 29.7%, median 4.5%). These findings were in contrast with those of the Ward group [Gao 2005], who had reported predominantly cell-surface expression in rat neutrophils.

Gerard and co-workers' findings [Bamberg 2010] were in agreement with those previously reported by Scola [Scola 2009] and discussed above. They reported that pre-incubation of human neutrophils with the mouse anti-human C5L2 monoclonal antibody (mAb) clone 1D9, previously shown to block C5L2, had no effect on I^{125}C5a binding, suggesting little or no surface expression of C5L2. Flow cytometry analysis of human neutrophils using another C5L2-specific mAb (clone 4C8) showed no increase in fluorescence over isotype control in intact neutrophils, but a substantial increase when cells were permeabilised with saponin before incubation with the primary antibody, both of which findings suggested an intracellular localisation for C5L2, at least in human neutrophils. Confocal microscopy supported this observation; C5aR showed a distinct peripheral staining pattern consistent with cell-surface expression in resting cells, with rapid redistribution to the cytoplasm following stimulation with C5a. C5L2, however, showed a diffuse cortical staining pattern in resting cells characteristic of intracellular localisation of the receptor.

The apparent inconsistency between the findings of Köhl *et al* [Otto 2004], who failed to demonstrate cell-surface C5L2 expression on human neutrophils, with the later studies may perhaps be explained on the basis of a predominantly intracellular location for C5L2 in human neutrophils, and the reported wide intersubject variation in cell-surface expression. Furthermore, while the Köhl group report that C5L2 labelling with the primary antiserum occurred at zero degrees, several of the other studies report incubating with antibody at room temperature, in which conditions antibody might be internalised and bind to intracellular receptors. It is more difficult to reconcile the findings of Ward *et al* [Gao 2005] in rat neutrophils with those of Monk and Gerard in human neutrophils [Scola 2009 and Bamberg 2010]. It is possible that C5L2 has a different subcellular localisation in rat compared with human neutrophils.

In summary, C5L2 has been shown to be expressed as a cell-surface protein in monocytes and macrophages as well as astrocytes and glial cells. It has been reported to be predominantly expressed at the cell surface in rat neutrophils, and predominantly intracellular in human neutrophils. In cells which exhibit surface expression, its abundance may vary significantly from one donor to another.

c. Ligand specificity

The status of the putative chemokine receptor C5L2 as a receptor for C5a was demonstrated by Cain and Monk [Cain and Monk 2002] who showed specific binding of C5a, C5adesArg, C4a and C3a to C5L2 using radiolabelled C5a competition assays. The affinity of C5L2 for C5a was similar to that reported for C5aR ($Kd=9.5$ nM for C5L2 compared with 7.7nM for C5aR) but the affinity of C5L2 for C5adesArg was approximately ten-fold higher than the affinity of C5aR ($Kd=36.5$ nM compared with 412 nM). Furthermore, the on-off rates of the two receptors are very different. Using a model of L1.2 lymphoblasts transfected with either C5aR or C5L2,

Gerard and co-workers demonstrated that C5a binding to C5aR reaches equilibrium within minutes whereas binding to C5L2 had not reached equilibrium by 2 hours. Similarly, the off-rate of C5L2 was estimated to be eight times slower [Okinaga 2003].

A detailed and comprehensive study published by the Monk group in 2007 [Scola 2007] addressed the issue of the molecular basis for the apparent preference of C5L2 for binding C5adesArg rather than C5a. As previously discussed, the C5aR's primary binding site for C5aR is a series of acidic and Tyr residues at the N-terminus of the peptide; a secondary binding site in the hydrophobic pocket formed by the transmembrane helices mediates agonist activity. However, a panel of C5a agonists that selectively bind to the hydrophobic pocket had relatively little effect on C5a or C5adesArg binding to C5L2, suggesting that this site was less important for C5L2 binding of its ligands. Hence it appears that acidic and tyrosine residues at the N-terminus are critical for C5a binding to C5aR, and for C5adesArg binding to C5L2, but that C5a binds to different residues on C5L2. The authors also identified sulphation of *N*-terminal tyrosine residues as critical for C5aR binding to either ligand, whereas inhibition of tyrosine sulphation had only a small effect on C5a binding to C5L2 and none on C5desArg binding.

Importantly, the authors also noted that rat and mouse C5aR and C5L2 (which are commonly-used experimental models) do not exhibit similar affinities for the two ligands to the human receptors. Mouse and especially rat C5L2 have a lower affinity for murine C5a and much higher affinity for C5adesArg, such that at concentrations of C5a observed *in vivo*, C5L2 is essentially a receptor for C5adesArg, with very little capacity to bind C5a [Scola 2007]. Hence a degree of caution is needed when generalising findings from these models to humans.

d. Signal transduction and function

Thirteen years after its initial description, the function of C5L2 is still a matter of controversy. Upon its discovery in 2000, the Gerard group noted that the receptor lacked the PKC phosphorylation site possessed by C5aR and C3aR on the third intracellular loop, which is important for intracellular signal transduction [Ohno 2000]. Furthermore, the third intracellular loop of most GPCRs contains a conserved Asp-Arg-X motif that is critical for G-protein binding. C5aR has Asp-Arg-Phe and C3aR has Asp-Arg-Cys, but C5L2 has Asp-Leu-Cys, suggesting that it is unlikely to couple to G-proteins. Consistent with this, Cain and Monk found that C5L2 couples only very weakly to classical GPCR signal transduction pathways [Cain and Monk 2002]. Supra-physiological stimulating concentrations of C5a, C5adesArg, C4a or C3a failed to induce Ca^{2+} mobilisation or granule enzyme release in RBL cells transfected with C5L2. The authors also found no evidence of ligand-mediated receptor internalisation, a typical consequence of GPCR activation and signalling, and so concluded that the likely function of C5L2 was as a scavenger receptor, acting as a 'sink' for activated complement fragments. Interestingly, however, priming C5L2-transfected RBL cells with C5a or C5adesArg before activation with IgE immune complexes (known to induce activation of nuclear factor-kappa B (NF-κB) and inflammatory cytokine release via the $F_c\epsilon$ Receptor1/Tyrosine Kinase pathway) did result in ~30% increase in granule enzyme release, in a pertussis toxin-dependent manner, which suggested a $G\alpha_i$-mediated effect of C5L2 on F_c receptor signal transduction [Ten 1999]. This raised the possibility that, regardless of its putative role in C5a signal transduction, C5L2 might exert a regulatory effect on the signal transduction of other receptors.

A further study by the Gerard group in 2003 produced results both concordant and contrasting with those of Cain and Monk [Okinaga 2003]. Consistently with previous findings, the authors

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were unable to demonstrate C5a-induced Ca^{2+} flux in cell lines transfected with C5L2, even when $G\alpha_{16}$ was co-transfected. A weak C5a-induced Ca^{2+} mobilisation was achieved by inducing $Leu^{132}Arg$ mutation at the critical G-protein-binding site in intracellular loop 3 (thus restoring the Asp-Arg-X motif critical for G-protein binding), which was enhanced by co-transfection with $G\alpha_{16}$. However, the relative weakness of the signal compared with that seen in a $C5aR^+$ $G\alpha_{16}$ co-transfectant model suggests that the AspLeuTyr mutation in C5L2 is not the only factor in uncoupling C5L2 from G protein signalling.

It is notable that the Ca^{2+} flux induced by this mutation was sustained rather than transient, which the authors interpreted as reflecting a lack of ligand-induced receptor deactivation. Ligand-induced deactivation of GPCRs is typically a consequence of C-terminal Ser/Thre phosphorylation by GRKs. Consistent with this, whereas C5aR was rapidly and strongly phosphorylated in response to C5a stimulation, C5L2 showed much lesser degree of, and slower phosphorylation. The authors also failed to observe C5a-induced ERK1/2 phosphorylation in C5L2-transfected L1.2 lymphoblasts. If the findings from this experimental model may be generalised, it would suggest that if C5L2 were able to modulate responses of cells to C5a it is more likely to reflect an effect on the kinetics of the C5a-C5aR interaction than signal transduction through C5L2 itself, which would be consistent with a role as a decoy receptor.

The Gerard group presented the first data generated using a C5L2-deficient C57/BL6 mouse model in 2005 [Gerard 2005], data which were consistent with the general hypothesis that the primary function of C5L2 was to attenuate C5aR-mediated pro-inflammatory responses by scavenging ligand. In a model of immune complex-mediated pulmonary injury (which is characterised by pulmonary complement activation), C5L2-deficient mice exhibited greater neutrophil infiltration and more severe histological pulmonary inflammation than wild type

mice. Lung tissue homogenates showed higher levels of the pro-inflammatory cytokines IL-6 and TNF-α, and isolated bone marrow cells from C5L2-deficient mice showed enhanced chemotaxis in response to C5a. These data indicating a more 'pro-inflammatory' phenotype in C5L2-deficient mice would suggest that C5L2 was exerting an inhibitory effect on C5a-mediated inflammatory responses in the wild-type animals.

An intriguing study conducted by Feinstein and co-workers in 2005 co-incidentally identified the presence of C5L2 in rat astrocytes [Gavrilyuk 2005]. Immunohistochemical staining of rat brain tissue for C5L2 showed that it was expressed throughout the rat brain in neurones and astroglia. Using generation of reactive nitrogen species (levels of nitric oxide synthase (NOS) and nitrites) as a marker for LPS-induced inflammation, the investigators showed an anti-inflammatory effect of C5L2: preincubation of cultured rat astrocytes with an antisense oligonucleotide to rat C5L2 resulted in a 2.5-fold increase in LPS-induced NOS mRNA and nitrite levels. Transfection of C6 glioma cells with C5L2 resulted in a significant reduction (>70%) of NF-κB activation in response to LPS treatment compared with cells not expressing C5L2. Interestingly, this effect was observed in the *absence* of exogenously administered C5a, raising the possibility that constitutive activation of C5L2 might have a modulatory effect on TLR-mediated inflammatory responses; this possibility is discussed in more detail in sections 1.5 and 3.8 (below).

The Klos group demonstrated that C5L2 is constitutively expressed by the epithelial HeLa cell line [Johswich 2006]. C5aR however was neither constitutively nor inducibly expressed in these cells. The myeloblastic cell lines U937 and HL-60 were found to express C5L2 constitutively, and inducibly upon stimulation with IFNγ and dibutyryl cAMP, but not TNF-α. However, no C5a-induced Ca^{2+} mobilisation could be shown in these cell lines. Thus the investigators concluded that the likely role of C5L2 is as non-signalling receptor or 'sink' for

C5a/C5adesArg, in which context it might inhibit C5a-induced inflammatory responses mediated by C5aR. They did not, however, exclude the possibility of Ca^{2+}-independent signalling via C5L2, nor did they address the question of why an epithelial cell might express a decoy receptor for C5a when it does not express the 'live' receptor, C5aR.

Up to this point, functional studies had consistently found that blockade or deficiency of C5L2 resulted in a stronger response to C5a or other inflammatory stimuli, suggesting an inhibitory effect of C5L2 on pro-inflammatory responses to C5a (and possibly other stimuli). However, data from independently-generated C5L2-deficient C57/BL6 and BALB/c mouse models were presented by Yeh and colleagues in 2007 [Chen 2007], suggesting a different and more complex function for C5L2 than that previously proposed. In a detailed and comprehensive study, the investigators presented findings that appeared to demonstrate a role for C5L2 in *potentiating* both C5a and C3a-mediated inflammatory responses. Neutrophils from wild-type mice showed similar sensitivity to C5a to those from C5L2-deficient animals, as measured by expression of activation markers and IL-6 release. However, C5a-induced MAPK signalling in cells from C5L2-deficient mice was found to be inhibited compared with wild-type cells. A corresponding effect on C5a-mediated chemotaxis was also reported in that recruitment to and activation of neutrophils in thioglycollate- and C5a-inoculated dorsal air pouches were reduced in C5L2-deficient mice compared with wild-type littermates. C5L2 deficiency was also associated with reduced airway hyper-responsiveness and lung inflammation in an OVA-induced asthma model. These findings suggested that in this experimental model, C5L2 was either potentiating signal transduction through C5aR or directly transducing C5a signals itself since its deficiency resulted in greatly attenuated MAPK phosphorylation; neither effect was consistent with its previously-proposed role as a decoy receptor for C5a.

An investigation published by the Ward group in 2008, conducted using the Gerard group's C5L2-deficient mice, produced the first conclusive evidence of C5L2 signal transduction independently of C5aR [Rittirsch 2008]. The investigators found that *in vitro* C5a stimulation of murine macrophages induced the release of high mobility group box protein 1 (HMGB1), a chromatin-binding protein that is mobilised from the nucleus and secreted in response to inflammatory stimuli, and functions both as a marker and late mediator of inflammation in sepsis [Wang 2008]. Sepsis also induced significant plasma levels of HMGB1. In both the *in vitro* and sepsis models HMGB1 secretion was unaffected by either blockade or deficiency of C5aR, but was abolished either by C5L2 deficiency or by treatment with a C5a antagonist, suggesting that HMGB1 secretion was dependent on the interaction of C5a with C5L2, rather than C5aR. The C5a-mediated release of HMGB1 *in vitro* could be blocked by inhibitors of specific MAPK pathways (ERK1/2 and JNK1/2 but not p38), indicating that C5L2 is in fact capable of coupling to the MAPK signal transduction pathways without any requirement for C5aR. The findings of this study are discussed in more detail in section 1.5 (below).

Since C5L2 has been shown to be incapable of signalling through G proteins in numerous model systems, it is possible that the effects described above are mediated by a G protein-independent signal transduction mechanism. Zaman and co-workers [Van Lith 2009] developed a β-galactosidase complementation assay that demonstrated C5a- and C5adesArg-induced co-localisation of C5L2 with β-arrestin 2 in a CHO transfectant system. Gerard *et al.* [Bamberg 2010] reproduced these findings in human neutrophils, demonstrating by confocal microscopy and co-immunoprecipitation that stimulation of neutrophils with C5a induces co-localisation of C5L2 with β-arrestin 1. The investigators also showed that C5aR at the cell surface is rapidly internalised to cytoplasmic vesicles upon C5a stimulation, where it associates with C5L2, raising the possibility of a C5aR ligation-dependent formation of a C5aR/C5L2/β-arrestin signalling complex. This would suggest that C5L2 ligation of C5a is a secondary event,

following C5aR ligation and internalisation, which would be consistent with the group's finding of a predominantly intracellular location for C5L2 in human neutrophils. Also consistent with this hypothesis, the investigators were able to block association of C5L2 with β-arrestin 1 by pre-incubating cells with an anti-C5aR antibody prior to C5a stimulation. Antibody blockade of C5L2 resulted in a dramatic enhancement of neutrophil chemotaxis but had no effect on Ca^{2+} signalling, consistent with employment of a G protein-independent signalling pathway. It did, however, enhance ERK1/2 phosphorylation.

These findings led the investigators to propose a model in which C5L2, through its association with β-arrestin 1, exerted a negative regulatory effect on C5aR-mediated ERK1/2 phosphorylation and chemotaxis. Since C5L2 had been shown to be predominantly intracellular, and since blockade of C5aR prevented co-localisation of C5a and C5L2, the authors proposed that C5L2 activation was a secondary event, strictly dependent upon C5aR ligation and internalisation. However, whilst explaining their own findings in human neutrophils, this hypothesis is not consistent with the earlier evidence provided by the Ward group that C5L2 is capable of inducing HMGB1 release by C5aR-deficient murine macrophages.

Köhl and co-workers [Zhang 2010] identified a role for C5L2 in the regulation of adaptive immunity in a mouse model of allergic asthma. C5a is reported to have biphasic effects in the context of allergy [Köhl 2006], while it is a key mediator of inflammation in the context of acute exacerbations of asthma, it has been reported to have a negative regulatory role during allergen sensitisation by moderating DC-mediated activation of naïve T cells. Zhang *et al.* found that in an ovalbumin-induced asthma model, C5L2-deficient BALB/c mice exhibited decreased airway hyper-responsiveness, histological airway inflammation and leucocyte infiltration as well as reduced serum levels of IgE compared with wild-type littermates. C5L2-deficient pulmonary cells harvested by bronchoalveolar lavage and then cultured overnight

secreted lower levels of the TH_2-promoting cytokines IL-4, IL-5, IL-13 and IL-10. Similar results were obtained using a house dust mite-induced model of asthma; in this model, cultured cells retrieved by bronchial lavage of C5L2-deficient mice secreted also much higher levels of IL-17 than wild-type mice. These findings suggested that C5L2 was responsible for the release of cytokines that promoted an allergenic TH_2 polarisation of $CD4^+$ T cells, and that its absence resulted in a cytokine milieu favouring TH_{17} polarisation.

An alternative role for C5L2 as a receptor for the inactive stable metabolite of C3a, C3adesArg[77], was proposed by the Monk and Cianflone groups in 2002 [Kalant 2002]. C3adesArg had previously been shown to be identical with acylation-stimulating protein, a regulator of lipid metabolism in adipocytes and other connective tissue cells [Baldo 1993]. C3adesArg has very low affinity for C3aR, and has not been shown to induce any immunological effects in haematopoietic cells that express C3aR. Kalant *et al.* found that both human adipocytes and fibroblasts express mRNA for C5L2, and that C5L2 bound both C3adesArg and C4adesArg. This study prompted a rapidly growing body of research into the potential function of C5L2 as a regulator of lipid metabolism, with possible roles in the pathogenesis of obesity and atherosclerosis. Whilst this is a fascinating field of investigation, a detailed discussion of this part that C5L2 may play in lipid metabolism lies beyond the scope of this thesis.

In summary, the issues of C5L2's function and signal transduction mechanism remain a subject of controversy. Most studies have shown that it fails to induce significant Ca^{2+} mobilisation in response to C5a stimulation, which suggests that C5L2 is unable to couple efficiently to G proteins, as expected on the basis of the Asp-Leu-Cys mutation in the third intracellular loop. However, this does not exclude the possibility that it may participate in a G protein-independent signal transduction mechanism, and indeed there is evidence that in human

neutrophils, it may at least modulate C5aR-mediated ERK1/2 signalling through its interaction with β-arrestin 1 [van Lith 2009, Bamberg 2010], while in murine macrophages it appears able to induce MAPK phosphorylation and HMGB1 release in response to C5a without the need for any interaction with C5aR [Rittirsch 2008], suggesting that it may be capable of signalling in its own right.

C5L2's status as a decoy receptor is uncertain, since studies in primary cells have questioned its cell-surface expression [Otto 2004, Bamberg 2010] and functional data have suggested that it may not antagonise the effects of C5aR [Rittirsch 2008]. Finally, the question of its ultimate function is unresolved: it is unclear whether or not it exerts a pro- or an anti-inflammatory effect, or whether it inhibits or potentiates C5aR-mediated inflammatory responses, since it is reported to have different effects on different inflammatory mediators in different cell types (and even in the same cell types but between different species).

1.4 Pathogen recognition by the cells of the innate immune system

The leucocytes of the innate immune system include neutrophils, (polymorphonuclear leucocytes, PMN), monocytes and macrophages, dendritic cells (DC) and natural killer cells (NK). PMN, monocytes/macrophages and DCs have a range of important homeostatic functions in addition to their role in pathogen recognition and the response to infection: they participate in the clearance of dead cells, promote tissue repair in response to sterile injury and are involved in lipid scavenging. DCs, in addition, regulate T cell tolerance to self-antigens and maintain the numbers and functional competence of lymphocytes [Sancho and Reis e Sousa 2012]. However, the major function of all these cells is chemotaxis to the site of infection and phagocytosis of pathogens, both in order to clear infecting organisms and to

present processed antigen to the B- and T-cells of the adaptive immune system [Murphy 2012]. They also play a key role in orchestrating the exaggerated secretion of pro-inflammatory chemokines and cytokines that is implicated in pathological inflammatory responses, as in the case of sepsis. The primary function of NK cells is the destruction of virus-infected and tumour cells [Murphy 2012].

Pathogen recognition by innate immune cells is accomplished mainly through several families of PRRs (Figure 1). These include cytoplasmic and cell membrane-associated PRRs.

1.4.1 Cytoplasmic PRRs

The cytoplasmic PRRs are located intracellularly and are typically not associated with membranous organelles. Their primary function is the detection of components of intracellular pathogens. They include retinoic acid-inducible gene-I-like receptors (RLRs), nucleotide binding domain leucine-rich repeat receptors (NLRs) and membrane-associated receptors of the collectin and scavenger receptor (SR) families as well as some members of the Toll-like Receptor (TLR) family.

1.4.1.1 Retinoic acid-inducible gene-I-like receptors (RLRs)

RLRs play an important role in the recognition of intracellular viral RNA, in response to which they promote the secretion of pro-inflammatory cytokines and type I interferons. Key RLRs include RIG-I, MDA5, and LGP2, which contain a helicase domain capable of unwinding double-stranded RNA (dsRNA). Ligation leads to assembly of a signalling complex that localises to the mitochondria and induces activation and translocation of NF-κB and Interferon Response Factors (IRFs) 3 and 7 (Kato 2011).

1.4.1.2 Nucleotide binding domain leucine-rich repeat receptors (NLRs)

NLR proteins belong to the Signal Transduction ATPases with Numerous Domains (STAND) sub clade of the AAAATPase superfamily. They are characterized by a centrally-located nucleotide-binding domain, a variable number of highly polymorphic C-terminal leucine-rich repeats (LRRs), and diverse N-termini [Bonardi 2012]. Human NLRs include the Class II Transactivator (CTA), a regulator of MHCII expression; NAIP, which triggers activation of the caspase-containing inflammasome protein complex in response to bacterial flagellin; nucleotide oligomerisation domain 1 (Nod 1, NLRC1) and Nod2 (NLRC2), which recognize bacterial proteoglycans and result in activation of NF-κB; and members of the NLRP (NALP) family, which are reported to exhibit negative regulatory functions on inflammatory responses [Robertson 2012 and Lamkanfi and Kanneganti 2011].

1.4.2 Cell Membrane-associated PRRs

These PRRs, which are expressed not only by innate immunocompetent cells but also by a wide range of cell types and tissues, include the collectins, scavenger receptors and the Toll-like receptor families.

1.4.2.1 Cell membrane-associated collectins

C-type lectin receptors (CLRs) are members of a superfamily of more than 1,000 proteins classified into 17 sub-groups on the basis of receptor structure [Sancho and Reis e Sousa 2012]. Their ligands include protein, carbohydrate and lipid components of pathogens and 'damaged self' molecules, and they trigger a range of responses including endocytosis, phagocytosis and secretion of pro- and anti-inflammatory cytokines. Ca^{2+}-dependent Group II CLRs such as DC-SIGN and Dectin-2 bind mannose and fucose residues from a range of viral,

bacterial, mycobacterial and protozoan pathogens. Some (Ca^{2+}-independent) Group V CLRs such as DCAL-1 bind endogenous ligands expressed on $CD4^+$ T cells and are involved in regulating the adaptive immune response. Others, such as DNGR-1 are involved in phagocytosis and clearance of necrotic host T cells. Others, including Dectin 1, are critical for the recognition and phagocytosis of mycobacterial and fungal pathogens, recognising microbial β-glucan motifs. Group VI CLRs include the macrophage mannose receptor, which mediates recognition and phagocytosis of a range of viral, bacterial, fungal and protozoan pathogens by binding to mannose residues [Sancho and Reis e Sousa 2012].

1.4.2.2 Scavenger receptors

SRs are expressed predominantly on macrophages and monocytes. They were first defined functionally, on the basis of their ability to bind and internalise modified low-density lipoproteins [Goldstein 1979]. They have been strongly implicated in the process of vascular inflammation that leads to the formation of atherosclerotic plaques, and it is in this context that they have been most extensively studied [Kzhyshkowska 2012], but they have also been shown to be involved in recognition of microbial ligands. SRs are divided into 8 classes (SR-A to -H) on the basis of the structure of their extracellular domains. Members of the SR-A class such as macrophage receptor with a collagenous structure (MARCO) have been shown to bind cell-wall components from both Gram-positive (e.g. Lipoteichoic acid, LTA) and Gram-negative (e.g. LPS) bacteria. Other SRs known to bind microbial ligands include CD36 (which recognises diacyl lipopeptides), oxidized low-density lipoprotein receptor 1 and scavenger receptor expressed by endothelial cell-I (which recognise outer membrane protein A of *Klebsiella pneumoniae)* [reviewed in Plüddermann 2006].

1.4.2.3 Toll-like receptors

Genetic research into the embryogenesis of the fruit fly *Drosophila Melanogaster* conducted during the 1980's, identified a protein, Toll, which was critical for induction of dorsal-ventral polarity in the *Drosophila* embryo [Anderson and Nüsslein-Volhard 1984 and Anderson 1985]. Toll was demonstrated to be activated by Spätzle, the end product of a proteolytic cascade, and to signal via a serine kinase (Pelle) to activate a member of the NF-κB family, Dorsal. A possible immunological function for this pathway was suggested in 1991 by Gay and Keith [Gay and Keith 1991], who observed that the signalling domain of the mammalian IL-1 receptor was homologous to the cytoplasmic domain of *Drosophila* Toll. IL-1 is part of the acute phase response to infection characterized by fever and the secretion of defense proteins into the circulation. This discovery suggested that this cytoplasmic domain, now known as the Toll-interleukin receptor homology (TIR) domain, was involved in signaling processes not only in the restricted context of insect development but also in the generation of initial responses to infection by mammalian/human immune system cells. The full significance of this discovery was demonstrated in 1996 when Le Maitre *et al*, noting earlier observations that the promoter regions of a number of *Drosophila* antimicrobial genes also contain NF-κB binding sites, showed that Toll was necessary for the *Drosophila* antifungal response, which involved production of the antimicrobial peptide drosomycin [Lemaitre 1996].

The first mammalian Toll-like receptor, later identified as TLR1, was cloned in 1991 [Nomura 1994] and mapped to human chromosome 4 [Taguchi 1996]. A human homologue of drosophila Toll, later identified as TLR4, was expressed in a monocytic cell line by Janeway in 1997, who also demonstrated that a constitutively active form of the receptor induced the expression of inflammatory cytokines and co-stimulatory molecules, clearly indicating a role in the immune response [Medzhitov 1997].

At this point, the investigation intersected with another long-running enquiry, namely the search for the identity of the receptor for bacterial endotoxin (lipopolysaccharide). When Beutler *et al* demonstrated by positional cloning analysis of the LPS-insensitive mouse strain C3H/HeJ that a point mutation in the TIR domain of TLR4 was responsible for the defect in LPS signal transduction [Poltorak 1998], and that another LPS-resistant mouse strain (C56BL/10ScCr) lacked the entire *tlr4* gene, it became clear that the gene previously identified as *lps* (coding for a putative LPS receptor) was in fact the *tlr4* gene [reviewed in Beutler and Rietschel 2003]. These findings were swiftly corroborated by Malo and colleagues [Qureshi 1999], using a similar genetic approach, and by the Akira group [Hoshino 1999] who recapitulated the LPS-resistant phenotype by targeted mutation of the *tlr4* gene. Identification of 9 other human TLRs and a still-growing array of the cognate ligands for the encoded receptors has followed in the subsequent years, bringing the total to 11, although *tlr11* has not been found to be expressed in humans [Chaudhury 1998, Rock 1998, Takeuchi 1999, Hemmi 2000, Hemmi 2002 and Zhang 2004].

a. Subcellular localisation and ligand specificity

TLR 1, 2, 4, 5, and 6 are predominantly expressed at the cell surface, whereas TLR 3, 7, 8 and 9 are predominantly associated with intracellular vesicles [Watts 2008 and Paludan 2011]. TLR7 and TLR9 are exclusively present in the endoplasmic reticulum of resting cells, but rapidly traffic to endolysosomes after ligand stimulation [Kim 2008]. This translocation is regulated by the chaperone proteins UNC93b1 and PRAT4a [Tabeta 2006]; PRAT4a is also responsible for the trafficking of TLR4 to the plasma membrane while gp96 - a member of the heat-shock protein family - appears to be a general chaperone for TLRs [Yang 2007]. Cell-surface TLRs are endocytosed following ligand binding; the endocytosed TLRs may be recruited to, and signal from, phagosomes containing endocytosed bacteria. The small GTPase Rab11a appears to

regulate this recruitment in the case of TLR4 endosomes [Husebye 2010]. The subcellular localisation of TLRs broadly reflects their ligand specificity: the intracellular TLRs are predominantly receptors for components of intracellular pathogens such as viruses and protozoa, while the extracellular TLRs recognise a diverse array of ligands found in the extracellular milieu (summarised in Table 1).

Table 1. TLR Recognition of microbial components

Microbial component	Species	TLR usage
Bacteria		
Lipopolysaccharide	Gram-negative Bacteria	TLR4
Diacyl Lipopeptides	Mycoplasma	TLR6/TLR2
Triacyl Lipopeptides	Bacteria and Mycobacteria	TLR1/TLR2
Lipoteichoic Acid	Gram-positive Bacteria	TLR6/TLR2
Proteoglycans	Gram-positive Bacteria	TLR2
Porins	*Neisseria spp*	TLR2
Lipoarabinomannan	Mycobacteria	TLR2
Flagellin	Flagellated Bacteria	TLR5
CpG-DNA	Bacteria and Mycobacteria	TLR9
ND	Uropathogenic Bacteria	TLR11 (mouse)
Fungi		
Zymosan	*Saccharomyces cerevisiae*	TLR6/TLR2
Phospholipomannan	*Candida albicans*	TLR2
Mannan	*Candida albicans*	TLR4
Glucuronoxylomannan	*Cryptococcus neoformans*	TLR2 and TLR4
Parasites		
tGPI-mutin	*Trypanosoma*	TLR2
Glycosylinositolphospholipids	*Trypanosoma*	TLR4
Hemozoin	*Plasmodium*	TLR9
Profilin-like molecule	*Toxoplasma Gondii*	TLR11 (mouse)
Viruses		
DNA	Viruses	TLR9
dsRNA	Viruses	TLR3
ssRNA	RNA viruses	TLR7 and TLR8
Envelope proteins	RSV, MMTV	TLR4
Haemagglutinin Protein	Measles virus	TLR2
Envelope glycoproteins	HCMV, HSV1	TLR2

HCMV, human cytomegalovirus; HSV1, herpes simplex virus 1; MMTV, mouse mammary tumour virus; ND, not determined; RSV, respiratory syncytial virus. Adapted from [Akira 2006] and [Lin 2011].

Bacteria are grouped according to the staining properties and thus the composition of their cell walls. A major component of the cell wall of Gram-positive bacteria is a thick layer of peptidoglycan (PG) composed of alternating residues of N-acetylglucosamine and N-acetylmuraminic acid, which is anchored to the underlying cell membrane by lipoteichoic acid (LTA) (Figure 11A). Both PG and LTA are recognised by TLR2. Gram-negative bacteria have a relatively thin PG layer, but are surrounded by an additional outer membrane composed largely of lipopolysaccharide (LPS) (Figure 11B). LPS is the prototypical TLR4 ligand, and is a potent inducer of immune responses. Mycobacterial cell walls also have a relatively thin PG layer that is reinforced by a thick, mycolate-rich hydrophobic layer (Figure 11C).Mycobacterial cell wall components are ligands of TLR2. DNA from all classes of prokaryotic organisms is a ligand for TLR9.

The structure of a typical fungal cell wall is shown in Figure 11D. A number of fungal polysaccharides have been identified as TLR ligands. TLR4 was shown to recognise mannans [Shoham 2001] and glucuronoxylmannan [Netea 2004]. Zymosan, a cell wall component of *Saccharomyces cerevisiae* has been shown to bind to TLR2 [Sato 2003]. Fungal DNA is also a ligand for TLR9 [Akira 2006].
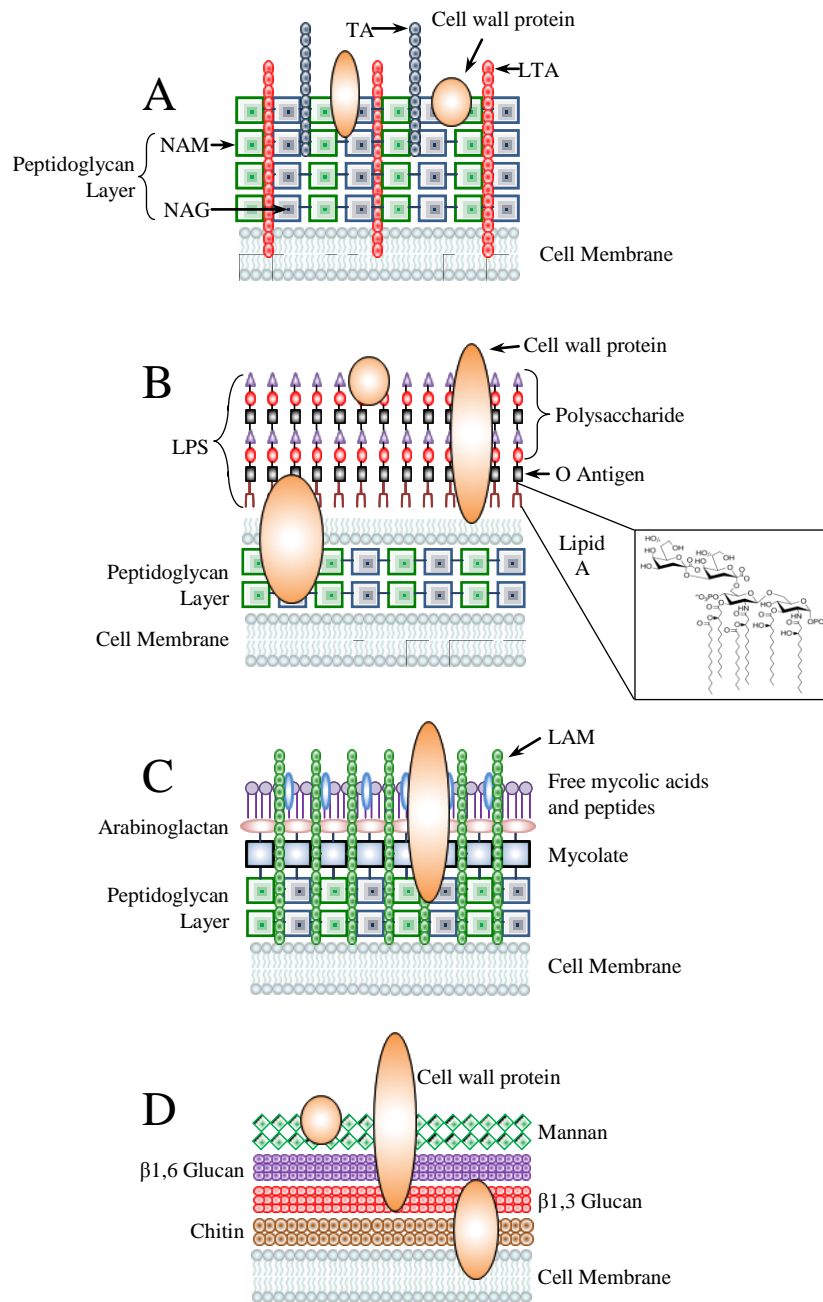
Figure 11. Structure of typical microbial cell walls. Simplified structure of typical **(A)** Gram-positive, **(B)** Gram-negative bacterial, **(C)** mycobacterial and **(D)** fungal cell walls. Inset in (B) shows structure of Lipid A from *E. coli* K12 strain. LAM, lipoarabinomannan; LPS, lipopolysaccharide; LTA, lipoteichoic acid; NAG, N-acetyl glucosamine; NAM, N-acetyl muraminic acid; TA, teichoic acid. Adapted from [Abdallah 2007, Murphy 2012 and Netea 2008].

TLRs are also involved in the recognition of viruses, both at the cell surface and in endosomal compartments. TLR2 and TLR4 have been shown to play a role in the sensing of viral envelope proteins. TLR2 has been shown to recognise human cytomegalovirus (HCMV) [Compton 2003], herpes simplex 1 virus (HSV1) [Kurt-Jones 2004] and vaccinia virus [Zhu 2007] and TLR4 to detect respiratory syncytial virus fusion protein [Kurt-Jones 2000] and mouse mammary tumour virus [Rassa 2002]; the viral ligand has not been identified in all cases.

Viral nucleic acids are detected by the intracellular TLRs 3, 7, 8 and 9. Double-stranded RNA (dsRNA) is recognised by TLR3 [Alexopoulou 2001) and the synthetic dsRNA analogue poly I:C is commonly used as a TLR3 ligand. TLR7 and 8 sense viral genomic single stranded RNA (ssRNA [Diebold 2004 and Heil 2004] and play a key role in innate immune responses against ssRNA viruses such as influenza virus [Diebold 2004 and Lee 2007]. TLR9 recognises unmethylated CpG motif-containing single strand DNA (ssDNA) oligodeoxynucleotides [Latz 2007] and detects genomic DNA of DNA viruses such as human herpes viruses [Lund 2004, Heil 2004 and Krug 2004].

Protozoan proteins may also be recognised by TLRs. Profilins are cytoskeletal proteins involved in actin polymerisation, which play a key role in the motility and thus the capacity to invade host T cells of parasites such as *Plasmodium, Toxoplasma, Cryptosporidium and Cyclosporum* species [Kucera 2012]. *Toxoplasma* and *Plasmodium* profilins have been shown to be ligands for mouse TLR11 [Rosenberg 2005 and Kursula 2008], while protozoan DNA is a ligand for TLR9. Host or microbial molecules capable of binding DNA may facilitate its transfer to the endosomes and thus enhance TLR9 activation. The malarial parasite *Plasmodium falciparum* digests host haemoglobin into a hydrophobic polymer known as hemozoin that was found to induce inflammatory responses in a TLR9-dependent manner [Coban 2005]. However, purified hemozoin is itself immunologically inert and its mode of action appears to be by the

presentation of protozoan DNA to TLR9 [Parroche 2007]. The endogenous nuclear protein high mobility group box protein 1 (HMGB1) may play a similar role [Ivanov 2007].

Endogenous molecules may also be TLR ligands; 'damaged self' or 'altered self' molecules (sometimes referred to as Danger-Associated Molecular Patterns or DAMPs) may be released as a result of sterile cellular injury or stress and are important mediators of inflammation, especially in the autoimmune diseases [Lin 2011]. Endogenous molecules that are reported to be TLR ligands are summarised in Table 2. However, the identification of endogenous molecules as TLR ligands is fraught with difficulties relating to the contamination of preparations of endogenous molecules by microbial TLR ligands, especially LPS [Marincek 2008].

Table 2. Proposed endogenous TLR ligands

TLR	Proposed Endogenous Ligand
TLR2	Heat Shock Proteins
	HMGB1
	Biglycan
TLR3	mRNA
TLR4	HMGB1
	Heat Shock Proteins
	Fibronectin Extra Domain A
	Minimally Modified Low-Density Lipoproteins
	Hyaluronan Fragments
	Heparan Sulphate
	Fibrinogen
	Lung Surfactant Protein A
TLR7	ssRNA
TLR9	Hypomethylated CpG-DNA
	HMBG1

HMGB1, High Mobility Group Box Protein 1; ssRNA, single-stranded RNA. Adapted from [Rifkin 2005].

b. Tissue distribution

As befits their primary role in early pathogen recognition, TLRs are predominantly expressed on phagocytic cells of the myeloid lineage. However, they are also found in non-myeloid leucocytes and in a range of other cells and tissues [Sandor and Buc 2005].

Professional phagocytes express the widest range of TLRs: PMN, monocytes and macrophages express all TLRs except TLR3 [Muzio 2000 and Hornung 2002]. DCs show a variable pattern of TLR expression; myeloid DCs express all TLRs except TLR7 and TLR9, which are expressed almost exclusively in plasmacytoid DCs. Additionally, the TLR expression pattern of DCs may depend to some extent on their stage of maturation: Visintin and colleagues reported that immature DCs express TLR1, 2, 4 and 5 but that expression tends to decrease upon DC

maturation [Visintin 2001]. By contrast, TLR3 mRNA is expressed by mature DCs only [Muzio 2000]. Eosinophils express TLR1, 2, 4, 6, 7 and 9 [Nagase 2003], Basophils express TLR2 and TLR4, and Mast Cells express TLR2 and TLR6 [Sabroe 2002]. NK cells, which are important in the early response to viral infections, have been shown to express mRNA for TLRs 1-8, with highest expression of TLR2 and 3. Accordingly, NK cells have been shown to be capable of being directly activated by ligands for TLR2, 3, 5 and 9 [reviewed in Yang 2011]

TLRs are also expressed by non-myeloid lymphocytes: B cells can be functionally divided into 'innate-like' B cells - found predominantly in the marginal zones of lymph nodes - which mediate a rapid, T cell-independent IgA and IgM response to infections; and adaptive or follicular B cells localised to germinal centres - which mediate slower, T-cell dependent and predominantly IgG responses. Innate-like B cells express high levels of TLRs 1, 2, 4, 6, 7 and 9, and both proliferate and secrete high levels of antibody in response to stimulation with their ligands [reviewed in Rawlings 2012].

A range of T lymphocyte populations have also been shown to express TLRs either constitutively or inducibly, and to respond to stimulation with their ligands [reviewed in Kabelitz 2007]. CD25^{high}CD4$^+$ regulatory T cells (Tregs) but not CD25$^-$CD4$^+$ naïve T cells were found to express TLR2 and TLR8 mRNA and protein [Peng 2005]. Purified tonsillar T cells were found to express mRNA for TLRs 1, 2, 3, 5, 9 and 10, with distinct differences in expression reported between CD4$^+$ and CD8$^+$ subsets [Mansson 2006]. Unconventional T cells that have the alternative γδ T-cell receptor (TCR) are reported to express TLRs 1,2,3,5 and 6 [Wesch 2006 and Bress 2006]. TLR expression has also been reported to be inducible on stimulation of a number of T cell subsets either solely by TLR ligands [Iwasaki and Medzhitov 2004], [Napolitani 2005, Pasare and Medzhitov 2003 and Pasare and Medzhitov 2004] or upon costimulation of the TCR [Komai-Koma 2004 and Liu 2006].

TLRs are also expressed in a variety of non-haematopoietic tissues. They are widely expressed in epithelial tissues including the skin, respiratory, intestinal and genitourinary tracts. Keratinocytes express TLR1-5 constitutively and mRNA for TLR2 and TLR4 has been detected in nasal mucosa and salivary glands. TLR2 is expressed throughout the respiratory epithelium, and TLR4 in alveolar epithelial cells. Epithelial cells of the lower digestive tract are in constant contact with microbes but only microbial invasion of the basolateral epithelial compartment induces an inflammatory response; consistent with this, expression of TLR2 and TLR4 on the apical surface of colonic epithelia is very low, but higher on the basolateral surface, which also expresses TLR5. The female genital tract epithelium has been shown to express mRNA for TLRs 1, 2, 3, 5 and 6 [Sandor and Buc 2005 and Kuroishi 2007].

Vascular endothelial cells appear to express TLRs 2, 4 and 5 [Sandor and Buc 2005 and Ward 2009]. Expression is not uniform, and TLR2 in particular appears to be inducible in regions of the vasculature that are exposed to turbulent flow (such as bifurcation of large vessels). Intriguingly, these are the regions of the vascular tree that are most prone to the development of atherosclerosis, which is also associated with upregulation of TLR2 expression in endothelial cells [Dunzendorfer 2004]. Thus, a role for vascular endothelial TLR2 is likely in the pathogenesis of atherosclerosis.

c. Accessory molecules and co-receptors

TLR recognition of their ligands often requires the action of a co-receptor or accessory molecule [reviewed in Lee 2012]. A summary of the known TLR accessory molecules is shown in Table 3. Of particular note are the TLR4 accessory molecules: lipopolysaccharide-binding Protein (LBP), CD14 and MD2. The hydrophobic LPS molecule tends to form micelles when in aqueous solutions (such as in blood plasma); these micelles are poorly recognised by TLR4. LBP is a 481-amino-acid acute phase response serum protein that binds LPS with high affinity and

causes the disaggregation of micelles of LPS, thus facilitating its transfer to CD14. CD14 is a 375-amino-acid glycoprotein composed of leucine-reach repeats (LRRs). It is present as a soluble form in the blood and other biological fluids or as a glycosylphophinositol-anchored membrane protein mainly on myeloid cells [Ferrero 1990, Dziarski 1998, Labéta 2000]. Its role is not limited to the recognition of LPS as it interacts with multiple ligands for both cell-surface and endosomal TLRs [Hailman 1994, Dziarski 1998, Baumann, 2010, Lee 2006, Nakata 2006, Georgel 2007 and Kurt-Jones 2000] and enhances their ability to activate TLRs by delivering the microbial component to the corresponding TLR [Frey 1992, Labéta 2000, and Gay and Gangloff 2007]. MD-2, also known as LY96, is a 160-amino-acid soluble glycoprotein that associates with the extracellular domain of TLR4 and is necessary for TLR4 expression at the cell surface, as well as the initial interaction of TLR4 with LPS [Shimazu 1999 and Nagai 2002]. Park *et al.* identified the crystal structure of the TLR4-MD-2-LPS complex [Park 2009], demonstrating that the LPS molecule buries five of its six lipid chains into a hydrophobic pocket on MD-2. Two MD-2-LPS complexes are necessary to bridge two TLR4 molecules and induce TLR4 homodimerisation, a critical event in TLR4 activation.

Table 3. TLR co-receptors and accessory molecules.

Name	Localisation	Interacting TLR	Ligand
LBP	Secreted	None demonstrated	LPS
MD2	Plasma membrane, extracellular fluid	TLR4	LPS
CD36	Plasma membrane, Golgi	TLR2, TLR4, TLR6	FSL1, LTA, oxLDL, amyloid-β fibrils
CD14	Secreted, Plasma membrane (GPI-linked), Endolysosomes	TLR2, TLR3, TLR4, TLR7, TLR8, TLR9	LPS, peptidoglycan, Pam_3CSK_4, polyI:C, CpG DNA
TRIL	Plasma membrane, early endosomes	TLR3, TLR4	LPS
Progranulin	Secreted, endolysosomes	TLR9	CpG-A, CpG-B, CpG-C and inhibitory ODNs
HMBG1	Nucleus, cytoplasm, can be secreted following TLR ligation	TLR9, possibly TLR3 and TLR7	CpG-A ODNs, CpG-B ODNs, DNA, RNA
LL37	Early endosomes	Possibly TLR7 and TLR9	Mammalian DNA, mammalian RNA

FSL1, S-(2,3-bispalmitoyloxypropyl)-CGDPKHSPKSF; GPI, glycosylinositolphosphate; HMGB1, High Mobility Group Box 1 Protein; LBP, lipopolysaccharide-binding protein; LPS, lipopolysaccharide; LTA, Lipoteichoic acid; ODNs, oligodeoxynucleotides; oxLDL, oxidised low density lipoprotein; Pam_3CSK_4, Tripalmitoyl-cysteinyl-seryl-(lysyl)$_3$-lysine; poly I:C, polyinosinic-polycytydylic acid; TRIL, TLR4 interactor with leucine-rich repeats. Adapted from [Lee 2012].

d. Structure

TLRs are single-pass transmembrane proteins comprised of an extracellular ligand-binding domain, a single transmembrane helix, and an intracellular signalling domain [Gay and Gangloff 2007]. The extracellular domains bind either directly to ligands or to ligand-coreceptor complexes, initiating ligand-mediated multimerisation of the receptor. The intracellular domains have substantial sequence homology with the IL-1 receptor and are together known as Toll/IL-1R homology (TIR) domains (Figure 12A) [Gay and Keith 1991]. TLR signalling adaptor proteins also contain TIR domains. These adaptor proteins are recruited to the TIR domains of TLRs by heterotypic TIR-TIR interactions as a consequence of ligand binding

Figure 12. TLR structure. (A) Schematic representation of TLR structure, showing the leucine-rich repeat (LRR) extracellular domain, transmembrane domain (TM) and intracellular Toll/IL-1R homology (TIR) domain. **(B)** Folding domains of TLR 1,2,4 and 6, showing the functional significance of structural boundaries. Adapted from [Kang 2011].

and receptor oligomerisation [Kang and Lee 2011]. Aggregation of TLRs and adaptor proteins activates signal transduction cascades that ultimately lead to activation of transcription factors such as NF-κB and IRFs, and the synthesis and secretion of mediators of inflammation.

The extracellular domains of TLRs belong to the LRR family of proteins, which contains nearly 20,000 members [Interpro 2012] and are involved in a diverse array of functions, mostly involving protein-protein interactions. LRR family members have a structure characterised by repeating LRR modules, each of which contains a 20 - 30-amino acid sequence that includes

the motif LxxLxLxxN. The side chains of the conserved leucines point inwards into the protein core and are involved in forming the hydrophobic core. The conserved asparagine residues are important in maintaining the overall shape of the protein, because they form a continuous hydrogen-bond network with backbone carbonyl oxygens of neighbouring LRR modules. The variable 'x' residues are exposed to the solvent, and may play important roles in interactions with the ligand. As a result, the roles of the different residues in the LRR motifs are distinct: the variable residues are important for ligand binding and protein-protein interactions, while the leucines and asparagines confer stability. All LRR family proteins have a characteristic horseshoe-like solenoid structure; the concave part of the structure is comprised of a central β-sheet built of parallel strands provided by the LxxLxLxxN motifs, whereas the amino acid residues outside this motif form either parallel helices or loops and constitute the convex part of the structure [Kang and Lee 2011].

The TLR extracellular domains belong to the 'typical' subfamily of LRR proteins, but they are unusual family members in a number of respects [Jin and Lee 2008a and b]. Most members of this family are involved exclusively in protein-protein interactions, and their ligand binding sites are located in the concave portion of the protein; the ligands of most TLRs are not proteins, and their binding sites are typically located on the convex curve of the protein. The typical LRR subfamily has 24 amino acids in its LRR with a conserved pattern of xLxxLxxLxLxxNxLxxLPxxxFx; however, the number of amino acids in the LRR modules of TLRs is very variable, ranging from 19 to 33. Additionally, the convex curve of the TLR extracellular domain contains atypical structures such as α-helices and irregular loops, which makes the convex surfaces of TLRs unusually 'bumpy' and thus suited to interactions with non-protein ligands [Kang and Lee 2011].

While the central β-sheet of most LRR proteins has a uniform radius and tilt, the central β-sheets of some of the TLRs show structural transitions that divide the proteins into three domains: N-terminal, central and C-terminal. These structural discontinuities seem to be the result of irregular LRR sequences in the central domain; the proteins lack the usual sequence of asparagine networks that stabilises the overall horseshoe-like shape, allowing structural distortions. These distortions appear to have functional significance: the ligand-binding pockets of TLR1, 2 and 6 coincide with the transition between the C-terminal and central domains, while one of the MD-2 binding sites of TLR4 is located close to the boundary between the N-terminal and central domain (Figure 12B) [Jin 2007, Kang 2009 and Kim 2007]. Crystal structures of TLR2- and 4-ligand complexes are shown in Figures 13 and 14.

Figure 13. TLR2 Ligand Binding. Models of extracellular domains (ECD) of **(A)** TLR1(green)/2(blue) and **(B)** TLR2/6(gold) heterodimers showing binding of Pam_3CSK_4 (TLR1/2) and Pam_2CSK_4 (TLR2/6). **(C and D)** Detail of proposed interaction between receptor hydrophobic pockets and fatty acid tails of Pam_3CSK_4 (C) and Pam_2CSK_4 (D). In (D) phenylalanine residues in positions 343 and 365 of the TLR6 ECD (orange) block the lipid-binding pocket present in TLR1. From [Kang 2011].

Figure 14. TLR4 ligand binding. Ribbon diagram **(A)** and crystal structure **(B)** of the TLR4-MD2-LPS complex. In (A) The N-Terminal, Central and C-Terminal domains of TLR4 (grey) are divided by broken lines. The A and B patches of the dimerisation interface with MD-2 (blue) are marked by broken red and orange surfaces, respectively. **(C)** Detail of the dimerisation interface between TLR4 and MD-2 showing the binding site of the R2 lipid chain of LPS (red). Other parts of Lipid A and core carbohydrates are shown in pink and orange, respectively. Hydrophobic residues forming the LPS binding site are shown in light green (TLR4) and light blue (MD2) and hydrophilic residues are shown in dark green (LPS) and dark blue (MD2). (CO), backbone carbonyl oxygen; (NH) backbone amide nitrogen. From [Kang 2011].

TLR3 belongs to the single-domain fold subfamily because its LRR domain has uniform β-sheets with a continuous asparagine network [Choe 2005]. The crystal structures of TLRs 5, 7, 8, 9 and 10 have not yet been reported, but sequence analysis suggests that the first four belong to the single-domain subfamily, whereas TLR10 is likely to belong to the three-domain subfamily [Matsushima 2005 and 2007]. Functional analysis of the TLRs agrees with this classification: the three-domain TLRs utilise hydrophobic internal pockets as their binding sites for hydrophobic ligands such as LPS, whereas the single-domain TLRs interact with hydrophilic ligands such as nucleic acids, via surface-exposed residues. The crystal structure of the TLR3-dsRNA complex is shown in Figure 15.

This image has been removed by the author for copyright reasons.

Figure 15. TLR3 ligand binding. Crystal structure of the TLR3-dsRNA complex. TLR3 C-terminal and N-terminal sites are indicated. From [Kang 2011].

e. Activation

Ligand-induced multimerisation is a key event in TLR activation. TLR2 forms heterodimers with TLR1 and TLR6; TLR4 typically homodimerises but may also heterodimerise with TLR5 [Mizel 2003] and with TLR6 in a CD36-dependent manner [Stewart 2010]; the other TLRs form homodimers [Kawai and Akira 2010]. The presence of a cross-linking ligand seems necessary to the formation of TLR4 homodimers [Kim 2007], whereas TLR1/2 and TLR2/6 may dimerise in the absence of ligand [Triantafilou 2006]. Although receptor cross-linking is a necessary step in

TLR activation, it is not sufficient: many TLR-specific antibodies cross-link TLRs without any evidence of an agonist effect [Shimazu 1999 and Matsumoto 2002]. It is thought that ligand-induced dimerization also results in a conformational change of the TLR extracellular domain, which in turn promotes the rearrangement of the transmembrane helices and permits the formation of a stable receptor-receptor signalling complex [Gay and Gangloff 2007].

f. Signal Transduction

After ligand binding, TLRs activate signalling pathways that provide specific immunological responses tailored to the nature of the microbial or endogenous ligand. The specific response initiated by a particular TLR depends upon the recruitment of a single or specific combination of TIR-domain containing adaptor proteins [Kawai and Akira 2011].

The TIR domain is between 135 and 160 amino acids in length, and is composed of a central five-stranded parallel β-sheet which is surrounded by 5 α-helices [Xu 2000]. Each TIR domain contains three conserved regions named Boxes 1, 2 and 3. The Box 1 sequence is common to all TLRs. Box 2 contains a large conserved surface present in all TLRs and the adaptor protein myeloid differentiation gene product 88 (MyD88), known as the BB loop. A point proline-histidine mutation of the TLR4 BB loop is responsible for the absence of TLR4 signalling in the C3H/HeJ mouse [Poltorak 1998]. It appears that the BB loop is of critical importance for the homodimerisation of TLRs [Nyman 2008], while an interaction between the BB loop of TLR1 and a death domain (DD) loop (located on the opposite side to the BB loop) of TLR2 is predicted [Gautam 2006].

The formation of a stable TIR-TIR platform by ligand-induced TLR dimerisation promotes homotypic protein-protein interaction with other TIR domain-containing adaptor proteins, resulting in the assembly of a functional signalling complex, which initiates a chain of phosphorylation and ubiquitination events culminating in the activation of NF-κB and other

transcription factors leading to the expression of a diverse array of genes related to the inflammatory response, particularly the expression of pro- or anti-inflammatory cytokines [O'Neill and Bowie 2007]. To date, five TIR domain-containing adaptor proteins have been identified [Gay 2011]: MyD88; MyD88 adaptor-like (MAL; also known as TIR domain-containing adaptor protein or TIRAP); TIR domain-containing inducing interferon β (TRIF; also known as TIR-containing adaptor molecule 1 or TICAM-1); TRIF-related adaptor molecule (TRAM or TICAM-2); and sterile-alpha and HEAT-Armadillo motifs-containing protein (SARM). TLR ligation may lead to activation of a MyD88-dependent signalling pathway, used by all TLRs (except TLR3) that predominantly results in the secretion of inflammatory cytokines, and a MyD88-independent pathway (used by TLR3 and TLR4) which proceeds via the adaptor TRIF and results in Type I interferon secretion [Kawai and Akira 2010].

The MyD88-dependent pathway

The critical role of MyD88 in TLR signalling has been demonstrated by a number of studies of MyD88$^{-/-}$ mice in the context of a number of disease models; these animals are resistant to endotoxic shock but highly susceptible to infections by a range of different pathogens [Takeuchi 2000, Adachi 2001, Scanga 2002, Edelson 2002, Henneke 2002 and Muraille 2003]. It is now known that all TLRs except TLR3 signal through MyD88 [Kawai and Akira 2011].

MyD88 contains a death domain (DD) at its N-terminus and a TIR domain at the C-terminus. The DD of MyD88 interacts with the BB loop of the TLR cytoplasmic domain [Gautam 2006] and forms a stable signalling complex. MyD88 recruitment to the TIR domain of the TLR4 homodimer is dependent on MAL/TIRAP; MAL possesses a binding site for phosphatidylinositol-4,5-bisphosphate (PIP$_2$), a major component of the plasma membrane and the interaction of MAL with PIP$_2$ facilitates the recruitment of MyD88 to the receptor complex. The other MyD88-dependent TLRs do not use MAL for recruiting MyD88, although it

has a role in recruiting other signalling intermediates to the TLR2 signalling complexes [Kagan and Medzhitov 2006 and Brikos and O'Neill 2008]. The DDs of MyD88 are also involved in the interaction with interleukin receptor-associated kinase (IRAK) 4; binding of MyD88 to the TIR domains of the TLR dimer induces a conformational change that releases the MyD88 DD from a repressed state and enables it to interact stably with IRAK4. The net result is the sequential recruitment to the TIR domains of the ligand-stabilised TLR dimer of a helical structure comprised of a first layer of 2 MyD88 monomers, then a second layer of four more MyD88 monomers, followed by a third layer of four IRAK4 monomers and a fourth layer of four IRAK2 monomers; this signalling complex is known as the Myddosome (Figure 16) [Lin 2010].
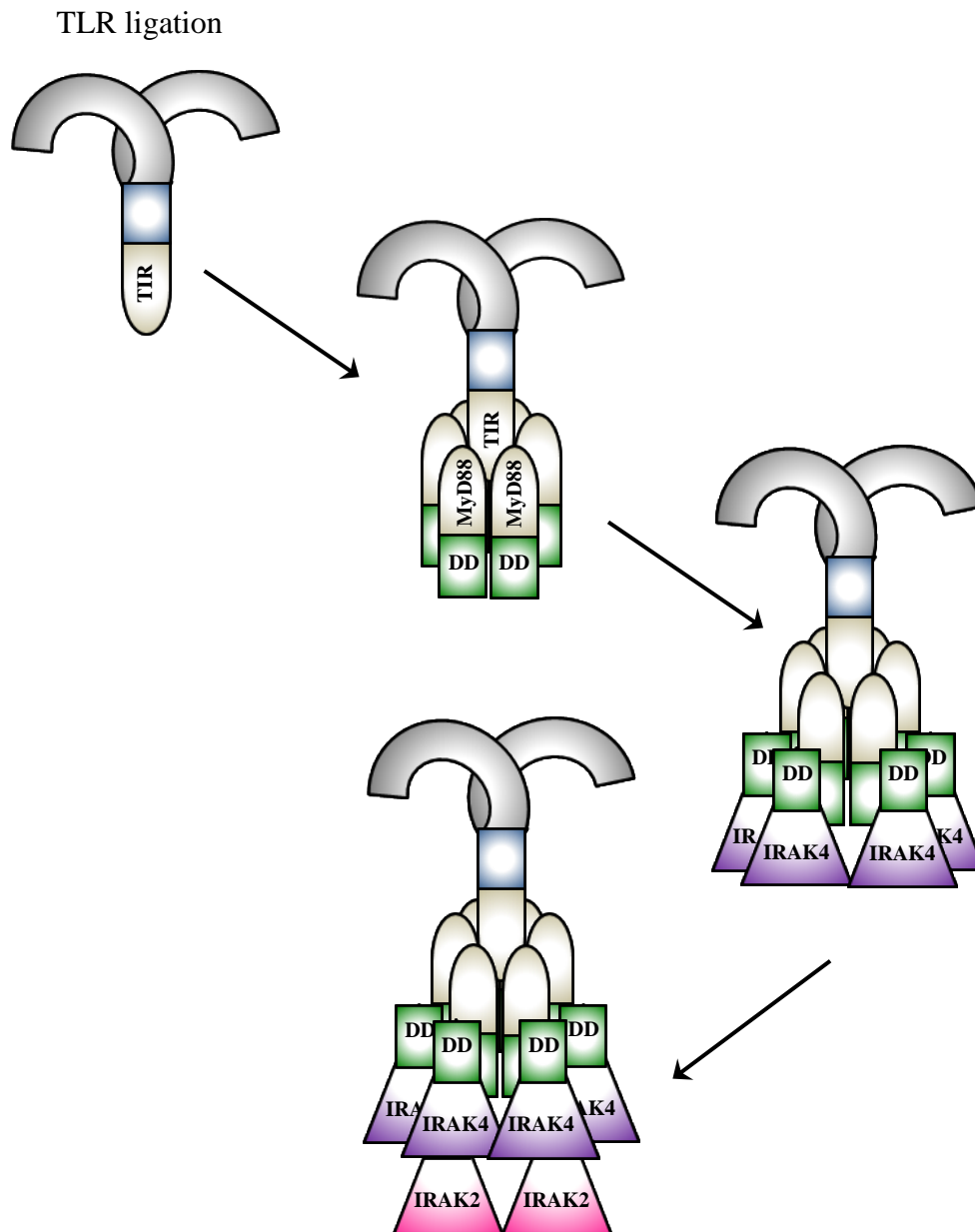
TLR ligation

Figure 16. Helical assembly of the Myddosome. TLR ligation leads to receptor dimerisation and the recruitment, via homotypic TIR-TIR interactions, of six MyD88 monomers. Sequential recruitment of four IRAK-4 and two IRAK-1 monomers results in assembly of the stable signalling complex. DD, death domain; MyD88, myeloid differentiation gene product 88; TIR, Toll/IL-1 receptor homology domain [Adapted from Kang 2011].

IRAK1 is next recruited to the Myddosome and activated; its activation results in dissociation from the Myddosome and association with TNF-α-receptor-associated factor 6 (TRAF6) [Kawai and Akira 2010]. TRAF6 is an E3 ligase that catalyses the polyubiquitination of Lys[63] (K63) of

IRAK1 in conjunction with the dimeric E2 ubiquitin conjugating enzymes Ubc13 and Uev1A. Activated TRAF6 associates with four downstream proteins via its K63-linked polyubiquitin chains: TGF-β-activated kinase1 (TAK1) and the TAK1-binding proteins 1-3 (TAB1-3) to form an active catalytic unit [Chen 2005]. TAK1 is then responsible for phosphorylating the inhibitor of NF-κB (IκB) kinase (IKK) complex. IKK is also ubiquitinated by the Uev1/Ubc13/TRAF6 complex [Doyle and O'Neill 2006]. The IKK complex consists of three subunits, IKKα, β and γ [Brikos and O'Neill 2008]; IKKγ (also called NF-κB essential modulator, NEMO) is a scaffold protein that holds the other subunits in proximity to IκB, whereas the α and β subunits are responsible for catalytic activity, phosphorylating IκB proteins. The function of IκB is the repression of NF-κB; quiescent NF-κB in the cytoplasm is associated with IκB. Phosphorylation of IκB by IKKα and β results in dissociation of IκB from NF-κB, which then translocates to the nucleus. The activated transcription factor plays a critical role in the induction of many inflammatory responses, including transcription of inflammatory cytokines [Kawai and Akira 2010 and Doyle and O'Neill 2006].The signal transduction pathway is summarised in Figure 17.
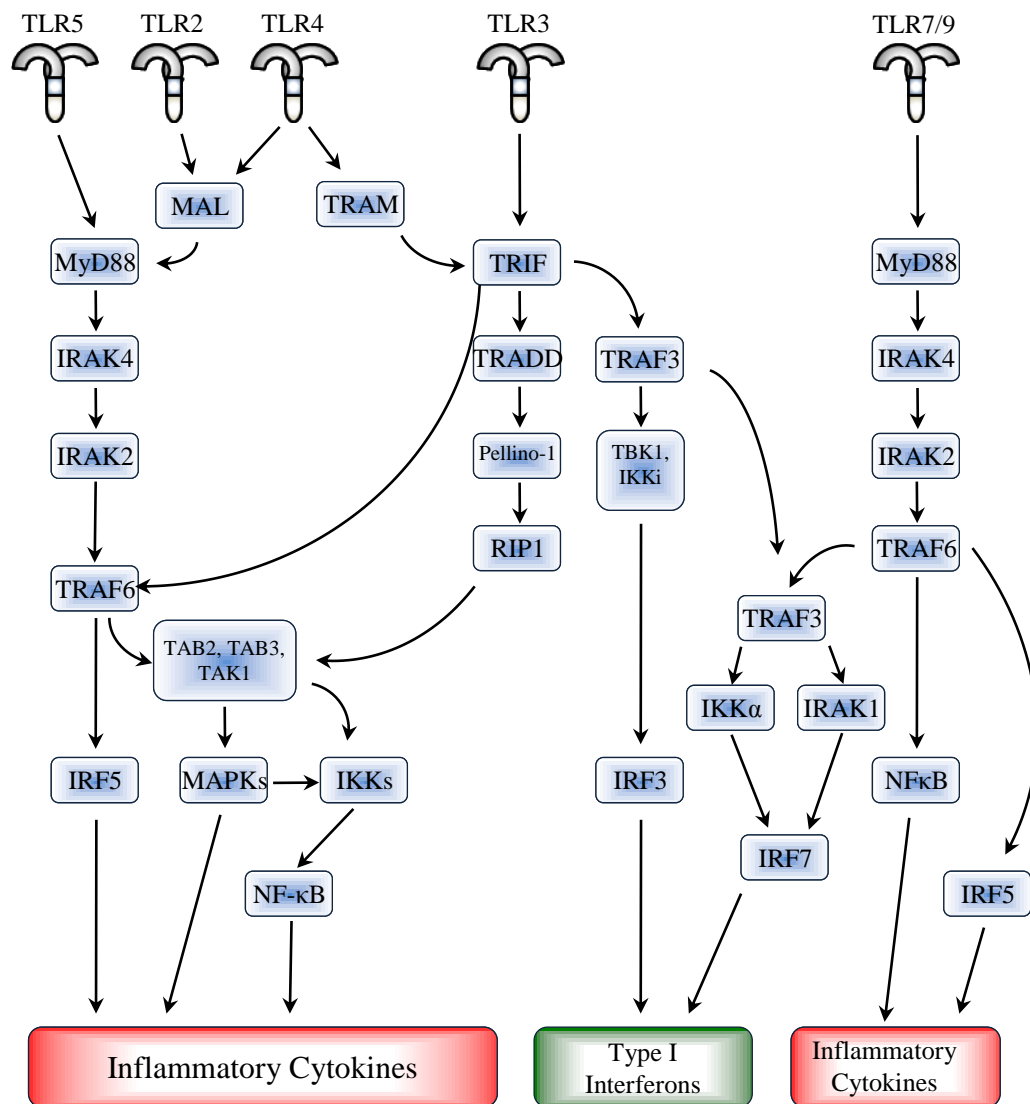
Figure 17. Overview of TLR signal transduction pathways. Key TLR signal transduction intermediates. IKK, inhibitor of NF-κB kinase; IRAK, interleukin receptor-associated kinase; IRF3/5/7, interferon response factor 3/5/7; MAL, Myeloid adaptor-like (also known as TIRAP); MyD88, myeloid differentiation gene product 88; NF-κB, nuclear factor kappa B; RIP1, receptor-interacting protein-1; SHP-1, protein tyrosine phosphatase, non-receptor type 6; TAB2/3, TGF-β-activated kinase binding protein 2/3; TAK, TGF-β-activated kinase ; TANK, TRAF family member-associated NF-κB activator ; TBK, TANK-binding kinase; TRADD, TNF receptor-associating via death domain; TRAF6, TNF receptor–associated factor 6; TRIF, TIR domain-containing inducing interferon beta (also known as TICAM-1). Adapted from [Kawai and Akira 2010].

TLR7 and TLR9 in plasmacytoid DCs also signal via MyD88, and may result either in activation

of NF-κB, leading to secretion of the pro-inflammatory cytokines IL-6 and IL-12p40 [Takaoka

2005] or of the transcription factor interferon response factor 7 (IRF7), leading to the secretion

of type I interferons. In this context, IRF7, which is constitutively expressed by plasmacytoid DCs, binds MyD88 and forms a signalling complex with IRAK4, TRAF6, IRAK1 and IKKα [Kawai and Akira 2008]. IRF7 is phosphorylated by IRAK1 and IKKα, dissociates from the signalling complex and translocates to the nucleus, where it binds to IFN-stimulated response element motifs and promotes the expression of type-1 interferons. Additional components that control the production of type-1 interferons in plasmacytoid DCs have also been identified, and illustrate the way in which the response to a given TLR ligand may be 'fine-tuned' in different cell types or in the presence of alternative stimuli: OPNi, a precursor of osteopontin, is a TLR9-inducible protein that is sequestered in the cytoplasm of plasmacytoid DCs and becomes a component of the MyD88-IRF7 complex [Shinohara 2006]; pharmacological inhibition of phosphoinositol-3-kinase (PI3K) and its downstream intermediates mTOR and p70S6K results in disruption of the interaction between TLR9 and MyD88 [Kagan and Medzhitov 2006], causing a reduction in nuclear translocation of IRF7 [Cao 2008].

It is thought that polarisation of the TLR7 and 9 response either towards IRF7 activation and interferon production or towards NF-κB activation and pro-inflammatory cytokine secretion is determined by the subcellular trafficking of the receptor. NF-κB activation predominates as a result of TLR7 and 9 ligation in early endosomes, whereas IRF7 activation is the result of subsequent trafficking of the TLRs to lysosome-related organelles under the control of the chaperone protein AP2 [Sasai 2010].

Given the critical role played by MyD88 in LPS-induced NF-κB activation, it would be expected that NF-κB activation would be abolished in MyD88-deficient mice. The observation that animals lacking MyD88 were still capable of mounting a response to LPS, albeit at a lower amplitude and after a delay, suggested that a delayed MyD88-independent TLR4 signalling apparatus might also exist [Yamamoto 2002].

The MyD88-independent pathway

The MyD88-independent pathway employs an alternative adaptor protein, TIR domain-containing adaptor inducing interferon β (TRIF) in a pathway that culminates not only in activation of NF-κB, but also the interferon β promoter. This pathway is initiated from an endosomal compartment, either via TLR3 (which is constitutively located in endosomes) or via TLR4, which is internalised to endosomes following ligand binding [Kagan 2008]. The role of TRIF was revealed by the fact that overexpression of TRIF resulted in constitutive activation of NF-κB and the IFN-β promoter, whereas TRIF^{-/-} animals showed a decreased response to TLR4 activation but an unchanged response to TLR2, TLR7 and TLR9. TRIF/MyD88 double knockout animals were unable to activate NF-κB at all [Brikos and O'Neill 2008]. This pathway has been shown to be utilised by TLR4 in conjunction with the MyD88 pathway (as described above), but exclusively by TLR3.

TRIF is recruited to the TLR following ligand binding. TRIF in turn recruits TRAF6, TAK1 and the adaptor (RIP1) through a distinct RIP homotypic interaction motif. RIP1 binds the adaptor TNF receptor-associating via death domain (TRADD) and undergoes K63-linked polyubiquitination by Pellino-1, a member of the Pellino family of RING-like domain-containing E3 ubiquitin ligases. The complex of TRIF with TRAF6, TRADD, Pellino1 and RIP1 activates TAK1, which in turn activates the NF-κB pathways.

As well as leading to NF-κB activation, the TRIF pathway leads to IRF3 activation. TRIF recruits a signalling complex involving the IKK-like kinase TRAF-family-member associated NF-κB activator (TANK)-binding kinase 1 (TBK1), the IKK homologue IKKε and IRF3 or IRF7. The formation of this complex leads to phosphorylation of IRF3 or IRF7 by TBK1 or IKKε, which bind to interferon sequence response elements and initiate transcription of IL-6, IL-12, TNF-α and interferon β [Kagan 2008].

g. Modulation of TLR signalling

As noted above, excessive cytokine responses to infection are implicated in a number of disease states. Thus, negative regulation of TLR activation pathways is of critical importance. Negative regulators of TLR activation have been identified at multiple levels, including soluble decoy receptors, inhibitors of adaptors or related proteins, ubiquitin ligases, deubiquitinases, transcriptional regulators and regulators of RNA stability [Kawai and Akira 2010]. They exist in a soluble form, either intracellularly or secreted into plasma or other bodily fluids. A consideration of some of the key inhibitors of TLR activation follows below (summarised in Table 4).

Soluble TLRs

A naturally-occurring soluble form of TLR2 (sTLR2) was first detected and characterised in our laboratory [LeBouder 2003, LeBouder 2006 and Raby 2009]. sTLR2 comprises the ECD of membrane-bound TLR2 (mTLR2) and is generated by post-translational modifications of mTLR2 rather than splice variants or mutations of the TLR2 gene. It is found in human plasma, breast milk, saliva, amniotic fluid [Kuroishi 2007 and Dulay 2009] and mouse peritoneal lavage fluid [Raby 2009]. It is secreted by monocytes in response to TLR2 ligation, most likely by proteolytic cleavage of endocytosed mTLR2 [LeBouder 2003]. It has been shown to negatively regulate mTLR2-mediated inflammation both by sequestering ligand (decoy receptor) and by disrupting the interaction between the co-receptor CD14 and the membrane-bound TLR [Raby 2009]. In human plasma, it appears to be a marker for systemic inflammation and has been found to predict disease progression in HIV/AIDS and mortality in congestive heart failure after myocardial infarction [Heggelund 2004 and Ueland 2006]. sTLR2 in breast milk may also have a role in promoting neonatal immunity [Henrick 2012].

A human soluble TLR3 has been generated by cloning the cDNA of the TLR3 ECD and transfection of HEK 293 cells [Sun 2006] although natural expression of the protein has yet to be demonstrated. A splice variant capable of producing a soluble product of the mouse *tlr4* gene was reported [Iwami 2000] and the existence of human soluble TLR4 has been demonstrated in saliva [Zunt 2009]. A naturally-occurring soluble form of TLR5 is found in fish [Tsujita 2006]. A naturally-occurring soluble form of human TLR9 has also been reported [Chockalingham 2011]. All of these gene products appear to be capable of negatively modulating inflammatory responses mediated by the relevant TLR.

Table 4. Negative regulators of TLR activation

Regulator	Expression and Induction	TLR	Possible mechanism
A20	LPS-induced expression in macrophages	TLR2, 3, 4, 9	De-ubiquitylates TRAF6
Atg16L1	Constitutively in macrophages	TLR4	Inhibits TRIF activation
IRAK-M	LPS-induced expression in monocytes	TLR4, 9	Inhibits phosphorylation of IRAK1
NOD2	ND	TLR2	Suppresses NF-κB
PI3K	Constitutively expressed by most cells	TLR2, 4, 9	Inhibits p38, JNK and NFκB function
RIP3	Constitutively expressed by most cells	TLR3, 4	Inhibits TRIF activation
SARM	Constitutively expressed by most cells	TLR3, 4	Inhibits TRIF activation
SHP-1	Macrophages	TLR4	Suppresses IRAK1
SIGIRR	Mainly expressed by epithelial cells and immature DCs but downregulated by activation	TLR4, 9	Interacts with TRAF6 and IRAK
SOCS1	LPS- and CpG-induced expression in macrophages	TLR4, 9	Suppresses IRAK
ST2L	LPS-induced expression in macrophages	TLR2, 4, 9	Sequesters MyD88 and MAL
sTLR2	Constitutively expressed in breast milk, saliva, plasma and amniotic fluid	TLR2	Decoy receptor, Blocks interaction with CD14
sTLR3	ND	TLR3	Decoy receptor
sTLR4	Saliva	TLR4	Blocks interaction with MD2
sTLR5	ND in human cells	TLR5	Decoy receptor
sTLR9	Constitutively expressed in HEK 293 cells	TLR9	Decoy receptor
sMyD88	LPS-induced expression, mainly in the spleen	TLR4	MyD88 antagonist
TANK	Constitutively expressed by most cells		
TOLLIP	Constitutively expressed by most cells	TLR2, 4	Phosphorylates IRAK1
TRAILR	Constitutively expressed by most cells	TLR2, 3, 4	Stabilises IκBα
TRIAD3A	Constitutively expressed by most cells	TLR4, 9	Ubiquitylates TLRs
Zinc-finger RNases	Constitutively expressed by most cells	All	Degrade cytokine mRNA

IRAK, interleukin receptor-associated kinase1; JNK, c-jun N-terminal Kinase; MyD88, myeloid differentiation gene product 8; ND, not determined; NF-κB, nuclear factor kappa B; NOD2, nucleotide oligomerisation domain-containing protein 2; p38, p38 mitogen activated protein kinase; PI3K, phosphoinostol-3-kinase; RIP, receptor-interacting protein; SARM, Sterile-alpha and HEAT-Armadillo motifs-containing protein; SIGIRR, single immunoglobulin IL-1R-related molecule; SOCS1, suppressor of cytokine signalling 1; TANK, TRAF family member-associated NF-κB activator; TOLLIP, toll-interacting protein; TRAF6, TNF receptor-associated factor 6; TRAILR, TNF-related apoptosis-inducing ligand; TRIAD3A, Triad-domain containing protein 3A;. s, indicates soluble form of protein. Adapted from [Liew 2005].

Soluble MyD88

Soluble MyD88 (sMyD88) is a naturally-occurring splice variant of MyD88 that contains its DD but lacks the binding domain for IRAK4. It is thought that sMyD88 promotes the formation of sMyD88-MyD88 complexes, effectively sequestering MyD88 and preventing it interacting with IRAK4 and initiating downstream signalling [Liew 2005].

IRAK-M

IRAK1, IRAK2 and IRAK4 are components of the MyD88-dependent pathway; IRAK-M, which is expressed only in monocytes and macrophages, is an inactive kinase due to a point mutation in its catalytic site. However, this mutation appears to have resulted in a change of function rather than a loss-of-function since IRAK-M$^{-/-}$ mice responded to an LPS or CpG DNA challenge with increased secretion of pro-inflammatory cytokines, suggesting an inhibitory function. Its exact mechanism of action is unclear [Wesche 1999 and Kobayashi 2002]

SOCS-1

The suppressor of cytokine signalling-1 (SOCS-1) plays a key role in the inhibition of pro-inflammatory cytokine secretion, as witnessed by the fact that SOCS-1$^{-/-}$ mice die within three weeks of birth because of multiorgan inflammation mediated by hypersensitivity to IFN-γ [Alexander 1999]. SOCS1$^{-/-}$mice are hypersensitive to LPS and other TLR ligands, responding with increased levels of the pro-inflammatory cytokines TNF-α, IL-6, IL-12 and IFNγ. SOCS-1 has been shown to inhibit responses to a range of inflammatory signals by increasing the degradation of the NF-κB p65 subunit [Ryo 2003 and Gingras 2004].

RIP-3

Receptor-interacting protein-3 (RIP-3) is an inactive member of the RIP-1 family, which inhibits TLR signalling either by sequestering TRIF and preventing RIP-1 binding, or by phosphorylating RIP-1 and preventing NF-κB activation [Meylan 2004].

TANK

TRAF family member-associated NF-κB activator (TANK) binds both TBK1 and IRF3. TANK$^{-/-}$ macrophages and B cells show exaggerated NF-κB activation and IL-6 production in response to TLR ligands compared with wild-type cells, and also showed enhanced TRAF6 ubiquitination, indicating that TANK may be an inhibitor of TRAF6 ubiquitination in both macrophages and B cells [Kawagoe 2009]. These animals spontaneously develop autoimmune glomerulonephritis that resolves with antibiotic treatment. The wild-type phenotype is rescued by deletion of IL-6 or MyD88.

TOLLIP

The Toll-interacting protein (TOLLIP) appears to have a complex regulatory role in TLR signalling. Didierlaurent and colleagues [Didierlaurent 2006] found no effect of TOLLIP knockout on the *ex vivo* response of DCs and lymphocytes to TLR3, 4, 5, and 9 agonists, but that TOLLIP-deficient mice challenged *in vivo* with a low dose of LPS responded with lower levels of IL-6 and TNF-α than wild-type, suggesting a positive regulatory role for TOLLIP. The effects of a lethal dose of LPS were unchanged as a result of TOLLIP knockout; these conflicting results are difficult to interpret. However, Shah *et al.* [Shah 2012] using targeted siRNA knockdown of TOLLIP found it to serve an inhibitory role in human monocytes, suppressing secretion of the pro-inflammatory cytokines IL-6 and TNF-α and enhancing secretion of the anti-inflammatory cytokine IL-10 in response to stimulation with TLR2 and TLR4 ligands. These

discrepancies suggest that the role of TOLLIP may be complex and vary between different species and cell types.

SARM

Sterile-alpha and HEAT-Armadillo motifs-containing protein (SARM) is the most recently-identified of the TIR-domain-containing TLR adaptor proteins. Unlike the other adaptors, it functions as a TLR-induced inhibitor of NF-κB and IRF activation. Its expression is up-regulated by TLR ligation, and small interfering RNA (siRNA) knockdown of SARM leads to enhanced cytokine production on stimulation via TLR3 or TLR4 [Carty 2006]. Thus, it is likely to function as an endogenous inhibitor of TLR-induced pro-inflammatory responses, probably by interfering with TRIF activation [Brikos and O'Neill 2008].

SIGIRR

The single immunoglobulin IL-1R-related molecule (SIGIRR) is a plasma membrane-bound TIR-domain-containing protein which has been shown to inhibit IL-1R and TLR signalling. SIGIRR$^{-/-}$ mice show enhanced inflammatory responses to LPS and CpG DNA, indicating that SIGIRR affects both TLR4 and TLR9 signalling. SIGIRR has been shown to interact with TLR4, TLR5 and TLR9 as well as the signalling intermediate TRAF6. Thus, it is thought that SIGIRR inhibits TLR signalling by interfering with the recruitment of TIR-domain-containing adaptors to the TLR [Wald 2003].

ST-2

Suppressor of tumorigenicity-2 (ST-2) is another TIR-domain-containing receptor. It has been found to interact with MyD88 but not TRIF; accordingly, macrophages from ST-2$^{-/-}$ mice were found to respond with higher levels of inflammatory cytokines to stimulation with LPS, lipopetides and CpG DNA, but not poly I:C, suggesting a role as an inhibitor of the MyD88-dependent pathway [Brint 2004].

TRAF1 and TRAF4

Unlike TRAF6, TRAF1 and TRAF4 have inhibitory roles in TLR signalling [Brikos and O'Neill 2008]. TRAF1 has been shown to bind to TRIF, and TRAF4 to both TRIF and TRAF6, resulting in their inactivation and inhibition of downstream signalling via both MyD88-dependent and -independent pathways. TRAF4 has been shown to inhibit NF-κB activation by TLRs 2, 3, 4, and 9 as well as TLR3- and TLR4-induced IFN-β promoter activation.

TRAILR

TNF-related apoptosis-inducing ligand (TRAIL) receptor (TRAILR) is a member of the TNF-receptor superfamily. TRAILR-deficient mouse macrophages produce increased levels of pro-inflammatory cytokines in response to TLR2, TLR3 or TLR4 activation. TRAILR seems to inhibit TLR signalling by stabilising IκB and thus reducing nuclear translocation of NF-κB [Diehl 2004 and Liew 2005]

Atg16L1

Atg16L1 is a mediator of autophagy, mutations of which have been linked to ulcerative colitis. Atg16L1-deficient mice macrophages show a TRIF-dependent enhancement of caspase 1 activation and production of IL-1β and IL-18 in response to LPS. They also exhibit a colitic

phenotype, with increased susceptibility to disease in models of ulcerative colitis. This suggests that Atg16L1 may be a negative regulator of TRIF in intestinal macrophages [Cadwell 2008].

Zinc-finger RNases

Zc3h12a is a TLR-inducible regulatory protein that contains a CCCH-type zinc finger domain and an RNase domain. It targets the 3' untranslated regions (UTR) of IL-6 mRNA for degradation. Zc3h12a-deficient macrophages produce prodigious amounts of IL-6 and IL-12p40, but normal amounts of TNF-α in response to TLR ligands, and Zc3h12a-deficient mice show enhanced autoantibody production [Matsuhita 2009]. Tristetraprolin (Xfp36), another zinc finger-containing protein, binds absorbance-unit rich elements (AUREs) in the 3' UTR of TNF-α mRNA and targets them for degradation. Xfp36 prevents the development of autoimmune rheumatoid arthritis in a mouse model [Carrick 2004].

A20

A20 is a TLR-inducible protein that has two enzymatic activities, acting both as an E3 ubiquitin ligase and a deubiquitinase. *In vitro* analysis has shown that A20 restricts NF-κB activation by modulating RIP1 and TRAF6. A20–deficient mice die prematurely from spontaneous multi-organ inflammation and cachexia, indicating that A20 has anti-inflammatory effects *in vivo*, perhaps by inducing tolerance to commensal bacteria, since the administration of antibiotics prevents cachexia, and A20/MyD88 double$^{-/-}$ animals do not exhibit the inflammatory phenotype [Turer 2008].

SHP-1

Mice bearing mutations in the tyrosine phosphatase Src homology-2 containing tyrosine phosphatase-1 (SHP-1) develop inflammatory lesions associated with aberrant macrophage

activation in response to TLR stimulation. MyD88 deficiency suppresses this inflammation, suggesting that SHP-1 negatively regulates the MyD88-dependent pathway; it has also been reported to suppress the functions of IRAK1 and IRAK2 [Croker 2008 and An 2008].

1.5 The TLR-C5a receptor interaction

The signal transduction pathways involved in the release of pro-inflammatory cytokines and chemokines in response to TLR and C5a receptor activation are complex and subject to a considerable degree of overlap (Figure 18). Furthermore, it is likely that *in vivo* the two systems are typically activated simultaneously, since during infection or sterile injury to tissues stimuli that activate TLRs are also likely to activate complement. Thus it is perhaps not surprising that there should be a functional interaction between the two receptor systems, and indeed a substantial body of literature demonstrates this to be the case.
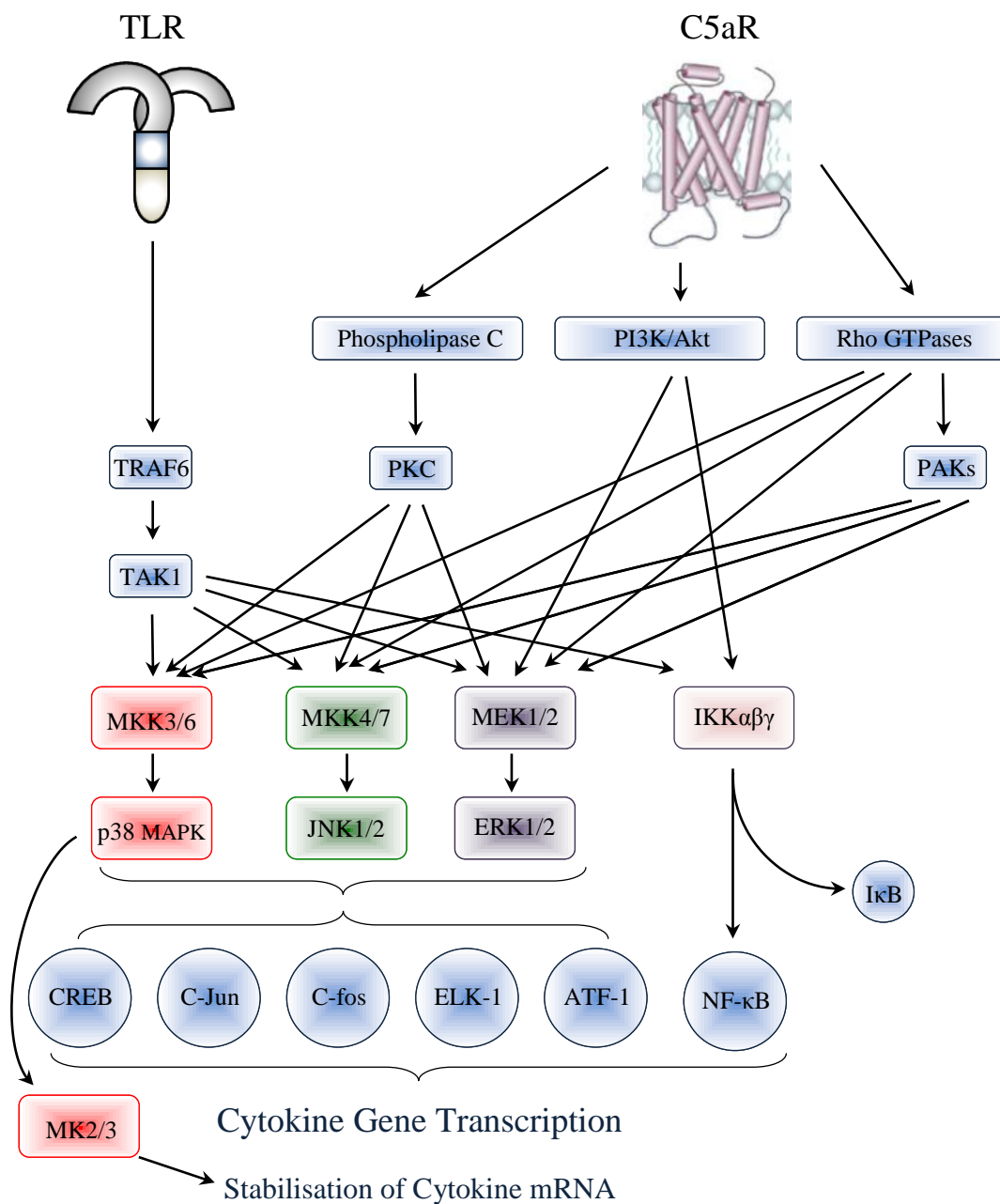
Figure 18. Overlapping signalling pathways involved in TLR- and C5aR-induced cytokine secretion. Complex and overlapping signal transduction pathways lead to cytokine secretion in response to TLR and C5aR activation, via activation of NF-κB and mitogen-activated protein kinase cascades. ATF-1, activating transcription factor 1; CREB, cyclic AMP responsive element binding protein; ELK-1, ETS-like tyrosine kinase-1; ERK1/2, extracellular signal related kinase 1/2; MEK1/2, MAPK/ERK kinase 1/2; JNK1/2, c-Jun N-terminal kinase 1/2; MK, MAPK-activated protein kinase; MKKs, MAPK kinase; PAKs, p21-activated kinases; PKC, protein kinase C; IκB, inhibitor of NF-κB; IKKαβγ, IκB kinase, subunits αβγ; PI3K, phosphoinositol-3-kinse; TAK-1, TNF receptor-associated kinase-1. Adapted from [Monk 2007, Flannery and Bowie 2010, and Kawai and Akira 2010].

1.5.1 Early studies demonstrating synergistic enhancement of pro-inflammatory cytokine release

A functional interaction between TLRs and C5a receptors was first explicitly demonstrated by Gelfand and co-workers [Okusawa 1987], who found that peripheral blood mononuclear cells (PBMC) exposed to C5a in combination with LPS released more IL-1 than the sum of that released by either stimulus alone (indicating a synergistic interaction between the two stimuli). A certain amount of controversy dogged this and other early studies, since C5a alone is a weak inducer of inflammatory cytokine release, and the detection methods for secreted cytokines were based on biological activity assays and so were less sensitive than modern antibody-based methods; many investigators concluded that while C5a alone could induce transcription of pro-inflammatory cytokines, a second signal (typically LPS) was necessary for inducing protein synthesis [Cavaillon 1990 and Schindler 1990]. However, it subsequently became clear that exposure of isolated leucocytes or whole blood to C5a induced an inflammatory response (including the transcription and secretion of IL-1, IL-6 and IL-8) that could be greatly potentiated by concurrent administration of LPS [Schindler 1990, Montz 1991, Czermak 1999 and Mack 2001] or other non-microbial TLR ligands [Kuhns 2007].

The synergistic effect of the TLR-C5a receptor interaction on the induction of cytokine release was not restricted to blood cells. Synergistic enhancement of inflammatory cytokine release by the combination of LPS and C5a has been reported in alveolar epithelial cells [Riedemann 2002a] and hepatic Kuppfer cells [Mack 2001]. The Ward group [Czermak 1999] also found that co-stimulation of rat neutrophils with LPS and C5a *in vivo* resulted in enhanced C5a-induced chemotaxis *ex vivo*, indicating that other functions of C5a as well as pro-inflammatory cytokine release were capable of being enhanced by co-incident TLR activation.

Thus, early reports indicated that concurrent activation of TLRs and C5a receptors resulted, in a number of different human and murine cell types, in enhanced inflammatory responses compared with those elicited by stimulating either receptor individually.

1.5.2 Findings from murine models of sepsis

Infection causes both complement activation (and thus generation of C5a) and the release of multiple TLR ligands. Hence, much information about the C5a-TLR interaction has been yielded by studies using animal models of infection, especially murine models employing targeted genetic deletions. Whilst a range of murine models has been used by investigators, it is generally accepted that the caecal ligation and puncture (CLP) model most closely represents the kind of infective challenge involved in sepsis in humans [Ward 2010a and b].

A number of studies published by the Ward group between 2001 and 2012 using the murine CLP model have firmly implicated C5a and its receptors in the pathophysiology of sepsis. In particular, these studies have identified the TLR-C5a receptor interaction as playing a critical role in the dysregulated inflammatory response that characterises sepsis. C5a was found to potentiate the inflammatory cytokine response to CLP sepsis, since the administration of a neutralising antibody to C5a had the effect of reducing the CLP-induced plasma levels of inflammatory mediators (IL-6 and TNF-α) as well as improving bacterial clearance, reducing histological evidence of multi-organ failure and improving survival [Huber-Lang 2001a and b, Riedemann 2002b].

The CLP model had a complex effect on C5aR expression: neutrophil C5aR expression was rapidly down-regulated by CLP sepsis; this down-regulation was strongly associated with increased septic lethality. The investigators found a corresponding deficit in *ex vivo* C5a-induced chemotaxis and oxidative burst metabolism in neutrophils from animals with CLP

sepsis [Huber-Lang 2001a, Huber-Lang 2002 and Guo 2003]; prevention of this deficit probably accounted for the enhancement in bacterial clearance seen in CLP animals treated with anti-C5a. On the other hand, CLP sepsis was found to up-regulate expression of C5aR in the vital organs (heart, lung, liver, and kidneys). This increased C5aR expression in non-haematopoietic tissues was also associated with histological evidence of multi-organ failure and with lethality, and was dependent on plasma IL-6 [Riedemann 2002b, Riedemann 2003]. The TLR-C5a receptor interaction was explicitly implicated in this picture by the finding that *in vitro,* C5a appeared to synergistically enhance LPS-induced release IL-6 by neutrophils. The contribution of neutrophils to the composition of the plasma cytokine milieu has elsewhere been reported to be relatively small compared with that of monocytes [Sabroe 2002 and Møller 2005], but neutrophil IL-6 appeared to play an important role in this model: the investigators found that neutrophil depletion before induction of CLP led to a similar decrease in plasma IL-6 to that observed in animals treated with anti-C5a. This suggested that the neutrophil TLR-C5a receptor interaction was critically involved in the elevated plasma IL-6 levels that had previously been shown to induce tissue C5aR up-regulation, which itself seemed to be at least partly responsible for organ failure and septic lethality.

The investigators proposed a mechanism that might account for the relationship between tissue C5aR expression and mortality [Laudes 2002]. A murine model of endotoxaemia showed that *in vivo* exposure to LPS induced the expression of C5aR in microvascular endothelial cells, raising the possibility that endothelial cells might be implicated in the harmful effects of C5a during CLP sepsis. An *in vitro* stimulation model revealed that consecutive TLR activation and C5a stimulation significantly enhanced the release of the neutrophil chemoattractants macrophage inflammatory protein-2 (MIP-2) (CXCL2) and MCP-1 (CCL2) compared with that induced by LPS alone (C5a alone did not induce chemokine release). Since neutrophil infiltration of vital organs during sepsis is known to have a critical role in inflammatory tissue

injury and organ failure, these findings suggested that the link between the previously observed increased tissue expression of C5aR and lethality in the CLP model was TLR /C5aR synergy in the release of neutrophil chemoattractants by vascular endothelia [Laudes 2002]. This study identified two key elements of the TLR-C5a interaction that are of particular relevance to the pathophysiology of murine sepsis. The first was the direct enhancing effect of the TLR-C5a receptor synergy in inducing cytokine release (neutrophil IL-6 and endothelial chemokines). The second was an indirect enhancing effect of TLR activation in CLP sepsis (mediated by IL-6 released from neutrophils) upon C5a-induced release of neutrophil chemokines through the upregulation of C5aR expression in vascular endothelia.

The effects of the TLR-C5a receptor interaction were observed to vary between different cell types. Rat alveolar epithelial cells responded to co-stimulation with LPS+C5a with a synergistic enhancement in the release of IL-1β, cytokine-induced neutrophil chemoattractant-1 (CINC-1), macrophage migration inhibitory protein-2 (MMIP-2) and TNF-α [Riedemann 2002a]. However, rat neutrophil MMIP-2 release was found to be attenuated by stimulation with LPS+C5a compared with that induced by either ligand alone [Riedemann 2004]. Similarly, mouse neutrophil TNF-α release was reported to be attenuated by LPS+C5a compared with LPS alone, whereas in alveolar macrophages LPS+C5a induced synergistic enhancement of TNF-α release. Murine alveolar macrophages and neutrophils exhibited a capacity for synergistic enhancement of CINC-1 and macrophage inflammatory protein (MIP, CXCL2), whereas PBMC did not [Guo 2006]. Thus it appeared that the effect of the TLR-C5a receptor interaction might be more complex than to globally enhance the release of pro-inflammatory cytokines, since its effects on TNF-α and other inflammatory mediators were positive in some cell types, but negative in others.

The signal transduction pathways involved in TLR ligand- and C5a-induced cytokine release were also investigated [Guo 2006]. Concurrent stimulation of murine neutrophils and alveolar macrophages with LPS and C5a induced a synergistic enhancement of MIP-2 release by neutrophils and alveolar macrophages. Perhaps unsurprisingly, NF-κB inhibition abolished secretion of MIP-2 in response to any stimulus. Inhibition of MAP Kinase JNK1/2 had no effect on MIP-2 secretion in either neutrophils or macrophages, whereas inhibition of p38 MAPK attenuated macrophage MIP-2 secretion and abolished neutrophil MIP-2 secretion. Interestingly, the investigators found that while TLR4 activation alone induced cytokine release, it did not induce significant phosphorylation of either ERK1/2 or p38 MAPK. On the other hand, in both alveolar macrophages and neutrophils, C5a induced rapid ERK1/2 and p38 phosphorylation, an effect that was enhanced by the addition of LPS. Thus, while TLR activation alone did not induce significant phosphorylation of ERK1/2 and p38, it did appear to up-regulate C5a-induced phosphorylation. This suggested that while the MAPK pathways were not critically involved in LPS-induced MIP-2 release, they did play a role in mediating the TLR-C5a receptor synergistic enhancement of MIP-2 release.

A later study by the Ward group also implicated the PI3K/Akt pathway in the regulation of cytokine release in response to C5a receptor and TLR activation [Riedemann 2004]. Macrophage migration inhibitory factor (MIF) is an immunomodulatory and pro-inflammatory cytokine released by macrophages, eosinophils, T cells and epithelia in response to TLR4 activation (amongst other stimuli). It is known to activate T cells and induce secretion of pro-inflammatory cytokines from macrophages, and is implicated in pro-inflammatory cytokine release and lethality in murine models of endotoxaemia as well as human sepsis [Bozza 1999, Gando 2001]. The investigators found that in murine CLP sepsis, neutrophils were the main source of plasma MIF, and that plasma MIF levels were reduced in response to either neutrophil depletion or C5aR mAb blockade, suggesting that neutrophil C5aR ligation was

critical to sepsis-induced MIF secretion. *In vitro,* the combination of LPS and C5a induced an increased level of MIF secretion from neutrophils compared with that induced by either stimulus alone. PI3K phosphorylation was observed in response to C5a stimulation, and enhanced by the concurrent administration of LPS with C5a but was not induced by LPS alone (although LPS alone did induce MIF secretion). All MIF release was abolished by addition of a PI3K inhibitor, indicating that PI3K signalling was necessary for MIF release induced by LPS alone, even though PI3K phosphorylation was not observed in response to LPS stimulation alone.

A further investigation by the Ward group into the effects of PI3K inhibition on CLP septic lethality [Wrann 2007] expanded and developed these findings. Mice that were treated with an inhibitor of PI3K before induction of CLP sepsis exhibited higher plasma levels of TNF-α, IL-1β, IL-6 and IL-10, as well as higher mortality than animals with intact PI3K signalling. In contrast with the group's previous reports, these findings indicated a protective, anti-inflammatory effect of PI3K in the setting of experimental sepsis. The use of PI3K inhibition *in vitro* revealed an intriguing effect of PI3K on the release of IL-1β and IL-8 by isolated neutrophils. In this model, the levels of cytokines released in response to stimulation with LPS+C5a were not substantially higher than those seen in response to LPS or C5a alone. However, when cells were stimulated with LPS+C5a (but not LPS or C5a alone) in the presence of a PI3K inhibitor, a marked enhancement of cytokine release was observed. These findings, while difficult to explain fully, suggest that in murine neutrophils, the capacity of C5a to synergise with LPS in the induction of IL-1β and IL-8 is subject to a strong inhibitory signal from PI3K. They also confirm that the concurrent engagement of TLRs and C5a receptors may have different effects on different inflammatory mediators.

A further study by the Ward group was the first to demonstrate the possible contribution of the second C5a receptor, C5L2, to the TLR-C5a receptor interaction [Gao 2005]. The investigators found that whereas mAb blockade of C5aR abolished the previously reported synergy between LPS and C5a in the induction of IL-6 release by rat neutrophils, the synergistic enhancement was greatly potentiated by blockade of C5L2. These findings suggested that the C5a receptors played antagonistic roles in the TLR-C5a receptor interaction, with C5L2 exerting a constitutive inhibition of C5aR's capacity to synergise with TLR ligands in inducing IL-6 release from neutrophils.

1.5.3 Findings from murine knock-out and gene silencing studies

The use of experimental models involving mice with targeted deletions or silencing of either (or both) of the two receptors for C5a has yielded much information about the differing roles of C5aR and C5L2 in the TLR-C5a receptor interaction.

As discussed in section 1.3.4.2 above, a study by Feinstein and colleagues identified an apparent anti-inflammatory effect of C5L2 on LPS-mediated inflammation in rat astrocytes [Gavrilyuk 2005]. Blockade of C5L2 synthesis induced an increase in LPS-induced NOS mRNA and nitrite levels in the *absence* of exogenously administered C5a. Transfection of C6 glioma cells (which do not express C5L2) with C5L2 resulted in a significant reduction (>70%) of NF-κB activation in response to LPS treatment compared with cells not expressing C5L2. These findings are intriguing, because they suggest a role for C5L2 in modulating TLR-induced inflammatory responses in the absence of exogenously administered C5a. A potential explanation for this is of course artefactual generation of C5a in the culture conditions, but the data are also compatible with the possibility of a regulatory effect of C5L2 on TLR signal transduction, although it is unclear how such an effect might be exerted.

The Yeh group's 2007 study using an independently generated C5L2-deficient mouse model[2] found a complex effect of C5L2 on the TLR-C5a receptor interaction [Chen 2007]. The group found that co-stimulation of wild-type neutrophils with LPS+C5a resulted in inhibition of LPS-induced TNF-α, and enhancement of IL-6 release. This apparent capacity of C5a to modulate the TLR response (either upwards or downwards) was lost in C5L2-deficient mice, suggesting that C5L2 was critically involved in whatever contribution C5a made to response to the combined TLR+C5a receptor stimulus. In contrast with previous reports and with the effect on TNF-α and IL-6 release in neutrophils, co-stimulation with LPS and C5a in macrophages was found to inhibit the LPS-induced release of both cytokines; again this effect was lost in C5L2-deficient cells.

The involvement of the PI3K/Akt, ERK1/2, p38 and JNK1/2 MAPK pathways was also investigated: consistent with the findings of the Ward group, Chen *et al.* found that C5a stimulation induced phosphorylation of Akt, ERK1/2 and p38 in neutrophils and macrophages, while LPS did not, but the addition of LPS to C5a tended to enhance the C5a-mediated phosphorylation of kinases. In both conditions, the C5a-induced phosphorylation was attenuated or lost in C5L2-deficient mice, suggesting again that the contribution of C5a to the signalling pathways implicated in the TLR-C5a receptor interaction was mediated by C5L2. Interestingly, in a model of lethal endotoxic peritonitis (which would inevitably also have induced substantial complement activation), the survival of the C5L2-deficient mice was markedly worse than that of WT littermates, perhaps suggesting that the overall effect of C5L2 (at least in this experimental model) was anti-inflammatory.

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[2] Two independently-generated C5L2-deficient mouse models are reported in the literature: the first was generated by the Gerard group and reported in 2005 [Gerard 2005]. This model was subsequently used in all of the other studies that report the use of a C5L2-deficient murine model, except that cited here which was generated by the Yeh group [Chen 2007].

A further study by the Ward group using Gerard's C5L2-deficient mice [Gerard 2005] produced findings both consistent and contrasting with the two previous reports, and also appeared to implicate C5L2 in the regulation of TLR signal transduction in the absence of concurrent C5a stimulus [Rittirsch 2008]. Consistently with the group's previous reports, deletion of C5aR had an anti-inflammatory effect on CLP sepsis, resulting in substantial decreases in the plasma levels of IL-1β, IL-6, MIP-1α and MIP-2. C5aR deletion had no effect on TNF-α levels. Deletion of C5L2 had a more complex effect: IL-1β, MIP-1α and MIP-2 levels were reduced and TNF-α unaffected, but IL-6 was markedly up-regulated. Blockade or deletion of either receptor tended to improve survival; in high-grade (100% lethality) CLP sepsis, blockade or deletion of both receptors was necessary to induce a protective effect. Thus, it would appear that the contribution of C5aR to the net inflammatory response to polymicrobial sepsis was to potentiate TLR-induced release of IL-1β, IL-6, MIP-1α and MIP-2, whereas C5L2 potentiated IL-1β, MIP-1α and MIP-2 but inhibited IL-6 release; the net effect on survival suggested a pro-inflammatory role for C5L2 in the context of murine CLP sepsis.

As discussed in section 1.3.4.2 above, another important finding of this study was that C5L2 appeared to regulate the release of the nuclear protein HMGB1. Peritoneal macrophages secreted HMGB1 in response to LPS (strongly), C5a (less strongly) and LPS+C5a (~the sum of each alone). C5aR deletion had no effect on the level of HMGB1 released by cells in response to any of the stimuli, indicating that the C5a-induced HMGB1 secretion was not mediated by C5aR. Intriguingly, however, the deletion of C5L2 greatly reduced HMGB1 release in response not only to C5a and C5a+LPS, but also to LPS alone. These findings are reminiscent of those of the Feinstein group [Gavrilyuk 2005], who also reported a regulatory effect of C5L2 on LPS-induced inflammatory responses, again without the addition of exogenous C5a.

A study by Song and co-workers [Zhang 2007] indirectly demonstrated a far-reaching interaction between complement and TLR activation by utilising a murine model of peritonitis in mice deficient in a key inhibitor of complement activation. The investigators demonstrated that mice with genetic deletion of the glycosylphosphoinositol-linked cell surface complement inhibitory protein, decay-accelerating factor (DAF, CD55) were hypersensitive to a sub-lethal intra-peritoneal LPS challenge, exhibiting worse symptoms of endotoxaemia and elevated plasma levels of IL-6, TNF-α and IL-1β compared with wild-type littermates. Since DAF accelerates the proteolytic breakdown of both C3 and C5 convertases, it was hypothesized that the DAF-deficient mice were exhibiting an exaggerated inflammatory phenotype as a consequence of enhanced LPS-induced complement activation. Accordingly, the plasma levels of activated C3 in the DAF-deficient mice were significantly higher than in wild-type mice, and the differences between DAF-deficient and DAF-sufficient mice were abolished in DAF/C3 double-knockout mice.

It was not possible, however, to attribute the greatly enhanced pro-inflammatory response in the DAF-deficient mice solely to the effects of increased complement activation in that population. The administration to wild-type mice of cobra venom factor (CVF), which causes massive complement activation, resulted in only a modest increase in plasma levels of IL-6; CVF administered in conjunction with LPS, however, resulted in a ~40-fold increase in plasma IL-6 levels compared with that induced by LPS alone, in effect reproducing the effect of DAF insufficiency. The effect could be partially blocked by concurrent administration of an antagonist to C3a (or C3a deficiency) and completely abolished by a C5a antagonist or by C5 deficiency, indicating that the TLR-C5a receptor interaction was primarily responsible for the enhancement in LPS-induced inflammatory cytokine release seen in the DAF-deficient mice.

Once again, the increased cytokine release seen in DAF-deficient mice was associated with increased activation of MAPK and NF-κB pathways. Splenocytes from DAF-deficient mice showed greater and more rapid phosphorylation of IκB after LPS injection, and lower levels of IκB overall. DAF-deficient RAW264.7 cells transfected with an NF-κB luciferase reporter showed a greater activation of NF-κB when stimulated with C5a+LPS than LPS or C5a alone, with a corresponding pattern of TNF-α release. Lysates of splenocytes from DAF-deficient mice showed increased ERK1/2 and JNK1/2 phosphorylation 30 minutes after *in vivo* LPS challenge compared with wild-type mice. Since the Ward and Gerard groups had previously reported that LPS alone did not induce MAPK phosphorylation in this time frame, but that LPS potentiated C5a-induced kinase phosphorylation, it is tempting to speculate that this effect was attributable to LPS potentiation of C5a in this model also; however, this hypothesis was not tested (for example by examining the effects of LPS + anti-C5a in the DAF-deficient mice).

DAF knockout was found to potentiate not only TLR4 but TLR2 and TLR9 as well. Intraperitoneal injection of zymosan, a TLR2/6 ligand also known to be capable of activating complement, induced an enhancement of plasma levels of IL-6 in DAF-deficient mice compared with wild type littermates. Again, the normal phenotype was restored by a DAF/C3 or DAF/C5aR double knockout. Similar findings resulted from intra-peritoneal challenge with the TLR9 ligand CpG DNA, indicating that TLR2 and 9 activation were similarly susceptible to modulation by the C5a receptor. Hence the effects of DAF deficiency were unlikely to be a result of an effect of DAF on the TLR4-LPS interaction (DAF has been reported to be capable of directly interacting with LPS [El-Samalouti 1999, Heine 2003]).

While the investigators undeniably demonstrated a far-reaching effect of the TLR-C5a receptor interaction, the indirect means by which they did so (via increased complement activation in

DAF-deficient cells) makes it difficult to formulate a clear theory as to the mechanism by which TLR and C5a receptor signal transduction might have affected cytokine release in their model.

1.5.4 The TLR-C5a receptor interaction and release of immunomodulatory cytokines

1.5.4.1 IL-12

IL-12 is a heterodimeric pro-inflammatory and immunomodulatory cytokine released by monocytes and macrophages in response to TLR ligands. It is comprised of p40 and p35 subunits, which are separate gene products; a heterodimer of p40 and p35 (IL-12p70) and a homodimer of p40 are released by cells. IL12 is known to drive polarisation of CD4$^+$ cells towards a TH$_1$ phenotype through its ability to induce proliferation and IFNγ secretion in T and NK cells. Its excess has been implicated in autoimmune diseases, and its deficiency in susceptibility to bacterial and viral infections; supplementing IL-12 has been investigated as a therapeutic strategy in such infections [Trinchieri 2003].

Werfel and colleagues [Wittmann 1999] found that IFNγ-primed human monocytes that were pre-exposed to C5a before addition of LPS manifested a C5a dose-dependent loss of intracellular staining for IL-12p40 and p70 compared with cells stimulated with LPS alone. This suggested that C5a receptor activation had an inhibitory effect upon LPS-induced secretion of IL-12-family cytokines. Similar results were reported by a number of groups, using a range of *in vitro* and *in vivo* experimental models [Braun 2000, La Sala 2005, Hawlisch 2005, Zhang 2007, Okazaki 2010, Wang 2010 and Liang 2010]. The inhibitory effect of C5a on TLR-induced IL-12 secretion was shown to be mediated by the PI3K/Akt, ERK1/2 and JNK1/2 pathways. It was also found to be dependent on autocrine secretion of IL-10, suggesting that the apparent inhibitory effect of C5a in TLR-induced inflammatory responses might in fact have been due to an enhancing effect of C5a receptor activation upon TLR-induced IL-10 release.

1.5.4.2 IL-17

Another study by the Song group in 2009 [Fang 2009] showed that the TLR-C5a synergy in the induction of IL-6 release also extended to the release of TGF-β, and created in plasma a cytokine milieu that favoured differentiation of naive $CD4^+$ T cells towards an auto-immunogenic TH_{17} profile. Incubation of naïve $CD4^+$ T cells activated by plate-bound anti-CD3/CD28 with serum from wild-type mice subjected to intraperitoneal challenge with LPS, CVF and/or C5a showed that the combination of LPS and CVF or LPS and C5a (but neither agent alone) induced a TH_{17} polarisation comparable with that seen when activated T-cells were incubated with IL-6 and TGF-β (the positive control for TH_{17} polarisation). The absence of IL-6 (due to antibody blockade in the secondary culture or genetic deletion in the mice given the *in vivo* challenge) resulted instead in an IFNγ-producing TH_1 phenotype, indicating that the TLR-C5a receptor synergy in the production of IL-6 was critical in determining this outcome.

There are accumulating reports in the literature that IL-17 isoforms (IL-17A and F) can be produced by cells of the innate immune system as well as by TH_{17}-polarised $CD4^+$ T cells. Substantial levels of IL-17A were released in the genetic absence of αβ T cells after CLP [Flierl 2008] and in a model of endotoxic shock, plasma IL-17A levels were not reduced by the genetic absence of αβ or γδ T-cells, or $CD4^+$ cells, whereas depletion of $F4/80^+$ macrophages did reduce IL-17A levels [Bosmann 2011]. The Ward group recently demonstrated that the release of the IL-17F isoform by peritoneal macrophages in response to LPS (but not TLR2, 3, 5 or 9 ligands) was subject to a synergistic enhancement by C5a in a manner that was dependent on MyD88, C5aR (but not C5L2) and PI3K/Akt.

1.5.5 The TLR-C5a receptor interaction and the lymphocytes of the innate immune system

The capacity of the TLR-C5a receptor interaction to modulate responses of innate immune lymphocytes was investigated by the Köhl and Mattner groups [Fusakio 2011] using a C57/BL6 mouse model of *E. coli* sepsis. Deficiency of C5aR but not C5L2 had a significant effect in reducing mortality. NK and NK T cells are able to rapidly secrete large amounts of IFNγ and TNF-α in response to microbial infections without prior sensitisation or clonal proliferation, and have been identified as key contributors to the harmful systemic inflammatory response in sepsis [Chiche 2011]. The investigators assessed the effects of either C5aR deficiency or genetic deletion of NK and NKT cells on the response to *E. coli* peritonitis. Deletion of C5aR (but not C5L2) or depletion of NK/NK T cells resulted in a substantial reduction in mortality and in serum levels of TNF-α and IFNγ. In an elegant series of *ex vivo* experiments, the investigators tested the capacity of splenocytes harvested from mice 24 h after induction of *E. coli* peritonitis to secrete TNF-α and IFNγ in response to ligands for TLR 1, 2, 4, 5, 6 and 9. Cells from C5aR-deficient mice showed a significantly reduced capacity to secrete TNF-α and IFNγ compared with wild-type littermates, suggesting that *in vivo* C5a-C5aR activation led to enhanced TLR-mediated cytokine release.

Next, the investigators harvested naïve splenocytes from wild-type, NK/NKT-deficient, C5L2-deficient and C5aR-deficient mice and stimulated them with TLR ligands alone or TLR ligands+C5a. The results are complex and intriguing: in wild-type cells, TLR 1/2, 2/6 and 9 ligands synergised with C5a in inducing IFNγ and TLR1/2, 2/6 and 5 with C5a in inducing TNF-α. TLR4 ligation+C5a failed to show statistically significant synergy in inducing either cytokine, and TLR5 activation failed to induce a significant IFNγ response either with or without C5a. These findings suggest that different TLR ligand/C5a combinations may preferentially induce different cytokines. The overall population of splenocytes would have contained a range of cell

types, including neutrophils, monocyte/macrophages, DCs, NK and NK T cells and of course B- and T-lymphocytes, and it is possible that the differential effects of different TLR ligand/C5a combinations on cytokine release simply reflect the presence of different cell types which predominantly express different TLRs and also have different capacities for secreting TNF-α and IFNγ. The investigators did not identify specific subpopulations for individual study but the involvement of NK/NK T cells in the TNF-α/IFNγ response was strongly implicated by data from NK/NKT-deficient animals, which showed a consistent reduction in the cytokine response to all TLR ligands both with and without C5a. Furthermore, the capacity of TLR ligands to synergise with C5a seen in wild-type splenocytes was in some cases lost in the NK/NKT-deficient animals, whilst the capacity to respond to TLR ligand alone was preserved (albeit with reduced sensitivity), suggesting that the NK/NK T cell population was at least partly responsible for the enhanced cytokine release observed in the wild-type animals.

Additional work by this group using an *ex vivo* co-culture model of bone-marrow-derived dendritic cells (BMDCs) with NK or NK T cells suggested that the TLR-C5a receptor interaction has an effect on the capacity of splenic DCs to drive NK/NKT secretion of TNF-α and IFNγ. Monocultures of BMDCs or NK cells from septic animals did not secrete detectable IFNγ in response to TLR ligands, whereas IFNγ release from BMDC+NK co-cultures was both substantial, and markedly attenuated by C5aR deficiency in the BMDCs. A similar BMDC C5aR-dependent effect was shown on NK T cell TNF-α release in response to ligands for TLR1/2, 2/6 and 5 (but not 4) in a corresponding co-culture model. TLR ligand-induced TNF-α and IFNγ secretion were found to be due not to direct stimulation of TLRs on NK/NKT cells, but to C5aR-dependent release of IL-12 and IL-18 in response to TLR ligation on co-cultured DCs. Hence the authors concluded that the elevated systemic levels of IFNγ and TNF-α, which are an important component of the 'cytokine storm' seen early in sepsis, may be a powerful but

indirect effect of the TLR-C5aR crosstalk in DCs, which then triggers massive pro-inflammatory cytokine release from NK and NK T cells.

A further level of complexity was suggested by Salmon *et al*. [Qing 2012], who demonstrated that TLR-C5a receptor synergistic enhancement of NK cell activation was not only indirectly mediated by DCs, but also that DC-mediated priming of NK cells was itself an indirect phenomenon, being the consequence of a TLR-C5aR mediated synergistic enhancement of TGF-β release from immature Gr1^{+} myeloid cells (pre-monocyte/neutrophils). Thus, it appears that the synergistic enhancement of inflammatory cytokines attributed to the TLR-C5a receptor interaction in *in vivo* models of sepsis is a complicated phenomenon involving interactions between a number of different cell types, and that NK and NKT lymphocytes may play a key role in the acute inflammatory response to infection.

1.5.6 Summary

The previously reported findings discussed here indicate a complex relationship between TLR and C5a receptor activation, with far-reaching effects. It appears that concurrent TLR and C5a receptor activation may dramatically amplify the release of a range of inflammatory mediators (particularly IL-6 and a number of chemokines), in many cases by several orders of magnitude over the responses that would be elicited by either stimulus alone. Thus, it is plausible that the TLR-C5a receptor interaction may play a key role in the efficient clearance of infections, but also in the excessive cytokine and chemokine response to infection that characterises the pathophysiology of sepsis.

On the other hand, the TLR-C5a receptor interaction may markedly inhibit the release of certain mediators compared with that induced by a TLR stimulus alone. The effect of concurrent C5a and TLR ligand activation on the release of TNF-α in particular appears to vary

in different cell types: neutrophils have been consistently reported to release less TNF-α if stimulated with LPS+C5a than with LPS alone, while resident tissue macrophages and epithelial cells typically showed an enhancement in TNF-α release in response to co-stimulation. The net effect of TLR+C5a receptor co-stimulation on systemic TNF-α release seems to be positive, since plasma levels were typically found to be inhibited in models of sepsis in which C5a signalling was interrupted by genetic deletions or antibody blockade of C5a.

An inhibitory effect of C5a on TLR-mediated IL-12 secretion was consistently found in both *in vivo* and *in vitro* models, and in a number of different cell types. However, in spite of some inconsistencies between reported studies, it is likely that this apparent inhibitory effect is secondary to changes in IL-10 secretion. Thus it might be more accurate to view the effect of the TLR-C5a receptor interaction on IL-12 release as the result of an enhancement of TLR- or C5a-induced IL-10 secretion, rather than as an inhibitory effect on IL-12 release *per se*.

Investigators have consistently found that the MAP kinases ERK1/2 and p38 and the PI3K/Akt pathway appear to be involved in mediating the effect of the C5a receptor-TLR interaction on cytokine release. A number of studies have demonstrated that C5a induces kinase phosphorylation, and that this is enhanced by concurrent TLR activation. This might indicate that TLR-mediated potentiation of the C5a-induced activation of these pathways is a key component of the TLR-C5a receptor interaction that results in modulation of cytokine release. The findings of the Ward group [Wrann 2008] in murine neutrophils are particularly striking in this respect, as they suggest that the PI3K/Akt pathway may be disproportionately and preferentially engaged when both TLRs and C5a receptors are activated in comparison with its level of engagement in response to either stimulus alone.

1.6 Hypothesis, Aims and Objectives

The capacity of TLR ligands + C5a to synergise and modulate pro-inflammatory and immunomodulatory cytokine production is well-established. This led to the conclusion that crosstalk between C5a receptors and TLRs occurs, which strengthens the innate host defense during infection. Most studies, however, have focused on the immunoregulatory effect of C5a on TLR-driven inflammation, neglecting potential effects of TLR activation on complement-mediated inflammation. In the present study, it was hypothesised that a genuine bi-directional signalling crosstalk between TLRs and C5a receptors exists by which not only may C5a receptor activation modulate TLR-mediated responses, but also TLR activation may modulate the extent and/or quality of cellular responses to C5a. This may guarantee a prompt, strong and efficient response to the microbial challenge and rapid clearance of infection. Thus the aim of the project was to evaluate the impact of TLR activation on C5a-driven cytokine and chemokine production *in vitro* and *ex vivo*.

Objectives

This study sought to:

1. Use an *in vitro* experimental model system to test the capacity of TLR activation to synergistically enhance inflammatory responses induced by C5a.

2. Conduct a detailed characterisation of the nature and extent of the putative modulatory effect of TLRs on C5aR-mediated pro-inflammatory responses.

3. Develop a suitable *in vivo* model to corroborate these findings.

4. Investigate the mechanism by which TLR activation may modulate C5a-induced cellular responses *in vitro* and *in vivo*.

5. Identify the key signal transduction intermediates involved in the effects of TLRs on C5a receptors.

Chapter 2: RESULTS

2.1 Modulation of cell sensitivity to C5a by TLR activation

2.1.1 Synergistic enhancement of pro-inflammatory cytokine release by co-stimulation via TLR and C5a receptors

In order to confirm the previously reported TLR-C5a receptor-mediated synergistic enhancement of pro-inflammatory cytokine release, human PBMC were stimulated with the TLR4 ligand LPS, recombinant human C5a or a combination of both ligands concurrently, and the levels of the prototypical neutrophil chemoattractant IL-8 (CXCL-8) released by the cells in each condition were estimated (ELISA) in the culture supernatants. Figure 19A shows that concurrent stimulation of PBMC with LPS+C5a resulted in a marked enhancement of IL-8 release compared with the estimated additive effect of each ligand alone. The synergistic effect of LPS+C5a was observed over a range of C5a concentrations tested, and resulted in IL-8 levels several-fold higher than those resulting from stimulation with the indicated concentrations of C5a alone (Figure 19B). The viability of cryopreserved PBMC after resuscitation and at the end of the culture period was ≥99% and >95%, respectively, as assessed by the trypan blue exclusion test.
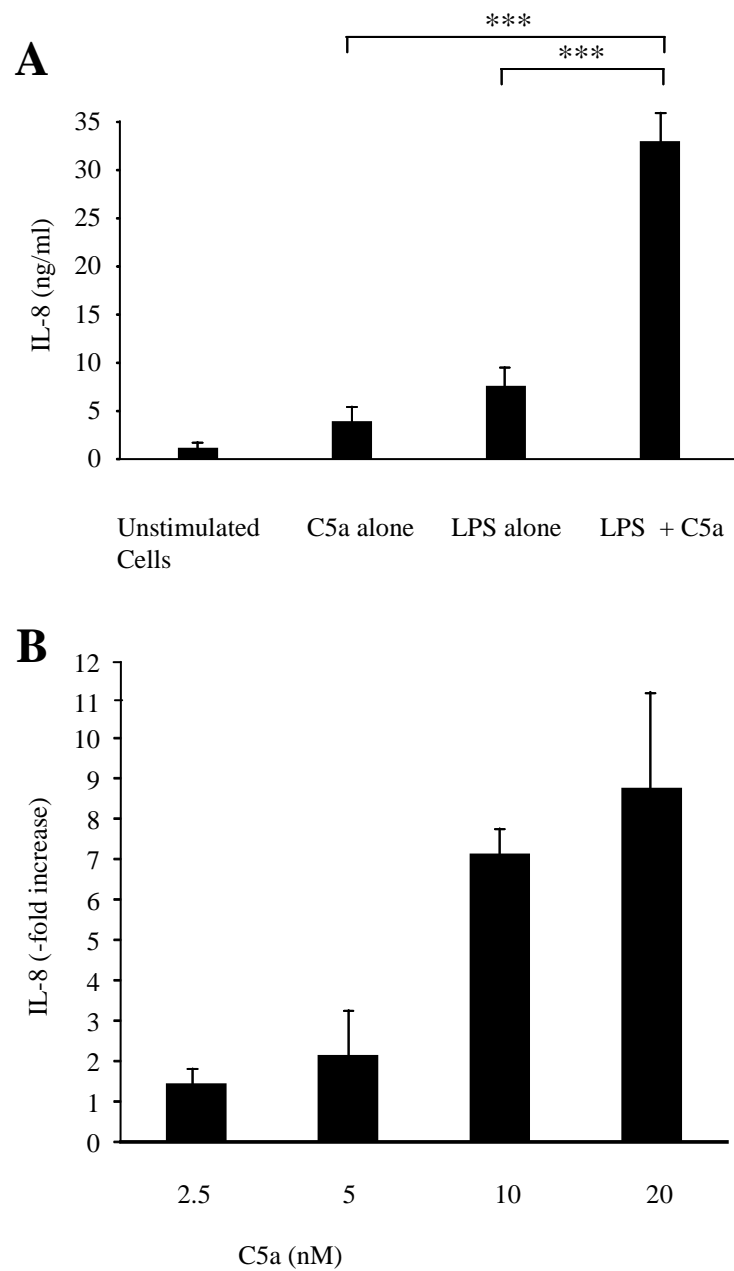
Figure 19. Effect of co-stimulation of PBMC with LPS and C5a. Human PBMC (1.5×10^5/well) were stimulated for 14 h with LPS (500 pg/mL), C5a (20 nM or as indicated) or LPS + C5a concurrently. **(A)** Overall IL-8 levels in culture supernantants. **(B)** –fold increase in IL-8 release over a range of stimulating concentrations of C5a. –fold increase was calculated as the ratio of IL-8 release induced by LPS+C5a to that induced by C5a alone, after subtraction of the relevant background. Results shown are mean (±SD) values of three independent experiments from a single donor representative of four. (*** p < 0.005).

2.1.2 Effect of TLR activation on cell sensitivity to C5a

In order to investigate the possibility that TLR activation modulates C5a-induced cytokine release, thus contributing to the synergistic effect observed and described previously, PBMC were first exposed to a typical TLR4 ligand (LPS), then washed thoroughly, and stimulated with C5a before estimation of cytokine levels in the cell culture supernatants (activation model illustrated in Figure 39A and discussed in section 5.2.2 below). Figure 20A shows that the levels of IL-8 released by C5a-stimulated cells that had been pre-exposed to LPS were significantly higher than those released by cells not pre-exposed to the TLR ligand. Thus, pre-exposing cells to LPS before C5a stimulation reproduced the LPS+C5a synergistic effect on IL-8 release observed upon co-stimulation (Figure 19A), indicating cell hypersensitivity to C5a following cell 'priming' by TLR activation. Notably, when the reverse cell treatment sequence was followed (pre-exposure to C5a followed by stimulation with LPS), the level of IL-8 released was only additive (Figure 20B). Indeed, it was not possible in this model to induce hypersensitivity of PBMC to LPS by pre-exposing them to C5a, as judged by the release of IL-8.

The possibility that the observed enhanced IL-8 release from cells pre-exposed to LPS was due to the presence of residual LPS during the C5a stimulation phase, carried over from the TLR activation phase, was evaluated – although the levels of IL-8 at the end of the culture in the supernatants of cells pre-exposed to LPS and not activated with C5a were extremely low (Figure 20A). PBMC were pre-exposed (or not) to a deliberately high concentration of LPS (10 ng/ml), then washed thoroughly, and cultured for 14h (no stimulation) in the presence and absence of polymyxin B – a cationic polypeptide that inactivates LPS by forming complexes with bacterial lipopolysaccharides [Jacobs and Morrison 1975, Morrison and Jacobs 1976]. Figure 20C shows that the levels of IL-8 released at the end of the culture following cell pre-exposure to LPS was unaffected by the presence of polymyxin B, suggesting that residual LPS
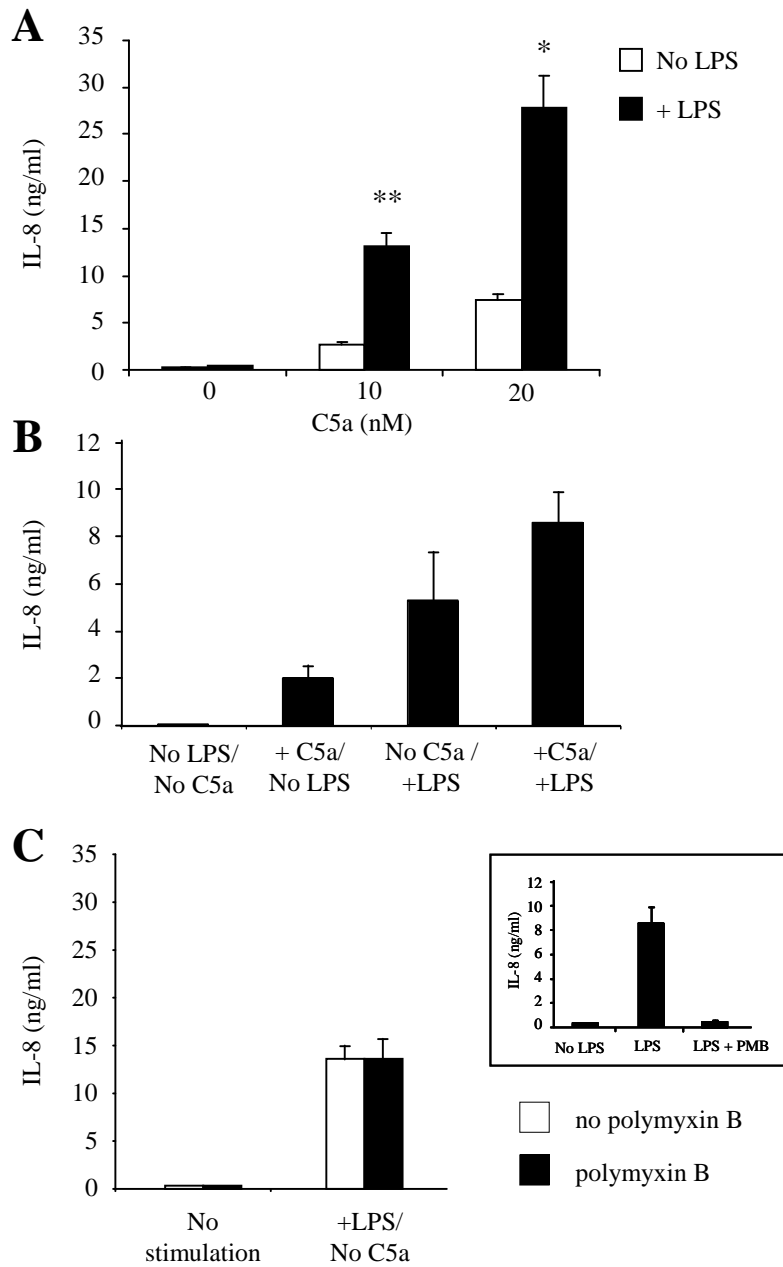
Figure 20. Effect of cell activation by TLR on PBMC sensitivity to C5a. Human PBMC (1.5×10^6/ml) were **(A)** activated (14h) with LPS (100 pg/ml) before stimulation (14h) with C5a (20 nM), **(B)** activated with C5a (20 nM) before stimulation with LPS (10 ng/ml), or **(C)** activated with LPS (10 ng/ml) before washing and culture (14h) in the absence or presence of polymyxin B (PMB, 10 μg/ml), before estimation of IL-8 levels in the supernantants by ELISA. Inset in (C) shows a control experiment, the effect of PMB (10 μg/ml) on LPS (10 ng/ml, 14h)-induced IL-8 release by PBMC. Results are means (±SD) of triplicate cultures of one experiment from a single donor representative of more than 20 (A) and two (C and D). (*$p<0.05$, **$p<0.01$, LPS-pre-exposed cells *vs.* cells not pre-exposed; NS, not significant, PMB *vs.* no PMB)

was not present following pre-exposure and washing, even when a relatively high priming concentration of LPS was used.

2.1.3 Hypersensitivity to C5a after activation of PBMC via different TLRs and other receptors

In order to test whether or not the capacity of TLR activation to enhance cell sensitivity to C5a was limited to TLR4, PBMC were pre-exposed to ligands for a range of other TLRs (Figure 21A) and for non-TLR receptors (Figure 21B) before C5a activation, including: Pam_3Cys-Ser-Lys_4 (TLR1/2), Zymosan (TLR2/6), poly I:C (TLR3), bacterial flagellin (TLR5), the antiviral compound imiquimod (TLR7/8), IL-1β (which utilises the MyD88-dependent signal transduction pathway, like TLR4), TNF-α and IL-6. In addition, the effect of non-receptor-mediated cell stimulation by phorbol 12-myristate 13-acetate (PMA) + ionomycin was also tested (Figure 21B). As seen in Figure 21A, cells pre-exposed to previously defined optimal concentrations of ligands for all TLRs tested except TLR3 showed variable degrees of hypersensitivity to C5a. Notably, and in contrast to the effects of ligands for other TLRs, pre-exposure to the TLR3 ligand poly I:C induced a marked reduction in cell sensitivity to C5a. Of the other stimuli tested, only IL-1β-induced cell stimulation resulted in hypersensitivity to C5a (Figure 21B).
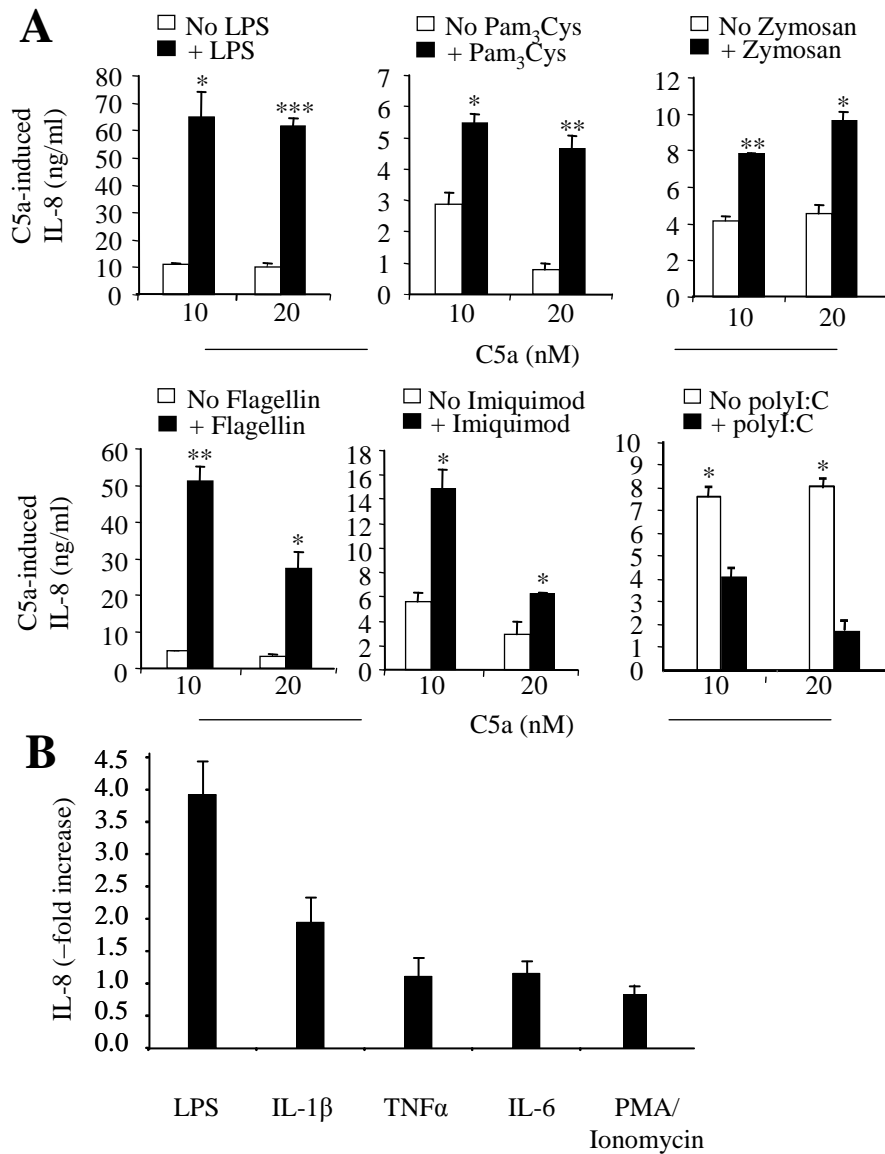
Figure 21. Effect of pre-exposure to a range of TLR and non-TLR ligands on PBMC sensitivity to C5a. (A) PBMC were pre-exposed or not (14h) to LPS (100 pg/ml), Pam$_3$Cys (100 ng/ml), Zymosan (1 µg/ml), Flagellin (5 µg/ml), Imiquimod (3 µg/ml) or poly I:C (80 µg/ml), before washing and stimulation with C5a at the indicated concentrations. Mean (±SD) C5a-induced IL-8 concentrations were estimated by subtracting the background levels of IL-8 present in cultures not activated with C5a and pre-exposed or not to TLR ligands from the corresponding C5a-activated samples (IL-8 background levels (ng/ml): No ligand/No C5a, 1.67±0.7; LPS, 2.3±1.2; Pam$_3$Cys, 1.5±0.6; Zymosan, 1.3±0.9; Flagellin, 6.9±2.5; Imiquimod, 5.3±1.1; poly I:C 1.8±0.4). (*p<0.05, **p<0.01, *** p < 0.01 pre-exposed cells *vs.* not pre-exposed). **(B)** Human PBMC (1.5 x 10^{6}/ml) were pre-exposed or not (14h) to LPS (10 ng/ml), IL-1β (5 ng/ml), TNF-α (10 ng/ml), IL-6 (10 ng/ml) or PMA/Ionomycin (50/500 ng/ml) before stimulation (14h) with C5a (20 nM). Mean (±SD) –fold increase in IL-8 release (primed cells compared with unprimed) is shown.   All results are from one experiment conducted in triplicate, representative of at least four for each ligand.

2.1.4 Effect of TLR ligand and C5a concentration on hypersensitivity to C5a

In order to investigate how sensitive human PBMC are to TLR-mediated modulation of responses to C5a, cells were pre-exposed to a wide range of LPS concentrations before washing and exposure to C5a. As Figure 22A shows, even LPS concentrations as low as 10 pg/ml, a concentration well below those found systemically in sepsis patients (~100 pg/ml to ~700 pg/ml) [Opal 1999], were able to induce a substantial enhancement (2-fold) of C5a-induced IL-8 release.

The response of cells to a wide range of C5a concentrations following TLR-mediated priming was also tested and compared with that of cells not primed. Notably, it was found that the C5a dose-response of cells pre-exposed and not pre-exposed to TLR ligands differed. Indeed, the C5a dose-response profile of PBMC that were stimulated with increasing concentrations of C5a without prior exposure to LPS exhibited a typical, saturable, dose-response relationship between the C5a concentrations and IL-8 release. However, when PBMC were pre-exposed to LPS before C5a stimulation, the C5a dose-response profile showed a peak of IL-8 release at relatively low stimulating concentrations of C5a (this concentration varied depending on the blood donor) followed by lower IL-8 levels that reached values lower than the plateau level seen in maximally-stimulated unprimed cells (Figure 22B). Cell viability in these experiments was assessed at the end of the culture period by trypan blue exclusion test and was found to be >95% in all conditions, with no significant differences between cells stimulated at higher and lower concentrations of C5a.
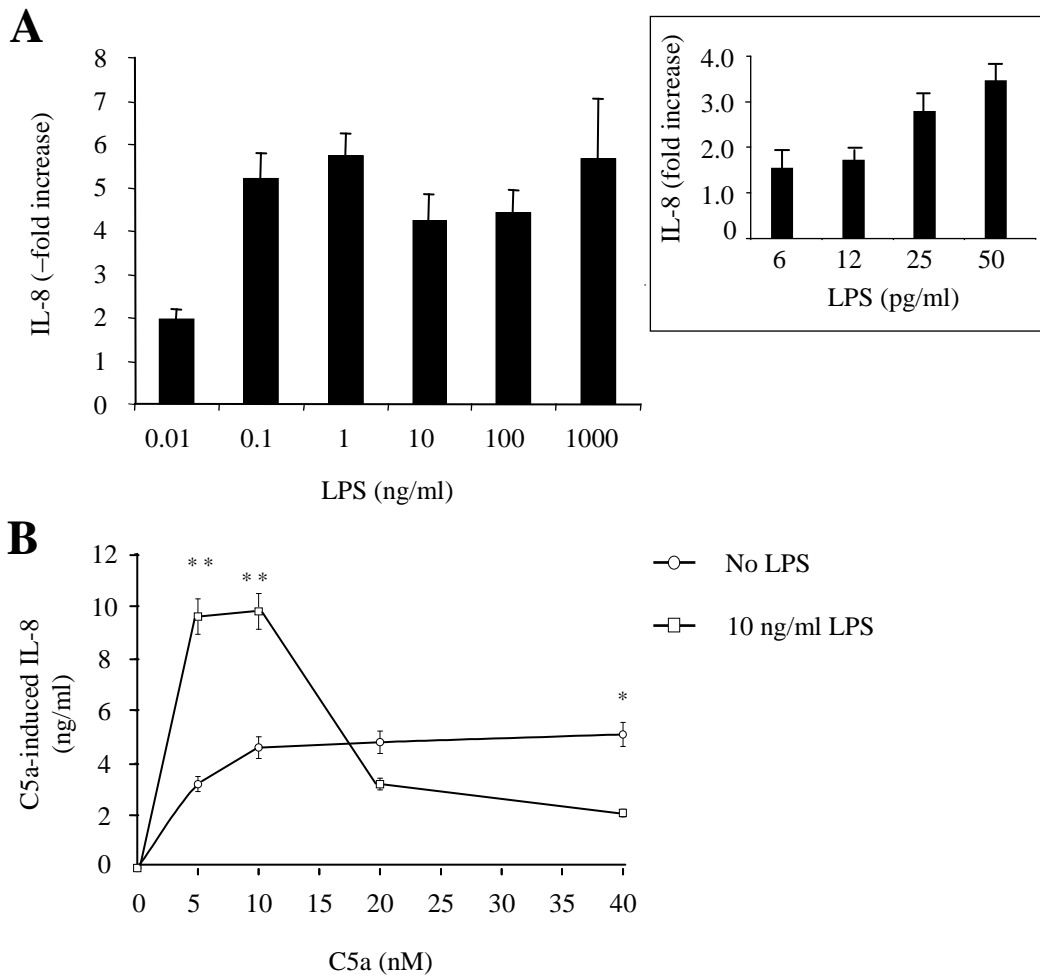
Figure 22. Effect of LPS and C5a concentration on TLR-induced hypersensitivity to C5a. (A) Human PBMC (1.5 x 10^5/well) were pre-exposed or not (14h) to LPS at the indicated concentrations before washing and stimulation (14h) with C5a (20 nM). Mean (±SD) –fold increase in C5a-induced IL-8 release is shown. **(B)** PBMC were pre-exposed or not (14h) to LPS (10 ng/ml) before stimulation (14h) with C5a at the indicated concentrations. Mean (±SD) C5a-induced IL-8 release is shown. Results in (A) and (B) are from one experiment, representative of three. (*$p<0.05$, **$p<0.01$, pre-exposed cells *vs.* not pre-exposed).

2.1.5 Effect of TLR activation on C5a-induced pro-inflammatory cytokine gene transcription

It was next tested whether the positive modulatory effect of TLR activation on C5a-induced inflammatory responses affected gene transcription or only the release of the pro-

inflammatory mediator. To this end, PBMC primed with LPS and subsequently activated with C5a were lysed at the end of the culture and mRNA levels for IL-8 as well as for IL-6 were analysed by RT-qPCR. Both IL-8 and IL-6 transcripts were found to be up-regulated in cells pre-exposed to LPS and stimulated with C5a compared with cells only pre-exposed to LPS or only stimulated with C5a (Figure 23A), indicating that TLR-induced cell hypersensitivity to C5a not only affects the release but also the transcription of pro-inflammatory mediators. Furthermore, the effect of TLR activation was not restricted to IL-8, as transcription of another potent pro-inflammatory cytokine, IL-6, was similarly affected. Consistent with this latter finding, the C5a-induced release of IL-6 protein was found to be enhanced following PBMC pre-exposure to LPS (Figure 23B).
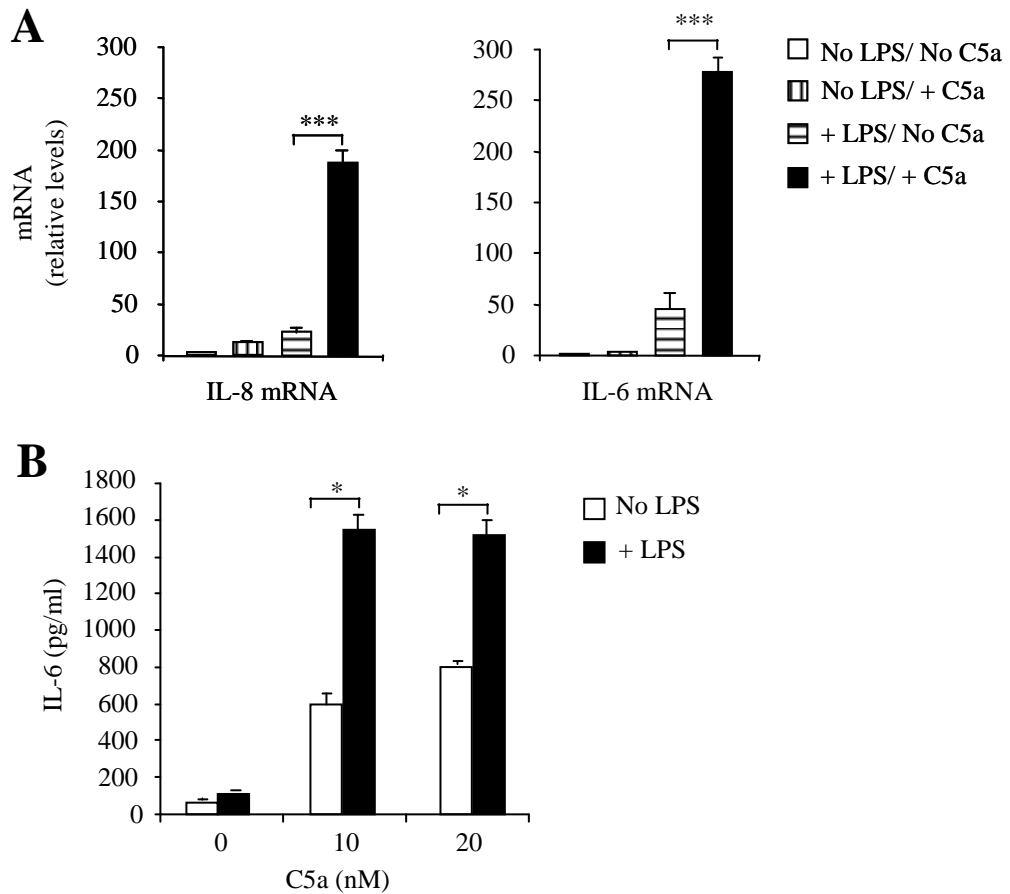
Figure 23. Effect of cell pre-exposure to LPS on C5a-induced expression of IL-8 and IL-6. (A) Human PBMC (1 x 10^6/condition) were pre-exposed or not (14h) to LPS (100 ng/ml) before washing and stimulation (14h) with C5a (20 nM). Cells were lysed and relative levels of mRNA for IL-8 and IL-6 estimated by RT-qPCR. **(B)** IL-6 levels in the culture supernatant corresponding to the experiment in (A). Mean (±SD) levels of IL-8 and IL-6 mRNA and IL-6 protein are shown from one experiment representative of three. (*$p<0.05$, *** $p< 0.01$ LPS pre-exposed cells *vs.* not pre-exposed).

To further study the effect of TLR activation on C5a-induced gene transcription, the effect of cell pre-exposure to LPS on the C5a-induced activation and nuclear translocation of the transcription factor NF-κB p65 (RelA) – a key regulator of immunoregulatory gene transcription – was investigated. Figure 24 shows that, similarly to the positive effect on IL-8 and IL-6 production and release, pre-exposure to LPS resulted in levels of NF-κB in nuclear extracts of C5a-stimulated PBMC that were markedly higher than in cells not pre-exposed to LPS, suggesting that TLR modulation of cell sensitivity to C5a might affect a wide range of

inflammatory mediators. Addition of the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) [Németh 2003] during the C5a stimulation phase abolished the enhancing effect of LPS on C5a-induced IL-8 release (Figure 24B). However, since NF-κB inhibition also abolished cytokine release induced by C5a alone, this strategy was not investigated further.
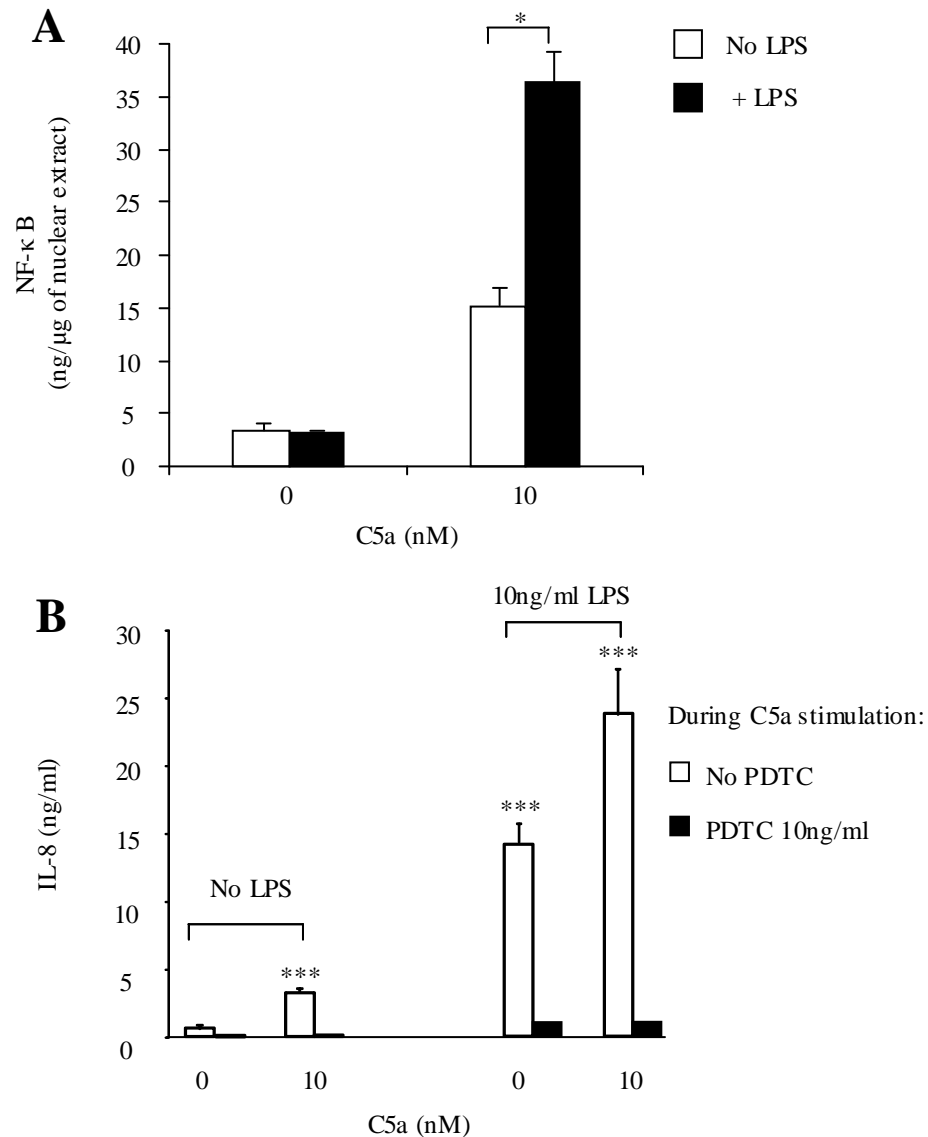
Figure 24. NF-κB activation and LPS-induced hypersensitivity to C5a. (A) Mean (±SD) NF-κB concentrations in nuclear extracts of PBMC (1.5 x 10^5/well) pre-exposed (14h) or not to LPS (10 ng/ml) before stimulation (14h) with 10 nM C5a. **(B)** Mean (± SD) IL-8 release from PBMC (1.5 x 10^5/well) upon stimulation with C5a in the presence and absence of the NF-κB inhibitor PDTC, after pre-exposure (14h) or not to LPS (10 ng/ml). Results are from one experiment, representative of three. (* $p<0.05$).

2.1.6 Modulation of blood cell sensitivity to C5a by TLR activation

In order to evaluate the *in vivo* relevance of the positive modulatory effect of TLR activation on cell sensitivity to C5a, a minimally perturbed experimental model was used in which anticoagulated human whole blood was pre-exposed to LPS or whole heat-killed *E. coli*. Following washing, the blood cells were re-suspended in heat-inactivated autologous plasma and stimulated with varying concentrations of C5a. Pre-exposure of whole blood to LPS or whole bacteria resulted in a substantially higher sensitivity of blood cells to C5a stimulation (Figure 25A), suggesting that TLR modulation of C5a sensitivity in peripheral blood immunocompetent cells might occur during infections *in vivo.*

The modulatory effect of TLR activation on blood cell sensitivity to C5a was investigated further in blood samples from a cohort of 15 healthy donors that were pre-exposed to a defined dose of LPS and stimulated with increasing concentrations of C5a (Figure 25B). Notably, the hypersensitivity to C5a of different donors varied widely, some donors exhibiting up to 60-fold increase in C5a-induced IL-8 release as a consequence of LPS pre-exposure, whereas others showed minimal or no augmentation. This indicated a marked intersubject variability in the capacity of TLR activation in different donors to amplify sensitivity to C5a. Furthermore, the shape of the C5a dose-response curve was variable, most donors showing a similar bell-shaped curve to that seen in PBMC (Figure 22B), with a peak hyperresponsiveness at relatively low stimulating concentrations of C5a and falling abruptly as the C5a concentration increased, while others – those showing a low capacity to augment C5a sensitivity in response to LPS priming – exhibited a sigmoid-like dose-response curve in which C5a hypersensitivity approached a plateau as the stimulating concentration of C5a increased (Figure 25B) .
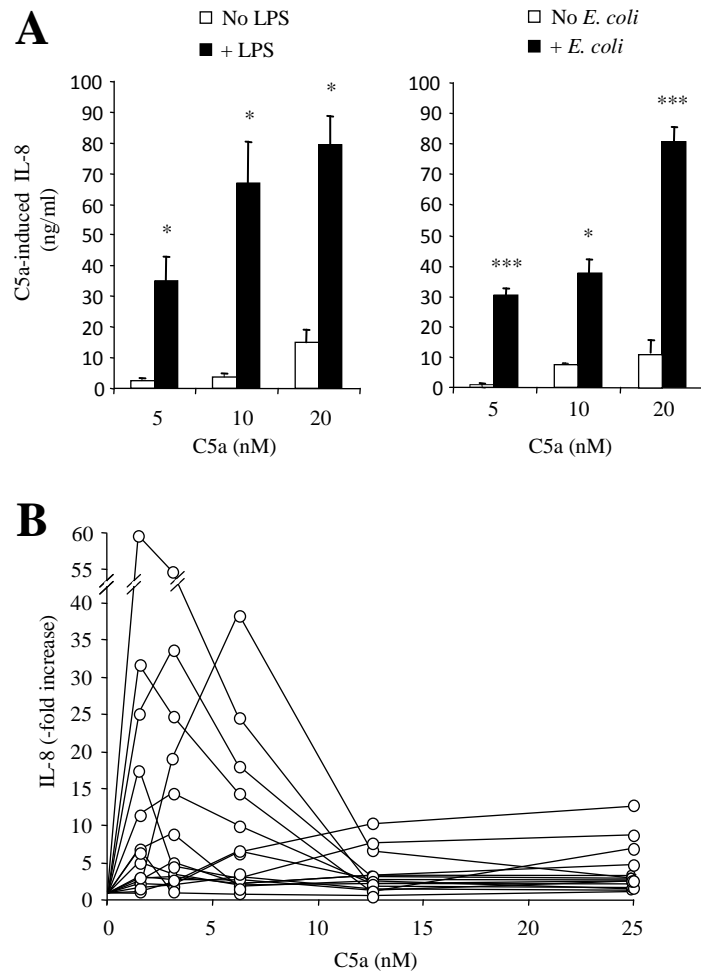
Figure 25. Sensitivity to C5a of human whole blood pre-exposed to LPS or *E. coli*. (A) Mean (±SD) C5a-induced levels of IL-8 in blood cell culture supernatants following pre-exposure (14h) of heparinised whole blood (100 μl/well) to LPS (500 pg/ml) or whole heat-killed *E. coli* (1×10^8 cfu/ml). **(B)** -fold increase in C5a-induced IL-8 secretion from heparinised whole blood from 15 healthy donors as a result of pre-exposure (14h) to LPS (500 pg/ml). Each line represents a single donor. Values in (A) are mean (±SD) of 3 independent experiments from a single donor representative of three, and in (B) are mean –fold increase of 3 experiments from each donor. (*$p<0.05$, ** $p< 0.01$, pre-exposed cells *vs.* not pre-exposed.)

2.1.7 Effect of TLR activation on blood cell sensitivity to C5a in TLR4 signalling-deficient mice

In order to confirm that microbial-induced hypersensitivity to C5a strictly depended upon TLR activation, a murine model of LPS-induced peritonitis was designed to mimic the *in vitro* model of cell priming and stimulation (illustrated in Figure 39A, and discussed in section 5.2.2 below). Wild-type (WT) mice (C3H/HeN) and mice deficient in TLR4 signal transduction (C3H/HeJ) were inoculated with LPS or mock-inoculated (PBS only) by i.p. injection. After 1 h, the animals were sacrificed, blood was drawn, and following washing, the blood cells were re-suspended in culture medium and stimulated *ex vivo* with increasing concentrations of recombinant mouse C5a or mock-stimulated (medium alone). Following stimulation, the blood cell sensitivity to C5a of WT and TLR4-signalling deficient mice was compared by testing the cell culture supernatants for pro-inflammatory chemokine levels (Figure 26).

The effect of pre-exposure to LPS on cell sensitivity to C5a observed *in vitro* in human PBMC and whole blood was reproduced in blood cells from WT mice. Indeed, blood cells from C3H/HeN animals pre-exposed to LPS *in vivo* responded to C5a with a greatly increased secretion of the prototypical neutrophil chemoattractant, keratinocyte-derived chemokine (KC, a murine functional counterpart of IL-8), and monocyte chemoattractant protein 1 (MCP1), compared with mock-injected animals. However, while blood cells from the TLR4 signalling-deficient mice (C3H/HeJ) retained the capacity to respond to C5a, they showed no capacity for augmentation of the chemokine response to C5a as a result of *in vivo* LPS pre-exposure.
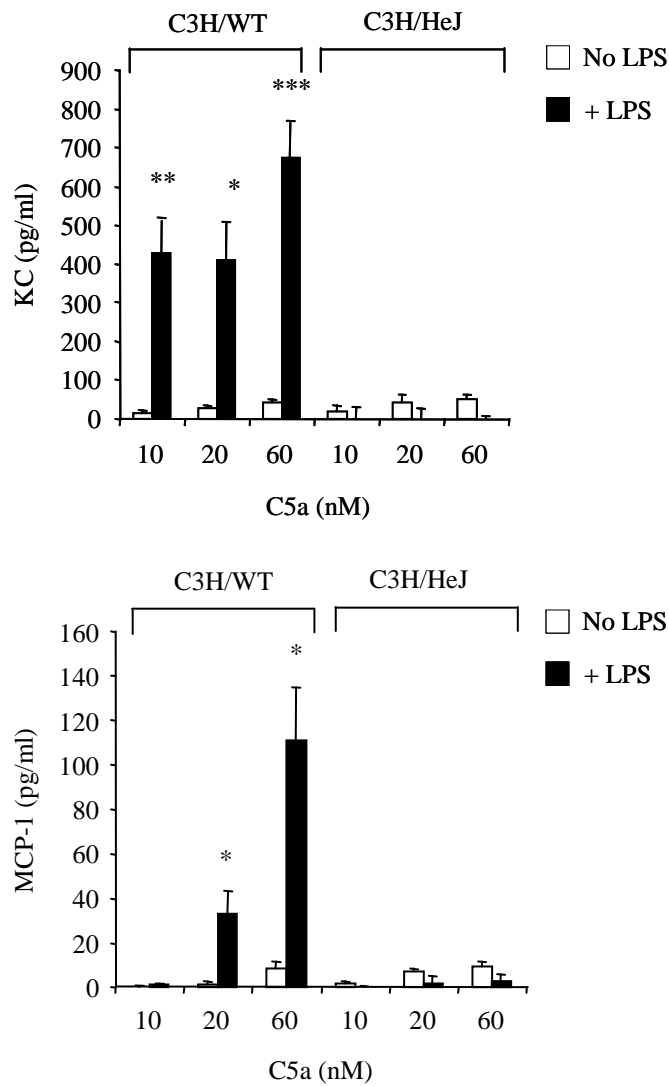
Figure 26. *Ex vivo* sensitivity to C5a of blood cells from TLR4 signalling-deficient and wild-type mice. C5a-induced levels of KC and MCP-1 in blood cell (100 µl/well) culture supernatants of C3H/HeN (WT) and C3H/HeJ (TLR4 signalling-deficient) mice stimulated *ex vivo* (14h) with the indicated concentrations of C5a following *in vivo* (i.p.) pre-exposure (1h) to LPS (50 µg/mouse) or PBS (no LPS). Chemokine concentrations shown are after background subtraction, as indicated in Figure 21. Values are expressed as the mean (±SEM) (*n*= 5 mice/condition). (*$p<0.05$, **$p<0.01$ *** $p< 0.005$, LPS-treated mice *vs.* untreated).

2.2 Study of the mechanism underlying TLR-induced cell hypersensitivity to C5a

The findings presented above indicated that prior TLR activation was capable of positively modulating inflammatory responses of isolated PBMC and whole blood to C5a in a manner similar to that previously reported for concurrent activation through both TLR and C5a receptors. This positive modulation, which appeared to be TLR-, MyD88- and NFκB-dependent, raised the question of its exact underlying mechanism. This was investigated next.

2.2.1 Duration of pre-exposure to a TLR ligand and cell hypersensitivity to C5a

In order to evaluate the impact that the duration of the pre-exposure phase had on TLR-induced cell hypersensitivity to C5a, PBMC were pre-exposed to LPS for different times between 30 min and 14h before washing and activation with C5a as described above. As shown in Figure 27, even a brief pre-exposure (30 min) to a low concentration of LPS (100 pg/ml) was sufficient to induce maximal hypersensitivity to C5a, suggesting that the mechanism underlying the TLR modulatory effect is relatively rapid.
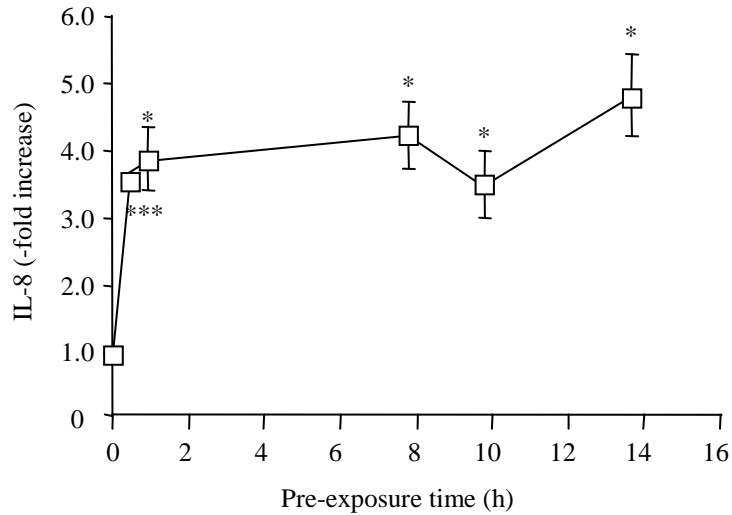
Figure 27. Effect of duration of cell pre-exposure to LPS on hypersensitivity to C5a. - Fold increase in C5a-induced levels of IL-8 released by PBMC (1.5 x 10^5/well) as a result of pre-exposure to LPS (100 pg/ml) for the indicated times (starting from 30 min), and subsequently activated (14h) with C5a (10 nM). Values shown are mean (±SD) of one experiment representative of 3. (*p<0.05, *** p< 0.005, LPS-treated cells *vs.* untreated.

2.2.2 TLR activation and cell-surface expression of C5aR

TLR-induced up-regulation of the cell-surface receptor for C5a (C5aR) might be a mechanism by which TLR activation resulted in increased sensitivity to C5a. In order to investigate this possibility, PBMC and whole blood were pre-exposed or not to LPS for 30 min before washing and stimulation with C5a for different intervals up to 12h. At each time point, C5aR expression on gated monocytes (PBMC) and neutrophils (whole blood) was estimated by flow cytometry (Figure 28A and B; representative examples of the fluorescence histograms are shown in the Appendix, Figure 46). C5aR expression on both monocytes and neutrophils was found not to be up-regulated after 30 min pre-exposure to LPS; in fact LPS pre-exposure resulted in >50% decrease in both monocyte and neutrophil C5aR expression, which was sustained over the following 12h in the absence of further stimulation with C5a. Stimulation of LPS-primed cells with C5a induced a further decrease in C5aR expression, followed by a partial recovery over time in monocytes but not neutrophils. This effect of C5a stimulation was also seen in

unprimed cells, and likely reflects ligand-dependent internalisation and recycling of C5aR back to the cell surface, as previously reported [Scola 2009]. Estimation of IL-8 levels in the culture supernatants confirmed TLR-induced hypersensitivity to C5a in PBMC from the same donors, subjected to the same experimental conditions (Figure 28A, inset).

mRNA extracted from samples of PBMC taken at the end of the experiment shown in Figure 28A was analysed by RT-qPCR for C5aR expression. Consistent with the LPS-induced down-modulation of C5aR cell-surface expression described above, LPS-primed cells were found to express lower levels of mRNA for C5aR than unprimed cells, irrespective of C5a stimulation (Figure 28C). Together, these findings indicated that TLR-induced hypersensitivity to C5a was not due to C5aR up-regulation, and indeed occurred in spite of a marked TLR-induced down-regulation of C5aR expression.
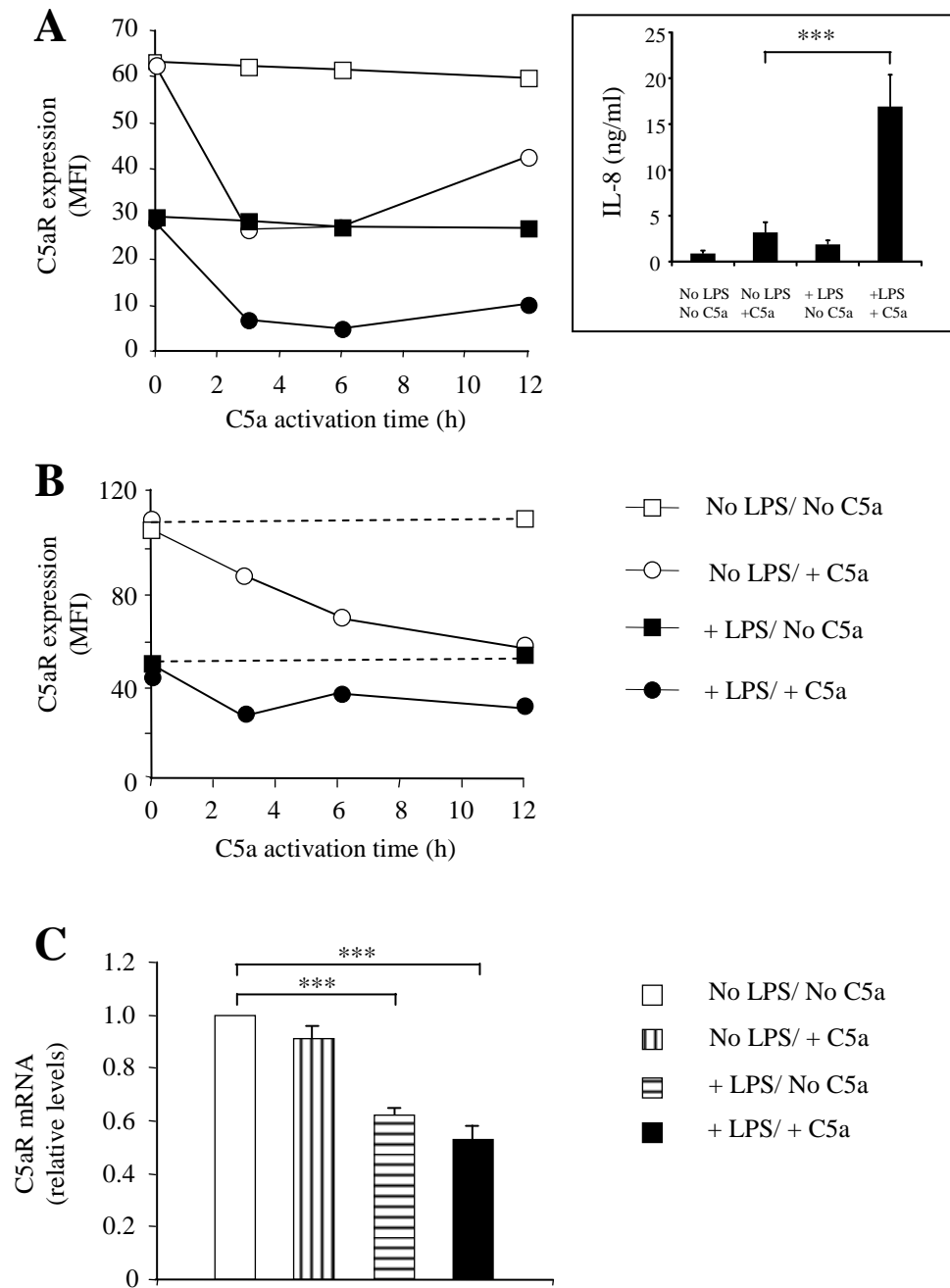
Figure 28. Effect of TLR activation and C5a stimulation on C5aR expression.
C5aR expression by flow cytometry on gated monocytes **(A)** and neutrophils **(B)** at different times following pre-exposure (30 min) of 1 x 10⁶ PBMC (monocyte C5aR) or 100 μl whole blood (neutrophil C5aR) to 100 pg/ml (PBMC) or 500 pg/ml (neutrophils) LPS. Inset in (A) shows the levels of IL-8 at the end of the culture in the cell culture supernatants of the PBMC used here for C5aR expression analysis. **(C)** C5aR mRNA levels estimated by RT-qPCR in RNA samples extracted from PBMC at the end of the experiment (12h in culture) shown in (A). Results are from one experiment (A inset and C, ±SD) representative of three. (*** $p < 0.005$. MFI, mean fluorescence intensity.)

2.2.3 TLR activation and C5a-induced Ca^{2+} mobilisation

C5aR G protein-dependent signal transduction is known to involve cytoplasmic Ca^{2+} mobilisation [Johswich 2006]. Therefore, in order to assess the possible involvement of Ca^{2+}-dependent signalling in the LPS-induced hypersensitivity to C5a, C5a-induced intracellular Ca^{2+} flux was estimated in PBMC pre-exposed or not to LPS for 3 min, 30 min and 14 h. As shown in Figure 29, pre-exposure of PBMC to LPS for any of the indicated periods of time did not induce any change in the kinetics (amplitude or duration) of C5a-induced Ca^{2+} mobilisation. This suggested that TLR-induced modulation of cell sensitivity to C5a either operates by affecting a G protein-dependent signal transduction pathway separate from Ca^{2+} signalling or a G protein-independent pathway used by C5aR.
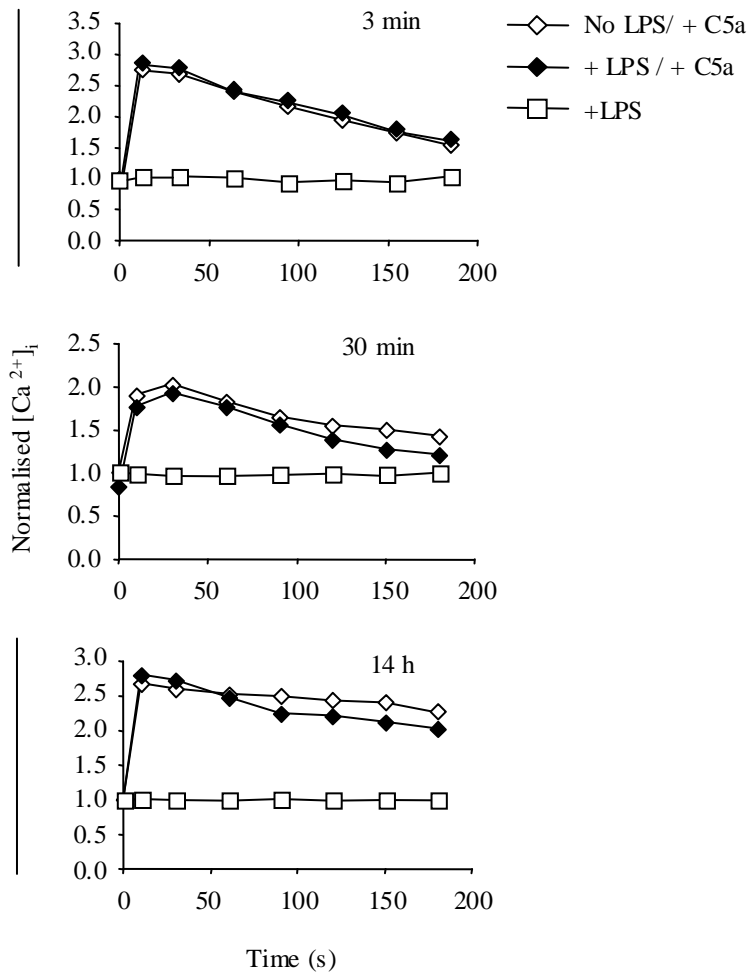
Figure 29. Effect of TLR activation on C5a-induced Ca^{2+} flux.
C5a (10 nM)-induced changes in monocyte cell fluorescence over 180 s
as a measure of intracellular Ca^{2+} mobilisation, detected by flow
cytometry following exposure of PBMC (1 x 10^6 per condition) to LPS
(100 pg/ml) or culture medium alone (No LPS) for the indicated times
and staining with the Ca^{2+}-chelating fluorescent dye, Fluo-3-AM.
Background fluorescence was determined before addition of C5a (time
0) in cells pre-exposed or not to LPS. Results are expressed as
normalised [Ca^{2+}]$_i$, the ratio between the mean fluorescence intensity at
time *t* after C5a addition and that observed at time 0. One experiment
representative of four is shown; representative examples of
fluorescence histograms used to generate the data are shown in Figure
47 (Appendix).

2.2.4 TLR activation and C5a-induced HMGB1 mobilisation

Seven transmembrane receptors, like those for C5a, also signal through a G-protein-independent pathway that involves the activity of β-arrestins, multifunctional adaptor proteins that mediate signalling and also control receptor desensitization and trafficking [Bamberg 2010 and Lefkowitz 2004]. Notably, C5aR signalling through the β-arrestin pathway was reported to be negatively modulated by the second C5a receptor, C5L2, a G protein-uncoupled receptor [Bamberg 2010]. Thus, in order to evaluate the possibility that a C5a-triggered G-protein-independent signalling event was the target of TLR modulation, it was tested whether TLR-induced hypersensitivity to C5a involved an effect on C5L2. As discussed in section 1.4 above, C5L2 (but not C5aR) has been reported to mediate the cytoplasmic mobilisation and secretion of the nuclear protein HMGB1 in response to stimulation with C5a via a G protein-independent signal transduction pathway [Rittirsch 2008]. Therefore, the effect of TLR activation on C5L2 activity was investigated by estimation of C5a-induced HMGB1 mobilisation. To this end, PBMC were pre-exposed (or not) to LPS and activated with C5a as described above. Cytoplasmic cell extracts were then tested by Western blotting for HMGB1 levels. Figure 30A shows that cell stimulation by C5a resulted in cytoplasmic mobilisation of HMGB1. However, when cells were pre-exposed to LPS before C5a stimulation, C5a-induced HMGB1 mobilisation was reduced, suggesting that TLR activation negatively affected C5L2 activity. This finding was in contrast to the positive effect of TLR activation on C5a-induced IL-8 secretion previously demonstrated (section 2.1.) and confirmed here, as the C5a-induced IL-8 levels in the PBMC culture supernatants from this experiment were substantially higher in LPS-primed compared with unprimed cells (Figure 30B).
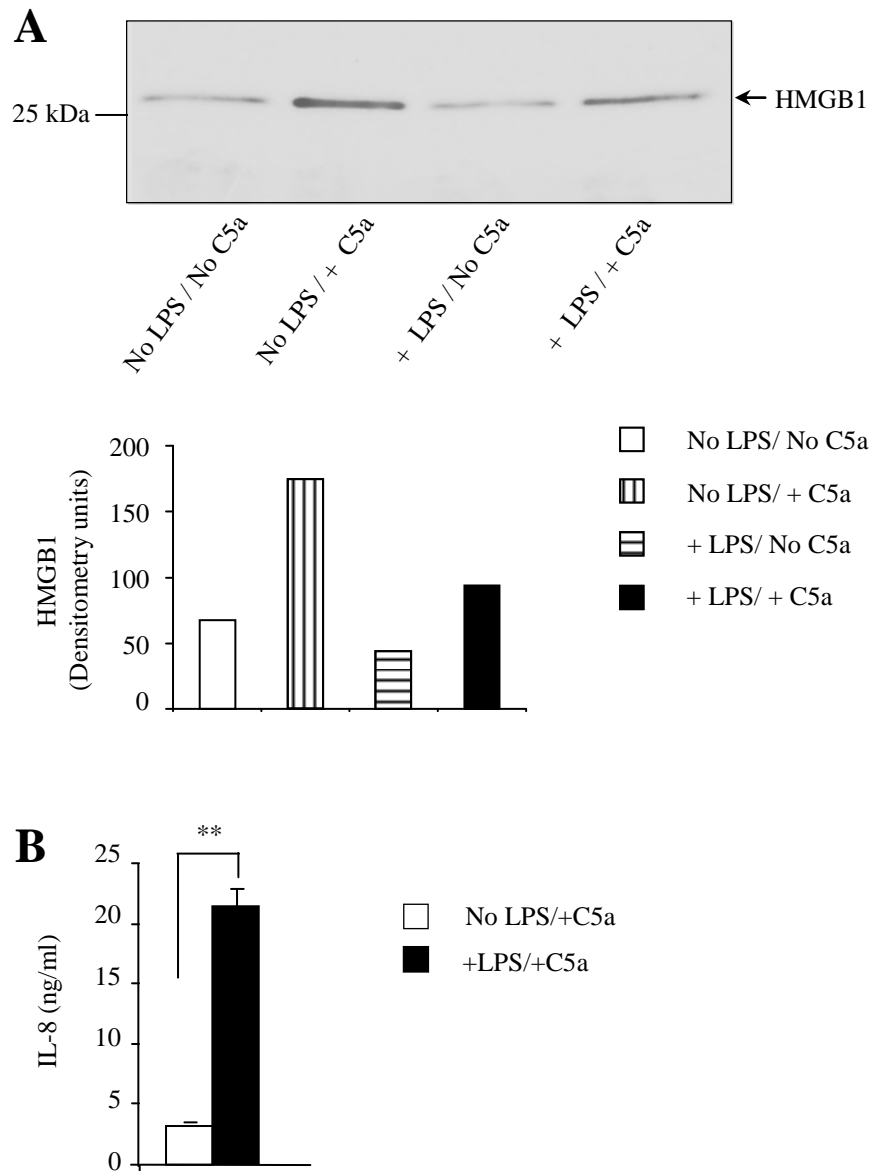
Figure 30. Effect of TLR activation on C5a-induced HMGB1 mobilisation.
(A) Western blot analysis and densitometric scanning of HMGB1 levels in cytoplasmic cell extracts of PBMC (0.5×10^6 per condition) pre-exposed (14h) or not to LPS (100 pg/ml) and subsequently stimulated (or not) with C5a (10 nM). **(B)** Mean (±SD) IL-8 levels in cell culture supernatants of the experiment shown in (A). Results are of one experiment representative of five. (**$p<0.01$).

Consistent findings were observed when WT and TLR4 signalling-deficient mice were challenged with LPS *in vivo.* Their blood cells were subsequently stimulated with C5a *ex vivo* and the culture supernatants analysed by Western blot for HMGB1 (Figure 31A). Blood cells from WT mice responded to C5a stimulation with release of HMGB1 into the culture supernatant (Figure 31A, WT, No LPS/+C5a). WT mice first inoculated with LPS before *ex vivo* cell stimulation with C5a showed greatly reduced levels of HMGB1 released (Fig 31A, WT, +LPS/+C5a). Notably, this LPS-induced inhibition of C5a-induced HMGB1 release was not observed in the TLR4 signalling-deficient mice (Figure 31A, C3H/HeJ mice, +LPS/+C5a compared with No LPS/+C5a). The negative effect on (WT) mouse blood cell HMGB1 release, like that observed in human PBMC, contrasted with the positive (inverse) effect on chemokine release by these cells (Figure 31B), and further supported the concept that TLR activation negatively affects C5L2 activity. Given that C5L2 has been reported to act as a negative regulator of C5aR, these findings raised the possibility that TLR-induced inhibition of C5L2 might be implicated in the enhancement of C5a-induced cytokine release.
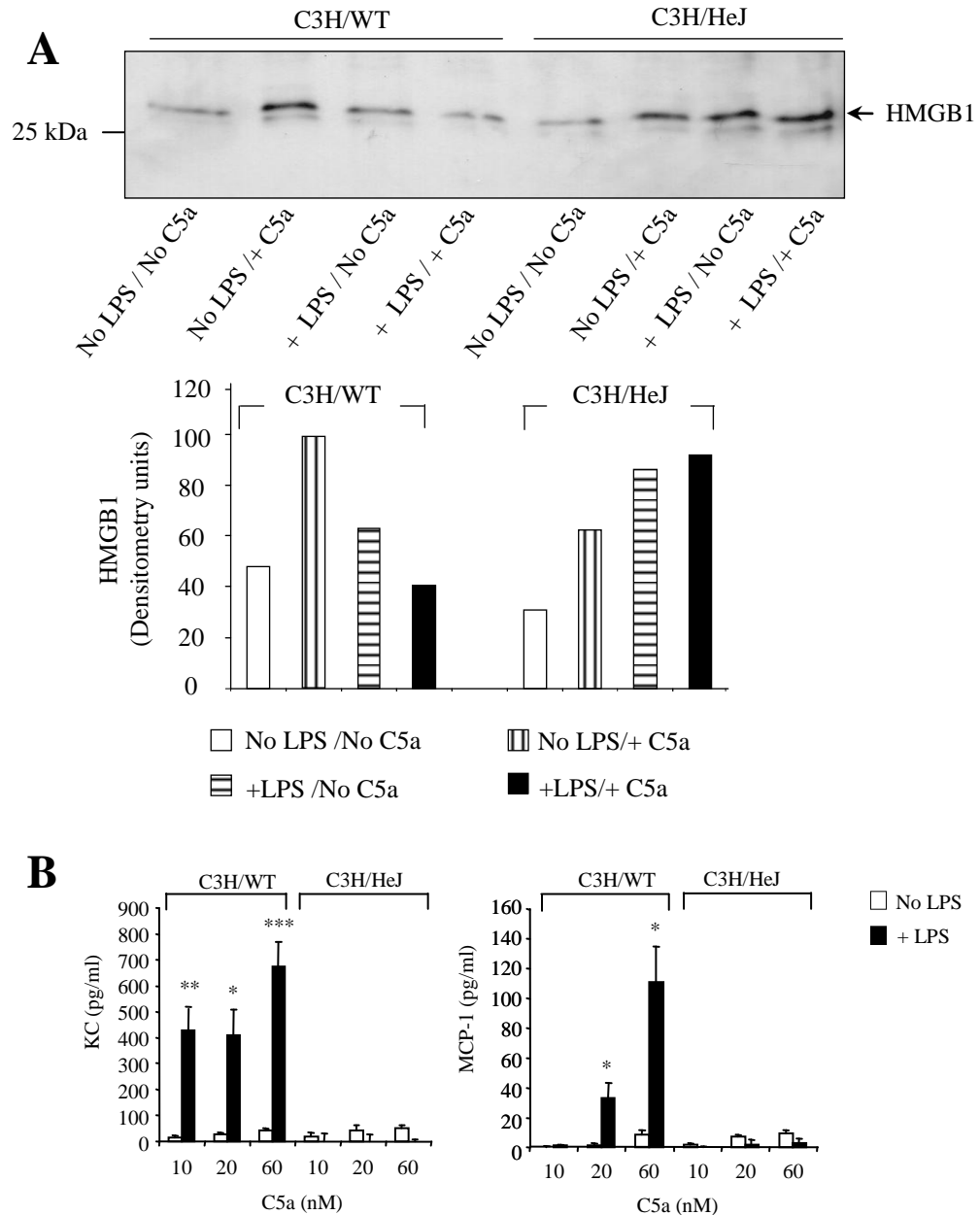
Figure 31. Effect of TLR activation *in vivo* on C5a-induced release of HMGB1 by mouse blood cells *ex vivo*. (A) Western blot and densitometric analysis of C5a (60 nM)-induced HMGB1 levels in culture supernatants of whole blood (100 μl/well) from wild-type (C3H/HeN) and TLR4 signalling-deficient (C3H/HeJ) mice (n= 5/condition) challenged (1h) i.p. with LPS (50 μg/mouse) or PBS (No LPS). Pooled supernatant from 5 animals was used in each experimental condition. **(B)** Mean (±SEM) chemokine levels in supernatants corresponding to the experiment described in (A), reproduced from Figure 26. (*$p<0.05$, **$p<0.01$, ***$p<0.005$ LPS-treated mice *vs.* untreated. Chemokine concentrations shown are after background subtraction).

2.2.5 TLR and C5a effect on C5L2 activity and expression

If the second C5a receptor, C5L2, were the target of TLR modulation, it might be expected that inhibition or blockade of C5L2 would reproduce the contrasting effects of TLR activation on both HMGB1 (negative) and IL-8 (positive) described above. To test this possibility, PBMC were stimulated with C5a in the presence and absence of a C5L2-specific mAb previously shown to be able to prevent C5L2 ligation by C5a [Bamberg 2010]. The cytoplasmic cell extracts and culture supernatants were tested for HMGB1 and IL-8 levels, respectively. As shown in Figure 32, PBMC stimulated with C5a alone showed a strong cytoplasmic HMGB1 band (Figure 32A) and a very modest increase in IL-8 release (Figure 32B). However, PBMC stimulated with C5a in the presence of the C5L2 blocking Ab showed a marked reduction in C5a-induced HMGB1 levels (Figure 32A), but a substantial increase in IL-8 release (Figure 32B). Since the presence of even trace amounts of a heat-stable TLR ligand such as LPS can induce a similar effect on IL-8 to that shown here by using the anti-C5L2 mAb, in control experiments the antibody was denatured by boiling for 30 min before addition to the culture. The denatured antibody lost the capacity both to inhibit C5a-induced HMGB1 release and to enhance IL-8 secretion (Figure 32, A and B), confirming its genuine effect. Thus, the effect of C5L2 blockade was similar to that of cell pre-exposure to a TLR ligand, suggesting that the positive effect of TLR activation on C5a-induced responses may involve inhibition of C5L2.

A

B
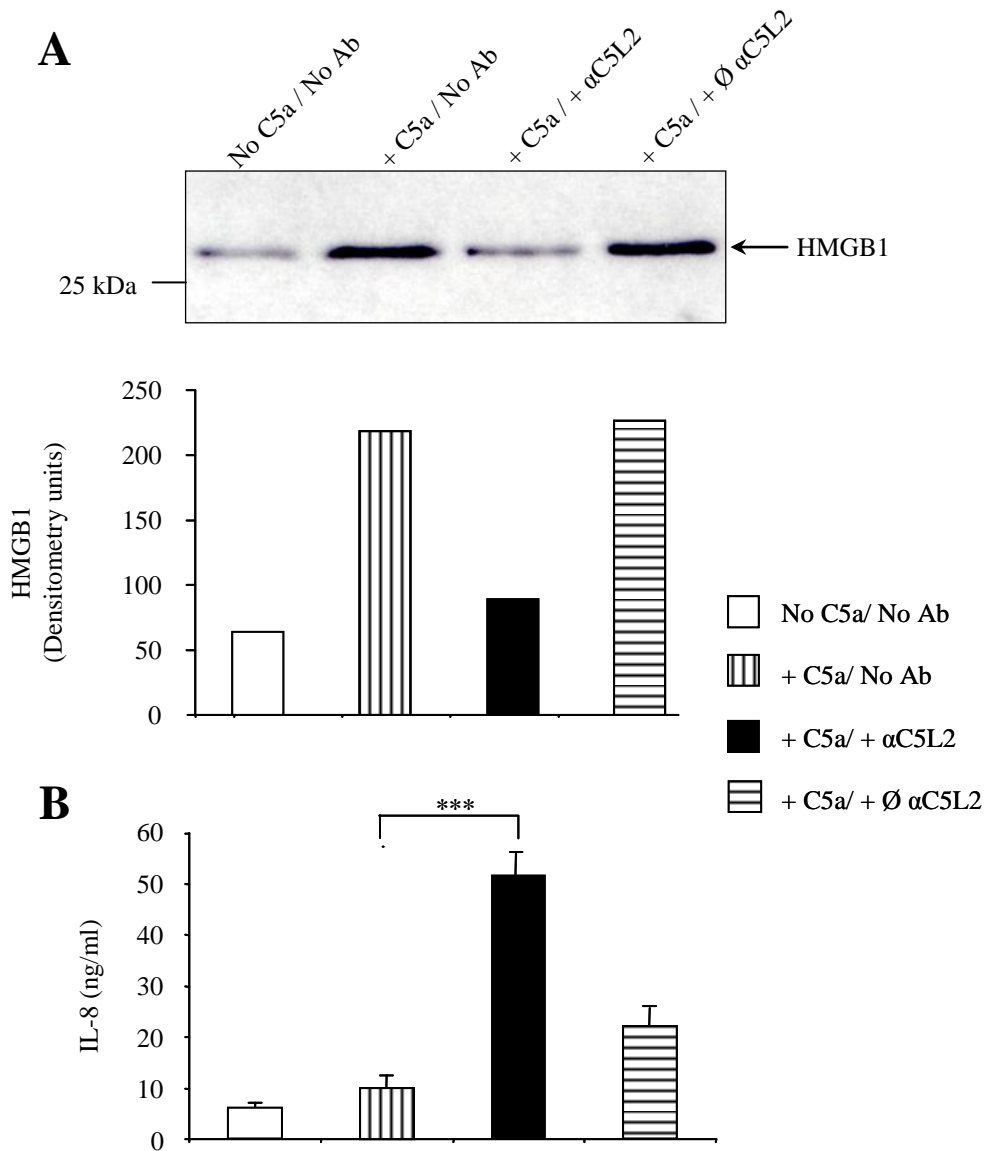
Figure 32. Effect of mAb blockade of C5L2 on HMGB1 mobilisation and IL-8 release. (A) Western blot and densitometric analysis of HMGB1 levels in cytoplasmic extracts of PBMC (0.5 x 10^6/condition) exposed (14h) to C5a (2.5 nM) in the presence and absence of an anti-C5L2 blocking mAb (5 µg/ml) or the same mAb denatured by boiling (Ø). **(B)** Mean (±SD) IL-8 levels in culture supernatants of the experiment shown in (A). Results are from one experiment representative of four (A) and three (B). (*** p <0.005)

In order to further test whether TLR-induced hyperresponsiveness to C5a involved inhibition of C5L2, the effect of cell pre-exposure to LPS on C5L2 protein expression was next examined (Figure 33). PBMC were stimulated with C5a after pre-exposure to LPS. At the end of the culture period, the cells were lysed and the expression levels of C5L2 in the cell lysates were estimated by Western blot. Pre-exposure to LPS did not result in any change in the expression of C5L2 at the end of the culture period in cells that were not subsequently stimulated with C5a (Figure 33, left panel, +LPS/No C5a). However, whilst stimulation of cells with C5a without prior exposure to LPS resulted in a slight increase in C5L2 expression (Figure 33, left panel, No LPS/+C5a), cells stimulated with C5a after LPS priming (Figure 33, left panel, +LPS/+C5a) showed a strong LPS dose-dependent reduction in C5L2 expression. This finding indicated that TLR activation negatively modulates C5L2 activity, at least in part, by reducing C5L2 expression. It is noteworthy, however, that this inhibitory effect of LPS occurred only upon subsequent C5a stimulation.
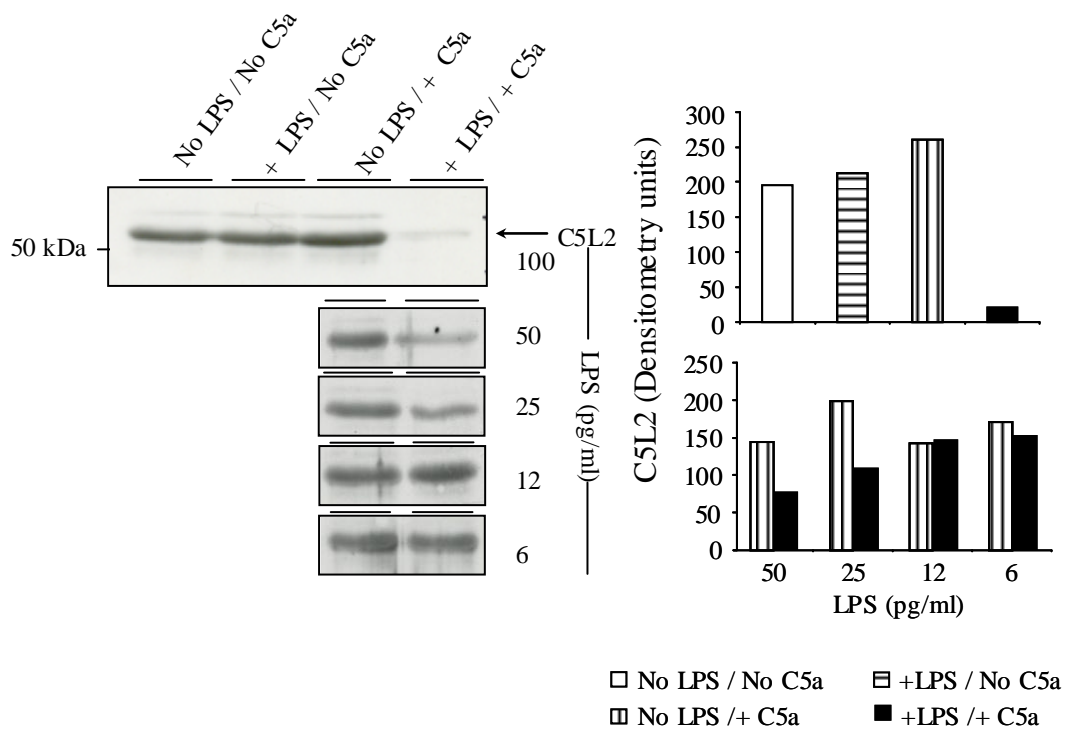
Figure 33. Effect of TLR activation on C5L2 expression. Western blot and densitometric analyses of C5L2 levels in cell lysates of PBMC (0.5×10^6) pre-exposed (14h) or not to the indicated concentrations of LPS and subsequently activated (14h) with C5a (10 nM). Data shown are from one experiment representative of four.

2.2.6 TLR activation and sensitivity to C5a in C5L2-deficient mice

In order to test further the involvement of C5L2 in the positive effect of TLR activation on cell sensitivity to C5a, a comparative analysis of the effect of *in vivo* pre-exposure to LPS on C5a-induced cytokine secretion by blood cells from WT and C5L2-deficient mice was conducted following challenge (i.p.) of mice for 1h with LPS (Figure 34). The C5a-induced release of KC was extremely low in both WT and C5L2-deficient mice that had not been challenged with LPS. Notably, pre-exposure to LPS resulted in a significant increase in cell sensitivity to C5a in WT but not in C5L2-deficient mice at all C5a concentrations tested. This finding further confirmed

the involvement of C5L2 in the modulatory effect of TLRs on C5a-mediated pro-inflammatory responses.
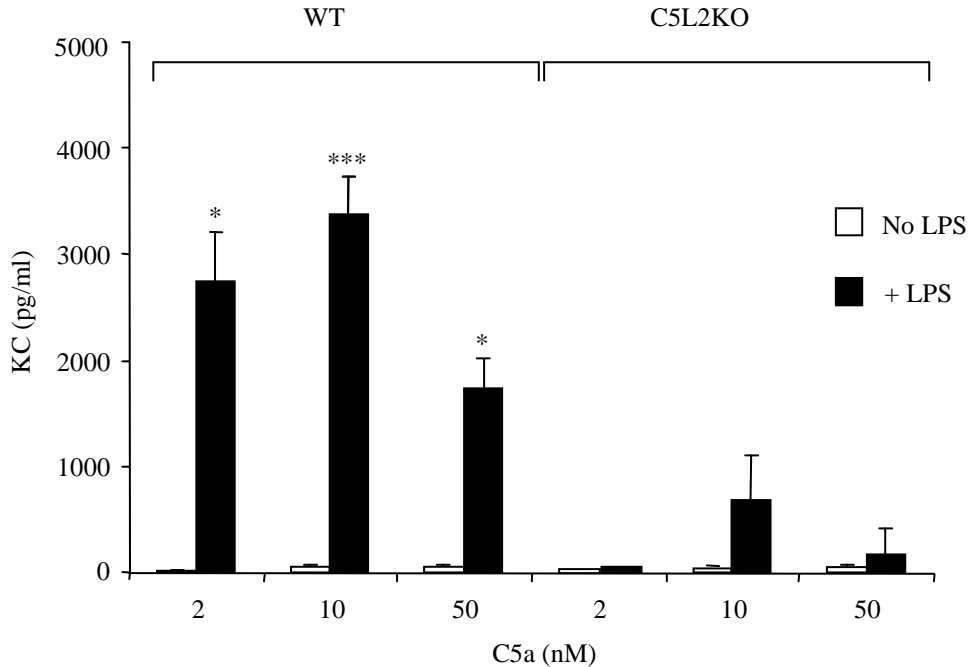
Figure 34. *Ex vivo* sensitivity to C5a of C5L2-deficient and wild-type mice. Mean (±SEM) C5a-induced levels of KC in blood cell (100 µl/well) culture supernatants following *in vivo* i.p. challenge (1h) of C5L2-deficient and WT mice (n=5 mice/condition) with LPS (50 µg/mouse) or PBS (no LPS). Chemokine concentrations shown are after background subtraction, as indicated in Figure 21. Values are expressed as the mean (±SEM) (*n*= 5 mice/condition. (*$p<0.05$, *** $p< 0.005$, LPS-treated *vs*. untreated.)

2.3 The effect of LPS and C5a on the transcription of genes involved in MAPK and NF-κB activation

It appeared that TLR activation induced cell hypersensitivity to C5a by inhibiting the C5a-induced expression of C5L2, which itself can exert a negative regulatory effect on C5aR-mediated pro-inflammatory responses. However, it was not clear by what mechanism TLR activation was able to affect C5L2 expression. Addressing this question has the added difficulty that it is also not clear how C5aR activity positively affects C5L2 expression, nor how C5L2 is

able to modulate C5aR-mediated signals. In an attempt to further investigate the mechanism underlying the effect of TLR activation on C5L2, a gene expression profiling strategy was used to identify candidate proteins for further investigation. Since TLR- and C5a receptor (C5aR and C5L2)-induced cytokine expression is known to involve activation of both MAPK and NF-κB signalling pathways (discussed in section 1.5), the mRNA expression levels of a panel of 176 genes known to be involved in MAPK and NF-κB signalling (see detailed list in Tables 10A and B, Appendix) were screened by RT-qPCR.

The relative expression levels of mRNAs in PBMC were quantified in each of the three experimental conditions used in this study to evaluate the effect of TLRs on C5aR-mediated responses (cells not pre-exposed to LPS and subsequently stimulated with C5a, cells pre-exposed to LPS and not stimulated with C5a, and cells pre-exposed to LPS and stimulated with C5a) by comparison with control cells (not pre-exposed and not stimulated with C5a). Genes that showed a less than 2-fold negative or positive change relative to control cells (*i.e.* relative expression from 0.51 to 1.99) were considered to be unaffected. Genes whose transcription was found affected by any of the three experimental conditions are listed in Tables 5A − G below. Positive changes (relative expression ≥ 2.00) in gene transcription are shown in green and negative changes (relative expression ≤ 0.50) in red.

Table 5A. Genes encoding pattern recognition receptors*

Gene Symbol**	Description	No LPS +C5a	+LPS No C5a	+LPS +C5a
NOD1	Nucleotide-binding oligomerization domain containing 1	0.53	**0.32**	**0.11**
TLR1	Toll-like receptor 1	1.06	**0.45**	0.52
TLR2	Toll-like receptor 2	**0.41**	**3.49**	0.69
TLR4	Toll-like receptor 4	0.75	**0.18**	**0.24**

*Expression levels in each experimental condition relative to the control condition No LPS/No C5a are shown. Red font indicates a greater than two-fold reduction, and green font a greater than two-fold increase in mRNA expression relative to unstimulated cells.

**HUGO Database Gene Nomenclature.

Table 5B. Genes encoding TLR signal transduction intermediates*

Gene Symbol**	Description	No LPS +C5a	+LPS No C5a	+LPS +C5a
IRAK1	Interleukin-1 receptor-associated kinase 1	**15.17**	**12.50**	1.67
IRAK2	Interleukin-1 receptor-associated kinase 2	**7.64**	1.07	**5.47**
TBK1	TANK-binding kinase 1	**2.58**	1.02	**2.06**
TICAM1	Toll-like receptor adaptor molecule 1 (TRIF)	**8.22**	0.77	**5.86**
TICAM2	Toll-like receptor adaptor molecule 2 (TRAM)	1.42	**0.17**	**0.19**
TRAF3	TNF receptor-associated factor 3	0.93	0.53	**0.28**
TRAF6	TNF receptor-associated factor 6	0.51	**0.34**	**0.06**

*Expression levels in each experimental condition relative to the control condition No LPS/No C5a are shown. Red font indicates a greater than two-fold reduction, and green font a greater than two-fold increase in mRNA expression relative to unstimulated cells.

**HUGO Database Gene Nomenclature.

Table 5C. Genes encoding MAPK signal transduction intermediates*

Gene Symbol**	Description	No LPS +C5a	+LPS No C5a	+LPS +C5a
ARRB1	Beta-arrestin 1	12.84	0.44	0.71
MAP3K1	Mitogen-activated protein kinase kinase kinase 1, MEKK1	0.39	0.17	0.44
MAPK10	Mitogen-activated protein kinase 10 (JNK3)	0.48	0.30	1.34
MAPK11	Mitogen-activated protein kinase 11 (p38β)	0.93	2.01	1.97
MAPK12	Mitogen-activated protein kinase 12 (p38γ)	0.55	0.47	0.42
MOS	V-mos Moloney murine sarcoma viral oncogene homolog	1.10	1.15	2.14
PAK1	P21 protein (Cdc42/Rac)-activated kinase 1	0.69	0.59	0.45
RAF1	V-raf-1 murine leukemia viral oncogene homolog 1	4.38	2.07	3.04
RHOA	Ras homolog gene family, member A	1.18	0.50	1.16

*Expression levels in each experimental condition relative to the control condition No LPS/No C5a are shown. Red font indicates a greater than two-fold reduction, and green font a greater than two-fold increase in mRNA expression relative to unstimulated cells.

**HUGO Database Gene Nomenclature.

Table 5D. Genes encoding NF-κB pathway signal transduction intermediates*

Gene Symbol**	Description	No LPS +C5a	+LPS No C5a	+LPS +C5a
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (IκB kinase β)	0.67	0.56	**0.30**
IKBKE	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon (IκB kinase ε)	1.72	**0.21**	0.94
IKBKG	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma (IκB kinase γ)	0.69	0.59	**0.25**
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (NF-κB p52/p100)	1.22	**0.47**	**0.45**
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα)	1.09	0.65	**0.45**
NFKBIB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta (IκBβ)	0.57	0.55	**0.12**
NFKBIE	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon (IκBε)	**2.19**	0.71	1.18
REL	V-rel reticuloendotheliosis viral oncogene homolog (C-Rel)	**0.30**	0.98	0.84
RELA	V-rel reticuloendotheliosis viral oncogene homolog A (Rel-A, NF-κB p65)	1.02	**0.39**	**0.28**

*Expression levels in each experimental condition relative to the control condition No LPS/No C5a are shown. Red font indicates a greater than two-fold reduction, and green font a greater than two-fold increase in mRNA expression relative to unstimulated cells.

**HUGO Database Gene Nomenclature.

Table 5E. Genes encoding transcription factors*

Gene Symbol**	Description	No LPS +C5a	+LPS No C5a	+LPS +C5a
ATF1	Activating transcription factor 1	0.79	1.18	**0.37**
STAT1	Signal transducer and activator of transcription 1, 91kDa	1.62	0.81	**0.47**
ELK1	ELK1, member of ETS oncogene family	**0.25**	1.02	0.73
IRF1	Interferon regulatory factor 1	0.56	0.59	**0.20**
JUN	Jun proto-oncogene, c-Jun	**3.29**	1.75	0.93
FOS	FBJ murine osteosarcoma viral oncogene homolog, c-Fos	0.79	**0.40**	**0.36**
MEF2C	Myocyte enhancer factor 2C	1.14	1.21	**3.69**

*Expression levels in each experimental condition relative to the control condition No LPS/No C5a are shown. Red font indicates a greater than two-fold reduction, and green font a greater than two-fold increase in mRNA expression relative to unstimulated cells.

**HUGO Database Gene Nomenclature.

Table 5F. Genes encoding cytokines*

Gene Symbol**	Description	No LPS +C5a	+LPS No C5a	+LPS +C5a
CCL2	Chemokine (C-C motif) ligand 2	6.46	5.52	14.74
CSF1	Colony stimulating factor 1 (macrophage)	0.26	0.73	0.28
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	2.44	5.17	4.27
CSF3	Colony stimulating factor 3 (granulocyte)	1.28	0.32	3.58
IFNG	Interferon, gamma	0.18	0.18	0.53
IL10	Interleukin 10	2.77	2.09	4.27
IL1A	Interleukin 1, alpha	1.07	35.91	118.20
IL1B	Interleukin 1, beta	3.22	61.61	64.30
IL8	Interleukin 8	2.61	7.41	8.54
LTA	Lymphotoxin alpha (TNF superfamily, member 1)	0.81	0.58	3.67
TNF	Tumor necrosis factor alpha	1.69	0.61	0.37
TNFSF14	Tumor necrosis factor (ligand) superfamily, member 14	0.36	1.68	1.86

*Expression levels in each experimental condition relative to the control condition No LPS/No C5a are shown. Red font indicates a greater than two-fold reduction, and green font a greater than two-fold increase in mRNA expression relative to unstimulated cells.

**HUGO Database Gene Nomenclature.

Table 5G. Genes encoding proteins related to control of cell cycle and apoptosis*

Gene Symbol**	Description	No LPS +C5a	+LPS No C5a	+LPS +C5a
CCNA1	Cyclin A1	1.01	1.89	**3.03**
RIPK1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	**0.23**	**0.39**	0.72
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	0.75	**0.41**	**0.50**
BIRC2	Baculoviral IAP repeat containing 2	1.24	**0.15**	**0.43**
BIRC3	Baculoviral IAP repeat containing 3	1.12	0.81	0.51
CARD11	Caspase recruitment domain family, member 11	**0.34**	**0.34**	**0.23**
CFLAR	CASP8 and FADD-like apoptosis regulator	0.72	0.87	**0.33**
CASP8	Caspase 8, apoptosis-related cysteine peptidase	0.92	0.75	**0.35**
BCL3	B-Cell Lymphoma Protein 3	0.93	**0.33**	**0.25**
BCL2L1	B-Cell Lymphoma 2-like 1	0.98	0.62	**0.24**
BCL2A1	BCL2-related protein A1	**4.02**	**3.08**	**4.79**
LTBR	Lymphotoxin beta receptor (TNFR superfamily, member 3)	0.76	**0.48**	**0.13**
CD40	CD40 molecule, TNF receptor superfamily member 5	1.67	**0.11**	**0.11**
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	0.65	0.56	**0.14**
TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a	1.35	**0.10**	**0.11**
TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	0.76	0.78	**0.16**
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	1.90	1.10	0.77
FADD	Fas (TNFRSF6)-associated via death domain	1.07	**7.80**	1.23
FASLG	Fas ligand (TNF (ligand) superfamily, member 6)	0.53	0.81	**0.10**

*Expression levels in each experimental condition relative to the control condition No LPS/No C5a are shown. Red font indicates a greater than two-fold reduction, and green font a greater than two-fold increase in mRNA expression relative to unstimulated cells.

**HUGO Database Gene Nomenclature.

Through the use of a gene array screening strategy it was possible to identify many gene transcripts that were affected by the different experimental conditions used. Many of them code for key signal transduction intermediates involved in inflammatory responses and thus would be worthy candidates for further study. However, given the impossibility of investigating all of them, β-arrestin 1 (Table 5C) was initially singled out for further examination for the following reasons: 1) β-arrestins are known to be involved in G-protein-independent GPCR signal transduction, and C5aR, like other GPCRs, is capable of signalling through a G-protein-independent pathway that involves β-arrestins [Bamberg 2010, Lefkowitz 2004, Rajagopal 2010]; 2) this pathway was reported to be negatively modulated by C5L2 [Bamberg 2010]; 3) the finding that TLRs exert positive modulation of cell sensitivity to C5a without affecting Ca^{2+} mobilisation (section 2.2.3) suggested that a G protein-independent signalling pathway was involved in the TLR effect; 4) β-arrestin 1 has been shown to couple to C5L2 in a C5a-dependent manner with a resulting inhibition of ERK-1 phosphorylation [Bamberg 2010]; 5) ERK1/2 phosphorylation is known to be critical in the transduction of C5aR-mediated pro-inflammatory signals (see section 1.3.4.1 *d.* above) Thus, it appears that β-arrestin 1 is involved not only in G-protein-independent C5aR signalling – which may result in up-modulation of C5L2 – but also in the C5L2-dependent constitutive negative regulation of C5aR-mediated pro-inflammatory signals [Bamberg 2010]. These findings raised the possibility that TLR-mediated inhibition of C5L2 expression activity may involve a negative effect on β-arrestin 1.

2.4 A Role for β-arrestin 1 in the TLR-C5a receptor crosstalk

In order to investigate a possible role for β-arrestin 1 in the TLR-C5L2-C5aR interaction, PBMC were primed or not with LPS and subsequently activated (or not) with C5a. After 4h of C5a activation, the cells were lysed and β-arrestin 1 mRNA expression estimated by RT-qPCR analysis using home-made primers, in order to confirm the findings by the gene arrays method. C5a enhanced, and cell pre-exposure to LPS inhibited β-arrestin 1 mRNA expression (Figure 35A); the effect of LPS was predominant, resulting in complete abolition of the C5a effect. These results were qualitatively similar to those previously observed (Table 5C).

Changes in β-arrestin 1 protein expression correlated with those in mRNA (Figure 35B). Western blot analysis of PBMC lysates following 14h stimulation with C5a showed an increase in β-arrestin 1, whereas cell pre-exposure to LPS before C5a stimulation reduced β-arrestin 1 to levels below those observed in unstimulated cells. As was observed at mRNA level, the effect of LPS was predominant, abolishing the C5a-induced enhancement of β-arrestin 1 protein expression.
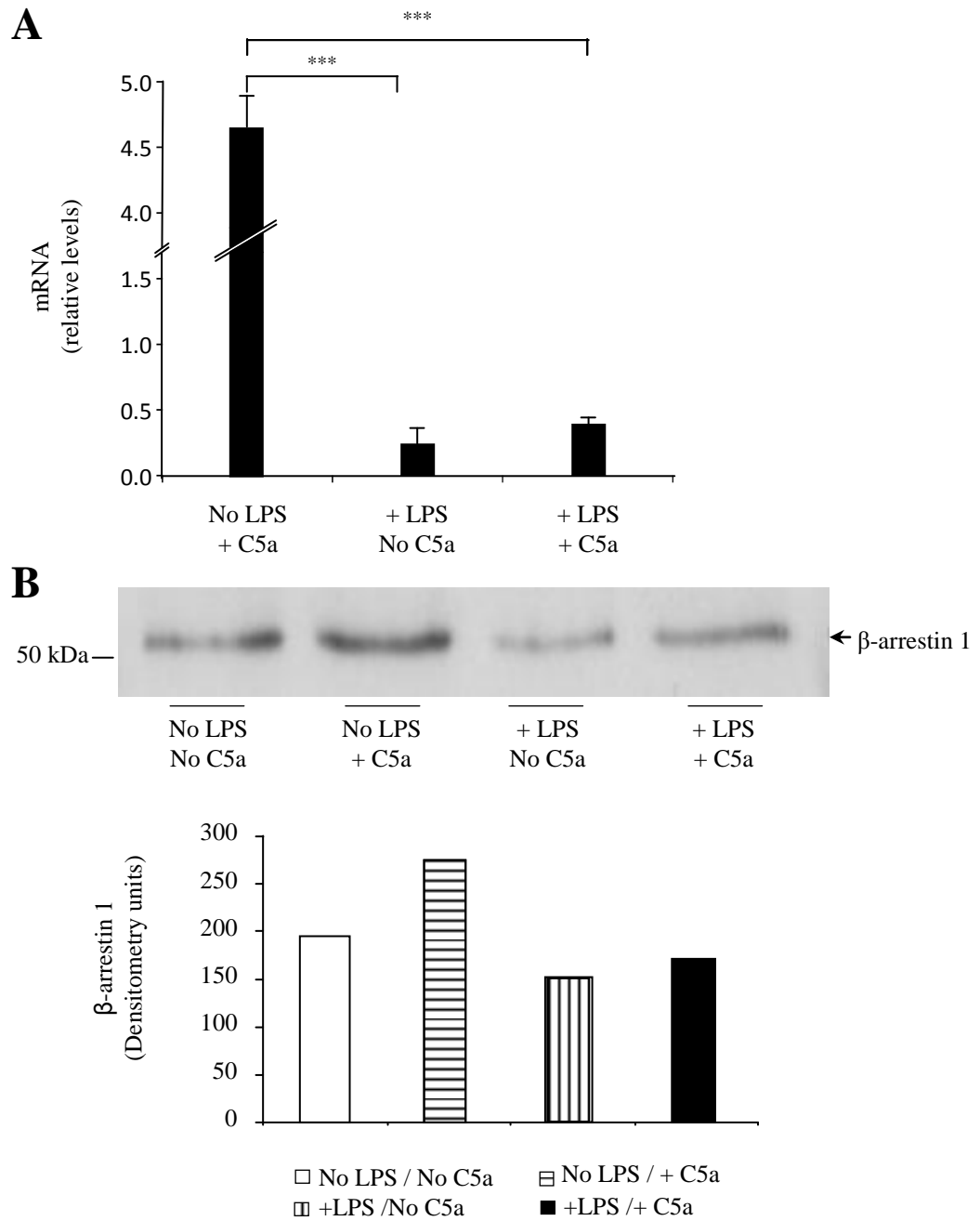
Figure 35. Effect of TLR activation on β-arrestin 1 expression in PBMC. (A) Mean (±SD) –fold change in β-arrestin 1 mRNA expression in PBMC pre-exposed (14h) or not to LPS (500 pg/ml) before being stimulated (4h) or not with C5a (1nM). **(B)** Western blot and densitometric analysis of β arrestin-1 expression in PBMC (0.5 x 10^6) pre-exposed or not (14h) to LPS (500 pg/ml) and subsequently activated (14h) with C5a (1 nM). Data shown are from one experiment representative of three.

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2.5 The physiological relevance of TLR-induced hypersensitivity to C5a.

The TLR-based cell priming model for testing cell sensitivity to C5a may not accurately reflect the sequence of events during infections *in vivo.* Rather, it was developed in order to address a specific question, namely that of whether an effect of TLR activation on C5aR-mediated inflammatory responses might contribute to the synergistic enhancement of cytokine release observed when cells were concurrently stimulated with both ligands, in which context the pathophysiological relevance of the model was not a primary concern. However, the fact that priming cells with a TLR ligand followed by C5a stimulation reproduced the effects of concurrently stimulating cells with both ligands tended to validate the experimental model used here.

In an attempt to investigate further the possible *in vivo* relevance of TLR-induced hypersensitivity to C5a in human subjects undergoing clinically significant infections, the *in vivo/ex vivo* model of TLR-mediated cell priming applied to mice in this study was extended to a cohort of patients with severe sepsis or septic shock (*n*=19) who presented to the intensive care unit (ICU) within 36 hours of the presumed onset of the infective insult (Table 8, section 5.2 below). It was anticipated that circulating leucocytes from these patients would have been exposed to a range of TLR ligands as a consequence of their infections, and thus would have been 'primed' for increased responsiveness to C5a. Anticoagulated blood was taken from patients on admission, and blood cells were washed and re-suspended in complete medium. The blood cells were then stimulated overnight with a range of concentrations of C5a before testing for IL-8 in the culture supernatants. Since it was not possible to test the *ex vivo* 'unprimed' cytokine response to C5a (the response of cells to C5a without preceding TLR

activation) in a pre-morbid sample from the same patients, the IL-8 response to C5a in blood taken from a cohort of healthy control donors (*n*=15) was used for comparison.

As can be seen in Figure 36A, and contrary to expectations, blood cells from the sepsis donors showed a uniformly reduced sensitivity to C5a compared with healthy controls. Furthermore, and also contrary to expectations, the basal IL-8 secretion by blood cells from sepsis patients not stimulated with C5a was no higher than that of blood from healthy control donors. Since it was inconceivable that the blood cells of patients with documented severe sepsis had not been exposed to TLR ligands during the course of their infection, the possibility was considered that the ability of the patients' leucocytes to mount a cytokine response was impaired. Indeed, sepsis-induced immunosuppression is a well-recognised phenomenon, and is characterised by lymphocyte apoptosis, neutrophil anergy, and by failure of monocytes to mount an appropriate cytokine response to inflammatory stimuli [Lendemans 2003, Opal 2010]. Therefore, in order to address this issue, the sensitivity of whole blood from the 19 sepsis patients shown in Figure 36A and healthy controls (n=15) to a range of TLR ligands and whole heat-killed bacteria was also tested. As shown in Figure 36B, the IL-8 response to TLR ligands and bacteria was also reduced in blood from sepsis patients compared with that of healthy control donors, indicating a global impairment of cytokine responses, which is characteristic of sepsis-induced immunosuppression.
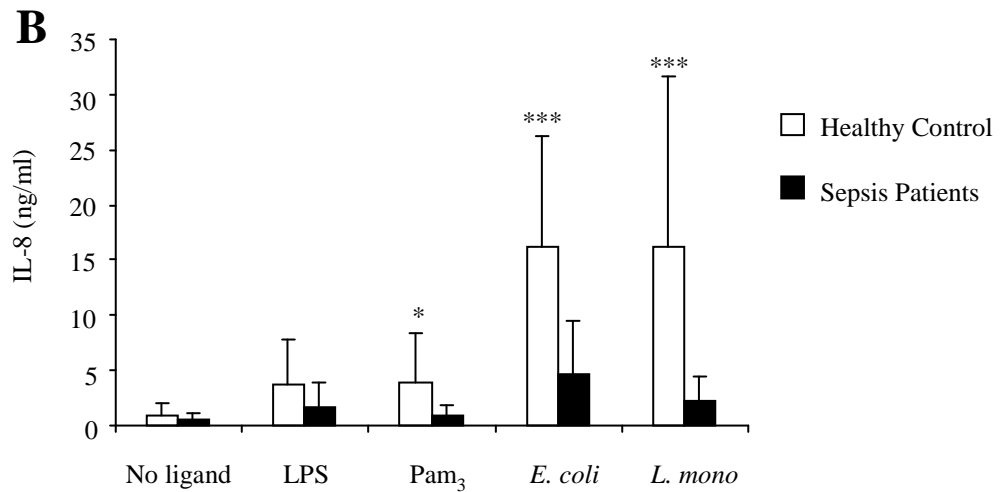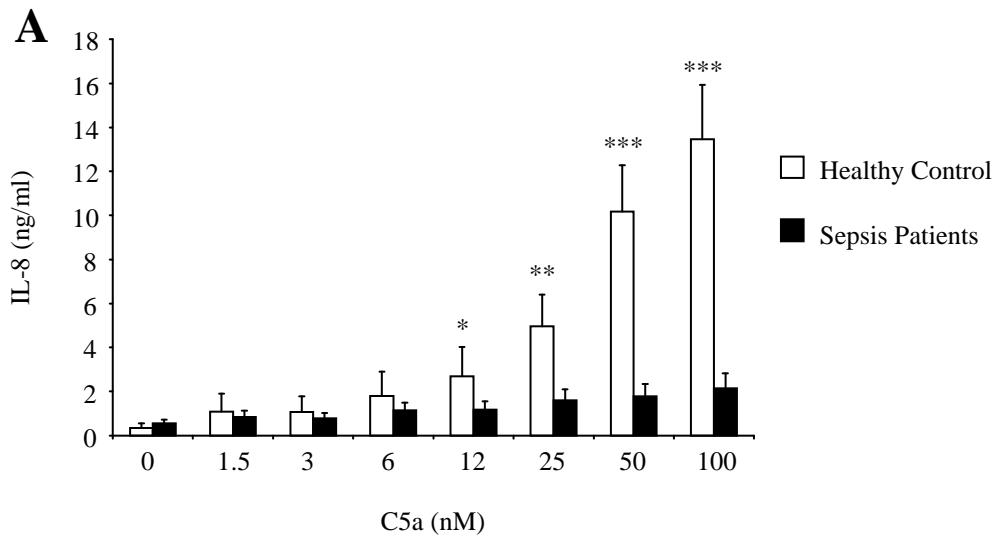
Figure 36. *Ex vivo* **sensitivity to C5a and TLR ligands of whole blood from sepsis patients. (A)** Mean (±SD) C5a-induced levels of IL-8 in blood cell (100 µl/well) culture supernatants from sepsis patients (n=19) and healthy control subjects (n=15) following *ex vivo* stimulation with C5a at the indicated concentrations. **(B)** Mean (±SD) IL-8 levels in culture supernatants in response to *ex vivo* stimulation of blood cells from the sepsis patients and controls described in (A) with TLR ligands LPS (1 ng/ml), Pam$_3$Cys (10 ng/ml) and whole heat-killed *E. coli* (2 x 10^4 cfu/ml) or *L. monocytogenes* (4 x 10^5 cfu/ml). (*p<0.05, *p<0.01 *** p< 0.005, healthy controls *vs.* sepsis patients.)

2.6 Intersubject variation in TLR-induced hypersensitivity to C5a and C5a receptor expression.

It is possible that the varying capacity of individuals to augment their cytokine response to C5a as a result of cell pre-exposure to TLR ligands (Figures 22B and 25B) is related to the activity of C5L2. The intersubject variability in LPS-induced hypersensitivity shown here might reflect variations in the constitutive expression level of C5L2 and the extent to which LPS pre-exposure (indirectly) induces its down-regulation. A preliminary flow cytometric analysis of monocyte C5a receptor expression in two of the human donors shown in Figure 25B is consistent with this hypothesis (Figure 37). Blood cells from Donor A showed a comparatively strong response to LPS pre-exposure, showing up to ~10-fold enhancement in C5a-induced IL-8 secretion, whereas Donor B showed a much more limited response to LPS pre-exposure, suggesting a high C5L2 activity (Figure 37A). Both C5aR and C5L2 were expressed at higher levels in Donor B (the 'TLR low responder') than in Donor A (the 'high responder'; Figure 37B and C). However, while the low responder exhibited higher absolute levels of C5L2 than the high responder, the ratio of C5L2 to C5aR was lower (0.41) in the low responder than in the high responder (0.63).
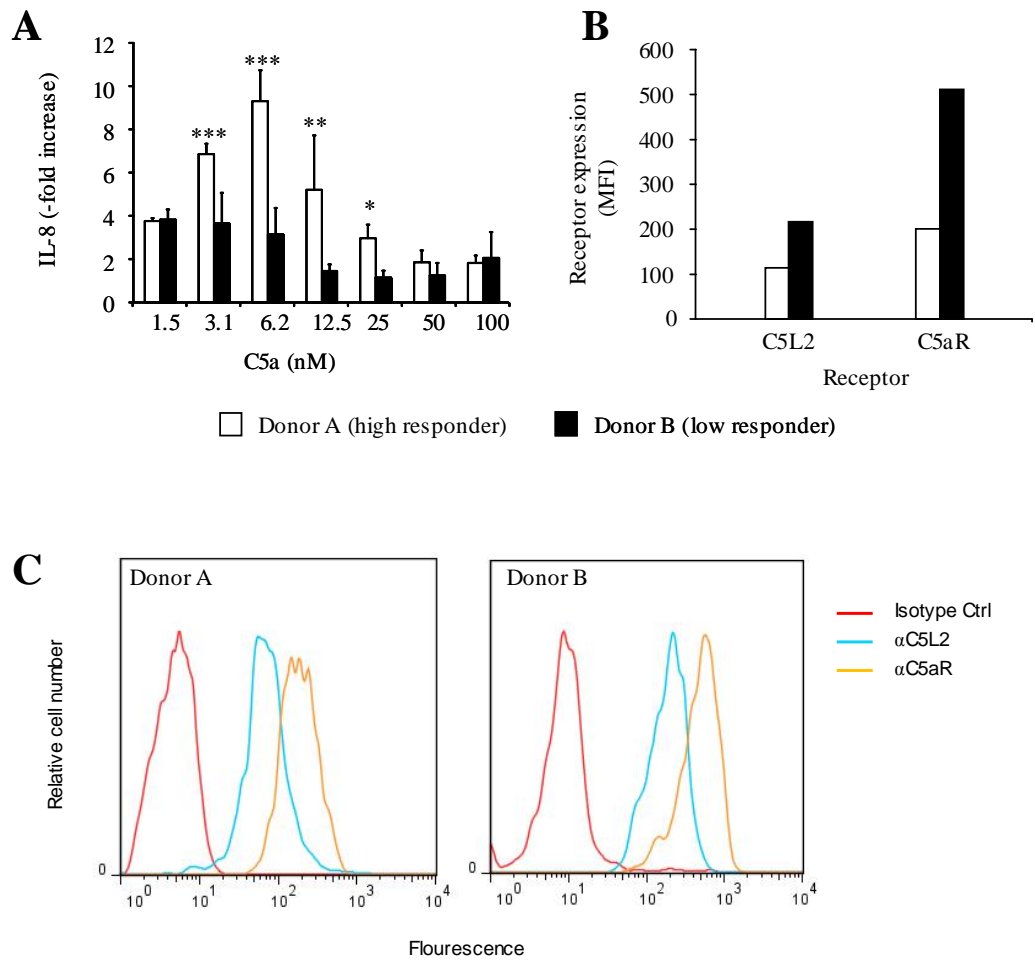
Figure 37. Intersubject variations in TLR-induced hypersensitivity to C5a and monocyte C5aR and C5L2 expression and activity. (A) Mean (±SD) -fold increase in C5a-induced IL-8 levels in cell culture supernatants as a consequence of LPS (500 pg/ml) pre-exposure (14h) of whole blood (100 µl/well) from individuals with high (Donor A) and low (Donor B) capacity for LPS-induced hypersensitivity to the indicated concentrations of C5a. **(B) and (C)** C5aR and C5L2 expression estimated by flow cytometry on gated monocytes in unstimulated blood from the two donors shown in (A)**.** Mean (±SD) –fold increases in IL-8 from 3 independent experiments are shown in (A), and (B) shows receptor expression (MFI) of C5aR and C5L2 from one experiment representative of three. (*$p<0.05$, **$p<0.01$, **$p<0.005$ Donor A *vs.* Donor B. MFI, mean fluorescence intensity).

Chapter 3: DISCUSSION

3.1 A genuine bi-directional signalling crosstalk

A mutually regulated and concerted activity of TLRs and the complement system would strengthen the efficiency of innate host defence against microbial pathogens. In support of this possibility, synergistic effects between TLRs and C5aR, and regulation of TLR-mediated pro-inflammatory and immunoregulatory responses by complement receptors have been reported, suggesting crosstalk between TLRs and complement receptors. However, the possibility of a modulatory effect exerted by TLRs on complement receptor-mediated pro-inflammatory responses, which would indicate a genuine bi-directional crosstalk, has not been directly investigated. The present study aimed at determining whether such a crosstalk exists by examining the capacity of TLR activation to influence inflammatory responses to the complement anaphylatoxin C5a, and demonstrated that activation of TLRs is capable of positively modulating inflammatory responses of mononuclear cells and whole blood to C5a, by a mechanism involving TLR-induced inhibition of the expression and activity of the second C5a receptor, C5L2 (Figure 38).
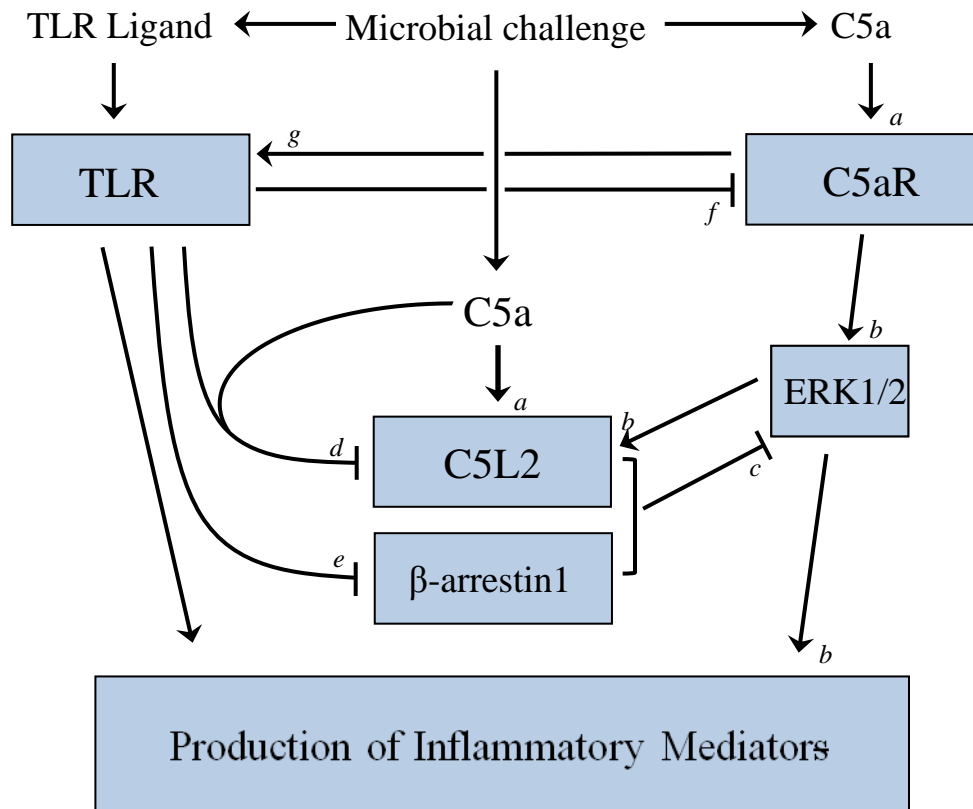
Figure 38. **Proposed** **model** **of** **TLR-C5L2-C5aR** **crosstalk.**
It appears that a complex interaction between C5aR, C5L2 and TLR activation exists. Microbial-induced complement activation results in generation of C5a, which binds to both C5aR and C5L2 (*a*). C5aR ligation activates signal transduction cascades including ERK1/2, resulting in synthesis and release of inflammatory mediators and positive modulation of C5L2 expression and activity (*b*). Binding of C5a to C5L2 recruits β-arrestin 1 to C5L2, resulting in inhibition of C5aR-mediated ERK1/2 signalling, attenuating the direct C5a-induced release of inflammatory mediators (*c*). Concurrent (or preceding) TLR activation results in indirect (C5a-dependent) down-regulation of C5L2 expression (*d*) and direct (C5a-independent) down-regulation of β-arrestin 1 expression (*e*). This attenuates the C5L2/β-arrestin-1 inhibitory signal on C5aR, resulting in synergistic enhancement of C5a-mediated release of inflammatory mediators, in spite of a direct TLR-induced down-regulation of cell surface C5aR expression (*f*). The TLR-mediated positive effects on pro-inflammatory production may be amplified by a similarly positive effect of C5aR activation on TLR signalling (*g*), as previously reported (discussed in section 1.5).

The previously-reported capacity of TLRs and C5aR, when simultaneously activated, to synergistically enhance pro-inflammatory cytokine release by immunocompetent cells was reproduced here in an *in vitro* PBMC stimulation model system (Figure 19). Importantly, an enhancing effect on pro-inflammatory cytokine release (and gene transcription) was similarly observed when TLR activation was induced by cell pre-exposure *in vitro* (Figures 20-25) or *in vivo* (Figure 26) to a number of TLR ligands before C5a stimulation. Of note, the inverse sequence of cell stimulation (first via C5aR and then via TLR, Figure 20B) was unable to induce a greater-than-additive effect on IL-8 cytokine release. This indicates that, in the model system described in the present study at least, the TLR/C5aR synergy in cytokine release is mainly due to a modulatory effect of TLR activation on C5a receptor sensitivity rather than *vice versa*. This is in contrast with previous reports claiming a modulatory effect of C5aR activation on TLR-induced inflammatory responses (discussed in section 1.5 above), but is not inconsistent with published data. Since TLR ligands (especially LPS, the ligand most commonly used in studies of TLR ligand-C5a synergy) are typically more potent stimuli of cytokine release than C5a, it has generally been assumed in previous reports that the synergistic enhancement of the cytokine response observed as a result of co-stimulation with both ligands was a consequence of a modulating effect of C5aR on a dominant TLR-mediated signal (see Section 1.5 above and related references). However, it has not been customary to test this hypothesis rigorously, for example by using a pre-exposure/exposure model system such as that described in the present study. The only published study which employed such a model [Laudes 2002] found a similar cell response to that reported here: vascular endothelial cells pre-exposed to LPS before activation with C5a responded with a synergistic enhancement of chemokine release, whereas the effect was less than additive if C5a was used as the priming agent. This previous observation however was not investigated further.

A potential confounding factor in the experimental model described in the present study was the possibility that the TLR-mediated enhancing effect on cell sensitivity to C5a observed here resulted from residual TLR ligands present during the C5a stimulation phase and carried over from the TLR activation phase. This is unlikely for the following reasons: 1) the levels of IL-8 at the end of the culture in the supernatants of cells pre-exposed to TLR ligands and not activated with C5a were extremely low (Figures 20A and 21A, see Figure legend); 2) polymyxin B added to the culture medium following cell pre-exposure to LPS and washing did not affect the levels of IL-8 released by the cells at the end of the cultures (Figure 20C), thus indicating the absence of residual LPS during the C5a activation phase. Of note, a 20- to 100-fold higher priming concentration of LPS than that typically used for the *in vitro* pre-exposure assays was deliberately used for this control experiment (10 ng/ml compared with 100-500 pg/ml) to maximise the possibility of residual traces of LPS being present after washing; 3) a TLR modulatory effect on cell sensitivity to C5a of similar intensity was observed over a wide range of LPS priming concentrations (0.1-1000 ng/ml, Figure 22A), which was inconsistent with the presence of residual LPS during C5a activation; 4) cells pre-exposed to LPS and then cultured in the absence of C5a did not show activation of NF-κB – a key regulator of cytokine/chemokine gene transcription that is activated by LPS – at the end of the culture (Figure 24), suggesting that there was no ongoing LPS stimulation of the primed cells during the C5a activation phase.

3.2 A potent but self-limiting mechanism for amplifying host defence

The high degree of leucocyte sensitivity to LPS pre-exposure is striking. Pre-exposure to concentrations of LPS as low as 10 pg/ml was able to double their sensitivity to C5a (Figure 22A). The median plasma levels of LPS reported during sepsis in humans are ~ 500 pg/ml [Opal

1999], a level much higher than that necessary to induce hypersensitivity of PBMC to C5a in this model system. Thus, TLR modulation of cell sensitivity to C5a appears to be an extremely sensitive mechanism that might operate during the course of mild as well as severe infections. It is plausible that the effects of TLR activation in enhancing sensitivity to C5a might contribute substantially to the excessive and dysregulated release of inflammatory cytokines that is the critical feature of the pathogenesis of inflammatory tissue injury and organ failure in septic shock. The TLR-mediated priming effect demonstrated in mononuclear cells was reproduced in the more physiological whole blood model (Figure 25) and in the *in vivo* (pre-exposure)/*ex vivo* (stimulation) mouse model systems (Figure 26), adding further support to the physiological relevance of microbial modulation of cell responses to C5a and confirming the crucial role that TLR activation plays in this phenomenon.

The capacity of LPS to enhance C5a-induced cytokine release was typically greatest at relatively low stimulating concentrations of C5a (Figures 22B and 25B) such as might occur in infected tissues at an early stage of infections. However, the synergistic enhancement of cytokine release tended to be self-limiting at higher concentrations of C5a – such as those reported systemically in severe sepsis and septic shock [Ward 2010a and 2010b] – in the donors that showed the greatest capacity for synergistic enhancement of cytokine responses (Figure 25B). It is possible that at higher C5a concentrations, such as those that might be generated *in vivo* during an acute infection, a more pronounced activation and ligand-induced down-regulation of C5aR may lead to C5L2 becoming proportionally more engaged. This may result in a stronger negative modulatory effect on C5aR responses that would counteract and limit the TLR enhancing effect. Thus, an individual's capacity to clear infection efficiently and successfully resolve inflammation might be determined, at least in part, by the extent of the TLR's enhancing effect relative to C5L2's capacity to counteract this positive effect.

3.3 A MyD88-dependent pathway

It appears that the capacity of inflammatory stimuli to induce hypersensitivity to C5a may be dependent on TIR domain-mediated, MyD88-dependent, signal transduction. Cell activation via TLRs and the IL-1β receptor, which was able to modulate C5a sensitivity (Figure 21B), requires TIR domain activation, whereas the other pro-inflammatory stimuli tested do not. Indeed, activation of a diverse array of pro-inflammatory pathways, the Jak/Stat (IL-6), FADD/TRAF2/RIP (TNF-α) and PKC/Ca^{2+} (PMA/ionomycin) pathways – all of which are able to induce inflammatory cytokine secretion in PBMC by a TIR domain-independent pathway – had no effect on subsequent sensitivity to C5a. The contrasting inhibitory effect of TLR3 activation on pro-inflammatory cytokine release is intriguing in light of the fact that, whereas the other TLRs and IL1βR all signal through the MyD88 signal adaptor, TLR3 does not. The precise nature of the different functional effect of TLR3 on C5aR sensitivity remains a subject of future investigation, as it may provide further insight into the mechanism underlying the effect of TLRs on C5a receptor-mediated responses.

3.4 Intersubject variability in the capacity of TLR activation to induce hypersensitivity to C5a

The degree of intersubject variation in the capacity of whole blood cells to respond to LPS priming by up-regulating their responsiveness to C5a is also striking (Figure 25B). Whilst many donors exhibited a marked capacity for LPS-induced hypersensitivity to C5a, others showed little or no LPS-induced augmentation in C5a sensitivity. The shape of the C5a dose-response curve was also subject to variation. Donors that exhibited a high degree of LPS-induced hypersensitivity to C5a tended to show maximal enhancement at low stimulating concentrations of C5a, followed by a decrease at higher concentrations. Other donors, who

showed overall lesser capacity for LPS-induced hypersensitivity to C5a, tended to exhibit a sigmoid-type response curve that reached a plateau at higher stimulating concentrations. These variations are intriguing; if reproduced in the context of *in vivo* infections, they might reflect a predisposition in different individuals towards a particular kind of chemokine response to a given infectious challenge. The subjects that showed greater LPS-induced hypersensitivity to relatively low C5a concentrations might represent a population capable of mounting a greatly enhanced chemokine response to low stimulating concentrations of TLR ligands released by pathogens, such as might occur in the early stages of infection when microbial numbers are relatively low. This might be beneficial to the host by amplifying the pro-inflammatory cytokine response to minor infections, preventing the pathogen from gaining a foothold. However, when faced with massive complement activation, such as might occur in disseminated infections or when the bacterial inoculum is massive (for example in gross faecal contamination of the peritoneum after a bowel perforation), attenuation of the inflammatory response might be protective against the cytokine-induced systemic inflammatory tissue injury that characterises the syndrome of septic shock.

The preliminary data shown in Figure 37 raise the possibility that the capacity of a particular individual to respond to TLR activation by increased sensitivity to C5a may depend upon the ratio of monocyte C5L2 to C5aR expression. It is possible that in the 'low responder' (who exhibited a lower C5L2:C5aR ratio), a proportionally weaker inhibitory signal from C5L2 (relative to the strength of the C5aR-mediated signal) was responsible for attenuating the C5aR-mediated pro-inflammatory responses in the absence of preceding TLR activation. This might result in TLR activation having less capacity to enhance C5a sensitivity by inhibiting C5L2 expression (i.e. there was less inhibition to remove). These findings, however, need to be corroborated in a larger study.

3.5 TLR-induced downregulation of C5L2 expression and activity

The study of the mechanism underlying the positive modulatory effect of TLR activation on cell sensitivity to C5a identified the second C5a receptor, C5L2, as a target for regulation by TLRs that appears to be critically involved in the TLRs' modulatory effects. Thus, TLR activation appears to increase cell sensitivity to C5a, at least in part, by reducing the inhibitory effect exerted by C5L2 on C5aR activity. As suggested by some studies [Gavrilyuk 2005, Gao 2005, and Gerard 2005] and confirmed by this work, the negative modulation of C5aR by C5L2 appears to be profound. This conclusion is supported by the finding reported here that TLR activation is capable of increasing C5aR sensitivity in spite of inducing a substantial down-regulation of C5aR cell-surface expression in both monocytes and neutrophils. The observed concomitant down-regulation of cell-surface C5aR might thus be a compensatory mechanism to prevent excessive release of inflammatory mediators in cells exposed to microbial components.

The relationship between TLR activation and C5L2 down-regulation appears to be complex, since pre-exposure of cells to LPS without subsequent C5a activation induced no change in cytoplasmic or secreted HMGB1 (Figures 30A and 31A, WT mice), and had no effect on cellular C5L2 expression (Figure 33). However, when LPS priming was followed by C5a stimulation, a marked down-regulation of HMGB1 (Figures 30A and 31A, WT mice) and a profound LPS dose-dependent inhibition of C5L2 expression occurred (Figure 33). Thus, it appears that TLR activation exerts a negative effect on C5L2 expression only upon C5a-induced C5aR activation, which itself has a positive effect on C5L2 expression. The observed TLR-induced down-regulation of cell-surface C5aR might thus at least contribute to the negative effect of TLRs on C5L2 expression.

The effect on pro-inflammatory cytokine release of C5L2 deficiency in mice was also somewhat more complex than expected. Although the mice did lose their capacity to up-regulate C5a sensitivity after LPS pre-exposure, confirming the crucial role of C5L2 in LPS-induced hypersensitivity to C5a, it was surprising to observe that blood cells from C5L2-deficient mice were no more sensitive to C5a than the WT (Figure 34). These findings were unexpected, as it was speculated that the absence of the putative negative regulator of C5aR, C5L2, should result in increased cell sensitivity to C5a. It is possible that an additional, compensatory, negative regulatory mechanism controlling responses to C5a operates only in the complete absence of C5L2 (C5L2KO mice). This would be compatible with the increased sensitivity to C5a resulting from C5L2 receptor inhibition by TLR activation or Ab blockade observed in this study, as such a compensatory negative regulatory mechanism would not be operational due to the presence of C5L2. In contrast to C5L2, the putative second regulatory mechanism would not be susceptible to negative regulation by TLRs, as the blood cells from C5L2KO mice pre-treated with LPS did not show increased KC production (Figure 34, C5L2KO, + LPS).

3.6 The role of β-arrestin 1 in LPS-induced hypersensitivity to C5a

The findings described in sections 2.3 and 2.4 above indicated that LPS has the capacity to down-regulate transcription and expression of β-arrestin 1, a signal intermediate involved in C5aR and C5L2 activities. The effects of C5a stimulation and LPS priming alone on β-arrestin 1 expression in PBMC deserve brief comment. C5a stimulation up-regulated β-arrestin 1 at both mRNA and protein levels, whereas cell exposure to LPS, with or without subsequent C5a stimulation, resulted in inhibition of β-arrestin 1 expression. The C5a-induced enhancement of β-arrestin 1 expression is consistent with an increased requirement for β-arrestin 1 in the normal process of ligand-dependent receptor deactivation, internalisation and recycling

[Shukla 2011]. However, if, as Gerard and colleagues proposed [Bamberg 2010], β-arrestin 1 mediates the inhibitory effect of C5L2 on C5aR-mediated inflammatory responses, C5a-induced C5aR-mediated enhancement of β-arrestin 1 might equally reflect the increased engagement of the C5L2/β-arrestin 1 inhibitory mechanism for controlling C5aR-dependent inflammatory signalling.

If this were the case, LPS-induced inhibition of β-arrestin 1 expression might be an important mechanism by which TLR activation down-regulates the inhibitory effect of C5L2 on C5aR-mediated pro-inflammatory responses. Alternatively, given that cell pre-exposure to LPS induced down-regulation of both C5aR (directly, Figure 28) and C5L2 (indirectly, Figure 33), it is possible that LPS-induced down-regulation of β-arrestin 1 is a reactive change, reflecting a reduced requirement for β-arrestin 1 by both receptors, either in the process of ligand-induced receptor deactivation (in the case of C5aR) or the inhibition of C5aR-mediated pro-inflammatory signalling (in the case of C5L2). Furthermore, since β-arrestins are involved in regulation of other 7 transmembrane region receptors as well as C5aR and C5L2, a role for LPS-induced inhibition of β-arrestin 1 in the regulation of other GPCRs (chemokine receptors, for example) cannot be excluded.

3.7. Gene Array screening of signal transduction intermediates

The gene microarray strategy that was employed to screen for potential mediators of the observed TLR-induced hypersensitivity to C5a has several limitations. RT-qPCR provides quantitative information about mRNA expression, but this may not directly reflect protein levels, especially when expression of the gene product is also subject to regulation at a translational or posttranslational level (as is the case with inflammatory cytokines).

Furthermore, findings may be very difficult to interpret; for example, in this study, mRNA for NF-κB p65, a critical inducer of cytokine gene expression, underwent a three-fold down-regulation as a result of stimulation with C5a after LPS priming. However, this change did not directly reflect its involvement in the LPS effect on C5a-induced cytokine responses, since it was previously shown that NF-κB p65 activation and nuclear translocation were dramatically enhanced by C5a after cell pre-exposure to LPS (Section 2.1.5 and Figure 24). A plausible explanation for the reduced abundance of mRNA transcripts for p65 in light of the data from Figure 24 is that the reduction in p65 transcription might be a compensatory effect to limit and control pro-inflammatory responses induced by large-scale NF-κB activation. However, in the absence of comparable data on expression level and activation status of many of the proteins whose transcription was either induced or inhibited by LPS or C5a, the gene array data may be difficult to interpret.

As a result of these concerns, the pathway-focussed qPCR arrays were employed only as a screening strategy to identify proteins as candidates for further study, especially where large changes in gene expression were detected. No more detailed inferences were drawn from any of the data.

Chapter 4: CONCLUDING REMARKS AND FUTURE WORK

4.1 The physiological relevance of TLR-induced hypersensitivity to C5a

As noted above, *ex vivo* testing of the sensitivity to C5a of blood cells from sepsis patients failed to reproduce the TLR-induced hypersensitivity to C5a observed *in vitro*; in fact, the capacity of blood from sepsis patients to produce a cytokine response to a range of inflammatory stimuli was globally impaired. This is perhaps not entirely surprising; in order to meet the diagnostic criteria for severe sepsis or septic shock, patients must display evidence of infection, a systemic inflammatory response to that infection, and failure of at least one organ system (for example, renal failure). Since a severe inflammatory tissue injury had already taken place (as indicated by the presence of organ failure, Table 8, section 5.2 below), it is plausible that at the time blood was sampled from these patients, a compensatory anti-inflammatory response was dominant, resulting in sepsis-induced immunosuppression. Hence, it appears that by the time the patients were admitted to the ICU, the window of opportunity for examining mechanisms regulating the initial excessive cytokine response had already passed. In light of this, the low cytokine response of sepsis patients to C5a reported above is not inexplicable.

However it is plausible that the TLR-C5a receptor crosstalk might nevertheless play an important role in determining the amplitude of the cytokine response to infection *in vivo*, but at an earlier point in the disease process; perhaps during the initial encounter between monocytes/neutrophils and microorganisms and at the time when the cytokine response that leads to systemic inflammation and organ failure is triggered. A different experimental model will be necessary to test this hypothesis further in humans. Possible models might include testing the *ex vivo* sensitivity to C5a of blood drawn from patients at an earlier stage of

infections; however, this approach would have a number of practical difficulties, including the difficulty of identifying and recruiting patients with such infections, the impossibility of standardising the infective challenge and of taking a control sample in order to assess each subject's 'unprimed' sensitivity to C5a. However, one possible *ex vivo* model overcomes most of these difficulties. The induction of low-level endotoxaemia by intravenous injection of purified LPS in human subjects is an established experimental model [Michie 1988]. Blood drawn before and immediately (30 min) after LPS inoculation will be stimulated with C5a in order to assess the effect of *in vivo* endotoxaemia on *ex vivo* sensitivity to C5a. The extent of endotoxaemia-induced enhancement of C5a sensitivity (if demonstrated) will be correlated with the relative monocyte expression levels of C5aR and C5L2 in samples taken at baseline and 30 min after induction of endotoxaemia. This will provide a necessary test of the *in vivo* relevance of the phenomenon identified in this study, and provide further valuable data regarding the relationship between C5aR/C5L2 expression and LPS-induced hypersensitivity to C5a.

It will also be interesting to test the sensitivity to C5a of blood cells of mice relatively late after being challenged *in vivo*, to test whether this model reproduces the sepsis-induced immunosuppression observed in humans. Reproducing sepsis-induced immunosuppression in an animal model would support the conclusion that the hyporesponsiveness observed in the sepsis patients was indeed due to the timing of the experiments. Furthermore, it would pave the way for evaluating novel strategies to rescue the pro-inflammatory response of these patients.

4.2 C5aR and C5L2 expression and TLR-induced hypersensitivity to C5a

It was found that the capacity of PBMC and whole blood cells to respond to TLR-mediated activation with enhanced sensitivity to C5a varied greatly between different donors (section 2.1.6 and Figure 25). These findings are interesting for a number of reasons.

As mentioned above, the inflammatory cytokine response to infection is something of a 'two edged sword'. It is vital that the presence of pathogenic microbes in a normally sterile tissue induces a rapid and robust local inflammatory response in order to contain, kill and clear the infective organisms before they establish a foothold. However, it is equally important that there be mechanisms that restrict the inflammatory response to the immediate vicinity of the infection in order to prevent systemic inflammation and injury to healthy tissues. It is often observed anecdotally that human subjects seem to vary in their susceptibility to an excessive, systemic inflammatory response to a given infective insult. An apparently similar microbial challenge (for example, a perforated bowel leading to faecal contamination of the peritoneum) might in one subject cause peritonitis that resolves promptly after surgical intervention, while in another it may result in septic shock and a prolonged period of multi-organ failure. It is of course impossible to test the veracity of this observation by experiment, since a 'standardised infectious challenge' does not exist, and many other confounding factors such as age and co-morbidity might also influence the outcome. However, it is plausible that intersubject variations in the mechanisms that regulate the magnitude of the pro-inflammatory cytokine response to infection might contribute to the different outcomes.

It is also plausible that intersubject variations in such a regulatory mechanism might be conserved as representing a survival advantage in different circumstances. An individual who

is prone to a more robust inflammatory response to infection might be better able to clear pathogens at an early stage of colonisation and thus prevent an infection from gaining a foothold, but be more susceptible to a fatal systemic inflammatory response if an infection becomes established. On the other hand, an individual prone to a less robust cytokine response might be more susceptible to infections but less likely to develop fatal sepsis. Either characteristic might represent a survival advantage depending on the prevalence of different infectious hazards in the environment. For example, an environment in which a particularly virulent pathogen was endemic might favour individuals prone to a more robust cytokine response, since infection might prove rapidly lethal if allowed to gain a foothold. Thus the characteristic might persist in the population in a similar way to the persistence of terminal complement pathway deficiencies in regions with a high prevalence of *Neisserial* infections [Walport 2001a] or sickle cell trait in areas where malaria is endemic [Rosenthal 2011].

As noted in section 2.6 above, it is possible that the varying capacity of individuals to augment their cytokine response to C5a as a result of cell pre-exposure to TLR ligands (Figures 22B, 25B and 37) is related to the relative expression levels of C5aR and C5L2 in monocytes. These findings, however, need to be corroborated in a larger study, and work is on-going to expand this study in a larger population of healthy donors to test the hypothesis that the absolute expression level of C5L2 or the ratio of C5L2 to C5aR expression might be associated with a differing capacity for TLR-induced hypersensitivity to C5a. If substantiated in a larger population, this would provide a valuable insight both into the mechanisms of C5a-induced cytokine induction and the pathophysiology of the cytokine response to infections.

4.3 The effect of C5L2 gene silencing on C5a sensitivity

As discussed in Section 3.5 above, it was surprising to find that genetic deletion of C5L2 did not have any (amplifying) effect on the *ex vivo* sensitivity of mouse blood cells to C5a in the absence of preceding TLR activation; this might be explained by the existence of an alternative regulatory pathway operative in the complete absence of C5L2. In order to provide further support for the proposed role of C5L2 in regulating C5aR-induced cytokine induction, a siRNA knockdown strategy may be used to assess the effect of C5L2 gene silencing upon C5a sensitivity. An *in vitro* model will be established in order to test the capacity of C5L2 gene silencing to reproduce the effects of C5L2 blockade.

4.4 The role of β-arrestin 1 in TLR-induced hypersensitivity to C5a.

β-arrestin 1 has been implicated in the inhibitory effect of C5L2 upon C5aR-induced pro-inflammatory responses, and its expression – like that of C5L2 – was found in this study to be down-regulated by cell pre-exposure to LPS. Therefore, it was concluded that TLR activation may also target β-arrestin 1 for negative modulation as part of the mechanism underlying TLR's positive effect on C5aR sensitivity (Section 2.4). The Gerard group [Bamberg 2010] found that the inhibitory effect of C5L2 on neutrophil responses to C5a was mediated by an inhibition of ERK1/2 phosphorylation, and inhibition of ERK1/2 has also been shown to be associated with reduction in C5a-induced responses [Hawlisch 2005, La Sala 2005 and Bamberg 2010]. In this study, the mRNA expression upon C5a stimulation of several key components of the MAPK pathway was found to be affected by cell pre-exposure to LPS (Section 2.3, Table 5C). Therefore, the pathway linking β-arrestin 1 and ERK1/2 deserves to be investigated in greater detail. Specific inhibitors and siRNA gene silencing of β-arrestin 1 may

be employed in order to investigate its role in this context. The expression levels of proteins whose mRNA expression was affected may be assessed by Western blot, and the phosphorylation (activation) states of key signalling intermediates may be studied using protein phosphorylation arrays. Specific inhibitors of key intermediates may also be employed to test their roles in TLR-induced C5a hypersensitivity.

4.5 The problem of autocrine effects of C5a

As noted in sections 1.3.4.2*d* and 1.5.3 above, gene silencing studies have raised the possibility of a modulatory effect of C5a receptors on TLR-mediated inflammatory responses *in the absence of* exogenous C5a [Gavrilyuk 2005, Rittirsch 2007, Zhang 2007 and Fusakio 2011]. Although artefactual generation of C5a from C5 in serum-supplemented culture media cannot be excluded as a possible explanation for this observation, it is unlikely in the culture conditions described in these studies. However, an autocrine/paracrine effect of C5a generated from C5 secreted and activated by the phagocytic cells themselves cannot be ruled out, since synthesis and secretion of complement proteins by a range of extrahepatic cell types is well-documented (see Table 6 below), as is the ability of phagocytic cells to activate C5 [Huber-Lang 2002]. If this were the case, it is possible that effects (on signal transduction and cytokine secretion) that were attributed to TLR activation alone were in fact due to the combination of TLR and C5a receptor activation. Although the extent of the contribution of the constitutively generated C5a cannot be estimated, (and indeed may be minimal compared with that of TLR activation) a careful consideration of the experimental designs of the studies cited in section 1.5 makes it difficult to exclude this possibility.

In order to design an experimental system in which the possibility of an effect of endogenous C5a on TLR-mediated inflammatory responses could be confidently excluded, it would be

necessary to prevent C5 secretion and/or activation, or to ensure that any endogenously generated C5a was prevented from interacting with either of its receptors. The latter possibility seems more likely to be successful (though by no means certain) and might be

Table 6. Extrahepatic synthesis of complement components

Type of Cell/Tissue	Complement Protein Detected
Myeloid/Lymphoid cells	
Monocyte	C1q, C2-9, B, D, I, P
Macrophage	C1-9
Neutrophil	C3
BMDC	C3
MDC	C1q, C3, C5, C9, I, H, B, D, P
NKC	C4
T Cell	C3
Organ/Tissue/Cell	
HUVEC	C3, C5-9, H
Intestinal Epithelial cell	C3, C4, B
Pancreatic Duct Epithelial Cell	C3, C4, B
Fibroblast	C2, C3, C5-9,
Keratinocyte	C3, B, H
Pneumocyte	C3-9, B, H, I
Mesothelial Cell	C3, C4
Muscle Cell	C3-5
Chondrocyte	C1r, C1s, C2-4
Adipocyte	C1qrs, C2-9
Astrocyte	C1-9, B, D, H, I
Microglia	C1q, C2-4
Neuroblastoma	C1q, C3-9, B, H
Oligodendrocyte	C1qrs, C2-9
Renal Biopsy	C1q, C2-4, B, D, H, P
Glomerular endothelial cell	C3
Glomerular epithelial cell	C1r, s, C2-4, B
Mesangial Cell	C3, C4
Renal tubule epithelial Cell	C2-4, H, B

BMDC, Bone-marrow-derived dendritic cell; HUVEC, human vascular endothelial cell; MDC monocyte-derived dendritic cell, NKC, Natural killer cell. [adapted from Li 2007].

achieved by the use of an excess of a neutralising antibody to C5a in experiments designed to

test the effects of TLR activation alone. This avenue of investigation will be pursued in future

work.

Chapter 5: MATERIALS AND METHODS

5.1 Antibodies and reagents

Antibodies and immunoreagents used and their sources are listed in Table 7 below. Recombinant human C5a (expressed in *E. coli*) was kindly provided by Dr P.N. Monk, Sheffield University, U.K (used in the experiments shown in Figures 20-30, 32-33, and 37-38). Purified natural human C5a was from CompTech (Tyler, TX, USA) and was used in the experiments shown in Figure 35 and Table 5. Preliminary experiments indicated that both sources of human C5a were free from detectable LPS contamination (representative experiment shown in Figure 40, Appendix). The relative potencies of recombinant and purified C5a were assessed and found to be similar (Figure 41, Appendix). Recombinant mouse C5a (Figures 31 and 34) was from Hycult Biotech (Plymouth Meeting, PA, USA).

RPMI-1640, L-glutamine and Fluo3-AM were supplied by Invitrogen Ltd (Paisley, U.K.). Low-endotoxin foetal calf serum (FCS) was from Hyclone (Logan, UT, USA; < 0.06 EU/ml endotoxin); Ficoll-Histopaque-1077, bovine serum albumin, sodium dodecyl sulphate (SDS), leupeptin, pepstatin A, molecular biology grade chloroform, ethanol and isopropanol, red blood cell lysis buffer, Nonidet P-40 (NP-40), polymyxin B, ammonium persulphate, sodium azide, DL-dithiothreitol (DTT), TRIS-hydrochloride, glycerol, phorbol myristate acetate (PMA) and ionomycin were from Sigma-Aldrich UK (Dorset, U.K.). Ultra-pure LPS (*Escherichia coli* O111:B4 strain), heat-killed *Listeria monocytogenes* and *Escherichia coli,* polyinosinic-polycytidylic acid (poly I:C), zymosan, flagellin, IL-1β, IL-6 and TNFα were from Invivogen (San Diego, CA, U.S.A.). The synthetic bacterial lipopeptide Pam_3-Cys-Ser-$(Lys)_4$ HCl (Pam_3Cys) was from EMC microcollections GmbH (Tübingen, Germany). Bicinchoninc Acid (BCA) protein quantification assay was from Bio-Rad (Hercules, CA, USA). Glycine, p-formaldehyde, bromophenol blue and phenylmethylsulfonyl fluoride (PMSF) were from Fisher Scientific UK (Loughborough, UK). All other chemicals were reagent grade.

Table 7. Antibodies and immunoreagents used

Antibody	Clone/Code	Isotype	Source
Human CD3	HIT3a-PE	Mouse IgG2a	eBioscience, San Diego, CA, USA
Human CD3	B-B11	IgG1	Diaclone, Besancon, France
Human CD14	MY4 (and MY4-FITC)	Mouse IgG2b	Coulter, Luton, UK
Human CD19	B-C3 -PE	Mouse IgG 1	Diaclone, Besancon, France
Human C5aR	S5/1 (and S5/1-FITC)	Mouse IgG2a	R&D Systems, Minneapolis, MN, USA
Human C5L2	1D9 M12 (and 1D9 M12-PE)	Mouse IgG2a	Biolegend, San Diego, CA, USA
Human/Mouse HMGB1	Ab18256	Rabbit Igs	Abcam, Cambridge, UK
Human β-arrestin 1	SC-74591	Mouse IgG1	Santa Cruz, CA, USA
Mouse IgG1	MOPC-21	Mouse IgG1	Biolegend, San Diego, CA, USA
Mouse IgG2	UPC-10	Mouse IgG2	Sigma-Aldrich, Dorset, UK
FITC-conjugated Rabbit anti-mouse Igs	F0313	Rabbit Igs	DAKO, Cambridge, UK
PE-conjugated Rabbit anti-mouse Igs	R0439	Rabbit Igs	DAKO, Cambridge, UK
HRP-conjugated Rabbit anti-Mouse Ig	A9044	Rabbit Igs	Sigma-Aldrich, Dorset, UK
HRP-conjugated Goat anti-Rabbit Ig	A6154	Goat Igs	Sigma-Aldrich, Dorset, UK

For RT-qPCR experiments, Tri-reagent was from Ambion (Austin, TX, USA), reverse transcription kit (High Capacity cDNA Reverse Transcription) and Sybr green/Rox qPCR mastermix (Power Sybr Green) were from Applied Biosystems (Foster City, CA, USA). For the pathway-focussed gene arrays, on-column RNA extraction kits (RNeasy Kit), nuclease-free water, DNAse, reverse transcription kits (RT^2 First Strand cDNA Synthesis Kit), Sybr green/Rox qPCR mastermix (RT^2 Sybr Green Mastermix) and gene arrays (RT^2 profiler PCR Arrays) were from Qiagen (Dusseldorf, Germany).

For Western blotting, Amersham Hybond nitrocellulose membrane was from GE Healthcare Life Sciences (Buckinghamshire, UK) and Pierce enhanced chemiluminescence (ECL Plus) reagents were from Fisher Scientific UK.

5.2 Cells and cell activations

Human whole blood samples were obtained from healthy volunteers and sepsis patients, and PBMC from the buffy coat fractions of blood donations provided by the Welsh Blood Service, in accordance with local NHS research ethics committee permissions (REC reference 10/WSE04/21). Patient samples were taken from adults with a diagnosis of severe sepsis or septic shock by consensus definitions [Levy 2003] on admission to the intensive care unit if within 36h of the presumed onset of the infective illness. Patient characteristics are shown in Table 8, below.

Table 8. Demographic and clinical characteristics of sepsis patients

Patient characteristics		Focus of Infection†*		Causative organism‡	
Male:Female (total)	7:12 (19)	**Respiratory tract**	6	**Gram +ve cocci**	
Age (yrs)	57±13	**Urinary**	4	*Streptococcus pneumoniae*	2
Admission APACHE II score	16.4 ±8.2	**Biliary tract**	3	*Enterococcus faecalis*	1
Outcome (lived:died)	14:5	**Peritonitis**	6	**Gram +ve bacilli**	
Days on ICU	15.7 ±14.2	**Soft tissue**	4	*Dermabacter hominis*	1
Duration of organ support (days)	9.4 ±8.5			**Gram -ve bacilli**	
†On admission *Some patients presented with more than one infection ‡Some samples were positive for more than one pathogen				*Escherichia coli*	6
				Enterobacter cloacae	1
				Pseudomonas aeruginosa	1
				Klebsiella pneumonia	1
				Haemophilus influenzae	2
				No organism isolated	10

5.2.1. Isolation, cryopreservation and resuscitation of peripheral blood mononuclear cells

PBMC were extracted by Ficoll density-gradient centrifugation of buffy coats. The blood was diluted 1:1 with room temperature RPMI-1640 medium, overlaid (12 ml) onto 8 ml of room temperature Ficoll-Histopaque (Sigma) to a blood: Ficoll final ratio of 3:2, and centrifuged at 750 x g for 20 min at room temperature. The upper (plasma) and lower (red cells and PMN) phases were discarded, and the interphase containing the PBMC was recovered and washed three times at room temperature with RPMI-1640 medium (first wash, 370 x g, 12 min; second, 190 x g, 10 min; third, 120 x g, 8 min). Monocytes comprised 5-10% and lymphocytes 90-95% of the PBMC, as assessed by CD3, CD14 and CD19 staining and forward- and side-scatter profile. Neutrophils accounted for less than 0.1% of the cell preparations. Following washing, the PBMC were resuspended in freezing medium (10% DMSO / 20% FCS / 70% RPMI-1640) at a concentration of ~20 x 10^6 cells/ml and frozen in 1 ml cryotubes immersed in isopropanol. Cells were stored at -70°C for up to 48h and thereafter in liquid nitrogen for up to

1 year. Cell aliquots were resuscitated by prompt defrosting at 37 °C, and drop wise dilution with RPMI-1640 medium followed by washing (3x) to remove the DMSO. Cell viability was always ≥ 99% as estimated by the trypan blue exclusion test.

5.2.2. Cell activation protocols

To test the effect of TLR activation on PBMC sensitivity to C5a *in vitro*, the experimental protocol shown in Figure 39A was used. Triplicate aliquots of PBMC (1.5×10^5 cells/well, unless stated otherwise) were cultured in phenol red-free RPMI-1640 medium supplemented with 10% heat-inactivated (56 °C, 30 min) FCS (HyClone) and 2 mM glutamine (complete medium), and stimulated (pre-exposed) at 37 °C for 14h or the time indicated with previously-defined optimal concentrations of LPS (100 pg/ml), Pam$_3$Cys (100 ng/ml), zymosan (1 µg/ml), flagellin (5 µg/ml), imiquimod (3 µg/ml) or polyI:C (80 µg/ml), or medium alone (mock-stimulation). The effect of cell pre-exposure to stimuli other than TLR ligands was also tested (Figure 21): IL-1β (5 ng/ml), TNFα (10 ng/ml), IL-6 (10 ng/ml) or PMA/ionomycin (50/500 ng/ml). Following incubation, cells were washed (3x, RPMI-1640 medium, room temperature), resuspended in complete medium, and activated for a further 12-14h with the indicated concentrations of C5a or mock-activated. In some experiments (Figure 20C), polymyxin B (10 µg/ml) was added during the C5a stimulation phase of the culture. Cell culture supernatants were then collected and tested for IL-6, IL-8, KC or MCP-1 by ELISA (Duoset, R&D Systems, Minneapolis, MN, USA). For C5L2 receptor blocking experiments (Figure 32), PBMC were preincubated (30 min at 37 °C) with the anti-human C5L2 blocking mAb, clone 1D9-M12 (5 µg/ml), before stimulation with C5a (2.5 nM). In control experiments the 1D9-M12 mAb was denatured by boiling for 10 min.

Whole blood cell sensitivity to C5a before and after pre-exposure to TLR ligands and whole bacteria was tested (Figure 25) using the same experimental protocol described above for

PBMC and in Figure 39A. Here, triplicate samples of heparinised (10 IU/ml) human whole

blood (100 µl/well) were
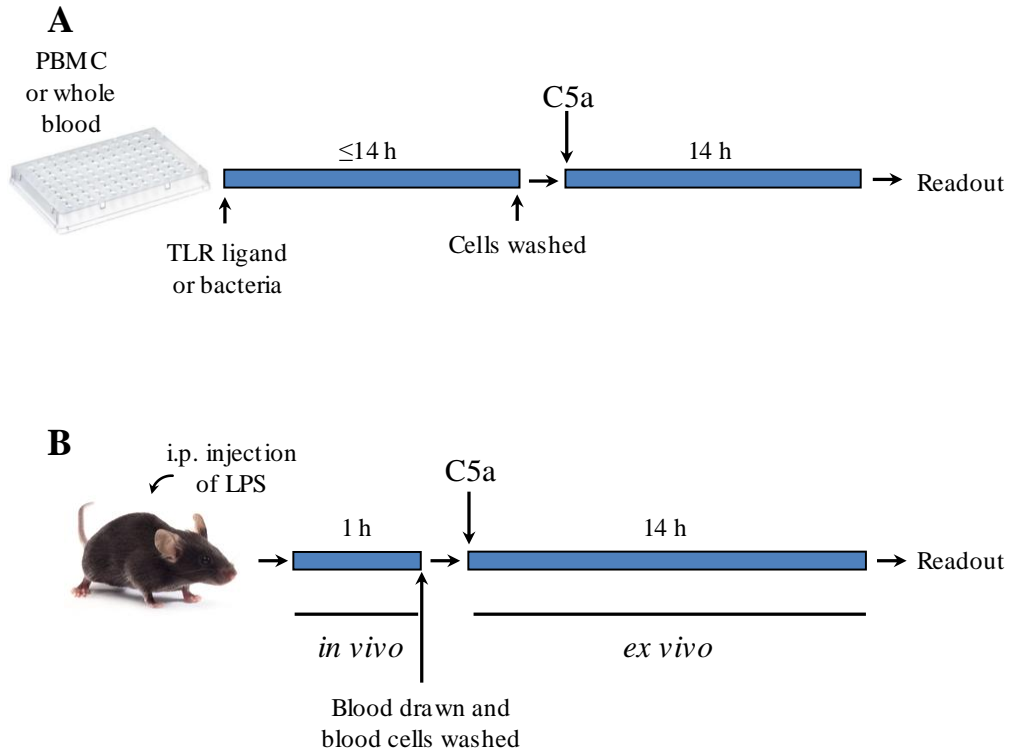
Figure 39. Experimental models of TLR/C5a receptor activation.
(A) *In vitro* model. Cells (PBMC or whole blood) were exposed to TLR ligands or whole heat-killed bacteria for variable durations before washing and stimulation with C5a for 14h. Following incubation, cell culture supernatants were tested for cytokine release (ELISA). Cell lysates were prepared and tested for NF-κB activation (ELISA) or HMGB1, C5L2 and β-arrestin 1 expression (Western blot). **(B)** *In vivo / ex vivo* model. Mice [C3H/HeN (WT), C3H/HeJ (TLR4 signalling deficient), BALB/c or C5L2KO (BALB/c background)] were pre-exposed *in vivo* to LPS by i.p. injection. After 1h, animals were sacrificed, blood drawn and the blood cells washed before being stimulated with C5a for 14h. After stimulation, culture supernatants were taken for chemokine determinations (ELISA) or HMGB1 levels (Western blot). Experimental details are described under Materials and Methods, sections 5.2 and 5.8.

pre-exposed to LPS (500 pg/ml) or heat-killed *Escherichia coli* (1×10^8 cfu/ml) for 14h.

Subsequently, samples were centrifuged (300 x g, 5 min, room temperature), the blood cells

washed (x3, RPMI-1640 medium, room temperature), resuspended in an equivalent volume of

heat-inactivated (30 min, 56 °C) 100% autologous plasma, and activated for 14h with the

indicated concentrations of C5a before cell culture supernatants were collected and tested for cytokine levels by ELISA.

To test the sensitivity of sepsis patients' whole blood to C5a or TLR activation (Figure 36), triplicate samples of heparinised whole blood (100 µl/well) were washed three times in RPMI-1640 and resuspended in equivalent volumes of complete medium before stimulation for 14h with the indicated concentrations of C5a or with LPS (10 ng/ml), Pam$_3$Cys (100 ng/ml), whole heat-killed *Escherichia coli* (2 x 10^4 cfu/ml) or *Listeria monocytogenes* (4 x 10^5 cfu/ml). Sub-optimal stimulating concentrations were chosen on the basis of dose-response titration experiments; a representative experiment is shown in Figure 42 (Appendix). IL-8 concentrations were estimated in the culture supernatant by ELISA.

In some experiments the C5a-induced IL-8 concentrations after background subtraction were shown. They were estimated by subtracting the background levels of IL-8 present in cultures not activated with C5a and pre-exposed or not to TLR ligands from the corresponding C5a-activated samples (background levels of IL-8 in the experiments described in Figure 21 – typical of all experiments – are shown in the figure legend). Fold changes in IL-8 release were estimated by comparing the C5a-induced IL-8 release in cells previously stimulated with TLR ligands or whole bacteria with that seen in cells not previously stimulated.

5.3 Enzyme-linked immunosorbent assay (ELISA)

Cytokine levels in cell culture supernatants were estimated by ELISA. Duoset ELISA kits for IL-6, IL-8, KC or MCP-1 were supplied by R&D systems (Minneapolis, MN, USA) and were used according to the manufacturer's instructions. Briefly, Costar 96-well stripwell plates (Corning Costar, Sussex, UK) were coated with a capture antibody to the protein of interest by overnight incubation at room temperature. After washing (x3 washing buffer: 0.05% Tween-20 in PBS), wells were blocked with 1% bovine serum albumin in PBS, followed by washing (washing buffer) and incubation with appropriately diluted samples and standards in PBS. Following washing, the wells were incubated with a biotinylated detection antibody, washed and incubated with streptavidin-HRP solution (The Jackson Laborotory Bar Harbour, MN, USA). Following a final wash, wells were incubated with colour development reagent (tetramethylbenzidine and hydrogen peroxide 1:1 v/v; TMB, SureBlue™, KPL) and the reaction stopped by addition of 1M HCl. Absorbance at 450 nM was measured (Benchmark microplate reader, Bio-Rad) and the cytokine concentration in the samples was estimated by extrapolating the absorbances of the samples in the standard curve prepared in parallel. All antibody concentrations and incubation times were optimised for the particular antibody pair for each ELISA.

5.4 Quantitative RT-PCR (RT-qPCR)

For RT-qPCR experiments (Figures 23 and 35), triplicate aliquots of PBMC (1 x 10^6 cells/ condition) were cultured in complete medium, stimulated or not with 100 pg/ml LPS, washed, and activated or not with C5a (10 nM), as described above. RNA was phenol/chloroform-extracted and precipitated with isopropanol overnight at -4°C. Precipitates were washed three times with 70% ice-cold ethanol and allowed to dry before reconstitution with nuclease-free

water. RNA concentration was quantified by spectrophotometry (Nanodrop Spectrophotometer, Thermo Scientific, Wilmington, USA) and RNA purity confirmed by $A_{260:280}$ (≥2.0) and $A_{260:230}$ (≥1.8) readings. Reverse transcription was performed on equal masses of RNA using random primers in a thermal cycler (MJ PTC-200, MJ Research, St Bruno, Quebec, Canada). qPCR was performed on the resulting cDNA using the Power SYBR Green PCR master mix (Applied Biosystems) and specific primers (Invitrogen; see Table 9 below). The primers were designed to avoid amplification of potential contaminant genomic DNA (one primer of each pair was designed to hybridise across two exons). In order to determine the primers' amplification efficiency, each pair of primers was titrated against varying amounts of cDNA. Efficiency for all primers was between 75 and 100%. A representative titration experiment is shown in Figure 43 (Appendix). PCR was carried out using the ABI 7900HT real-time PCR system (Applied Biosystems) with a thermal cycle profile of 10 min at 95°C followed by 40 cycles of 15s at 95 ^{o}C and 45s at 60 ^{o}C. The results were analysed by the ΔΔCt method [Livak and Schmittgen 2001], using β-glucuronidase as a housekeeping gene. Dissociation curves of the amplicons generated by all primer pairs showed a single clear dissociation peak; a representative example is shown in Figure 44 (Appendix).

Table 9. RT-qPCR Primers

Gene Symbol*	Forward (5′ − 3′)	Reverse (5′ − 3′)
IL6	CAGTTCCTGCAGAAAAAGGC	GAATGAGATGAGTTGTCATG
IL8	GAACTGAGAGTGATTGAGAGTGGA	CTCTTCAAAAACTTCTCCACAACC
C5AR1	GGAGACCAGAACATGAACTC	ATCCACAGGGGTGTTGAGGT
ARRB1	CCTGACCTTTCGCAAGGACC	CAAGCCTTCCCCGTGTCTTC
GUSB	TCTGTATTCATTGGAGGTGC	AAGGTTTCCCATTGATGAGG

*HUGO database gene nomenclature. IL6, interleukin-6; IL8, interleukin-8; C5AR1, C5aR/CD88; ARRB1, β-arrestin 1; GUSB, β-glucuronidase.

5.5 NF-κB assays

PBMC (1 x 10^7 cells/ condition) were pre-exposed to 100 pg/ml LPS, washed, and activated with C5a (10 nM) as described above (section 5.2.2 and Figure 39). In some experiments (Figure 24), the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC, 10 ng/ml) [Németh 2003] was added during the C5a stimulation phase. At the end of the culture, nuclear extracts were prepared (Nuclear Extract kit, Active Motif, Carlsbad, CA, USA) by lysing the cells with 0.5% NP-40 in a phosphatase inhibitor buffer (125 mM NaF, 250 mM β-glycerophosphate, 250 mM p-nitrophenyl phosphate, 25 mM $NaVO_3$) followed by centrifugation (all at 4 °C). The nuclear pellet was lysed using the manufacturer's proprietary lysis buffer containing a cocktail of protease inhibitors, and protein concentration was then determined (ProStain Fluorescent Protein Quantification, Active Motif). Five micrograms of total protein/sample were used to determine NF-κB p65 concentrations (ELISA, TransAM NF-κB p65, Active Motif).

5.6 In vivo model of TLR activation

Inbred 8- to 12-week-old C3H/HeN, C3H/HeJ (Harlan laboratories, Carshaltan, UK), BALB/c (The Jackson Laboratory, Bar Harbour, MN, USA) and C5L2-deficient mice (on BALB/c background, kindly provided by Prof. Jörg Köhl, Institute for Systemic Inflammation Research, University of Lübeck, Lübeck, Germany) were maintained under barrier conditions and pathogen free. All experimental procedures were carried out under Home Office (U.K.) or the Ministerium für Landwirtschaft, Umwelt und Ländliche Räume (Kiel, Germany) project licenses. In order to test the effect of TLR activation *in vivo* on blood cell sensitivity to C5a *ex vivo*, the experimental protocol described in figure 41B was used. Mice (*n*= 5/condition) were i.p. injected with a previously-defined dose of LPS (50 µg/mouse) or phosphate-buffered saline (PBS). After 1h, blood was collected by cardiac puncture and samples (100 µl/condition) were

washed (x3, RPMI 1640), resuspended in complete medium, and stimulated (14 h) with the indicated concentrations of mouse C5a. The cell culture supernatants were tested for KC and MCP-1 levels by ELISA (R&D Systems), and for HMBG1 by Western blot. The *in vivo* phase and *ex vivo* blood cell stimulations of the experiment involving C5L2-deficient mice were conducted at Prof. Jörg Köhl's Laboratory, and the cell culture supernatants were transported to our laboratory (Cardiff University, School of Medicine) on dry ice for chemokine and HMGB1 determinations.

5.7 Flow cytometric analysis

The expression levels of C5aR and C5L2 in monocytes and neutrophils were assessed by flow cytometry as previously described [Rey Nores 1999]. Here, PBMC (1 x 10^6 cells/condition) or whole blood (100 µl/condition) were pre-exposed for 30 min to 100 pg/ml (PBMC) or 500 pg/ml (whole blood) LPS or mock-stimulated (PBS). Following incubation, cell aliquots were collected for C5aR receptor expression, and the remaining samples were washed and activated or not with C5a (10 nM) for the indicated times, before aliquots were taken and also analysed for C5aR expression. In preparation for flow cytometric analysis of monocyte C5aR expression, PBMC were washed twice and resuspended in binding buffer (PBS, 0.5% BSA, 0.05% NaN$_3$). Fc receptors were blocked by incubating the samples with a 20% normal rabbit serum solution in binding buffer (100 µl/ 5 x 10^5 cells) for 15 min at room temperature. Subsequently, cells were incubated with the human C5aR-specific mAb S5/1-FITC or its isotype control (mouse IgG2a, UPC-10, Sigma), (1 µg mAb per 1 x 10^6 cells in 100 µl binding buffer) for 30 min at 4°C, washed (2 x 2 ml binding buffer), before being fixed in 2% p-formaldehyde. Neutrophil C5aR expression was analysed in aliquots of anticoagulated whole blood. At each time point, aliquots of whole blood (100 µl) were incubated with the S5/1-FITC anti C5aR mAb or isotype control (UPC-10, 30 min at 4°C) before red cell lysis and fixation. C5L2 expression was analysed

on monocytes and neutrophils from whole blood samples incubated (30 min at room temperature) with the human C5L2-specific mAb 1D9 M12-PE or its isotype control (mouse IgG1, MOP C-21, Sigma) at 1 µg mAb per 1×10^6 cells in 100 µl binding buffer, before red cell lysis and subsequent cell fixation. The stained cells were analysed by flow cytometry, 10,000 events being acquired for each sample using a FACSCalibur cytometer (Becton Dickinson, San Jose, CA, USA), equipped with the CellQuest™ Pro software. Data were analysed using the FlowJo software package (Treestar, OR, USA). C5aR or C5L2 receptor expression was estimated on gated monocytes or neutrophils identified by their CD14$^+$ staining and forward-and side-scatter profiles; the gating strategy is shown in Figure 45 (Appendix) and was used for the experiments shown in Figures 28A and 29. Representative examples of flow cytometric estimation of C5aR expression (corresponding to data presented in Figure 28) are also shown in Figure 45 (Appendix).

5.8 Calcium mobilisation assays

Calcium mobilisation in gated monocytes was analysed by flow cytometry following a previously described method [Schepers 2009] with some modifications. PBMC (1×10^6 cells/condition) were activated or not with LPS (100 pg/ml) for 30 min or 14h before staining (30 min, room temperature) with the Ca^{2+}-chelating fluorescent dye Fluo3-AM (10 µM) or first stained with Fluo3-AM before a 3-min activation with LPS (no calcium mobilisation was detected in response to LPS alone). An aliquot was taken from each sample to measure the background fluorescence at time 0. C5a (10 nM) was subsequently added to the remaining samples, and fluorescence was first measured 10 s after addition of C5a, and thereafter every 30 s for a total of 3 min. Results are expressed as normalized [Ca^{2+}]$_i$, as a measure of the fold increase in intracytoplasmic Ca^{2+} concentration at each time point after the addition of C5a, by

determining the ratio between the mean fluorescence intensity at time *t* and that at time 0. A representative experiment is shown in Figure 47 (Appendix).

5.9 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

HMGB1, C5L2 and β-arrestin 1 levels in cytoplasmic preparations from PBMC and in culture supernatants (20 µl) of mouse blood cells (HMGB1) were evaluated by Western blot analysis using a previously described technique [LeBouder 2003]. Cultured PBMC (0.5 x 10^6 cells/condition) were pre-exposed (14 h) or not to LPS (100 pg/ml or as indicated), then washed and stimulated (14 h) or not with C5a (10 nM). To test the effect of blocking C5L2 on HMGB1 levels (section 2.2.5 and Figure 32), the PBMC were stimulated with C5a (2.5 nM) in the absence or presence of an anti C5L2 mAb (1D9 M12, 5 µg/ml) or the same mAb denatured by boiling, before cell lysis. Cells were lysed (0.5% (v/v) NP-40, 50 mM Tris-HCl, 150 mM NaCl, 1 µg/ml leupeptin and pepstatin, 1 mM PMSF, pH 7.4 buffer) for 1h on ice, and the protein content of the cytoplasmic cell extracts was estimated (BCA assay, Bio-Rad).

SDS-PAGE was carried out using the Bio-Rad Mini Protean II gel apparatus. Equal amounts of cell extracts (5 µg protein /condition) and 25 µl of mouse blood cell culture supernatants were diluted with Laemmli [Laemmli 1970] reducing sample buffer (2% SDS, 100 mM DTT, 50 mM Tris-HCl, pH 6.8, 10 % glycerol and 0.1% bromophenol blue) and boiled for 4 min prior to SDS-PAGE (10% acrylamide gels for C5L2 or β-arrestin 1 and 12.5% for HMGB1). Pre-stained molecular weight markers (Precision Plus™ Protein Standards, Bio-Rad) were run in parallel. Electrophoresis (200 volts) was carried out using SDS containing running buffer (25mM Tris base, 192 mM glycine, 0.1% SDS). After electrophoresis, the gels were briefly washed twice with PBS before proceeding to Western blotting.

The gels were incubated (with shaking) with transfer buffer (48 mM Tris base, 39mM glycine, 20% (v/v) methanol) at room temperature for a total of 20 min with 3 changes of buffer. Extra thick filter paper (Bio-Rad) and the nitrocellulose membrane (GE Healthcare) used for electrotransfer were kept in transfer buffer for 20 min before use. Electrotransfer was carried out in a semi-dry transfer cell (Transblot SD, Bio-Rad) for 30 min at 13 volts. Following transfer, the membranes were blocked for 1 h at room temperature with blocking buffer consisting of PBS/0.1% Tween-20 (PBS-T) supplemented with 5% BSA (for C5L2 and β-arrestin 1) or 5% skimmed milk (for HMGB1). Membranes were then washed (1 x 15 min and 2 x 5 min at room temperature in PBS-T) and incubated with the primary antibody (all at 1 μg/ml) overnight at 4˚C with gentle rocking. HMGB1 was detected by using a human and mouse HMGB1-specific polyclonal Ab (Ab18256); β-arrestin 1 using the mouse anti-human mAb SC-74591; C5L2 using the mouse anti-human mAb 1D9-M12. Following incubation and washing (detailed above), the membranes were incubated (1 h, room temperature) with the appropriate horseradish peroxidase-conjugated secondary antibody in 2% BSA (C5L2 and β-arrestin 1) or milk (HMGB1) in PBS-T. Subsequently, the membranes were washed (PBS-T, 1 x 15 min and 4 x 5 min at room temperature) before detection by enhanced chemiluminescence (ECL, Pierce). Densitometric measurements were performed on scanned films using the ImageJ software package (National Institutes of Health, Bethesda, MD, USA). The specificity of all Western blots was confirmed by blotting duplicate samples with isotype control antibodies; representative examples are shown in Figure 48 (Appendix).

5.10 Pathway-focussed gene arrays

Pathway-focussed gene arrays were from Qiagen and were used according to the manufacturer's instructions. Briefly, PBMC (1×10^6 cells/ condition) were cultured in complete medium, pre-exposed (14 h) or not to 500 pg/ml LPS, washed, and activated or not with C5a (1 nM, 4 h), as described above. Cells were lysed and RNA extracted on-column (RNEasy kit). RNA concentration was quantified and RNA integrity was assessed using the Agilent 2100 Bioanalyzer; all samples had RNA Integrity Numbers ≥7. Reverse Transcription (RT kit, Qiagen) was performed on 500 ng of RNA after on-column DNAse treatment to remove genomic DNA. cDNA was diluted according to the manufacturer's instructions, and PCR was carried out using SYBR Green/Rox qPCR mastermix (Qiagen) in the ABI 7900HT real-time PCR system (Applied Biosystems). The thermal cycle profile was 10 min at 95 ^{o}C followed by 40 cycles of 15s at 95 ^{o}C and 45s at 55 ^{o}C, in 96-well plates pre-coated with primers for a panel of 172 genes associated with the pathways of interest (Table 10A and B, Appendix). Results were analyzed by the ΔΔCt method [Livak and Schmittgen 2001]. Gene expression changes were normalised to a panel of housekeeping genes including β-2-microglobulin, hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13a, glyceraldehyde-3-phosphate dehydrogenase and β actin. Internal controls for genomic DNA contamination and failure of reverse transcription were negative in all samples.

5.11 Statistical analysis

Statistical analysis of the data was performed using Microsoft XL 2010. Mean values were compared using Student's t test for paired samples. p values <0.05 were considered significant.

CHAPTER 6: APPENDIX

Table 10A. Pathway-focussed gene arrays: MAPK pathway

Gene Symbol*	Description	Gene Symbol*	Description
ARAF	V-raf murine sarcoma 3611 viral oncogene homolog	CDK4	Cyclin-dependent kinase 4
ATF2	Activating transcription factor 2	CDK6	Cyclin-dependent kinase 6
BRAF	V-raf murine sarcoma viral oncogene homolog B1	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CCNA1	Cyclin A1	CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)
CCNA2	Cyclin A2	CDKN1C	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)
CCNB1	Cyclin B1	CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
CCNB2	Cyclin B2	CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)
CCND1	Cyclin D1	CDKN2C	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
CCND2	Cyclin D2	CDKN2D	Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)
CCND3	Cyclin D3	CHUK	Conserved helix-loop-helix ubiquitous kinase
CCNE1	Cyclin E1	COL1A1	Collagen, type I, alpha 1
CDC42	Cell division cycle 42 (GTP binding protein, 25kDa)	CREB1	CAMP responsive element binding protein 1
CDK2	Cyclin-dependent kinase 2	CREBBP	CREB binding protein

*HUGO Database Gene Nomenclature.

Table 10A. Pathway-focussed gene arrays: MAPK pathway (continued)

Gene Symbol*	Description	Gene Symbol*	Description
DLK1	Delta-like 1 homolog (Drosophila)	KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
E2F1	E2F transcription factor 1	KSR1	Kinase suppressor of ras 1
EGFR	Epidermal growth factor receptor	MAP2K1	Mitogen-activated protein kinase kinase 1
EGR1	Early growth response 1	LAMTOR3	Late endosomal/lysosomal adaptor, MAPK and MTOR activator 3
ELK1	ELK1, member of ETS oncogene family	MAP2K2	Mitogen-activated protein kinase kinase 2
ETS1	V-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	MAP2K3	Mitogen-activated protein kinase kinase 3
ETS2	V-Ets erythroblastosis virus E26 oncogene homolog 2 (avian)	MAP2K4	Mitogen-activated protein kinase kinase 4
FOS	FBJ murine osteosarcoma viral oncogene homolog	MAP2K5	Mitogen-activated protein kinase kinase 5
GRB2	Growth factor receptor-bound protein 2	MAP2K6	Mitogen-activated protein kinase kinase 6
HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog	MAP2K7	Mitogen-activated protein kinase kinase 7
HSPA5	Heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	MAP3K1	Mitogen-activated protein kinase kinase kinase 1
HSPB1	Heat shock 27kDa protein 1	MAP3K2	Mitogen-activated protein kinase kinase kinase 2
JUN	Jun proto-oncogene	MAP3K3	Mitogen-activated protein kinase kinase kinase 3

*HUGO Database Gene Nomenclature.

Table 10A. Pathway-focussed gene arrays: MAPK pathway (continued)

Gene Symbol*	Description	Gene Symbol*	Description
MAP3K4	Mitogen-activated protein kinase kinase kinase 4	MAPK9	Mitogen-activated protein kinase 9
MAP4K1	Mitogen-activated protein kinase kinase kinase kinase 1	MAPKAPK2	Mitogen-activated protein kinase-activated protein kinase 2
MAPK1	Mitogen-activated protein kinase 1	MAPKAPK3	Mitogen-activated protein kinase-activated protein kinase 3
MAPK10	Mitogen-activated protein kinase 10	MAX	MYC associated factor X
MAPK11	Mitogen-activated protein kinase 11	MEF2C	Myocyte enhancer factor 2C
MAPK12	Mitogen-activated protein kinase 12	MKNK1	MAP kinase interacting serine/threonine kinase 1
MAPK13	Mitogen-activated protein kinase 13	MOS	V-mos Moloney murine sarcoma viral oncogene homolog
MAPK14	Mitogen-activated protein kinase 14	MST1	Macrophage stimulating 1 (hepatocyte growth factor-like)
MAPK3	Mitogen-activated protein kinase 3	MYC	V-myc myelocytomatosis viral oncogene homolog (avian)
MAPK6	Mitogen-activated protein kinase 6	NFATC4	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4
MAPK7	Mitogen-activated protein kinase 7	NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
MAPK8	Mitogen-activated protein kinase 8	PAK1	P21 protein (Cdc42/Rac)-activated kinase 1
MAPK8IP2	Mitogen-activated protein kinase 8 interacting protein 2	PRDX6	Peroxiredoxin 6

*HUGO Database Gene Nomenclature.

Table 10A. Pathway-focussed gene arrays: MAPK pathway (continued)

Gene Symbol*	Description	Gene Symbol*	Description
RAC1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	SFN	Stratifin
RAF1	V-raf-1 murine leukemia viral oncogene homolog 1	SMAD4	SMAD family member 4
RB1	Retinoblastoma 1	TP53	Tumor protein p53

*HUGO Database Gene Nomenclature.

Table 10B. Pathway-focussed gene arrays: NF-κB pathway

Gene Symbol*	Description	Gene Symbol*	Description
AGT	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	CCL5	Chemokine (C-C motif) ligand 5
AKT1	V-akt murine thymoma viral oncogene homolog 1	CD27	CD27 molecule
ATF1	Activating transcription factor 1	CD40	CD40 molecule, TNF receptor superfamily member 5
BCL10	B-cell CLL/lymphoma 10	CFLAR	CASP8 and FADD-like apoptosis regulator
BCL2A1	BCL2-related protein A1	CHUK	Conserved helix-loop-helix ubiquitous kinase
BCL2L1	BCL2-like 1	CSF1	Colony stimulating factor 1 (macrophage)
BCL3	B-cell CLL/lymphoma 3	CSF2	Colony stimulating factor 2 (granulocyte-macrophage)
BIRC2	Baculoviral IAP repeat containing 2	CSF3	Colony stimulating factor 3 (granulocyte)
BIRC3	Baculoviral IAP repeat containing 3	EGFR	Epidermal growth factor receptor
CARD11	Caspase recruitment domain family, member 11	EGR1	Early growth response 1
CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	ELK1	ELK1, member of ETS oncogene family
CASP8	Caspase 8, apoptosis-related cysteine peptidase	F2R	Coagulation factor II (thrombin) receptor
CCL2	Chemokine (C-C motif) ligand 2	FADD	Fas (TNFRSF6)-associated via death domain

*HUGO Database Gene Nomenclature.

Table 10B. Pathway-focussed gene arrays: NF-κB pathway (continued)

Gene Symbol*	Description	Gene Symbol*	Description
FASLG	Fas ligand (TNF superfamily, member 6)	IL8	Interleukin 8
FOS	FBJ murine osteosarcoma viral oncogene homolog	IRAK1	Interleukin-1 receptor-associated kinase 1
HMOX1	Heme oxygenase (decycling) 1	IRAK2	Interleukin-1 receptor-associated kinase 2
ICAM1	Intercellular adhesion molecule 1	IRF1	Interferon regulatory factor 1
IFNA1	Interferon, alpha 1	JUN	Jun proto-oncogene
IFNG	Interferon, gamma	LTA	Lymphotoxin alpha (TNF superfamily, member 1)
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	LTBR	Lymphotoxin beta receptor (TNFR superfamily, member 3)
IKBKE	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	MALT1	Mucosa associated lymphoid tissue lymphoma translocation gene 1
IKBKG	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	MAP3K1	Mitogen-activated protein kinase kinase kinase 1
IL10	Interleukin 10	MYD88	Myeloid differentiation primary response gene (88)
IL1A	Interleukin 1, alpha	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
IL1B	Interleukin 1, beta	NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)
IL1R1	Interleukin 1 receptor, type I	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

*HUGO Database Gene Nomenclature.

Table 10B. Pathway-focussed gene arrays: NF-κB pathway (continued)

Gene Symbol*	Description	Gene Symbol*	Description
NFKBIB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	TICAM2	Toll-like receptor adaptor molecule 2
NFKBIE	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	TIMP1	TIMP metallopeptidase inhibitor 1
NOD1	Nucleotide-binding oligomerization domain containing 1	TLR1	Toll-like receptor 1
PSIP1	PC4 and SFRS1 interacting protein 1	TLR2	Toll-like receptor 2
RAF1	V-raf-1 murine leukemia viral oncogene homolog 1	TLR3	Toll-like receptor 3
REL	V-rel reticuloendotheliosis viral oncogene homolog	TLR4	Toll-like receptor 4
RELA	V-rel reticuloendotheliosis viral oncogene homolog A	TLR6	Toll-like receptor 6
RELB	V-rel reticuloendotheliosis viral oncogene homolog B	TLR9	Toll-like receptor 9
RHOA	Ras homolog gene family, member A	TNF	Tumor necrosis factor
RIPK1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
STAT1	Signal transducer and activator of transcription 1, 91kDa	TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a
TBK1	TANK-binding kinase 1	TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b
TICAM1	Toll-like receptor adaptor molecule 1	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A

*HUGO Database Gene Nomenclature.

Table 10B. Pathway-focussed gene arrays: NF-κB pathway (continued)

Gene Symbol*	Description	Gene Symbol*	Description
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	TRAF2	TNF receptor-associated factor 2
TNFSF14	Tumor necrosis factor (ligand) superfamily, member 14	TRAF3	TNF receptor-associated factor 3
TRADD	TNFRSF1A-associated via death domain	TRAF6	TNF receptor-associated factor 6

*HUGO Database Gene Nomenclature.
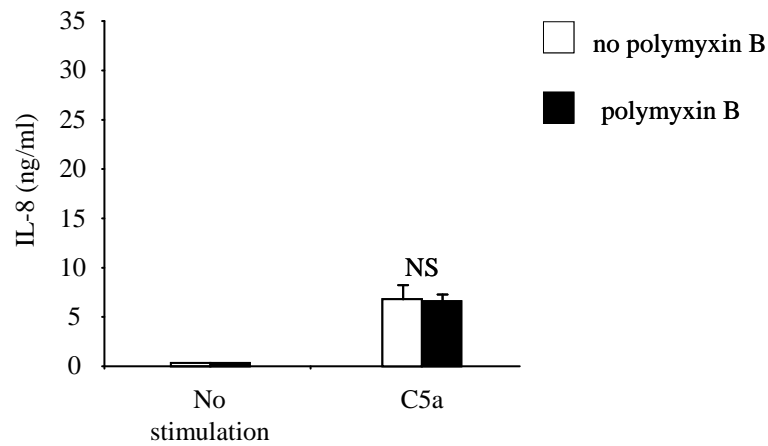
Figure 40. Effect of polymyxin B on sensitivity of PBMC to purified natural human C5a. Mean (±SD) IL-8 released by human PBMC (1.5 x 10^5/well) stimulated (14 h) with the indicated concentrations of purified natural human C5a. Data are shown from one experiment representative of three. (NS, not significant)
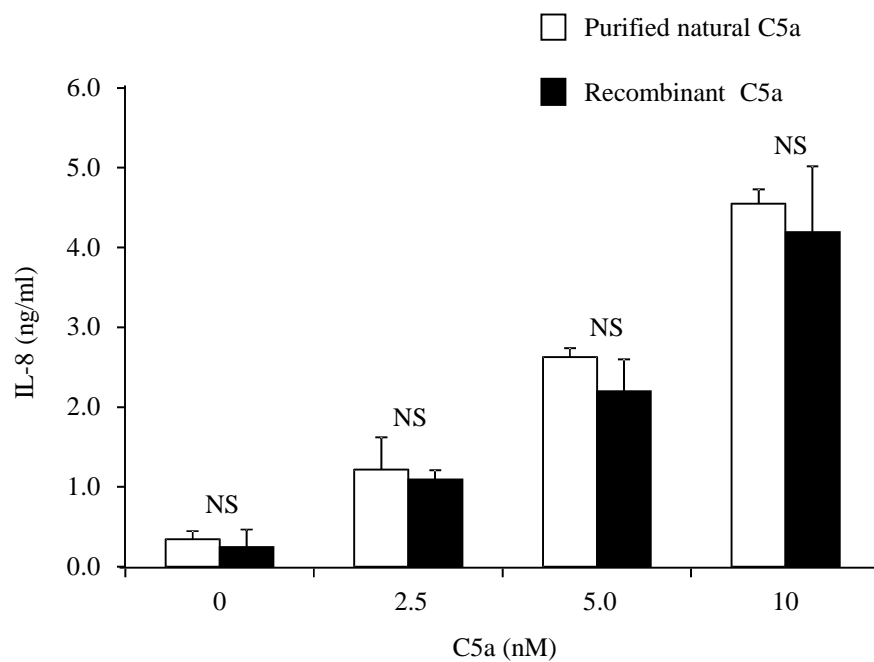
Figure 41. Relative potencies of recombinant and purified natural human C5a. Mean (±SD) IL-8 released by human PBMC (1.5 x 10^5/well) stimulated (14 h) with the indicated concentrations of recombinant or purified natural human C5a. Data are shown from one experiment representative of three. (NS, not significant).
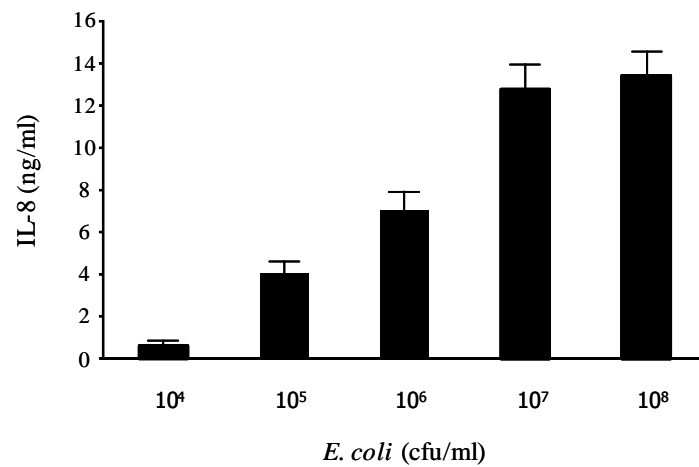
Figure 42. Optimisation of bacterial stimulating concentration. Representative example of titration experiments to establish sub-maximal stimulating concentrations of heat-killed *E. coli*. Whole blood (100 μl/well) from a sepsis patient (corresponding to one of the patients whose data are shown in Figure 36) was washed and re-suspended in complete medium before stimulation (14h) with heat-killed *E. coli* at the indicated concentrations. Mean (±SD) IL-8 concentration in culture supernatants is shown. A sub-maximal E. coli concentration of 2 x 10^4 cfu/ml was selected for the experiment
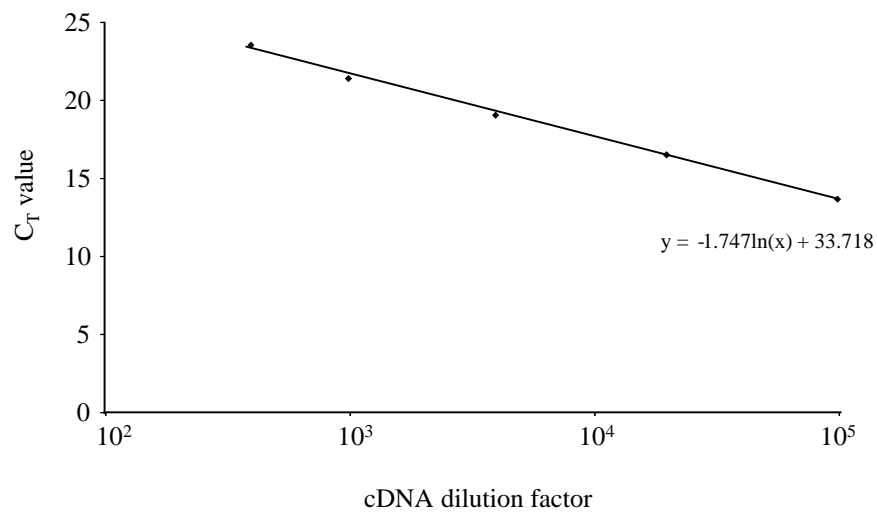
Figure 43. IL-8 primer efficiency titration. C_T values against cDNA concentrations for RT-qPCR conducted on serial dilutions of cDNA transcripts (10^0 indicates undiluted) of mRNA extracted from PBMC (1×10^6 cells) stimulated with LPS (10 ng/ml) for 14 h. Primer efficiency is calculated as ($10^{(-1/\ln(10) \times (\text{gradient}))}$) x 100 = 77% for this primer pair.
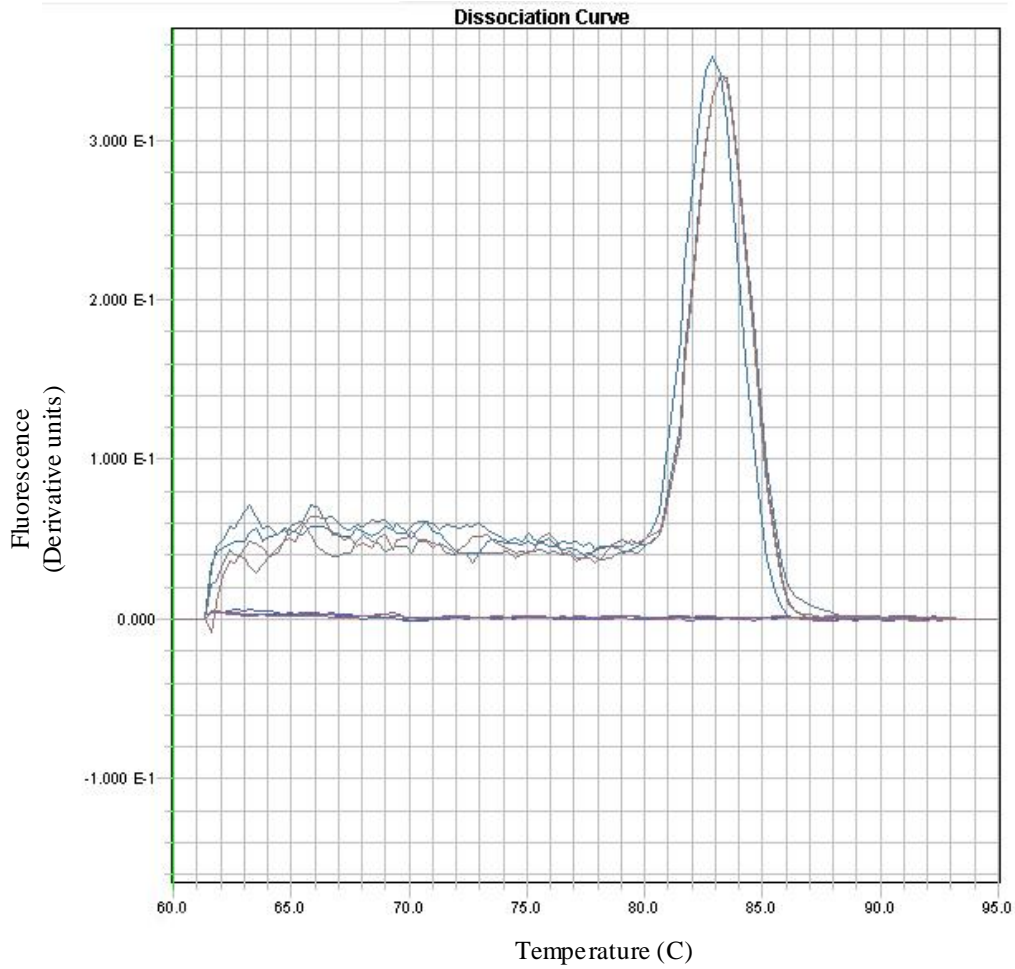
Figure 44. Dissociation curve of β-arrestin 1 amplicon. Representative dissociation curve of the β-arrestin 1 amplicon (typical of all primer pairs) after RT-qPCR performed on cDNA (500 ng) extracted from PBMC (1×10^6 per condition) pre-exposed or not to LPS (500 pg/ml, 14 h) and stimulated or not with C5a (1 nM, 4h). Data correspond to the experiment shown in Figure 35, and genomic DNA controls.
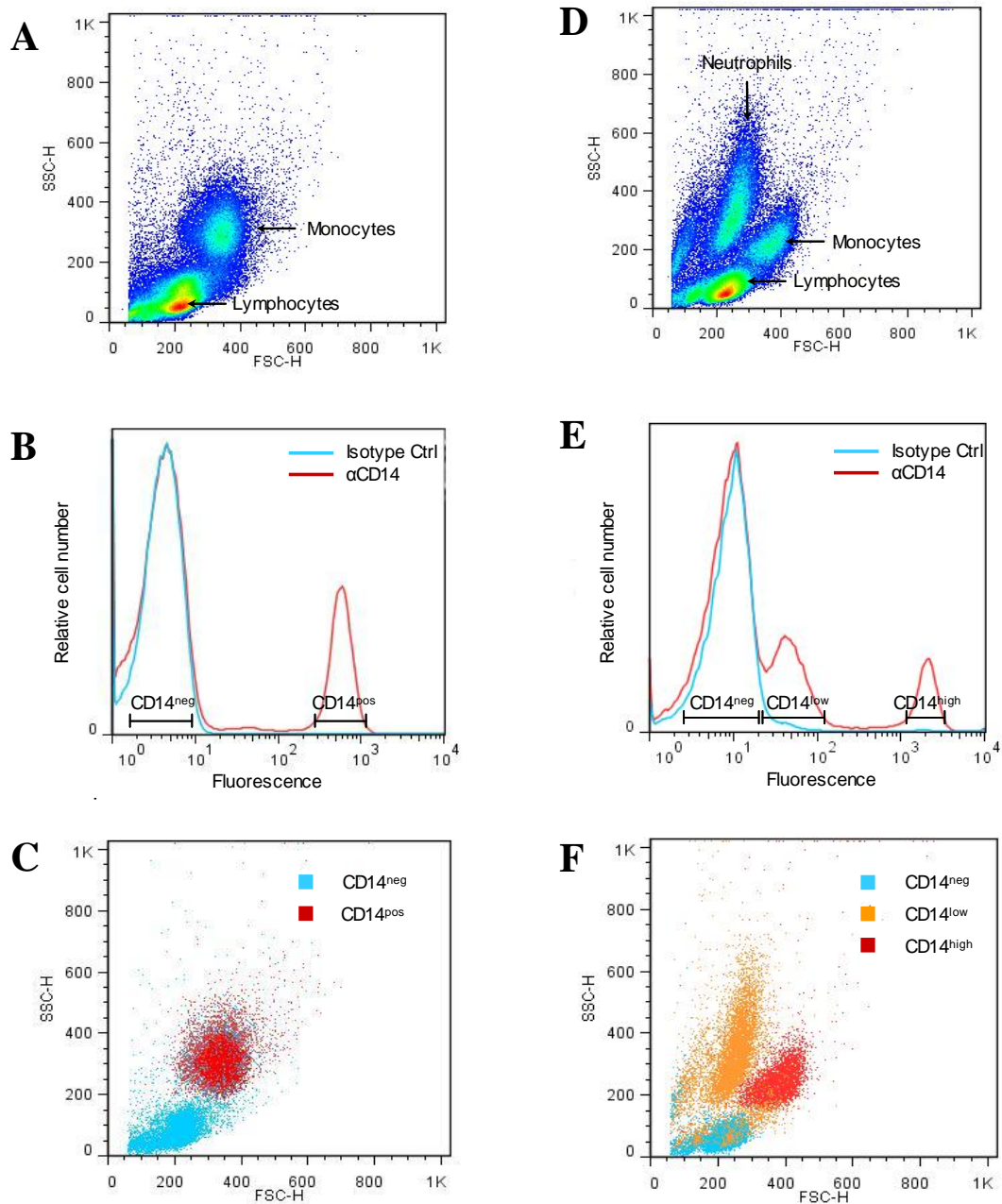
Figure 45. Flow cytometric gating strategies. Identification of the monocyte population in PBMC **(A-C)** and neutrophil and monocyte populations in aliquots of whole blood after red cell lysis, **(D-F)**, on the basis of forward and side-scatter **(A and D)** and CD14 staining profiles **(B and E)**. Projection of cell populations identified as expressing CD14 onto scatter cytograms confirms their identification as monocytes **(C)** and neutrophils **(F)** respectively**.** One representative example of each of the flow cytometric experiments conducted on PBMC (Figures 28A and 29) and whole blood (28B and 37B) is shown.
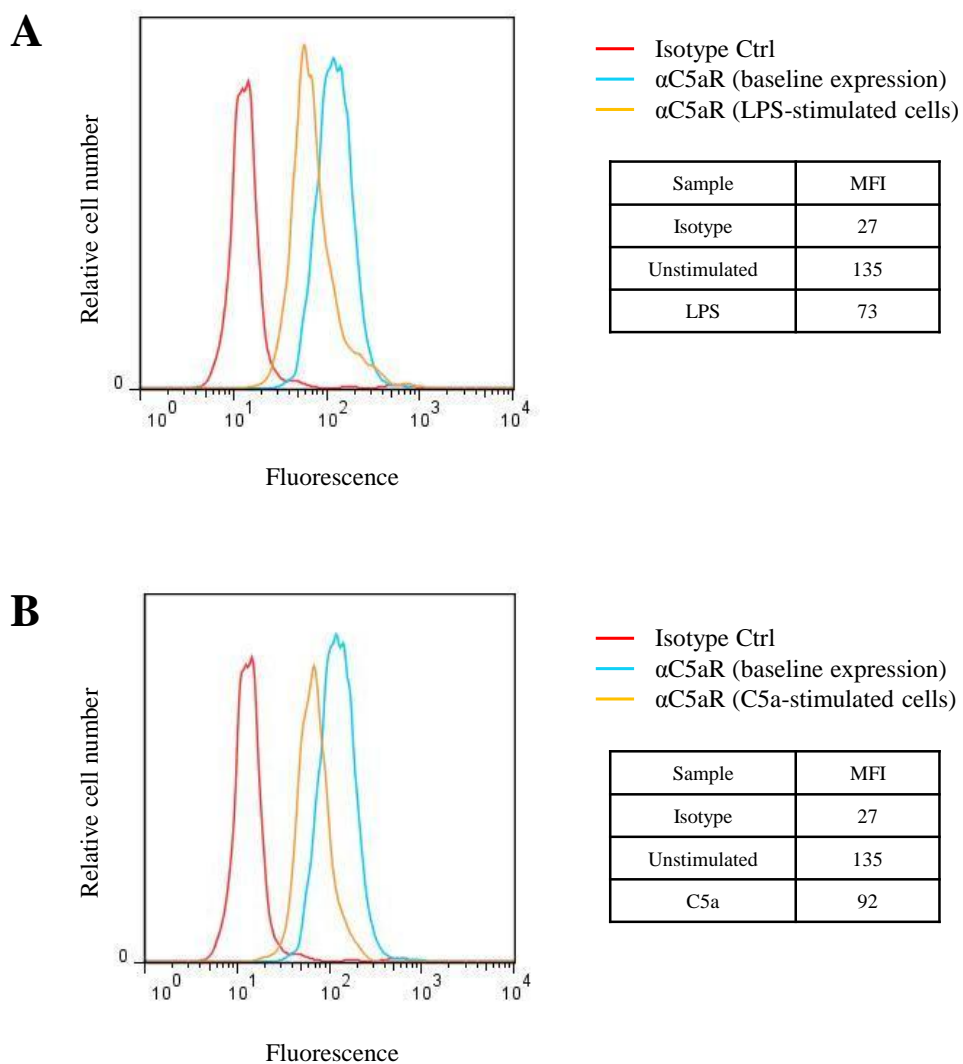
Figure 46. Representative examples of fluorescence histograms from experiments showing C5aR expression (Figure 28). C5aR expression on gated neutrophils from whole blood samples as shown in Figure 28. Figures shown correspond to the MFI data presented for C5aR expression in unstimulated neutrophils at time zero compared with cells pre-exposed to LPS **(A)** and compared with cells not pre-exposed to LPS and stimulated with C5a for 6h **(B)** after background fluorescence subtraction.

	Sample	MFI
	No Stimulation	467
	C5a 10nM 15s	1007
	C5a 10nM 30s	1097
	C5a 10nM 60s	1092
	C5a 10nM 90s	1063
	C5a 10nM 120s	1006
	C5a 10nM 150s	986
	C5a 10nM 180s	967
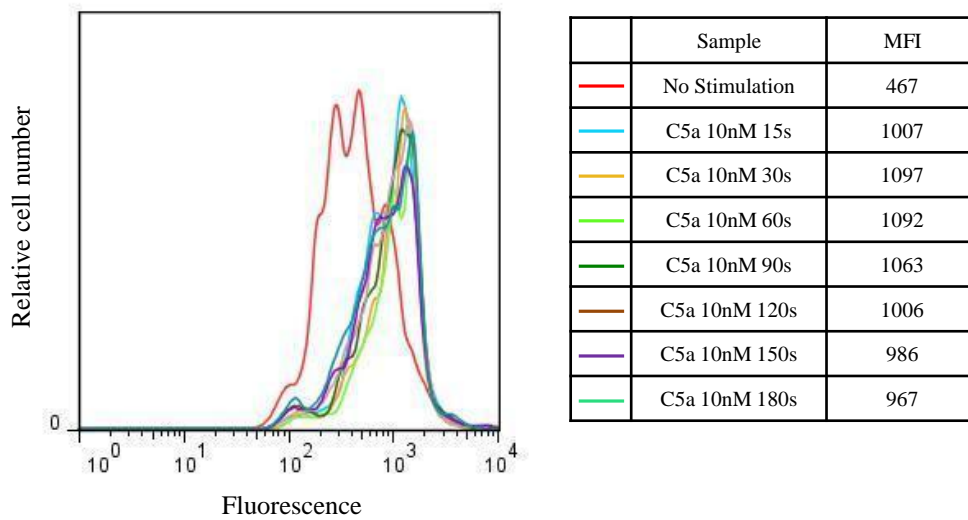
Figure 47. Calcium signalling assay. Representative example of fluorescence histogram from calcium signalling experiments shown in Figure 29. PBMC were loaded with Fluo3-AM and fluorescence was measured by flow cytometry on gated monocytes at baseline and at the indicated intervals after addition of C5a (10 nM) as described in Figure 29. MFI at each time point was divided by MFI at time 0 (unstimulated cells) to produce an estimate of normalised Ca^{2+} flux $[Ca^{2+}]_i$ at each time point.
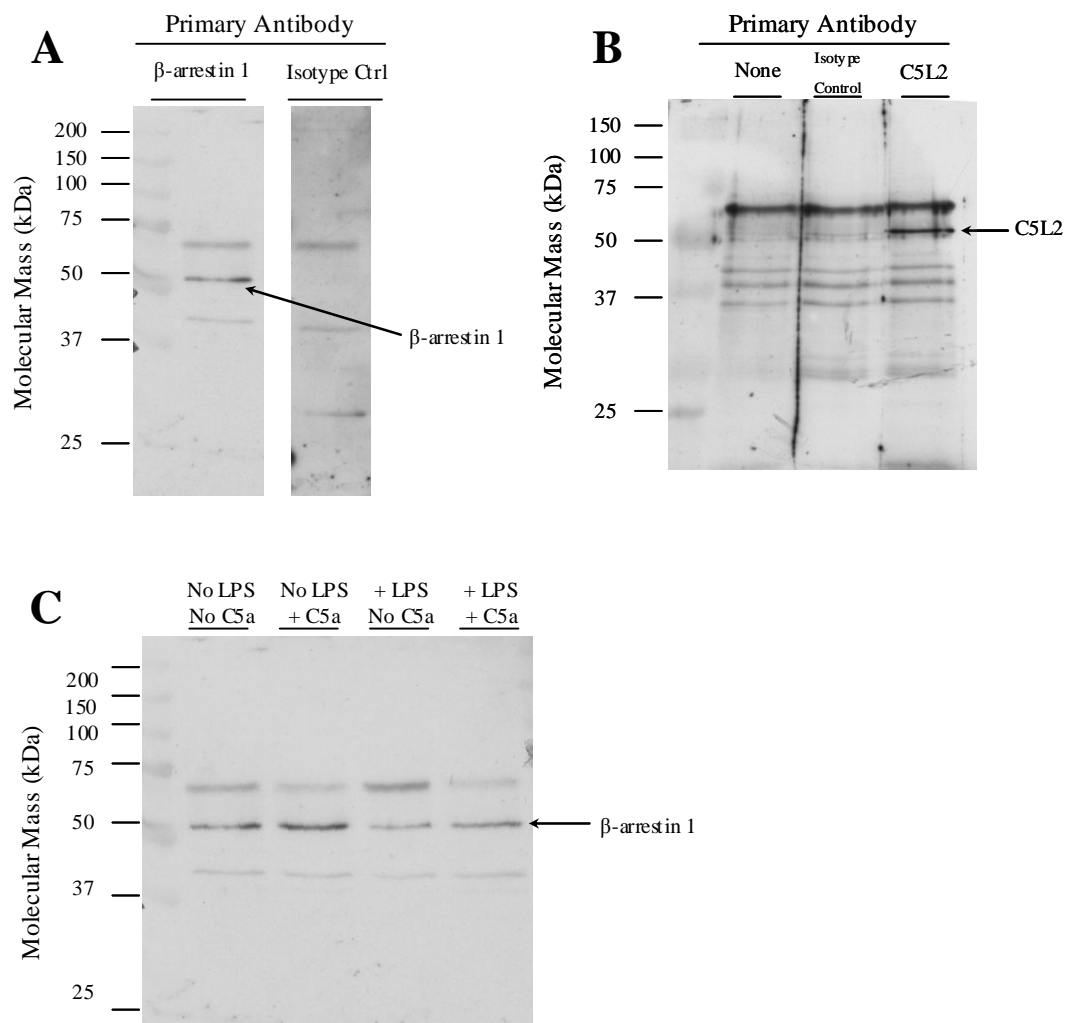
Figure 48. Control experiments for Western blots. Representative examples of Western blots showing the specificity of monoclonal antibodies used to detect **(A)** β-arrestin 1 and **(B)** C5L2 in cell lysates of PBMC. Equal aliquots of the same cell lysate were run in the same gel and, after electrotransfer, the membranes were incubated with the relevant primary antibody or isotype control (IgG1, clone MOPC-21 for β-arrestin 1 and IgG2a, clone UPC-10 for C5L2) as shown above before washing, incubation with identical secondary antibodies and ECL as described in section 5.8. A representative example of a full-length Western blot for β-arrestin 1 **(C)** is also shown (a duplicate experiment of that shown in Figure 35).

Chapter 7: REFERENCES

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