5 Signatures of the streptomycin mouse model: the complement system in mucosal *S.* Typhimurium infection

5.1 Introduction

5.1.1 The complement system in the immune response to infection

The complement system is a collection of > 30 proteins, including both soluble proteins activated through a cascade of proteolytic activity, and receptors for detection of activated complement protein fragments. Three pathways of activation converge to generate effector functions essential in immunity to many common pathogens. As a powerful defence mechanism tight controls upon the complement system prevent excessive activation and collateral damage to the host. The system is evolutionarily ancient and many functions in innate immunity have been gradually uncovered [274]. Complement has also been shown to participate in the stimulation of adaptive immunity [275]. Complement proteins are present at high concentrations in blood plasma and are produced rapidly in the acute phase response (APR) to inflammation; consequently the liver was originally thought to be the sole producer of biologically significant levels of complement proteins. However recent findings suggest a role for locally produced complement in inflammation and infection.

5.1.1.1 Three complement activation pathways converge upon a C3 convertase

The complement system undergoes activation via three pathways and activation by different proteins facilitates responses to a wide range of pathogen triggers. The first pathway to be described, known as the 'classical pathway', is founded around a complex of three complement proteins, C1q, C1r and C1s, that are together called the C1 complex. Upon binding of C1q to the Fc region of antigen-bound 'complement-fixing' antibodies (primarily IgG and IgM subclasses), the C1 complex serine proteases C1r and C1s are autocatalytically activated. Protease activation leads to cleavage of complement proteins C2 and C4. The larger fragments of protein resulting from these cleavage reactions associate to form the complex C4bC2a. Described as a C3 convertase, C4bC2a cleaves C3 into 9 kDa and 185 kDa fragments; C3a and C3b respectively.

The lectin pathway is similar to the classical pathway although dependent upon PRRs rather than antibodies for the initial triggering of serine protease activity. Upon recognition of

PAMPs, mannose binding lectin (MBL) and other lectins associate with serine proteases analogous to those in the C1 complex of the classical pathway. Here also serine protease activation leads to C2 and C4 cleavage, and formation of the C3 convertase C4bC2a.

The alternative pathway is mechanistically different to the classical and lectin pathways, and leads to formation of an alternative C3 convertase. Spontaneous hydrolysis of C3 produces an activated thioester bond to which the protein Factor B binds, resulting in cleavage of Factor B into fragments Bb and Ba catalysed by Factor D. The Bb fragment bound to hydrolysed C3 possesses C3 convertase activity, cleaving C3 into C3a and C3b. Subsequently where C3b associates with pathogen surfaces it binds to Bb forming C3bBb, the predominant convertase of the alternative pathway. C3bBb requires stabilisation by an additional factor called properdin or Factor P. The alternative pathway is considered a dual system with a role as a recognition and activation pathway similar to the classical and lectin pathways, and a role as an amplification system for these [276].

The three activation pathways described above are represented in the upper part of Figure 5.1. Whilst the initial triggers and complement proteins involved are different, all three pathways result in formation of a C3 convertase complex for cleavage of C3 to C3a and C3b fragments. Cleavage of C3 initiates the three major effector arms of the complement response; mediated by the membrane attack complex (MAC), opsonins, and anaphylotoxins.

5.1.1.2 The three major effector functions of the complement system

The MAC is an organised assembly of activated complement proteins, capable of forming a stable pore of up to 10 nm in diameter in bacterial outer membranes. Pore formation destroys the osmotic stability of the target, inducing cell lysis. Upon cleavage of C3, the C3b fragment is deposited on bacterial cell membranes where it interacts with C4bC2a and C3bBbC3b, forming C5 convertases. C5 cleavage exposes a binding site for C6, leading to a C5bC6 complex which serves as the foundation for pore assembly. Integration of the complement protein complex into the phospholipid bilayer occurs upon joining of C7, and fully stable pore formation is completed upon association of further complement proteins C8α, C8β and C9.

Host cell complement receptors enable phagocytes to bind and ingest material bound by activated complement fragment opsonins. Complement receptors on erythrocytes are

important for transport of immune complexes to the macrophage system for clearance, while receptors on neutrophils and monocytes mediate phagocytosis on binding of complement protein fragments C4b, C3b, iC3b, C3dg and C1q. In addition to directly activating phagocytes through complement receptors, complement on pathogen surfaces provides sites for antibody binding to further encourage clearance.

The complement protein fragments C3a, C4a, and C5a are collectively called anaphylotoxins and act as potent pro-inflammatory signalling molecules. Highly homologous in amino acid sequence, their many overlapping functions include promoting release of other inflammatory mediators to amplify the response, increasing vascular permeability, inducing smooth muscle contraction, and recruitment of leukocytes.

In addition to the innate immune functions described, more recent evidence demonstrates the importance of complement for inducing adaptive immunity, both in B and T cell-mediated processes [275]. Studies in which mice were depleted of systemic C3 demonstrated that complement is involved in shaping the B cell repertoire [277]. Multiple mechanisms linking complement proteins and B-lymphocytes have now been uncovered. The formation of a complex between the B cell receptor (BCR), complement receptor 2 and C3 activation fragments has been shown to influence several aspects of B cell biology, in particular by substantially lowering the threshold required for B-lymphocyte stimulation through the BCR [278]. Complement interaction with T cells has also been demonstrated however the mechanisms are less well understood than for B cells [279].

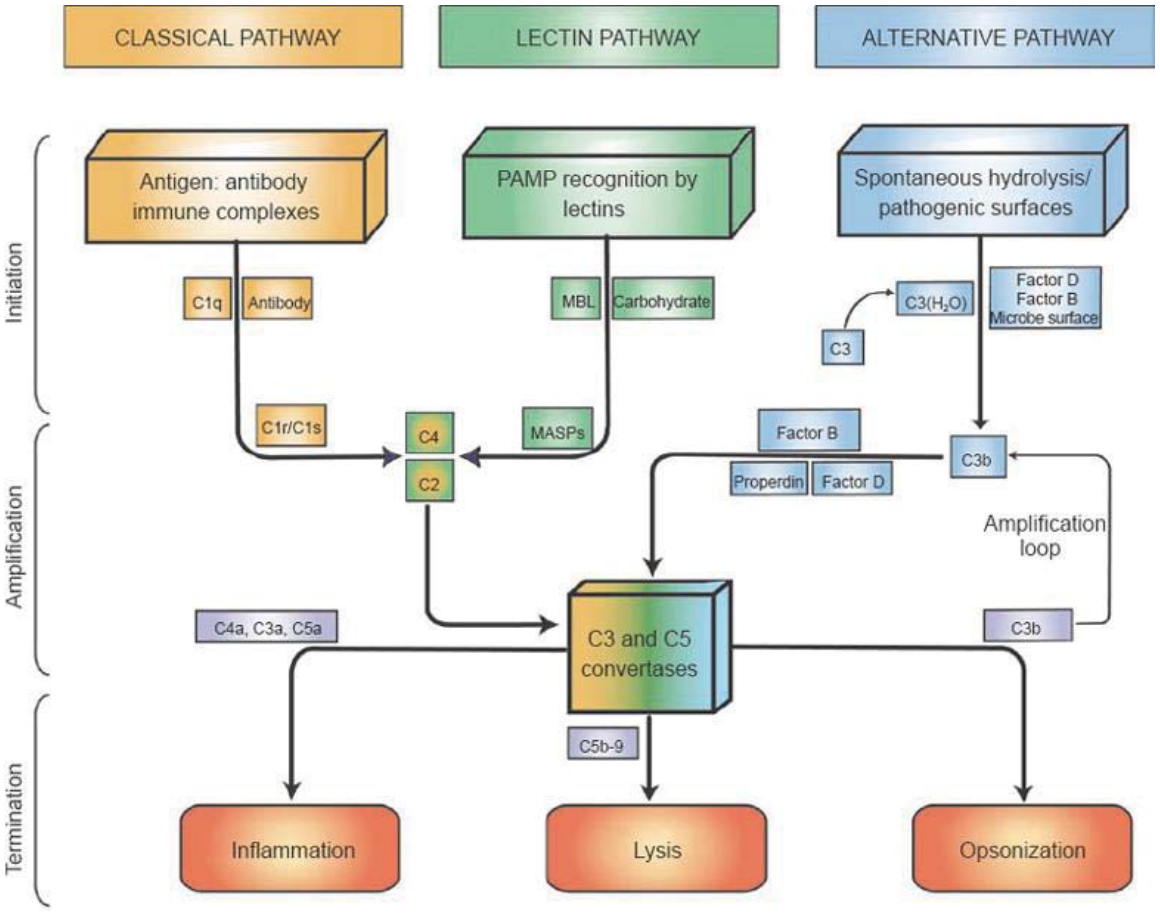


Figure 5.1. Complement activation pathways and major effector functions of activated complement. Three pathways of complement activation, the classical, lectin and alternative pathways converge in formation of a C3 convertase, leading to three major effector functions. In the classical pathway C1q in the C1 complex binds the Fc region of antigen-bound antibody, activating the serine proteases C1r and C1s to cleave C2 and C4. Activation of the lectin pathway is initiated by binding of MBL to pathogen surface carbohydrate motifs, activating the MBL-associated serine proteases (MASPs), again leading to cleavage of C2 and C4. Finally the alternative pathway is initiated by spontaneous hydrolysis of C3, an early C3 convertase forming as a result of cleavage of Factor B by the constitutively active serum protease Factor D, stabilised by properdin. C3b formed by this convertase binds available Factor B creating new C3bBb in a positive feedback loop. The three effector functions triggered by C3 convertase production are performed by different collections of complement proteins. Small protein fragments produced in complement cleavage; C3a, C4a and C5a; are potent inflammatory signalling molecules. C5b-9 associate to form a membrane-spanning pore for target cell lysis and opsonins such as C3b bind surfaces of foreign targets to facilitate phagocytosis and antibody binding. Figure taken from [280].

5.1.1.3 Regulation of the complement cascade

Tight control of the complement pathway is required to ensure the restriction of activation to surfaces of foreign cells, and generation of a proportionate response rapidly terminated on resolution of infection to prevent damage to nearby host cells. Regulatory

mechanisms act at multiple stages to inhibit the complement cascade, examples of which are outlined in Figure 5.2.

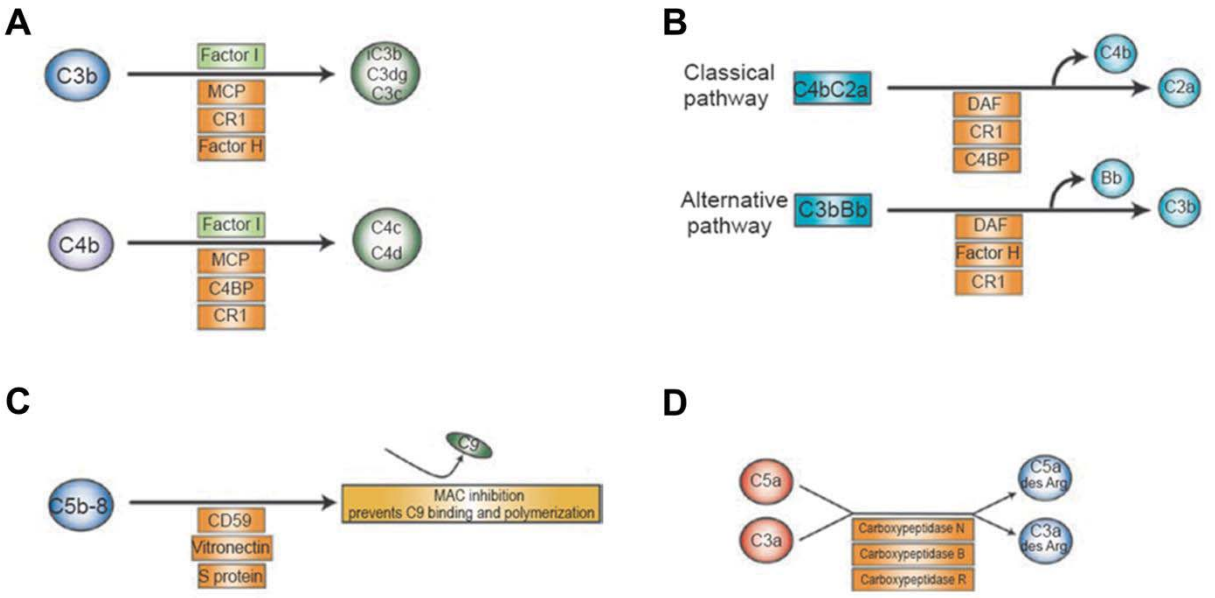


Figure 5.2. Complement activity is controlled by multiple regulatory mechanisms. (A) Serine protease Factor I cleaves complement activation products C3b and C4b into inactive fragments in the presence of proteins which identify host cells; including host cell membrane intrinsic proteins such as MCP, and soluble proteins which bind components specific to host membranes such as Factor H. Cleavage prevents the formation of the C3 convertase on host cells. (B) Convertase inhibitors and decay accelerating factors inhibit assembly and shorten the half-life of existing convertases. (C) During extensive complement activation MAC inhibitors limit complex assembly and pore formation. (D) Serum carboxypeptidases remove N-terminal arginine residues from anaphylotoxins when these are no longer required to limit their interaction with cognate receptors.

5.1.1.4 Proteolytic cleavage of the major complement protein C3

C3 is central to the three complement activation pathways and therefore critical to the effector functions of the complement system, making it an important target of complement regulatory mechanisms as outlined above. C3 is also the most highly abundant complement protein, present in blood plasma from healthy individuals at around 2 mg/ml. The proteolytic processes which act upon C3 during complement activation have been studied in detail. Awareness of the functional roles of C3 fragments, generated during processing both to activate and terminate C3 activity, is essential in order to understand the impact of C3 in a particular tissue or condition. Figure 5.3 outlines the steps involved in the processing of C3 and the activities of the intermediate forms produced.

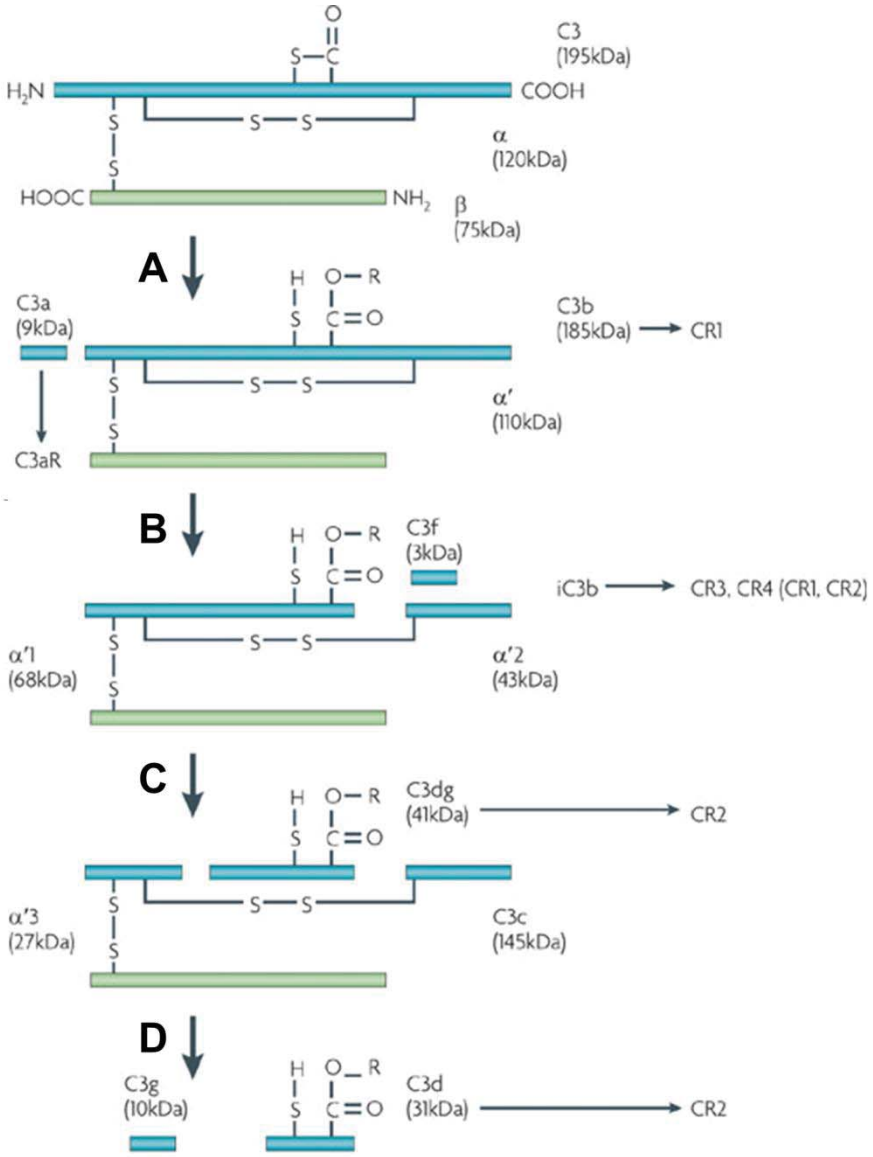


Figure 5.3. Proteolytic processing of C3 is important for both activation and termination of effector functions. Circulating C3 is constructed of disulphide bonded α and β chains. C3 convertase activity in (A) produces the 9 kDa anaphylotoxin C3a, and the larger fragment C3b with an exposed thioester bond susceptible to hydrolysis or bonding with surfaces including those of microbes. C3b interacts with Factor B to stimulate the alternative pathway, with complement receptor 1 (CR1) for activity as an opsonin, or with C3 convertases to form C5 convertases. When inhibition of complement activity is required C3b undergoes cleavage by Factor I to release the small fragment C3f and produce iC3b. Although unable to form convertases, the cleavage product iC3b retains the ability to be bound by complement receptors to mediate phagocytosis of complement-coated pathogens. In (C) Factor I mediates further degradation of iC3b to C3c which is released and C3dg, which remains bound to antigen and interacts with B cells and dendritic cells through CR2. Finally, inflammatory proteases mediate degradation of C3dg to C3g, which is released while C3d remains bound to antigen and, like C3dg, also mediates activation though CR2. Figure adapted from [281].

5.1.1.5 Complement control of *Salmonella*

Both cell-free complement-mediated killing and opsonization of bacteria by complement fragments have been shown to be important in the control of *Salmonella* infection in certain contexts. A study into the mechanisms controlling *S.* Typhimurium in human blood investigated the kinetics of complement activation, terminal complex formation and phagocytosis. Following rapid opsonization of *Salmonella* by C3 and specific antibodies phagocytosis was shown to be initiated immediately whilst the onset of terminal pathway activity is more delayed. This work suggests that slow formation of the membrane attack complex results in significant numbers of bacteria avoiding complement-mediated lysis, instead being taken up by phagocytes including macrophages and transported to an intracellular environment more favourable for their survival [282]. A further discovery was the requirement of *Salmonella*-specific antibody for complement mediated killing mechanisms. This finding is in line with work showing specific antibody is essential for protection of African children against iNTS infection, in part explaining the observed age distribution of childhood iNTS [283].

Studies have shown that the structure of the *Salmonella* O surface antigen is important in determining the rate at which activation of the alternative complement pathway, and subsequent opsonization of *Salmonella* by C3 occurs. In mice infected with *S.* Typhimurium by IP injection more rapid opsonization of bacteria in an O-antigen dependent manner leads to faster uptake of *Salmonella* by phagocytic cells, resulting in enhanced control of infection. This effect is absent when *Salmonella* are delivered intravenously due to the large excess of C3 in the blood [284, 285].

Important differences between humans and mice have been demonstrated in activity of the complement cascade. Over half a century ago it was shown that mouse serum lacks bactericidal activity against many microorganisms effectively killed by complement-dependent lysis following treatment with serum from humans or other mammals [286]. Recent work to investigate the absence of *Salmonella* killing by mouse serum demonstrated restoration of bactericidal activity by the addition of human complement factors, showing that the lack of killing arises from differences in the complement factors themselves rather than the *Salmonella*-specific antibody response [287]. Despite the apparent absence of cell-free complement-mediated killing in mice, $C1qa^{-/-}$ mice are less able to control *S.* Typhimurium following IP and intravenous infection, indicating that other effector mechanisms mediated by

the classical complement activation pathway are important in the murine response to *Salmonella* infection [288]. Of note, differences in the degree of opsonization of *Salmonella* by complement may contribute to observed mouse strain-specific differences in control of *Salmonella* [289].

5.1.1.6 Local complement protein synthesis

The high concentrations of complement proteins in plasma and rapid stimulation of production in the APR led to the view of complement as a systemic system of proteins synthesised in the liver [290]. However, studies demonstrating the presence of complement proteins at sites where penetration of plasma is poor have suggested that local synthesis of complement may also be important, opening a new area of research. A huge range of cell types have been shown to be capable of synthesising complement components *in vitro*, for example production of MAC has been demonstrated in cultured monocytes [291]. Yet the *in vivo* contribution of individual cell types and the functional importance of complement produced outside the liver is poorly understood. In strong support of local complement synthesis is a study finding intense staining for complement in a type of glial cell in and around areas of pathology in inflammatory brain disease. A hepatic origin for complement here is unlikely due to exclusion of plasma by the blood brain barrier [292]. Multiple studies also support the kidney as a site of local complement production with kidney cell types shown to produce complement during inflammation, in addition to infiltrating monocytes and macrophages [293]. Local complement production has also been implicated in the autoimmune condition rheumatoid arthritis [294].

5.1.1.7 The role of complement in the gastrointestinal mucosa

Relatively little work has addressed the possibility of local complement production in the intestinal mucosa, or has investigated the function of intestinal complement in health and disease. Evidence for local production of complement in the intestine is largely provided by IBD studies, with the effect of bacterial infection upon complement production receiving minimal attention [295]. Two studies of complement in the host response to *C. rodentium* infection described later in this section find an important protective role. However more work is needed to understand how these findings relate to enteric infections more generally.

Support for the local production of complement proteins in the intestinal mucosa is reviewed in [295]. Briefly, several studies have provided evidence for expression of complement components at the transcriptional level using immortalised intestinal cell lines, primary cells extracted from intestinal tissue and human tissue biopsies [296, 297]. Further studies confirm the presence of complement pathway proteins in intestinal cells or tissue, including [298] (MBP), [299] (C3b and MAC proteins) and [300] (C3 and C3d). The presence of MAC components in intestinal tissue is a subject of conflicting reports. However, the majority of studies which address this topic report MAC proteins are not detected, and as such lytic functions of complement do not play a major role in the mucosa. Local production of MAC proteins has been detected at extraintestinal locations [301]. In contrast to terminal pathway components, the presence of both C3 and C3 activation fragments is relatively well supported, as is increased concentrations of these proteins in IBD.

Despite the studies described above the precise cellular sources of intestinal complement proteins *in vivo* remain unresolved. During infection and inflammation vascular permeability is increased and the normal barrier function of the epithelium impaired. Passive leakage of serum proteins, including those of the complement cascade, into surrounding tissue and the gut lumen is likely. Further work is required to determine the contributions of serum complement leakage and active intestinal protein production toward the total complement protein present. Further, complement proteins are produced by infiltrating leukocytes, for example properdin is stored in neutrophil granules for release on cytokine or anaphylotoxin stimulation, and the relative contribution of complement produced by immune cells is also an important question [302].

Whilst studies of complement in IBD are in agreement regarding increased production of complement proteins in inflammation, both protective roles and detrimental effects of complement have been proposed. For example *C3*$^{-/-}$ mice were protected against DSS-induced colitis, and deficiencies in the anaphylotoxin receptors C3aR and C5aR were also protective [303, 304]. Conversely C5aR deficiency was shown to result in more severe disease in a DSS colitis chronic inflammation model. Similarly C5-deficient mice also displayed more extensive inflammation compared with wild type controls in DSS-induced colitis [305, 306]. Deficiencies in early complement pathway proteins (C1 - C4) are associated with inflammation and autoimmune disease as a result of defective immune complex clearance

[307]. Additional work is required to understand the basis of these seemingly contradictory findings.

The relative importance of the three major complement activation pathways in the intestine is also unknown. Factor B is one of the most transcriptionally-induced proteins in epithelial cells in IBD, suggesting the alternative pathway may be important under inflammatory conditions [308]. The interaction of IgG antibodies with C3-coated bacteria in [309] indicates the classical activation pathway may also be important.

Two published works have investigated the role of complement in infection with an intestinal pathogen, *C. rodentium*. Both studies suggest the presence of complement-mediated protective effects. The earlier of these studies indicates a potential role for luminal complement in conjunction with complement fixing-isotypes of IgG. Binding of C3 and serum IgG to *C. rodentium* in the intestinal lumen was demonstrated by flow cytometry, and colocalisation of C3b and IgG at the epithelial surface of the mucosa by immunofluorescence staining. Finding C3 and IgG inhibited the growth of *C. rodentium in vitro* led to a model in which classical pathway activation leads to opsonization of *C. rodentium* by complement, in turn directing Th1-dependent antibody responses against *C. rodentium* antigens, in particular production of IgG2c subtypes. Using $C3^{-/-}$ mice and depletion of systemic C3 by cobra venom factor the presence of complement was shown to be an important determinant of infection severity.

Published earlier this year, a study of *C. rodentium* infection in properdin-deficient mice demonstrated a protective role for this C3/C5 convertase-stabilising factor. Properdin was shown to be important for generation of the anaphylotoxin C5a and subsequent stimulation of epithelial cell IL6 production. In the absence of properdin mice displayed exacerbated colitis and increased bacterial colonisation [310].

In summary, evidence from studies of autoimmune disease and infection support important biological functions for the complement system in the intestinal mucosa. However the source of intestinal complement, pathways involved in activation, and effects upon susceptibility to intestinal pathology are areas requiring attention.

5.2 Aims of the work described in this chapter

Regulation of complement during *S.* Typhimurium infection in the streptomycin mouse model was examined in RNAseq and proteomic datasets described in Chapters 3 and 4. Upregulation of C3 in caecal tissue was verified by Western blotting. Further, Western blotting was also employed to detect activation of C3 in caecal tissue and plasma from *S.* Typhimurium-infected mice. The localisation of C3 protein (including C3 fragments) in caecal tissue was investigated using immunofluorescence staining approaches.

5.3 Results

5.3.1 Complement upregulation during *S.* Typhimurium infection in the gastrointestinal mucosa

A highly significant association of complement-related pathways with transcripts and proteins upregulated in *S.* Typhimurium SL1344 infection in caecal tissue was described in Chapter 4. Abundance values, and fold changes compared with naïve tissue with associated p-values, for the four complement pathway genes significantly upregulated at both the level of RNA and protein are displayed in Table 5.1.

	RNAseq			**MS**		
	Transcript abundance	**Log2 fold change**	**Adjusted p-value**	**Protein abundance**	**Log2 fold change**	**Moderated T-test p-value**
C3	78079	4.50	1.29E-48	0.77	4.52	1.66E-06
C4b	16675	3.12	1.73E-39	0.09	Infected only (6)	-
Cfb	31644	3.05	2.73E-52	0.02	Infected only (5)	-
Cfh	3309	2.21	1.25E-27	0.08	Infected only (13)	-

Table 5.1. Complement pathway genes upregulated both in RNA and protein in *S.* Typhimurium-infected caecal tissue. Transcript abundance is the baseMean output from DESeq2 (the average of the normalised count values over all samples). Protein abundance is the normalised summed top three peptide intensities for each protein averaged over all samples. For proteins detected only in *S.* Typhimurium SL1344-infected samples the number in brackets in the column 'log2 fold change' denotes the number of MS runs in which the protein was detected.

C3 is the most highly abundant complement protein in blood. We find C3 to be the most abundant complement protein and most highly expressed transcript in caecal tissue. Furthermore of all complement and complement pathway factor genes detected by RNAseq, C3 is the most highly upregulated transcript. C3 is also highly upregulated at the level of protein; of proteins quantified in both naïve control and *S*. Typhimurium SL1344-infected samples, C3 was the fifth most highly upregulated. The dramatic increase in levels of C3 upon infection led us to investigate C3 further by Western blotting and immunofluorescence staining.

Significantly increased both in protein and RNA in addition to C3 are the C3 convertase-forming components C4b, Complement Factor B (Cfb), and the convertase inhibitor and decay accelerating factor, Complement Factor H (Cfh). Highly upregulated at the level of RNA, the proteins encoded by these three genes were present in ≥ 5 of the total 20 MS runs for infected caecal tissue extracts, and were below the threshold of detection in naïve samples.

Complement proteins C1qb, C2, and C8b, and negative regulators of complement activation, Decay Accelerating Factor (CD55) and Vitronectin were also detected by MS. These proteins were detected in ≤ 4 of the 40 MS runs in total for naïve and infected samples, however detection of C1qb and Vitronectin exclusively in infected samples (≥ 3 runs), and the non-significant upregulation of C2 in infection are in line with the upregulation of complement activity in infection. C8b and CD55 were detected in a single MS run only.

5.3.2 Detection of C3 and C3d by Western blotting

Western blotting for C3 was performed on intestinal content and faeces from *S.* Typhimurium-infected and naïve control mice to investigate possible translocation into the intestinal lumen as reported previously in *C. rodentium* infection [309]. An anti-human C3c antibody with cross-reactivity for mouse protein was used, reported to bind C3c (~ 145 kDa) and larger forms of C3 which contain this fragment (C3, C3b and iC3b). Caecal tissue protein extracts from *S.* Typhimurium SL1344-infected and naïve control mice at day 4 PI were investigated by Western blotting with the C3c antibody also. Extracted liver proteins and serum from untreated mice were used as positive controls. Tissue extracts and plasma from *S.* Typhimurium SL1344-infected and naïve control mice at day 4 PI were tested for complement activation by blotting with an anti-C3d antibody. The C3d antibody is reported to

bind the stable activation product C3d (~ 31 kDa) and larger C3 fragments containing the polypeptide chain liberated as C3d by proteolytic processing.

5.3.2.1 Failure to detect C3 in luminal content or faeces by Western blot

Multiple attempts were made to detect C3 in luminal content and faeces obtained from *S.* Typhimurium-infected and naïve control mice by Western blotting with anti-C3c antibody. An example blot is shown in Figure 5.4. While serum controls consistently produced a polypeptide between 100 and 150 kDa, no polypeptide of this size or smaller was detected in intestinal content or faecal protein extracts. As SDS-PAGE was performed under reducing conditions, the disulphide bond linking the 120 kDa α-chain and 75 kDa β-chain of C3 should be broken. Accordingly the polypeptide detected in serum is the correct size for the C3 α-chain. In extracts from naïve control mice a single polypeptide in the region of 250 kDa was detected, significantly larger than full length C3 (~ 195 kDa). The detection of this product exclusively in naïve control mice, which have a comparatively rich microbiota, suggests the product might potentially arise from cross-reactivity of the polyclonal antibody with a microbial protein. Immunoprecipitation and MS identification could be performed for confirmation of the band identity. The polyclonal C3c antibody used in Figure 5.4 consistently produced high background and dark marks on blots making interpretation difficult. Based on scrutiny of several blots performed with this antibody the diagonal lines in lanes 4 and 8 appear to be regions of non-specific binding.

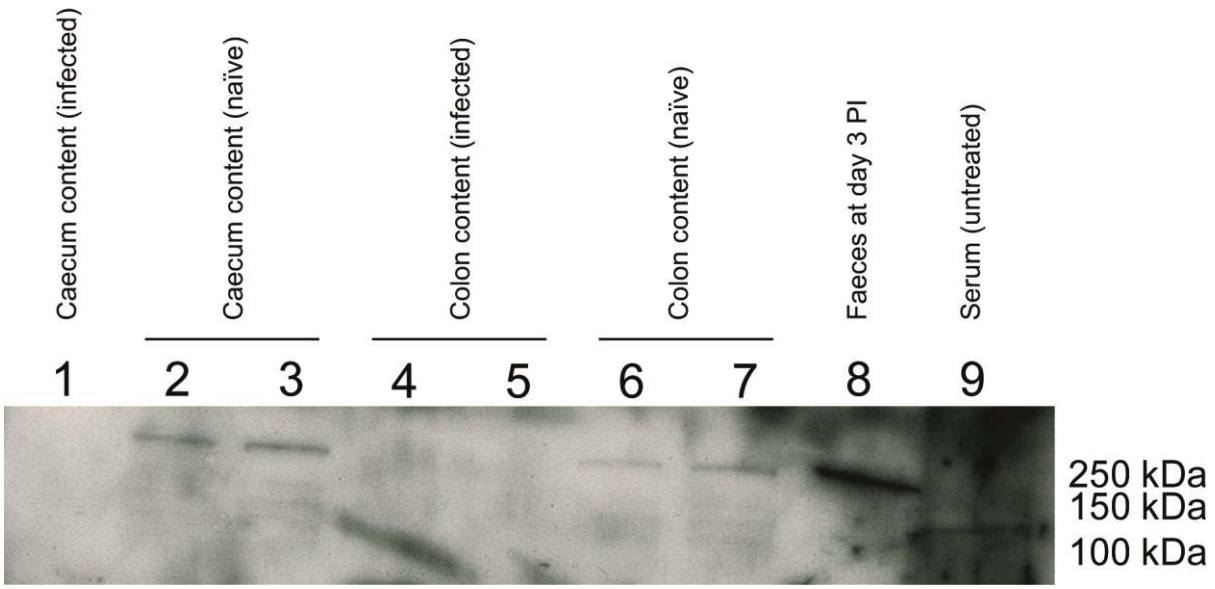


Figure 5.4. Western blotting for C3 in intestinal content and faeces. Western blot produced using Dako human C3c antibody. Lanes are numbered as follows: 1 = caecum content extract from an *S.* Typhimurium SL1344-infected mouse, 2 & 3 = caecum content extracts from individual naïve mice, 4 & 5 = colon content extracts from individual *S.* Typhimurium SL1344-infected mice, 6 & 7 = colon content extracts from individual naïve mice, 8 = protein extract from faeces collected at day 3 PI from an *S.* Typhimurium SL1344-infected mouse, 9 = serum from an untreated mouse. All lanes were loaded with 40 µg protein/lane with the exception of lane 9 in which 20 µg serum protein was loaded. Blot image was produced with a 2 min exposure.

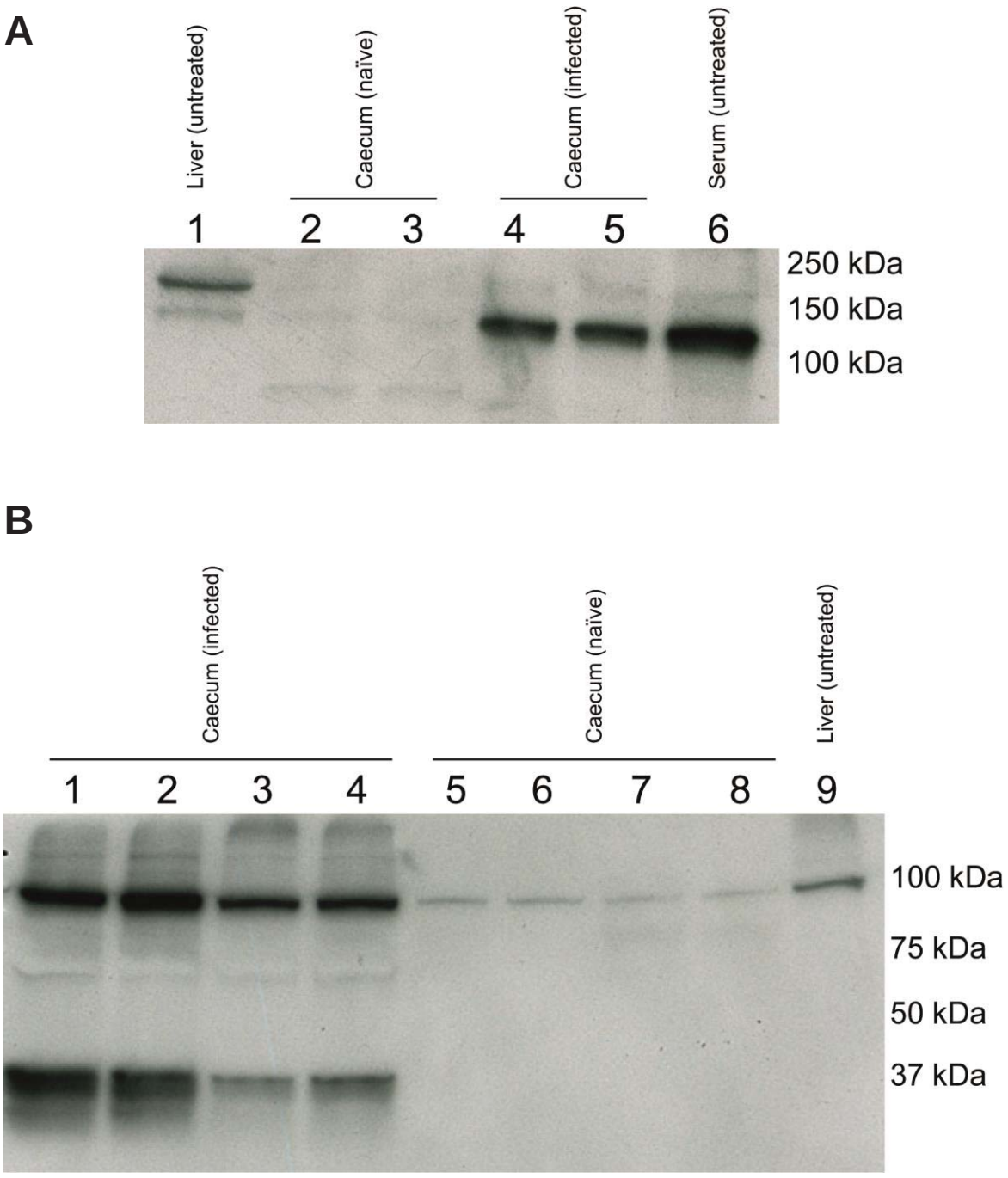
5.3.2.2 C3 and C3 activation fragments in caecal tissue and plasma

Western blotting was performed on caecal tissue extracts from *S.* Typhimurium-infected and naïve control mice using anti-C3c antibody (Figure 5.5A). A dark product at ~ 120 kDa was detected in *S.* Typhimurium-infected tissue extracts whereas in naïve control extracts this was barely visible. These findings are in agreement with the MS data.

Having demonstrated the presence of significant quantities of C3 in infected caecal tissue we next investigated the activation state of caecal C3 using an anti-C3d antibody (Figure 5.5B). At around 100 kDa a product corresponding to full-length C3 was detected in all samples. As with the C3c antibody the C3 polypeptide was clearly and consistently more concentrated in caecum extracts from mice at day 4 PI with *S.* Typhimurium compared with naïve controls. At around 30 kDa a polypeptide corresponding to the C3 activation product C3d was detected exclusively in *S.* Typhimurium-infected caecum extracts. The C3d product

was observed to be consistently fainter than the signal corresponding to full-length C3, indicating that much of the C3 present in infected tissue remains as the full-length form.

We were interested to determine whether C3 activation could be detected in blood upon *S.* Typhimurium infection. Figure 5.5C shows a blot produced with anti-C3d antibody and plasma samples from *S.* Typhimurium-infected and naïve control mice at day 4 PI. The signal intensity for full length C3 appeared highly similar between *S.* Typhimurium-infected and naïve control plasma samples. Densitometry analysis indicated slightly lower intensity for naïve control mice however this was not statistically significant (student T-test). C3d signals were dramatically darker in plasma samples from infected mice; densitometry analysis indicated levels of C3d approximately 100-fold higher in infected plasma versus naïve plasma for which a C3d signal was visible. Both full-length C3 and the C3d fragment were detected at higher levels in plasma compared with tissue.


A
Liver (untreated)
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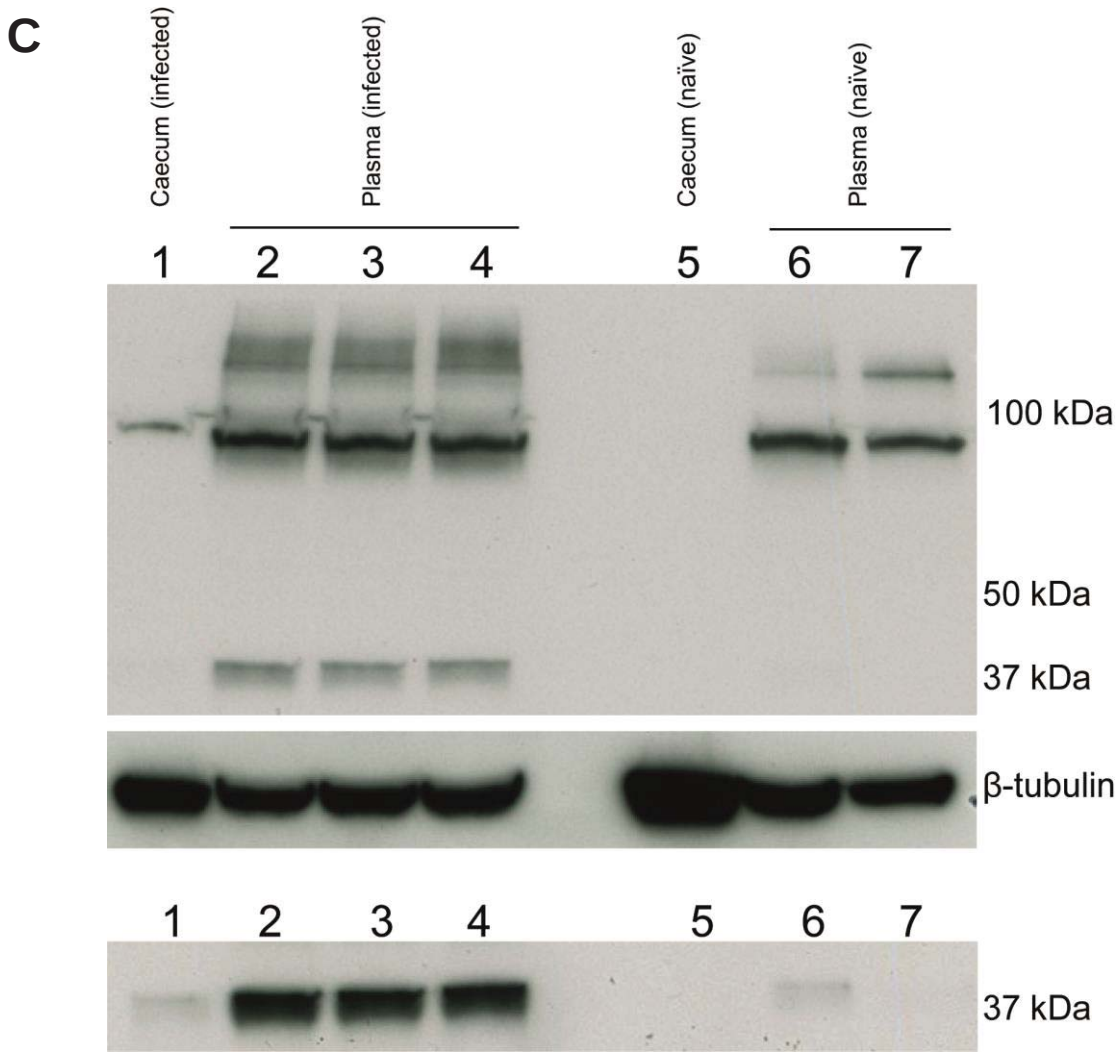

Figure 5.5. Western blotting for C3 and the activation fragment C3d. (A) Western blot produced using Dako human C3c antibody to compare effect of *S.* Typhimurium infection on C3 in caecal tissue. Lanes are numbered as follows: 1 = liver protein extract from an untreated mouse, 2 & 3 = caecal tissue extracts from individual naïve mice, 4 & 5 = caecal tissue extracts from individual *S.* Typhimurium SL1344-infected mice, 6 = serum from an untreated mouse. All lanes were loaded with 30 µg protein/lane with the exception of lane 6 in which 20 µg serum protein was loaded. Blot image was produced with a 1 min exposure. (B) Western blot produced using R & D systems mouse C3d antibody to compare effect of *S.* Typhimurium infection on C3 and C3d in caecal tissue. Lanes are numbered as follows: 1 - 4 = caecal tissue extracts from individual *S.* Typhimurium SL1344-infected mice, 5 - 8 = caecal tissue extracts from individual naïve mice, 9 = liver extract from an untreated mouse. All lanes were loaded with 30 µg protein/lane. Blot image was produced with a 2 min exposure. (C) Western blot produced using R & D systems mouse C3d antibody to compare effect of *S.* Typhimurium infection on C3 and C3d in plasma, with β-tubulin loading control. Upper blot image was produced with a 1 min exposure. Lower blot image shows ~ 30 kDa region of the same blot following a 2 min exposure. Lanes are numbered as follows: 1 = caecal tissue extract from an *S.* Typhimurium SL1344-infected mouse, 2 - 4 = plasma from individual *S.* Typhimurium SL1344-infected mice, 5 = caecal tissue extract from a naïve mouse, 6 & 7 = plasma from individual naïve control mice. All lanes were loaded with 20 µg protein/lane.

5.3.3 Localisation of C3 in caecal tissue by immunofluorescence staining

Immunofluorescence staining was performed to investigate the localisation of C3 in caecal tissue from *S.* Typhimurium SL1344-infected and naïve control mice. 5 µm sections of frozen tissue were stained with C3 and C3d antibodies for the detection of C3 and activation fragments. Staining for the endothelial cell marker CD34 was used to indicate the position of caecal vasculature, and staining for *Salmonella* common structural antigens (CSA) for visualisation of *Salmonella*.

Figure 5.6 shows typical images of co-staining for C3 and CD34. C3 staining was more intense and extensively distributed in *S.* Typhimurium-infected mice compared with naïve controls, in line with the detection of increased C3 in infected caecal tissue by MS and Western blotting. Strong colocalisation of C3 and CD34 was clearly observed in infected tissue. In places C3 staining appeared to extend a little beyond the CD34-stained regions, although these inconsistencies could be the result of differences in the intrinsic brightness of the fluorophores used. Staining performed with C3d antibody showed a distribution indistinguishable from the C3 antibody staining.

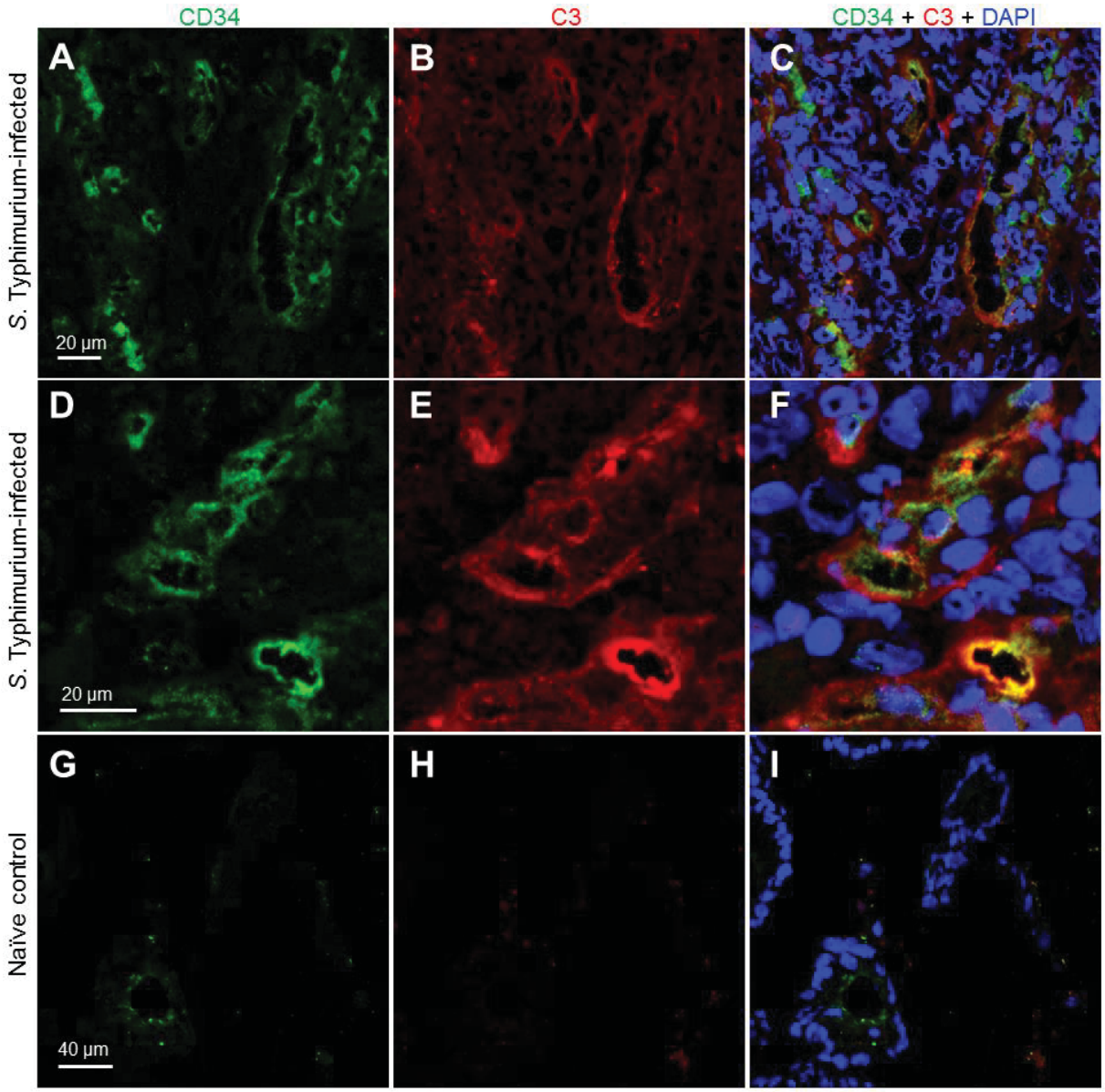

Figure 5.6. Immunofluorescence staining of C3 and CD34 in caecal tissue. 5 µm sections of frozen caecal tissue were stained with C3 and CD34 primary antibodies with appropriate secondary antibodies for C3 staining in red and CD34 staining in green. DAPI was used to stain DNA. Confocal images to the left and in the centre display fluorescence in the individual green and red channels respectively. Images to the right display the overlay of the red, green and blue channels. A - C = *S.* Typhimurium-infected caecum, low magnification, D - F = *S*. Typhimurium-infected caecum, high magnification, G - I = naïve control caecum.

Salmonella CSA staining of 5 µm sections was performed to determine the distribution of complement relative to *Salmonella* within infected caecal tissue. Regions of complement staining were often observed in the proximity of *Salmonella* bacteria but CSA and C3 staining did not appear to be directly overlapping. Individual host cells, possibly

macrophages, brightly stained with CSA antibody were observed in infected caecal tissue though their presence was relatively rare. These cells were located in regions of tissue with bright staining for C3 and were stained for C3 themselves.

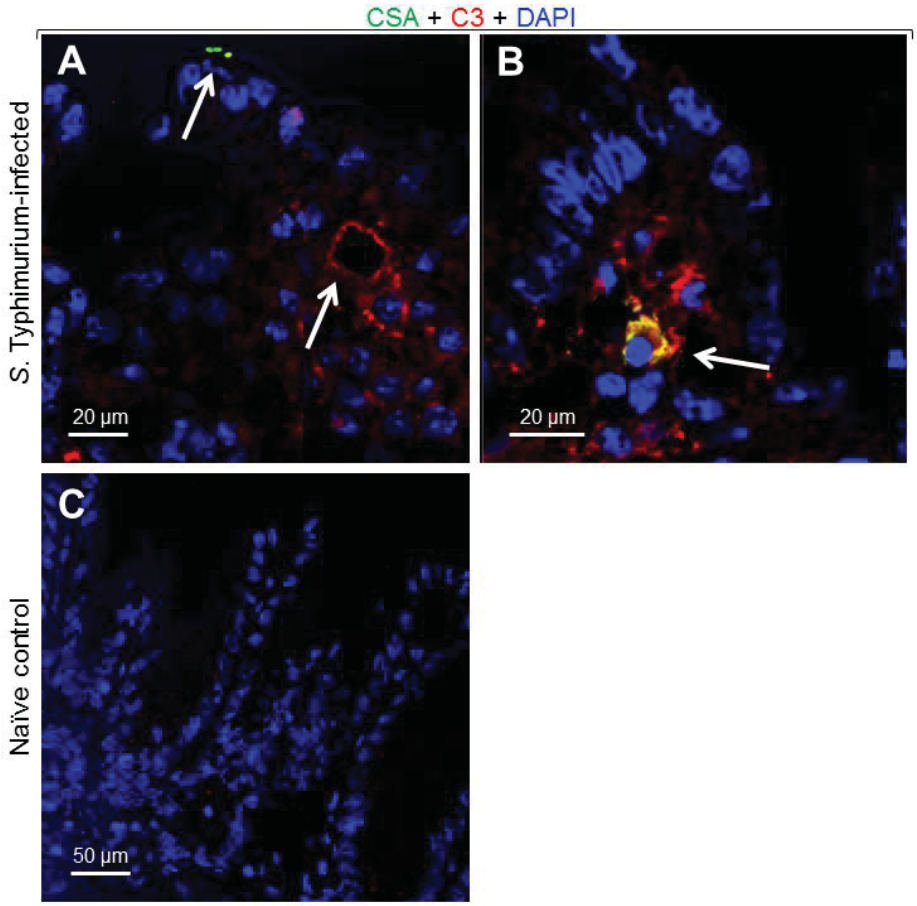

Figure 5.7. Immunofluorescence staining of C3d and CSA in caecal tissue. 5 µm sections of frozen caecal tissue were stained with C3d and CSA-FITC primary antibodies with appropriate secondary antibody for C3d staining in red. DAPI was used to stain DNA. All images are the overlay of red, green and blue channels detected by confocal imaging. A = *S.* Typhimurium-infected caecum, upper arrow points to *Salmonella*, lower arrow indicates a region of bright C3d staining. B = *S.* Typhimurium-infected caecum, arrow points to a cell brightly stained by both C3d and CSA antibodies. C = naïve control caecum.

Whole-mount three-dimensional imaging of caecal tissue demonstrated a greater degree of overlap between C3 and *Salmonella* compared with the staining of 5 µm tissue sections (Figure 5.8). *S.* Typhimurium bacterium associated with epithelial cells at the luminal surface, and in the lamina propria, were brightly stained for C3. Regions of more diffuse C3 staining were also present throughout caecal tissue.

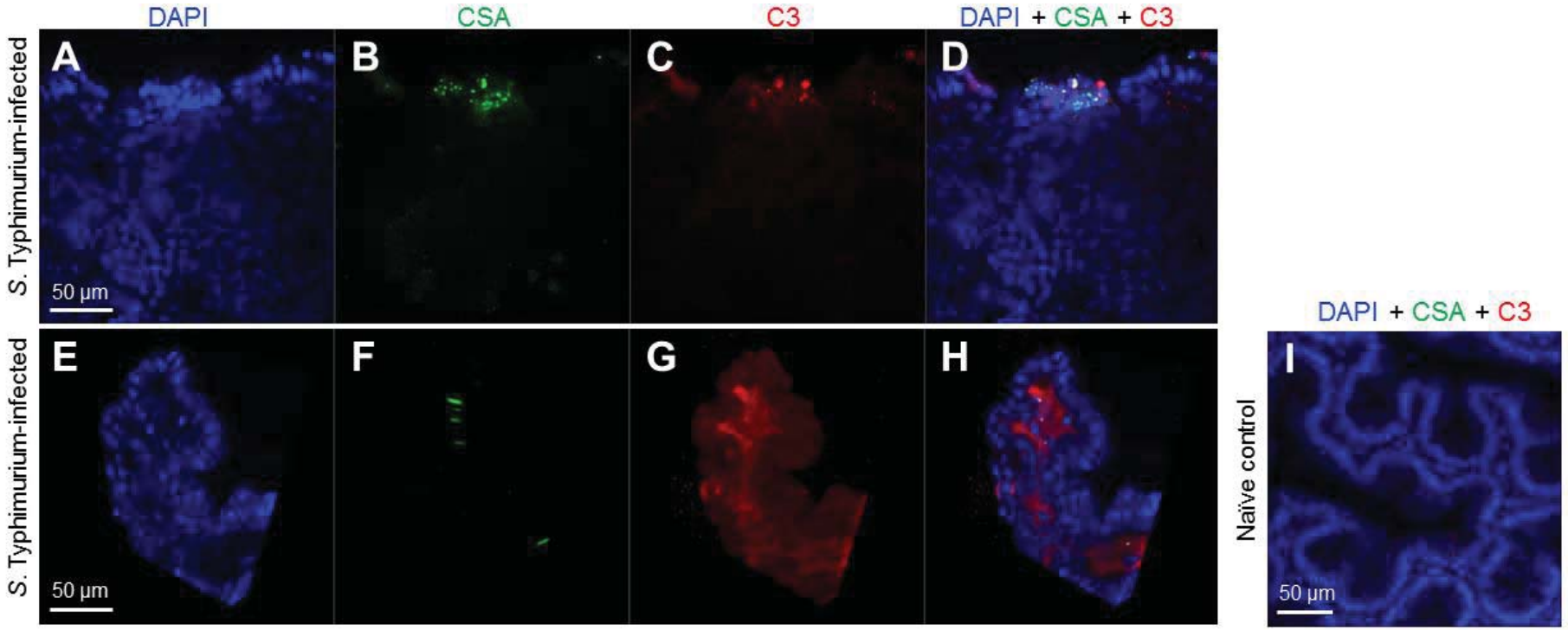

Figure 5.8. Three-dimensional confocal imaging of C3 and CSA in caecal tissue. PFA-fixed caecal tissue was stained with C3d and CSA-FITC primary antibodies with appropriate secondary antibody for C3d staining in red. DAPI was used to stain DNA. From left to right images display fluorescence captured in the individual blue green and red channels. Images D, H and I, display the overlay of the three channels. A - D = *S.* Typhimurium-infected caecum with *Salmonella* associated with the epithelial surface. E - H = villus-like projection in *S.* Typhimurium-infected caecum with *Salmonella* inside the central core. I = naïve control caecum.

5.4 Discussion

The increased abundance of several complement pathway proteins in caecal tissue during *S.* Typhimurium infection suggests complement may play an important role in enteric infection. The source of these proteins may be the intestinal tissue itself, as a result of increased local production. Alternatively, translocation of circulating liver-derived complement proteins into caecal tissue may take place during infection. Moreover the increase in complement pathway proteins likely arises from a combination of these two effects. Past studies support an increase in local complement protein production during inflammation of the intestinal mucosa [311]. Our finding that mRNAs encoding complement pathway proteins are dramatically increased in *Salmonella*-infected caecal tissue suggests an increase in complement production within the caecal tissue may be taking place. However a contribution from systemic complement to total tissue complement protein is not excluded. Given the increase in vascular permeability which occurs upon inflammation, leakage of blood complement proteins into tissue seems likely. Additionally, as MS and Western blotting were performed upon protein extracted from whole caecal tissue, protein from inside blood vessels within the tissue makes an unknown contribution to total complement pathway protein. The impact of increased vascular permeability might be assessed by measuring levels of a stably-produced blood protein similar in size to C3 in naïve and *Salmonella*-infected caecal tissue. An example of such a molecule is creatinine (113 kDa), commonly used as an indicator of renal function in medical practice.

The source of intestinal complement in infection was partially addressed by staining caecal tissue for C3 and C3 stable activation products. The colocalisation of C3 and the endothelial cell marker CD34 indicates C3 is deposited on the walls of blood vessels within the intestinal mucosa, and diffuses into surrounding tissue. However C3 appeared also to be present at sites remote from vessels, perhaps indicating either diffusion or transport of C3 through the tissue, or alternatively the production of C3 *in situ*. Individual cells interspersed throughout the caecum which were brightly stained for CSA also stained for C3. C3 was also observed diffusely localised in the surrounding region. In the CSA-positive cells rod-shaped structures or obvious bacteria were not visible, therefore these cells may represent phagocytes which have ingested and lysed *Salmonella* causing release of CSA into the cytoplasm or its display on the cell surface. Activated immune cells including polymorphonuclear cells and

dendritic cells have been shown to be producers of complement proteins [312, 313], therefore we suggest these cells may represent activated phagocytes, possibly macrophages. Co-staining of intestinal tissue for immune cell surface markers and C3 would confirm this and aid understanding of the contribution of immune cells to C3 production. The work presented in this chapter has provided insight into potential sources of the complement protein C3 in intestinal infection. Further work is required to determine if similar patterns are observed with other components of the complement pathway.

Cell isolation experiments may be a means by which to further address the source of increased C3 in the caecum during infection [314, 315]. Isolation of individual intestinal cell types followed by protein extraction and MS or blotting would allow the contribution of different cell types to be quantified. Early trials of separating intestinal cell types were performed, however contamination of epithelial cell fractions with $CD45^+$ cells was a recurring problem, and prohibited the analysis of pure cell populations. Ensuring cells are washed sufficiently to remove complement associated with the cell surface or in surrounding fluid would be essential but difficult to determine.

Cobra venom factor has been used extensively to deplete systemic C3 [309, 316, 317]. Systemic complement depletion could provide insight into the contribution of locally-produced complement protein in *S.* Typhimurium infection. The efficacy of cobra venom factor is not precisely defined and therefore systemic C3 may contaminate tissue extracts. However, examining the differential impact of systemic C3 depletion on naïve and infected tissue may help to further determine the sources of C3.

A previous study demonstrated the presence of C3 on the surface of a bacterial pathogen in faeces [309]. In the study, C3 was detected on the surface of luminal bacteria in $C3^{-/-}$ mice treated with wild type mouse serum, indicating C3 was able to translocate into the lumen from the circulatory system. C3 was also detected on the surface of luminal bacteria from mice treated with cobra venom factor, suggesting locally produced complement may contribute in addition to complement from a systemic source. Despite multiple attempts we were unable to detect C3 in intestinal content or faeces by Western blotting, irrespective of infection. In light of the greater epithelial damage incurred in infection with *S.* Typhimurium compared with *C. rodentium* it seems highly unlikely that systemic leakage of C3 occurs exclusively in *C. rodentium* infection or indeed intestinal production of complement is stimulated specifically by *C. rodentium*. C3 is susceptible to heat degradation and is degraded

by certain species of bacteria, most likely as an immune evasion strategy [318, 319]. The intestinal lumen is rich in enzymatic activity, mediated by both bacterial and host-derived digestive enzymes. Therefore following translocation into the intestinal lumen C3 may be quickly broken down. Future work to investigate the presence of luminal C3 might include flow cytometry analysis of faeces from mice infected with labelled *Salmonella* using C3 antibodies, as previously described for *C. rodentium*.

By Western blotting we detected the stable product of C3 activation C3d in *S.* Typhimurium-infected caecal tissue. C3d contains a highly reactive thioester bond through which it covalently attaches to pathogen surfaces. The importance of an interaction between C3d on pathogen surfaces and the B-cell CR2 receptor in directing the adaptive immune response is well described [275, 320]. The differential intensity of signatures corresponding to C3 and C3d in Western blots indicates the majority of C3 protein present in *Salmonella*-infected caecal tissue remains in the full length form. This is likely the result of a situation in which a massive influx and/or upregulation of C3 floods the tissue with the complement protein, while activation is restricted to the locations of bacteria within the caecum. Indeed we detected colocalisation of C3 and/or activation fragments and *Salmonella* using three-dimensional confocal imaging, but also complement in regions of tissue without bacteria. Regions of colocalisation likely represent sites of C3 activation, though the pathway by which activation may occur is unknown. The apparent lack of commercial antibodies which specifically recognise activated forms of C3 is regrettable; ideally the location of activation products within tissue would be examined by immunofluorescence and compared with the distribution of full length C3. Further work could include co-staining for C3 and complement-fixing Ig subtypes in the intestinal mucosa to investigate the possibility of complement activation by the classical pathway.

Western blotting demonstrates a vast increase in C3d in the plasma of *S.* Typhimurium-infected mice compared with naïve controls at day 4 PI in addition to the increase in caecal tissue. Indeed C3d detected in plasma was shown to form a significantly larger proportion of total plasma protein compared with the proportion of C3d in protein extracted from caecal tissue. On the one hand the observed increase in systemic C3d could occur through the transport away from the tissue of C3d fragments produced by activation of C3 in the intestine. Alternatively C3d detected in plasma may have been generated by C3 activation in the liver and spleen, organs which by day 4 PI in the streptomycin mouse model

carry a considerable burden of *Salmonella* (section 3.3.1). Given that C3 is thought to translocate from vessels into caecal tissue upon infection C3d may travel similarly into the caecal tissue. Therefore the detection of activated C3 fragments in the caecum cannot be attributed conclusively to C3 activation in tissue. Although the presence of a high bacterial burden in the caecum suggests complement activation likely is taking place, similar to C3 we cannot determine the proportion generated *in situ* and outside the tissue. As C3d is reported to be covalently bound to pathogen surfaces, C3d moving from the circulation to the intestinal tissue would likely be accompanied by bacteria. Based on the much greater signal intensities observed for plasma compared with caecal tissue from *S.* Typhimurium-infected mice this might suggest that more C3 activation is occurring in the blood than within the intestinal mucosa.

A statistically significant increase in blood plasma C3 at day 4 PI with *S.* Typhimurium compared with uninfected mice was not detected. For confirmation blotting should be repeated with loading of reduced amounts of protein. Relative to the high concentration of C3 in plasma in the naïve condition a small change in the total amount of C3 protein will be difficult to detect.

The systemic spread of *S.* Typhimurium in the later stages of the streptomycin mouse model is a significant deviation from the most commonly observed outcome of *S.* Typhimurium infection in humans; self-limiting gastroenteritis. The streptomycin mouse model, and in particular later time points in the infection, is poorly suited for studying the presence and role of complement activation in such cases of gastroenteritis. It might be argued that the course of infection in the mouse model more closely approximates bacteremic and extraintestinal focal *Salmonella* infections caused by non-typhoidal *Salmonella* species. Invasive infections occur in approximately 5% of people infected with non-typhoidal *Salmonella* serotypes and are more prevalent in certain groups such as the elderly and those with immunosupressive conditions. *C. rodentium* studies can provide some insight into the role of complement in a non-invasive pathogen but studying an invasive enteric pathogen is more difficult. A variation of the streptomycin mouse model in which an *sseD* mutant derivative of *S.* Typhimurium gives rise to a self-resolving infection might be used to study complement in intestinal *Salmonella* infection. Alternatively infection of *Salmonella* resistant mouse strains (*Nramp1*$^{+/+}$) in which intestinal inflammation occurs without development of systemic infection might provide a more appropriate system for these studies. [50].

In summary the work presented in this chapter raises the possibility of important roles for complement in bacterial infection of the intestinal mucosa. Further experiments to explore these roles will include *S.* Typhimurium infection of *C3*$^{-/-}$ mice. C3-deficient mice were previously reported to be highly susceptible to *C. rodentium* infection [309]. Infection of *C3*$^{-/-}$ mice in the streptomycin mouse model will determine if the increase in C3 in the caecum upon *S*. Typhimurium infection is required for protection from inflammation. However due to the eventual systemic spread of *S*. Typhimurium in the streptomycin mouse model, study of multiple time points or passive transfer of C3 in serum to *C3*$^{-/-}$ mice, may be required to separate the effect of C3 deficiency on intestinal inflammation, from effects upon systemic control of *Salmonella*.