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Membrane attack by complement: the assembly and biology of terminal complement complexes

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Abstract Complement system activation plays an important role in both innate and acquired immunity. Activation of the complement and the subsequent formation of C5b-9 channels (the membrane attack complex) on the cell membranes lead to cell death. However, when the number of channels assembled on the surface of nucleated cells is limited, sublytic C5b-9 can induce cell cycle progression by activating signal transduction pathways and transcription factors and inhibiting apoptosis. This induction by C5b-9 is dependent upon the activation of the phosphatidylinositol 3-kinase/Akt/FOXO1 and ERK1 pathways in a Gi protein-dependent manner. C5b-9 induces sequential activation of CDK4 and CDK2, enabling the G1/S-phase transition and cellular proliferation. In addition, it induces RGC-32, a novel gene that plays a role in cell cycle activation by interacting with Akt and the cyclin B1-CDC2 complex. C5b-9 also inhibits apoptosis by inducing the phosphorylation of Bad and blocking the activation of FLIP, caspase-8, and Bid cleavage. Thus, sublytic C5b-9 plays an important role in cell activation, proliferation, and differentiation, thereby contributing to the maintenance of cell and tissue homeostasis.

Keywords C5b-9 terminal complement complex · Membrane attack complex · Cell cycle · Signal transduction · Transcriptional regulation · Apoptosis

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Introduction

The complement system comprises more than 15 soluble proteins in body fluids, a number of receptors, and several soluble and membrane proteins with regulatory functions. When activated, it defends the host against infection and acts as an immune effector and regulator. The system was first described more than a century ago by Hans Buchner as a heat-labile "bactericidal principle" in the blood. Jules Bordet then demonstrated that the bactericidal activity of serum, when destroyed by heating, could be restored by adding fresh serum and named the active component "alexine." Paul Ehrlich called this serum factor "complement" [1]. In 1901, Bordet and Gengou developed the complement fixation assay to measure the antigen–antibody reaction. The hemolytic assay, which uses the lysis of erythrocytes to determine the complement activation endpoint, has been used effectively for nearly half a century to unravel the activation cascade and to elucidate the protein–protein and protein–membrane interactions that underlie this cascade system [2, 3].

The complement system is activated in three ways: via the classical pathway, which includes the proteins C1, C4, C2, and C3; the alternative pathway, with the participation of C3 and protein factors B, D, and P; and the lectin pathway, with the participation of mannose-binding lectin

(MBL and MBL-associated proteases) (Fig. 1). All three pathways lead to the activation of the C5, C6, C7, C8, and C9 proteins, resulting in the sequential assembly of C5b-7, C5b-8, and C5b-9 (membrane attack) complexes on the target cell (Fig. 2).

Activation by the classical pathway

Classical pathway activation occurs when a minimum of two globular regions of C1q interact with the C_H2 domains of an IgG duplex or with the C_H3 domains of a single IgM in immune complexes. In the absence of antibody, other substrates such as viral envelope membranes, Gram-negative bacterial cell walls, C-reactive protein, cardiolipin DNA, cytoskeletal intermediate filaments, and central nervous system myelin can also activate the classical pathway. The C1q bound to the activator allows the ternary complexes $C1_r2$–$C1_s2$ to become active when they are bound to the collagenous portion of C1q in the presence of Ca^{2+}. Both C1q and C1s are proenzymes that possess serine esterase catalytic domains, and the sequential auto-activation of C1r and C1s can lead to the activation of C4 and C2 through cleavage by C1s. The function of activated C1 is to bind and cleave C4, the next protein in the classical pathway activation sequence. Cleavage of the α chain of C4 releases a 9-kDa peptide, C4a, while the remaining 190-kDa C4b protein binds covalently to target molecules. Activation of C4 exposes a thioester-containing binding site on C4b that allows the covalent attachment of C4b to the target. Once C2 has bound to the C14b complex, the C1 subunit C1s cleaves C2 to produce C2b and C2a. C2 binding to C4 requires the presence of Mg^{2+}. C2a, which has a catalytic site, then complexes with C4b to form C4b2a, the C3 convertase, which can bind and cleave C3, generating C3b and C3a. The C3a fragment has anaphylatoxic and proinflammatory activity. C3a is able to cause mast cell degranulation, with resulting histamine release [4]. C3b and its split products interact with specific complement receptors, CR1 for C3b, CR2 for C3dg or C3d, and CR3 for iC3b. When C3b binds to the C3 convertase, the C4b2a3b complex (C5 convertase) is generated. The binding of C3b to form a C4b2a3b complex generates a new binding site for C5, allowing the cleavage of the complex into C5a and C5b by C2a [5].

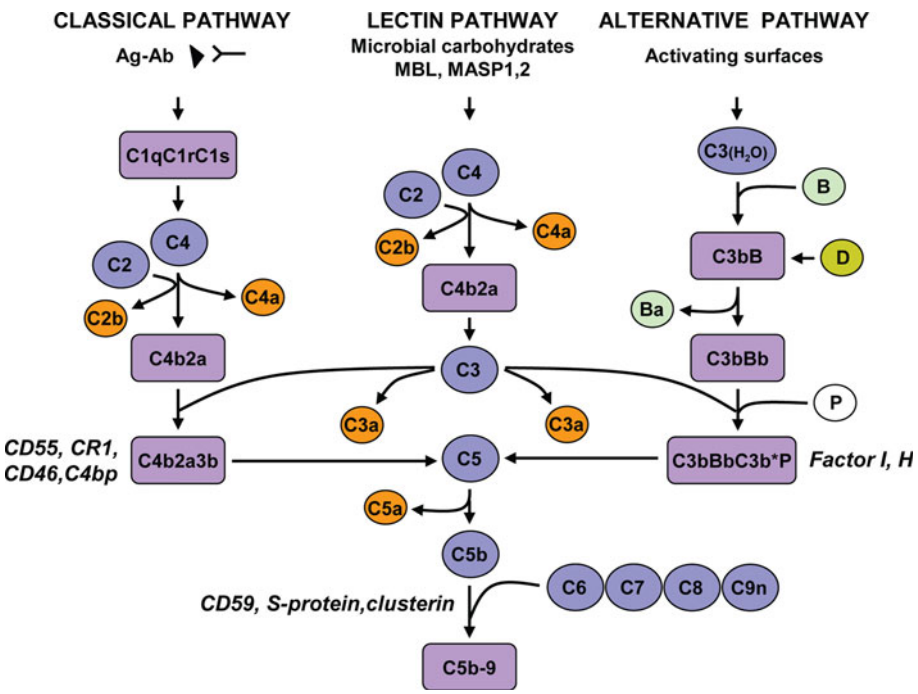

Fig. 1 Complement activation pathways and assembly of the terminal pathway. The classical pathway is initiated by the binding of the C1 complex to antibody already bound to antigen, leading to the formation of the C4b2a enzyme complex (the C3 convertase). The lectin pathway is activated by the binding of either MBL or ficolin and MASP 1, 2, and 3, respectively, to an array of mannose groups on the surface of bacterial cells and the generation of C3 convertase of the classical pathway. The alternative pathway is initiated by hydrolyzed C3 and factor B and the subsequent formation of the alternative pathway C3 convertase, C3bBb. Generation of the C3 convertase allows the formation of the C5 convertase enzyme, which initiates the formation of the C5b-9 terminal complement complex. The complement system is regulated at several levels: CD55, CR1, CD46, C4bp, and factors I and H regulate the activity of the C3 convertase and C5 convertase, and other proteins such as CD59 block the final assembly of the pores by preventing the binding of C9. The S-protein/vitronectin binds to C5b-7 and leads to the formation of a cytolytically inactive SC5b-9 complex

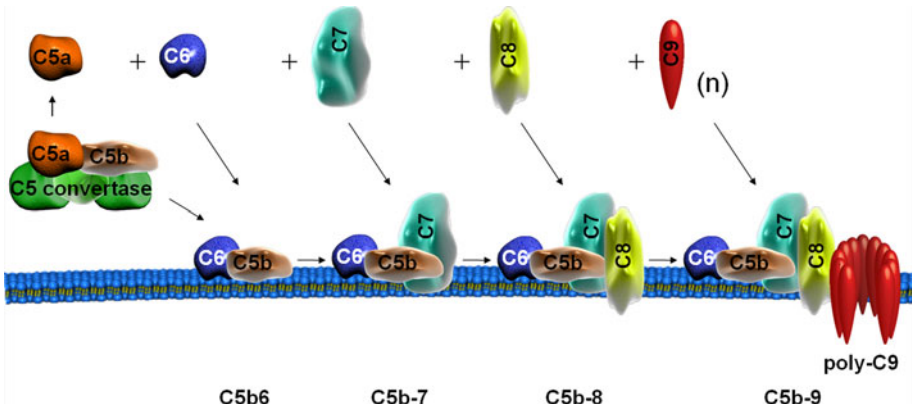

Fig. 2 Assembly of terminal complement complex. The formation and assembly of C5 convertase occur via either the classical, alternative, or mannose-binding lectin pathway. C5 convertase binds to C5 and cleaves it to generate C5a and C5b. After binding to C5b, C6 acquires the ability to interact with the lipid bilayer and form a C5b6 bimolecular complex. C7 and then C8 sequentially bind to C5b and further insert into the lipid bilayer, forming C5b-7 and C5b-8 complexes, respectively. One molecule of C9 binds to membrane-inserted C8, after which the polymerization of multiple C9 molecules occurs to form the C5b-9 complex

Activation by the alternative pathway

In the alternative pathway, C3 is itself the recognition molecule [5]. C3 has two chains, α and β, with an internal thioester joining a cysteine at position 988 to a glutamine at position 991 in the α-chain backbone. Activation of the alternative pathway begins when factor D, a serine protease in serum, cleaves factor B; this cleavage occurs only when factor B is bound to C3b derived from the classical pathway or when factor D is associated with C3(H_2O), the spontaneously hydrolyzed form of C3. Cleavage of factor B generates C3bBb or C3(H_2O)Bb—known as the alternative pathway C3 convertase—in which Bb, like C2a, exposes a catalytic site. This enzyme complex is rapidly inactivated by factors H and I in serum. Factor H competes with Bb for binding to C3b, which dissociates Bb and allows factor I to cleave C3b and produce iC3b. Properdin (P) increases the stability of this enzyme complex by forming C3bBbP. It has recently been suggested that properdin bound to a substrate can also bind C3b and initiate alternative pathway attack [5]. Like the classical pathway convertase, the alternative pathway convertase requires Mg^{2+}. C3bBbP is converted to a C5 convertase by the binding of an additional C3b, which provides a binding site for C5. These convertases, when formed in the fluid phase, are not as effective as when assembled on a solid phase such as the cell membrane. Activators of the alternative pathway include zymosan, high molecular weight dextrans, plastic surfaces, peripheral nerve myelin, endotoxin, and other surface structures of microbial and tumor cells. The alternative pathway activators function by protecting the activator-bound C3 convertases from factors H and I and increasing the binding affinity of Bb for C3b [6].

Activation of the lectin pathway

The lectin pathway is initiated by the binding of mannose-binding lectin (MBL) and ficolins to carbohydrate groups on the surface of bacterial cells [7]. MBL has a structure similar to C1q, with a central core and a series of radiating arms composed of a flexible triple helix, each ending in a binding structure. Unlike C1q's helix, the helix in MBL contains three copies of a single chain. MBL circulates as a series of multimers and can have two, four, or six arms. MBL and ficolins are typical pattern recognition molecules that serve to attach the MBL-associated serine proteases (MASP) 1, 2, and 3, thus activating their esterase activity. MASP2 is believed to be the principal serine protease involved in the activation of the complement cascade. Once activated, MASPs cleave and activate C4 and C2, thus generating the C3 convertase C4bC2a [8, 9]. MASP-2 is the enzyme component that, like C1s in the classical pathway, cleaves the complement components C4 and C2 to form the C3 convertase C4b2a, a common step in the activation of both the lectin and classical pathways. Alternatively, MASP-1 can cleave C3 directly [9–11], resulting in the activation of the alternative pathway [10].

The activation of C5–C9 and assembly of terminal complement complexes

Assembly of the C5b-9 complex in a membrane lipid bilayer starts with the cleavage of an Arg–Leu bond in the C5 α-chain to generate C5a and C5b (Fig. 2). Although an association also occurs between native C5 and C6, C5b6 complexes are formed when C5b fragments associate with C6. The C5b6 complexes can then bind reversibly to the

membranes through ionic as well as hydrophobic interactions. The subsequent interactions of C7, C8, and C9 with C5b6 and the membrane result in the formation of heteropolymeric transmembrane pores. This pore assembly takes place via distinct phases of intermediate formation, namely C5b-7, C5b-8, and C5b-9. These complexes are collectively referred to as terminal complement complexes (TCCs), while C5b-9, the final complex and the most effective at inducing cell death, is referred to as the membrane attack complex (MAC) [2, 12–14]. The mechanisms of the TCC forming steps will be reviewed below.

Proteins of the terminal complement cascade

C5

C5 is a 190-kDa protein and is structurally homologous to C3, C4, and α2-macro-globulin. The predicted cDNA sequences of human and mouse C5 reveal that the pro-C5 molecule begins with the β-chain at the N-terminus [15–17]. Although most C5 synthesis occurs in hepatocytes, C5 is also made by macrophages and alveolar epithelial cells. The major polypeptides C5a and C5b are produced by the cleavage of the C5α chain by C5 convertases. The small fragment released, C5a, has one glycosylation site, and 50% of the peptide is in an α-helical conformation [18]. C5a is the most potent inflammatory peptide released by complement activation, has strong neutrophil chemotactic activity, and is also an anaphylatoxin.

C6 and C7

C6 is unusual because it is a mosaic protein that consists of several homology modules found in many other proteins (Fig. 3) [19, 20]. C6 has a C-terminal Cys-rich domain that is partly homologous to regions of thrombospondin 1, the LDL receptor, and the EGF receptor. C6, C7, C8α, C8β, and C9 are all homologous, and all contain N-and C-terminal modules and an intervening 40-kDa segment referred to as the membrane attack complex/perforin (MACPF) domain [21]. C6 and C7 have several domains with homologies to the short consensus repeats (a characteristic motif shared among large numbers of proteins, including complement-regulatory proteins and receptors). The Cys-rich C-terminal region of C6 binds to the α-chain of C5b to form C5b6.

C8

C8 consists of three polypeptides: The α-chain (64 kDa) and the β-chain (64 kDa) share extremely close homology and are noncovalently linked, and the γ-chain (22 kDa) is linked to the α-chain by a disulfide bond. C8α, C8β, and C8γ are encoded by three different genes. The C8 α and β subunits contain a pair of N-terminal modules (thrombospondin type 1 and low-density lipoprotein receptor class A) and a pair of C-terminal modules (epidermal growth factor and thrombospondin type 1). The middle segment is referred to as the membrane attack complex/perforin domain (MACPF). C8α and C8β share significant homologies with other TCC proteins [22, 23]. C8γ belongs to the lipocain family of proteins that display a β-barrel fold and bind small hydrophobic ligands [21]. Crystal structures of the human C8α MACPF domain have recently been reported, and both display a fold similar to those of the bacterial pore-forming cholesterol-dependent cytolysins. Several hundred proteins with MACPF domains have been identified on the basis of sequence similarity; however, the structure and function of most are unknown [21]. The MACPF domains of both C8α and C8β are required for lysis, but membrane insertion has been attributed only to C8α-MACPF (the TMH1 and TMH2 subregions which are exposed at the surface), which is predicted to undergo

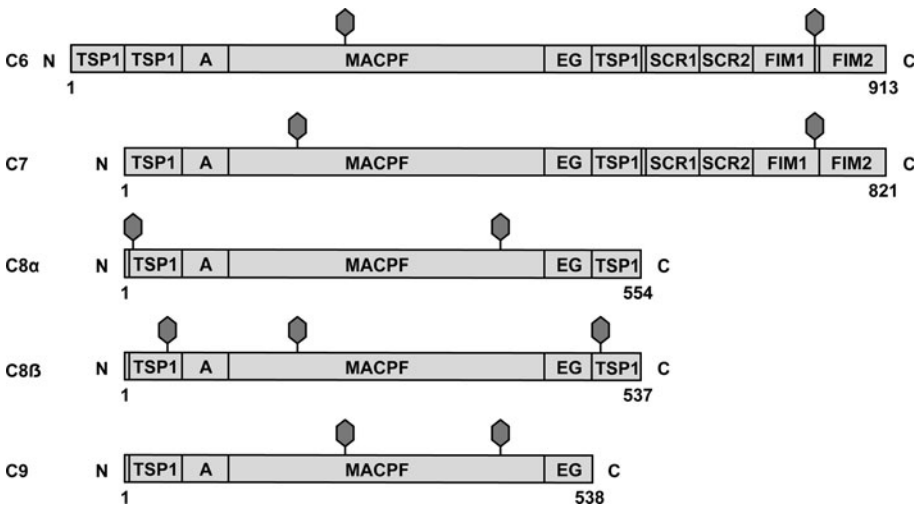

Fig. 3 Structural organization of the terminal complement system proteins. Six different types of structural modules are common to proteins of the human terminal components: the membrane attack complex/perforin domain (MACPF), the LDL receptor module (A), the EGF receptor module (EF), the thrombospondin 1 module (TSP1), the short consensus repeat (SCR), and the factor I module (FIM). Gray hexagonal symbols indicate the asparagine-linked glycosylation sites

conformational change and insert into the bilayer. C8β-MACPF is presented on the opposite face for assembly into the larger C5b-7 complex, while C8γ projects away from the C8$\alpha\beta$ core [24].

C9

C9, a single-chain globular protein (71 kDa) which is similar to the C6 from residues 56–540, also reveals conserved domains that are shared with other TCC proteins [25, 26]. The C9 sequence is 27 and 34% homologous to that of C6 and C8α/C8β, respectively. C9 can be cleaved at His–Gly (residues 244–245) by α-thrombin to yield C9a and C9b. C9b's cytolytic activity is mediated through its interaction with the membrane lipid bilayer [27]. C9 expression, which is highest in hepatocytes, is modulated under many pathologic conditions [28].

Assembly of the C5b-7 complex

Upon binding to the α-chain of C5b, C6 undergoes conformational changes and acquires the capacity to interact with the hydrophobic domains of the lipid bilayer [29]. The interaction of C7 with the α-chain of C5b in C5b6 results in a C5b-7 complex, with an amphiphilic transformation of the C7 molecule to produce a complex with high affinity for lipids. This affinity has been demonstrated by the C5b-7-induced release of the phospholipids from the liposomes, where binding to the photoreactive probes in the membrane increased conductance across 35-Å thin planar black lipid membranes (BLM) [29–32]. C5b-7 monomers and dimers become anchored to the membrane and allow the binding of C8 and polymerization of C9. C5b-7 associates with the outer lipid surface and only minimally penetrates the membrane and therefore does not induce cell lysis. Insertion of C5b-7 into the membrane, however, does stimulate the cells, as evidenced by the activation of signal transduction pathways, enhanced elimination of C5b-7 from the cell surface, and the ability of C5b-7 to induce the hydrolysis of myelin basic protein [33–35]. C5b-7 complexes formed in the solution become complexed to inhibitory proteins in the serum; however, these C5b-7 complexes, designated SC5b-7, do not interact with the membrane [36].

Assembly of the C5b-8 complex

The C8 β-chain binds to the C5b of membrane-associated C5b-7. An amphiphilic transition in C8 occurs in both the α- and β-chains as previously described for C7, allowing the insertion of hydrophobic peptides from the α- and β-chains. C8α in the C5b-8 complex serves as the binding site for C9 to form C5b-9; C8γ is not essential for the molecule's cytolytic activity. Both the α- and β-chains of C8 have multiple functional domains. C8α has a β-chain binding domain, a domain that associates with the γ-chain via disulfide linkage, a membrane insertion domain, and a domain that binds and activates C9. C8β has a C5b-binding domain, a C8α-binding domain, and a domain that interacts with membrane lipids [23]. Pore formation by C5b-8 in the membrane has been demonstrated in the form of an increased conductance across BLM and by marker release/ion flux through resealed erythrocyte ghosts. The C5b-8 pores, ranging from 0.4 to 3 nm in diameter, are unstable and have a finite life span [37]. To lyse an erythrocyte, a large number of C5b-8 complexes are required [38]. The kinetics of hemolysis by C5b-8 is slower than those of C5b-9. Lytic C5b-8 pores have been demonstrated in M21 human melanoma cells [39], the U937 human histiocytic cell line [40], and *Giardia lamblia* [41]. Sublytic C5b-8 activates target cells by increasing cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and generating other signal messengers [42].

Assembly of the C5b-9 complex

A single C9 binds to the C8 α-chain in both membrane-bound C5b-8 and C5b-8 formed in the solution. C9 rapidly interacts with C5b-8 and initiates the transformation of a globular C9 (8 nm in length) into an elongated C9 (16 nm in length). The rapid binding of C9 to C5b-8 produces the C5b-8,9_1 complex, which is then followed by the slower incorporation of multiple C9 molecules to form C5b-8,9_n through C9–C9 polymerization, which can incorporate as many as 16 molecules of C9 [43–46]. C9 polymerization, while not required for erythrocyte lysis or nucleated cell killing, is necessary for the killing of Gram-negative bacteria [47]. C9 polymers with more than six molecules of C9 form an SDS-resistant C5b-8,9_n complex of tubular structure, also called poly-C9 [13, 32]. The C5b-9 complex has an annular ring structure with an external diameter of 20 nm, an internal diameter of 5 nm, and a height of 15 nm. C9 polymers with fewer than six molecules of C9 form an SDS-dissociable C5b-8,9_{1-6} complex that does not show the characteristic ultrastructure of poly-C9. The functional size of the C5b-9 channel ranges from 1 nm to 11 nm, and the pore size increases with increasing quantity of C9 molecules [48, 49]. The diameter of tubular poly-C9 without C5b-8 has been reported to be 10 nm [50].

Regulation of TCC assembly

S-protein/vitronectin

S-protein, an 80-kDa multi-functional glycoprotein, was first identified as a component of C5b-9 complexes

activated in serum. Using molecular cloning approaches, the S-protein was found to be identical to vitronectin. Purified S-protein inhibits C5b-9-mediated hemolysis by preventing the association of C5b-7 with the membrane [51]. S-protein/vitronectin binds to metastable sites on the nascent C5b-7 and produces water-soluble SC5b-7, which is unable to interact with the membrane. SC5b-7 can bind one C8 or three C9 molecules to form soluble SC5b-8 and SC5b-9, respectively. All of these complexes are unable to bind to membranes. Therefore, they are lytically inactive and are cleared from the circulation. S-protein also inhibits C9 polymerization and channel formation by perforin, thereby limiting not only the complement-generated pores but also the pores produced by cytotoxic lymphocytes. Recently, SC5b-9 was found to mediate upregulation of osteoprotegerin in endothelial cells (EC), possibly contributing to enhanced inflammation in rheumatoid arthritis [52].

Clusterin

Clusterin, a 70-kDa glycoprotein, was first identified in rete testis fluid on the basis of its ability to cause the aggregation of a variety of cells. It is found in the plasma in association with lipoproteins. Clusterin gene is expressed in cells that are directly involved in epithelial differentiation and morphogenesis [53]. Clusterin inhibits the assembly of C5b-7, C5b-8, and C5b-9 by interacting with a structural motif common to C7, C8α, and C9b [44]. Clusterin inhibits C9 assembly on C5b-8 and C5b-9 and also binds to C5b-7 to prevent membrane attachment. The impact on C5b-9 assembly is the most potent [54]. Clusterin is also associated with the hemolytically inactive SC5b-9 complexes formed in the solution together with S-protein/vitronectin.

CD59

Human CD59 is expressed as a glycosylphosphatidylinositol (GPI)-linked protein on the membrane surface of many cell types. It inhibits the MAC by interacting with C8α and C9 during the assembly of the complex on the same cell to which it is attached. This interaction limits the number of C9 molecules bound by C5b-8 and restricts the formation of a fully functional MAC. In paroxysmal nocturnal hemoglobinuria (PNH), membrane expression of CD59 is reduced or absent [55]. The genetic defect in PNH cells involves abnormal transcription of the phosphatidylinositol-glycans (PIG-A) gene, which belongs to a group of genes called PIG that are involved in the biosynthesis of GPI-anchored proteins. PIG-A encodes an early protein required for anchoring GPI to the protein backbone, near its C-terminus. Transcriptional and/or splicing defects result in small PIG-A transcripts or transcripts of normal size that lack activity as the result of a T-to-A mutation in the coding region [56].

In addition to its expression as a membrane protein, CD59 is present in soluble form in the blood, urine, and other body fluids [57]. CD59 inhibits the binding of C9 to C5b-8 by affecting the association of a C8α domain with C9b [58]. CD59 competes with C9 for binding to a nascent epitope on C8 that is exposed during C8 activation. CD59 can not only function as an inhibitor of the formation of large MACs but can also allow cells to eliminate newly formed MACs by blocking the early, functional channel formation of C5b-8 and C5b-9 complexes. Moreover, neither SC5b-9, inactive complexes isolated from complement-activated serum, nor poly-C9 binds to CD59. CD59 and other GPI-anchored proteins have been reported to contribute to signal transduction by increasing tyrosine kinase activity and initiated an increase in intracellular free Ca^{2+} concentration [59]. CD59 associated with CD58 also has a stimulatory effect on T cells after CD2 cross-linking [59].

Cell death and recovery after complement attack

While a single channel is sufficient to lyse an erythrocyte, the killing of nucleated cells is a "multi-hit" process requiring multiple C5b-9 complexes [60]. Given that limited C5b-9 complexes are efficiently eliminated, the formation of multiple channels is required to overcome this repair process and induce cell death. These data suggest that C5b-9-induced killing is affected by the ability of nucleated cells to eliminate C5b-9 complexes and repair cellular membranes.

Mechanism of cell death induced by C5b-9

During a limited complement attack, the MAC functions as a stimulus to eliminate TCCs, as previously discussed. However, when TCC formation exceeds the elimination rate from the cell membrane, cell death increases. In the presence of a large number of C5b-9, the rate and extent of cell death are independent of cell-volume regulation. It has been shown that a single channel in the membrane of erythrocytes is enough to lyse the cell through colloid osmosis [60]. Complement-mediated lysis of nucleated cells requires the presence of multiple MACs on the cell surface ("multi-hit characteristics") [60]. C5b-8,9_1 can cause nucleated cell death, although C5b-8,9_4 is twice as effective. Loss of ATP, ADP, AMP, and mitochondrial membrane potential has been shown to occur during the prelytic phase of complement attack [61]. The rate of cell death rises with an increase in intracellular Ca^{2+} level. An influx of Ca^{2+} through lytic C5b-9 channels is responsible

for the massive increase in Ca^{2+} and the rapid loss of inner mitochondrial membrane potentials, both of which are followed by acute cell death [61]. These findings collectively indicate that C5b-9 induces Ca^{2+}-dependent acute cell death and also negatively affects mitochondrial function. Lytic C5b-9 also induces morphological changes in the cells that are typical of apoptosis [62]. In addition, DNA fragmentation and TUNEL assay positivity have been reported after 30 min of exposure to a lytic dose of complement [63]. These studies have shown that this death is primarily necrotic, but with secondary features that resemble nuclear apoptosis and most likely occur as a result of extracellular DNase entering through the disrupted membrane. C5b-9-induced cell death has been found to be dependent on Bid cleavage and caspase activation [64]. In addition, both JNK1 and JNK2 have cytotoxic potential; however, JNK2 is the primary signal transducer [65].

The mechanism of Gram-negative bacterial killing by C5b-9 is much less well understood than that of mammalian cell death. Gram-positive bacteria characteristically possess a thick cell wall that prevents the MACs from reaching and breaching their inner plasma membrane. Gram-negative organisms, however, lack this thick wall, and lysis by C5b-9 has been found to play an important role in killing Gram-negative organisms of the genus *Neisseria*. Experimental evidence indicates that bacterial cell death may occur as a result of inner membrane dissolution induced by C5b-9 through the activation of metabolic processes such as oxidative phosphorylation [47].

Repair mechanisms implicated in cell survival after complement attack

In order for cells to survive limited complement attack, they must rapidly eliminate potentially lytic C5b-9 from their surface. Elimination of the C5b-9, as studied under conditions of limited complement attack, occurs via endocytotic processes or membrane shedding. The half-life of C5b-8,9_n channels remaining on the cell surface ranges from 1 to 3 min for nucleated cells and can be as much as 72 h on erythrocytes [31]. C5b-8 elimination from the cell surface is slower than C5b-9 removal, and C5b-7 is eliminated at the slowest rate. In Ehlrich cells, immunotracing of C5b-8,9_n with colloidal gold revealed the membrane-bound TCC entering multivesicular bodies (MVB) through endocytotic-coated vesicles [33], whereas in polymorphonuclear leukocytes, 35% of the entry occurred via endocytosis and 65% via membrane shedding [66]. Complement assembly generates an increase in cytosolic calcium as a result of Ca^{2+} influx through C5b-8 or C5b-9 pores. The rate of TCC elimination rises with the increasing $[Ca^{2+}]i$. Calcium influx through the channel also activates PKC, and this signaling is responsible for the membrane vesiculation and the internalization of TCC [67]. Inhibition of PKC reduces the endocytosis of C5b-9, but ERK inhibition has no effect [68]. In oligodendrocytes, nonlethal complement attack leads to reversible cell injury, with recovery following a transient rise in intracellular calcium and a fall in ATP in the absence of membrane permeabilization by propidium iodide [69]. C5b-9 complexes deposited on glomerular epithelial cells in the kidneys of rats with experimental membranous nephropathy have been found in clathrin-coated pits and MVBs [70]. C5b-9 complexes are endocytosed, packed into MVBs, and then released as exocytotic vesicles into the urine. In addition, mortalin/GRP75 promotes the shedding of membrane vesicles loaded with complement MAC and protects cells from complement-mediated lysis [71]. Other components that seem to play a role in membrane vesiculation are GPI-membrane-anchored proteins [35].

Cell activation induced by the TCC in nucleated cells

Signal transduction pathways induced by C5b-9 and required for cell cycle activation

A complex signaling mechanism is induced by TCC assembly and insertion into the plasma membrane. An increase in cytosolic Ca^{2+} and PKC activities, which are responsible for some functions of the TCC, is induced primarily by the pore-forming complexes C5b-8 and C5b-9 [67, 72]. However, the generation of cAMP and lipid-derived signal messengers such as *sn*-1,2-diacylglycerol (DAG) and ceramide is achieved at the stage of C5b-7 membrane insertion. Moreover, the assembly of C5b-8 and C5b-9 further increases the level of DAG and ceramide [35, 67]. Activation of membrane phospholipases at the C5b-7 level, when neither channel formation nor Ca^{2+} influx occurs, suggests that this effect is dependent on the insertion of C7-C9 peptides into the membrane lipid bilayer. The TCC-mediated production of DAG is specifically inhibited by the pretreatment of cells with pertussis toxin (PTX) [35]. Further studies have demonstrated that in the plasma membrane, TCC activates heterotrimeric *G* proteins of the G_i/G_o subfamily [73]. The ability of C5b-7, C5b-8, and C5b-9 to form coprecipitable complexes with active G proteins seems to be a critical component of the signal transduction pathways initiated by TCC [42] (Fig. 4).

C5b-9 has also been shown to activate the small G-protein Ras, which induces Raf-1 translocation to the plasma membrane and triggers ERK pathway activation (Fig. 4). PTX treatment inhibits both Raf-1 and ERK1 kinase activity, indicating the involvement of the Gi protein in this process [74]. In human aortic smooth muscle cells, sublytic C5b-9, but not C5b6, activates

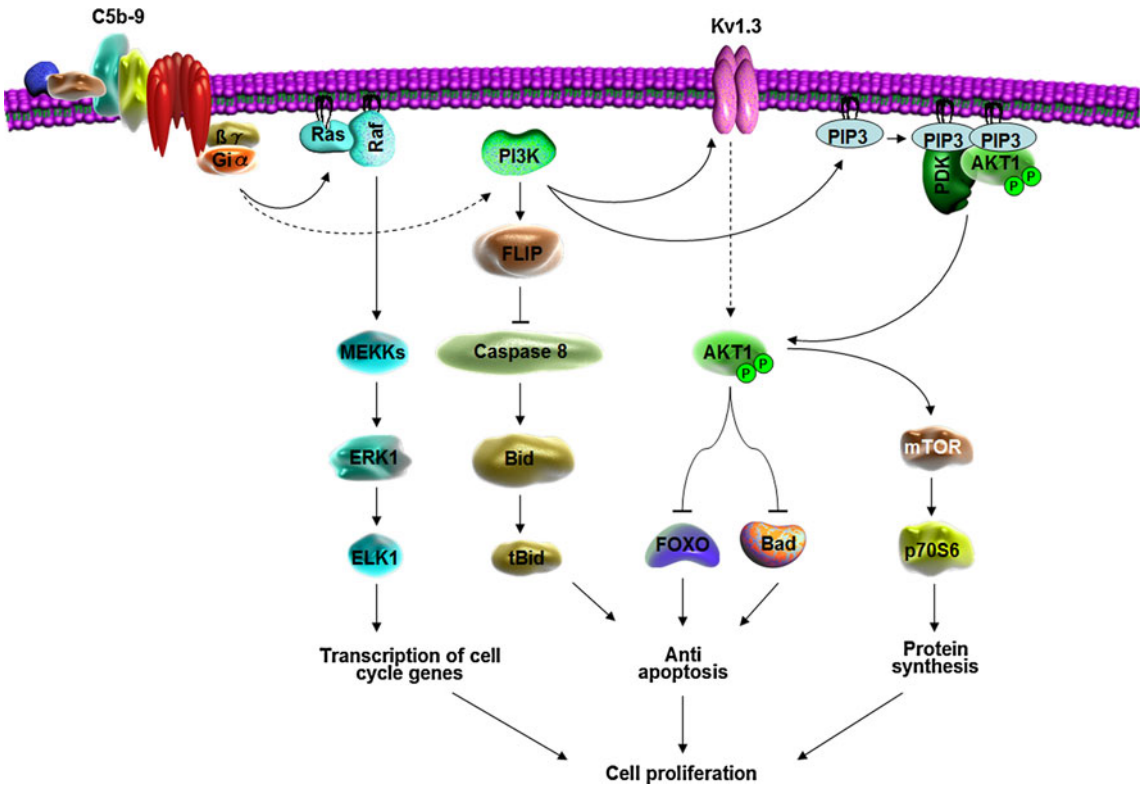

Fig. 4 Signaling pathways induced by C5b-9 that play a role in cellular proliferation and protection from apoptosis. C5b-9 interacts with the Gi protein, inducing activation of Ras. Ras then activates Raf-1, which in turn activates MEK1 and ERK1. C5b-9 also activates PI3K and induces the activation of PDK1 and Akt. PI3K also participates in the activation of ERK1 and p70S6 kinase, leading to the activation of the cell cycle. $K_v1.3$ is also involved in cell cycle activation and is induced by C5b-9 via the modulation of Akt activation. Activation of PI3K induces upregulation of FLIP and inhibits caspase-8 processing. C5b-9-induced Akt activation also inhibits apoptosis by regulating FOXO1, Bad phosphorylation and inhibiting Bid cleavage

phosphatidylinositol 3-kinase (PI3K). Pretreatment with the PI3K inhibitor wortmannin inhibits the C5b-9-mediated activation of ERK1, indicating potential cross-talk between these two pathways. Both ERK1 and PI3K activity are required for C5b-9-stimulated thymidine incorporation and cell cycle activation [75] (Fig. 4). C5b-9 activates ERK1, JNK1, and p38 MAPK pathways [75–79]. In the case of human aortic smooth muscle cells, their activity was only transiently increased and was not involved in DNA synthesis [75]. However, in rat glomerular epithelial cells, p38 MAPK activation increased significantly in response to C5b-9 and was required for the cytoprotective effects of sublytic complement [80]. In Schwann cells, C5b-9 induced cell proliferation and was dependent upon ERK1, Gi protein, and PKC [81].

Using in vitro kinase assays and detection of Ser-473 phosphorylation, we have shown that C5b-9 activates Akt. In our system, C5b-9-induced cell cycle activation was inhibited by pretreatment with LY294002 (PI3K inhibitor) or SH-5 (Akt inhibitor), or by transfection with Akt siRNA. These data suggest that the PI3K/Akt pathway is required for C5b-9-induced cell cycle activation. FOXO1, a member of the forkhead transcription factor family, was phosphorylated at Ser-256 and inactivated after C5b-9 stimulation, as indicated by a decrease in DNA binding and cytoplasmic relocalization. Silencing FOXO1 expression using siRNA stimulated EC proliferation and regulated angiogenic factor release. Our data indicated that C5b-9-mediated regulation of cell cycle activation through the Akt pathway in these cells is dependent on the inactivation of FOXO1. Taken together, these data indicate that the activation of the Gi protein/PI3K/Akt and ERK1 pathways plays a critical role in the cell proliferation induced by C5b-9 and that this effect is due in part to the regulation of cell cycle-specific genes (Fig. 4). In addition, PI3K inhibitors were able to inhibit p70 S6 kinase activation and cell cycle activation [82, 83]. C5b-9 induced tyrosine phosphorylation of JAK1 and STAT3, as well as the translocation of STAT3 to the nucleus; this translocation was independent of G-protein activation [84]. STAT3 also plays an important role in the activation of the cell cycle by C5b-9.

Activation of the cell cycle by C5b-9

Sublytic C5b-9 has been implicated in cellular proliferation, and this response may be important for cell survival in

an inflammatory milieu [82]. Sublytic C5b-9 induces cell proliferation in many cell subtypes [81, 83, 84]. In aortic smooth muscle cells, C5b-9 induces an initial increase in CDK4, followed by an increase in CDK2 activity. The mRNA and protein levels of the p21 cell cycle inhibitor are significantly reduced during the G1/S transition [75].

In oligodendrocytes (OLGs), the terminally differentiated cells that myelinate the axons of the central nervous system, C5b-9 assembly leads to S-phase activation [76, 77] but not cellular proliferation. C5b-9 activation is associated with an increase in CDC2 kinase activity [76]. This increase in CDC2 activity in early G_1 cells suggests a role for CDC2 in the G_1/S transition. Although CDC2 plays a major role in the G_2/M transition and mitosis, evidence exists to suggest it also functions in either the G_1 or the G_1/S transition [85–87]. Recent evidence indicates that the current paradigm of CDK2 being essential for the G_1/S transition and S-phase entry may be inaccurate, given that CDK2 knockout mice are fully viable and without developmental or cell cycle defects [88]. Recently, we have shown that C5b-9 induces the expression of the potassium channel $K_v1.3$ in oligodendrocytes and that the inhibition of $K_v1.3$ expression leads to an inhibition of DNA synthesis, indicating an important role for $K_v1.3$ in C5b-9-induced cell cycle activation (Fig. 4) [89].

As is true for oligodendrocytes, C5b-9 induces DNA synthesis in terminally differentiated glomerular epithelial cells without inducing proliferation [90]. There is also a delay in the G_2/M phase progression that is associated with DNA damage and increased p53 and p21 levels [91]. Therefore, proliferation may not occur in glomerular epithelial cells because of DNA damage and subsequent G_2/M delay.

C5b-9-mediated growth factor release may play an important role in cell cycle activation [92]. In EC, C5b-9 induces the release of the growth factors PDGF and bFGF [92, 93]. Addition of anti-PDGF and anti-bFGF antibodies to conditioned medium abolishes DNA synthesis, indicating that PDGF and bFGF are involved in cell cycle activation [93]. C5b-9 induces the release of IGF-1, which protects smooth muscle cells from apoptosis via an autocrine mechanism [94].

As part of a screen to identify novel genes that are induced by complement activation, we initially cloned response gene to complement (RGC)-32 from rat oligodendrocytes by differential display [95]. RGC-32 has been detected in most tissues examined and is therefore assumed to be involved in cell cycle activation [95]. RGC-32 forms a complex with cyclin B1/CDC2 and increases its kinase activity [83]. Overexpression of RGC-32 in the OLGxC6 cell line increases DNA synthesis in response to serum growth factors or C5b-9 [95]. In addition, overexpression in human aortic smooth muscle cells leads to S-phase and G_2/M entry of unstimulated cells, and C5b-9 further increases G_2/M progression [83]. RGC-32 silencing in aortic EC abolishes the DNA synthesis induced by C5b-9 and serum growth factors, indicating a requirement for RGC-32 activity for S-phase entry. RGC-32 siRNA-mediated knockdown also significantly reduces the C5b-9-induced CDC2 activation and Akt phosphorylation. RGC-32 has been found to be physically associated with and be phosphorylated by Akt in vitro. In addition, RGC-32 regulates the release of growth factors from these cells. Taken together, these findings suggest that cell cycle induction by C5b-9 is RGC-32-dependent and that this process occurs in part through the regulation of Akt and growth factor release [96].

Transcriptional and post-transcriptional regulation of gene expression by C5b-9

Regulation of proto-oncogene expression

Induction of immediate-early gene expression following exposure to extracellular stimuli represents the first major transcriptional event that precedes changes in other cellular responses. Regulation of proto-oncogene and transcription factors plays a major role in C5b-9-mediated cell cycle progression. C5b-9 is able to induce a sustained increase in proto-oncogene mRNA, while the induction of DNA synthesis is c-*jun* dependent [76, 77, 97]. In response to sublytic C5b-9, activating protein-1 (AP-1) DNA binding activity is increased, and this increase is Gi protein-dependent [74, 76]. To assess the effects of sublytic C5b-9 on transcription, we investigated the regulation of *c-fos* gene expression in myotubes by C5b-9 [98] and found that C5b-9 activated *c-fos* primarily through transcriptional activation [98]. Three transcription factors—serum response factor, Elk1, and Sap1—act synergistically to trans-activate the *c-fos* serum response element [98]. In our study, Elk1, a member of the ternary complex factor family [99], was phosphorylated at Ser383 in response to C5b-9 in an ERK1-dependent manner [98]. Thus, our data suggest that the activation of ERK1 plays a critical role in the initiation of C5b-9-induced *c-fos* transcription. C5b-9 also activates the transcription factor NF-κB [100, 101]. NF-κB activation induces the production of IL-6 [101] as well as IL-8 and MCP-1 [100]. NF-kB may serve not to activate the cell cycle but to induce pro-inflammatory cytokines.

Regulation of gene expression in terminally differentiated cells

Terminally differentiated cells such as oligodendrocytes and Schwann cells are cells that have withdrawn from the cell cycle and express cell type-specific genes. During oligodendrocyte differentiation, the expression of three

Table 1 Presence of C5b-9 neoantigens in human tissue

Organ	Disease	Reference
Brain	Multiple sclerosis	[109, 110]
	Neuromyelitis optica	[111]
	Meningitis	[112]
	Rasmussen's encephalitis	[113]
	Prion disease	[114]
	Alzheimer's disease	[115]
	Pick disease	[116]
	Parkinson's disease	[117]
	Stroke	[118, 119]
	Trauma	[120]
	Temporal lobe epilepsy	[121, 122]
	Closed head injury	[123]
Spinal cord	Disc disease	[124]
Muscle and nervous tissue	Muscle necrosis	[125, 126]
	Guillian–Barré syndrome	[127, 128]
	Muscular dystrophy	[129, 130]
	Colchicine myopathy	[131]
Kidney	SLE	[132–134]
	Diabetes mellitus	[135, 136]
	Membranous and membranoproliferative GN	[137–139]
	Hemodialysis	[140, 141]
Skin	Cutaneous SLE	[142]
	Diabetes mellitus	[143]
	Pemphigus	[144, 145]
	Dermatomyositis	[146, 147]
	Henoch–Schönlein purpura	[145, 148]
	Scleroderma	[149]
	Porphyria cutanea tarda	[143]
Cardiovascular	Atherosclerosis	[150, 151]
	Myocardial infarction	[152–154]
	Cardiomyopathias	[155, 156]
	Cardiopulmonary bypass	[157, 158]
	Cardiac transplant	[159]
	Aortic valves	[160]
Lung	Asthma	[79]
	Bronchiolitis obliterans syndrome	[145]
Gastrointestinal	Ulcerative colitis	[161–163]
	Crohn's disease	[162]
Liver	Fatty liver	[164]
	Ischemia	[165]
	Liver cirrhosis	[166, 167]
	Hepatitis C	[168]
	Transplant	[159, 169]
Breast, stomach, bladder	Cancer	[170–172]
Eye	Macular degeneration	[173]
	Glaucoma	[174]
	Diabetic retinopathy	[175]
Joints	Rheumatoid arthritis	[52, 176, 177]
	Osteoarthritis	[178]
	Gout	[179]
Spleen	Splenectomy	[180]
Placenta	Trophoblasts	[181]
Chest, Abdomen	Injury	[120]

myelin-specific genes, proteolipid like protein (PLP), myelin basic protein (MBP), and 2′ 3′-cyclic nucleotide 3′-phosphodiesterase (CNP-ase) has been found to increase [76]. Sublytic C5b-9 significantly reduces the accumulation of mRNA for genes encoding PLP and MBP but not CNP-ase [76]. This mRNA decay observed for PLP and MBP in the presence of C5b-9 is post-transcriptionally regulated and also dependent on Ca^{2+} influx in the case of PLP gene expression. In Schwann cells, C5b-9 significantly reduces myelin-specific protein P_0 gene expression, in part through the modulation of P_0 transcription [102]. Post-transcriptional regulation of the P_0 gene is also involved in down-regulation of expression. These changes induced by C5b-9 in myelin gene expression decrease the ability of OLGs and Schwann cells to myelinate and may contribute to pathologic demyelination.

In skeletal muscle cells, sublytic C5b-9 exposure has been shown to be able to reduce the expression of muscle-specific genes such as α-actin, troponin I_s, aldolase A, and acetylcholine receptor α [97]. In that study, troponin promoter activity was reduced by 50% in response to sublytic C5b-9, and the decay in muscle-specific genes was increased in the presence of sublytic complement attack. These data suggest that C5b-9 is able to inhibit the transcription of muscle-specific genes and increase the rate of decay of their mRNA [97].

In conclusion, these changes in gene expression may represent a mechanism by which differentiated cells can respond to limited complement attack and thereby survive in an inflammatory milieu.

C5b-9 complex-mediated protection from apoptotic cell death

Complement activation has been implicated in the pathogenesis of multiple sclerosis (MS), and oligodendrocytes are susceptible to C5b-9-mediated cell lysis in vitro. However, sublytic doses of the C5b-9 have been found to promote the survival of these cells [103, 104]. Apoptosis initiated in oligodendrocytes by serum withdrawal is associated with a rapid decline in PI3K/Akt activity,

together with the release of cytochrome c, activation of caspase 9, and cleavage of caspase-3 [103, 104]. All these apoptosis-associated activities are inhibited by the activation of complement and the assembly of sublytic C5b-9 complexes. Studies of upstream signaling have shown that C5b-9 induces strong PI3K/Akt activation and phosphorylation of Bad. These complexes increase the phosphorylation of Bad at Ser112 and Ser136, resulting in the dissociation of Bad/Bcl-x_L complexes [104]. Both processes can be reversed by inhibiting PI3K. Therefore, sublytic complement attack appears to increase the survival of oligodendrocytes, in part by activating signaling pathways that are important for Bad phosphorylation and the subsequent alteration of the association between Bcl-x_L and Bad (Fig. 4). In addition, Bim is required for the oligodendrocyte cell death caused by serum withdrawal, and C5b-9 prevents this association by promoting the rapid dissociation of preformed Bim/Bcl-x_L complexes [105]. Similarly, sublytic MACs can rescue Schwann cells from apoptosis via the activation of PI3K/Akt, BAD phosphorylation, and increased expression of Bcl-x_L.

We found that both TNFα and FasL are able to induce the apoptosis of OLGs and that C5b-9 inhibits FasL- and TNF-α-induced cell death [103, 105]. This C5b-9 effect is mediated through the inhibition of caspase-8 activation and Bid cleavage [106]. Exposure to C5b-9 also caused a significant increase in c-$FLIP_L$ expression [106]. These results suggest that C5b-9 prevents caspase-8 processing through a c-$FLIP_L$-dependent mechanism.

Thus, sublytic C5b-9, acting through PI3K signaling, is able to rescue oligodendrocytes from apoptosis by upregulating c-$FLIP_L$ and preventing mitochondrial insertion of the pro-apoptotic proteins Bad, Bid, and Bim (Fig. 4). These data indicate that sublytic C5b-9 detected on oligodendrocytes and Schwann cells in vivo during demyelination may facilitate the survival of those cells that are essential for remyelination.

Role of C5b-9 in health and disease in humans

The presence of C5b-9 neoantigens in human tissues in healthy individuals and controls (see Table 1) indicates in situ complement activation and MAC assembly. C5b-9 deposits have been found to be associated with cell debris or localized to the plasma membranes of cells adjacent to areas of necrosis and sclerosis. Many of these cells carrying C5b-9 complexes in atherosclerotic fibrous plaques are macrophages, suggesting a role for both inflammatory cells and complement activation in vascular tissue injury [107, 108]. In some cases, C5b-9 deposits were colocalized with S-protein/vitronectin, suggesting that some of the complexes might be cytolytically inactive. It is reasonable to assume that C5b-9 complexes directly participate in the pathogenesis of chronic inflammation and tissue healing by inducing cell lysis. However, sublytic C5b-9 plays an important role in modulating a variety of metabolic activities in target cells, including cell proliferation and differentiation, and is involved in maintaining cell and tissue homeostasis during acute and chronic inflammation.

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