Outside in: Roles of complement in autophagy

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The complement system is a well-characterized cascade of extracellular serum proteins that is activated by pathogens and unwanted waste material. Products of activated complement signal to the host cells via cell surface receptors, eliciting responses such as removal of the stimulus by phagocytosis. The complement system therefore functions as a warning system, resulting in removal of unwanted material. This review describes how extracellular activation of the complement system can also trigger autophagic responses within cells, up-regulating protective homeostatic autophagy in response to perceived stress, but also initiating targeted anti-microbial autophagy in order to kill intracellular cytoinvasive pathogens. In particular, we will focus on recent discoveries that indicate that complement may also have roles in detection and autophagy-mediated disposal of unwanted materials within the intracellular environment. We therefore summarize the current evidence for complement involvement in autophagy, both by transducing signals across the cell membrane, as well as roles within the cellular environment.

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1 | AUTOPHAGY: A BRIEF DESCRIPTION

Autophagy, literally “self-eating,” is a process by which cells break down intracellular contents, often for recycling or turnover of nutrients and material, regulation of organelle function or removal of misfolded proteins and inclusion bodies (Dikic & Elazar, 2018). In general, autophagy is a homeostatic process that protects against cellular stress and, accordingly, can be up-regulated when cells face certain pressures. Three main pathways exist: microautophagy, chaperone-mediated autophagy and macroautophagy. Micro-autophagy describes lysosomal membrane invagination and vesicle scission into the lumen, in a process similar to the formation of multivesicular bodies, which leads to the digestion of the vesicles and their contents. While this is a constitutive process of membrane homeostasis, it becomes up-regulated during nitrogen restriction (Li, Li, & Bao, 2012). Chaperone-mediated autophagy describes direct translocation of soluble cytosolic proteins across the lysosomal...
membranes, due to their recognition and selection by cytosolic chaperones such as heat shock cognate protein 70 (HSC70 or Hsp70) (Chiang, Terlecky, Plant, & Dice, 1989), which are then translocated across the lysosomal membrane via interaction with lysosome-associated membrane protein type 2A (LAMP2A) (Kaushik & Cuervo, 2018). Substrates of chaperone-mediated autophagy may consist of proteins from other subcellular compartments, which are transported into the cytosol for degradation as part of a quality control mechanism, for example in the removal of misfolded proteins. This can occur via HSC70-mediated recognition of a KFERQ-like sequence present in some 40% of the mammalian proteome, highlighting the importance of chaperone-mediated autophagy in protein turnover (Dice, 1990).

The term “autophagy” is however often used as shorthand for macroautophagy, which describes the process of degradation of larger portions of the cytosol, which may include targeted organelles such as mitochondria, by the formation of a double-membraned vesicle known as the autophagosome. This is also a homeostatic process required for removal and recycling of damaged organelles and aggregated and misfolded proteins, but is also up-regulated by hypoxia, oxidative or endoplasmic reticulum stress, protein aggregation, cell damage or nutrient starvation in many cell types (Dikic & Elazar, 2018). Autophagosome formation begins with nucleation of a pre-autophagosomal structure, called the omegasome, from the endoplasmic reticulum. Cellular stress causes activation of Unc-51-like kinase (ULK1) complex, which then phosphorylates the class III PI3K (PI3K) complex, made up of PI3K, Beclin 1, VPS34 and ATG14L (Dikic & Elazar, 2018). Autophagy genes (ATG) were first discovered and numbered in yeast, and their mammalian homologues are often named ATGL for “ATG-like.” Local production of phosphatidylinositol 3-phosphate (PI3P) by the PI3K complex recruits PI3P-binding proteins such as WD-repeat domain phosphoinositide-interacting protein 2 (WIPI2) to the growing phagophore membrane. This is then extended, enclosing and partitioning a portion of the cytosol containing autophagy substrates. An important step in this process is the recruitment of ATG16L1, which occurs via lipid interactions and binding to WIPI2. ATG16L1 itself binds to a conjugate of ATG12 and ATG5 (Otomo, Metlagel, Takaesu, & Otomo, 2013). The ATG12–ATG5 complex has E3 ligase-like activity and is involved in lipidation of the ubiquitin-like cytoplasmic protein LC3 (a homologue to yeast ATG8), by coupling it to phosphoethanolamine, thus relocating LC3 from the cytosol and into the growing phagophore membrane (Kabeya et al., 2000). The observation of these LC3-positive structures by fluorescent microscopy, as well as the gel motility shift observed on lipidation of LC3 (from soluble LC3-I to membrane-associated lipidated LC3-II), is often used to assess cellular autophagy induction (Klionsky et al., 2016). Membrane-associated LC3-II is involved in sequestering targeted material to the growing phagophore, via interaction with receptor proteins such as the stress-inducible autophagy substrate p62/Sequestosome 1 (SQSTM1), which itself binds to intracellular ubiquitinylated material and protein aggregates targeted for destruction (Bjorkoy et al., 2005; Ichimura et al., 2008). Other ubiquitin-independent LC3-binding receptors can be stabilized on the surface of organelles such as damaged mitochondria, marking them as cargo for inclusion within the growing phagophore and subsequent recycling by autophagy (Koentjoro, Park, & Sue, 2017). LC3-II is required for optimal growth of the forming autophagosome, but LC3 and related ATG8-family GABARAP proteins are also essential for autophagosome completion, an incompletely understood process by which the phagophore membrane fuses to entirely surround and enclose the sequestered cytosol and cargo (Nguyen et al., 2016; Weidberg et al., 2011). This is an important step, without which the autophagosome does not subsequently fuse with lysosomes. Premature fusion of a lysosome with an unsealed autophagosome would result in leakage of lysosomal contents into the cytosol.

Once the autophagosome is sealed, ATG proteins must be removed from the cytosol-facing surface of the autophagosome membrane for recycling, to avoid being degraded within the lysosome after fusion. It is possible that presence of some of these proteins on the autophagosome inhibits lysosome fusion. In yeast, phospholipid phosphatases and the protease ATG4 are involved in removal of PI3P and ATG8 from the autophagosome surface, respectively, without which lysosomal fusion does not occur (Reggiori & Ungermann, 2017). As ATG8/LC3-II and PI3P presence in the membrane recruits many other ATG proteins to the autophagosome, their removal likely induces dissociation of the remaining autophagosome-forming machinery and therefore allows subsequent lysosomal fusion. Completed autophagosomes are transported along the cytoskeleton and fused with lysosomes via the action of tethering proteins and soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (Nakamura & Yoshimori, 2017), which help to physically fuse the lipid membranes together, allowing lysosomal enzymes to mix with the enclosed cytosolic compartment, degrading its contents. Liberated amino acids and nutrients are then exported from the completed autolysosome back into the cytosol for recycling.

Although the function of autophagy was first identified as a mechanism required for both homeostatic and responsive turnover of cellular contents, it has also been identified as an intracellular defence mechanism against pathogens. Whereas viral particles can be small enough for destruction by the proteasome, intracellular bacteria need to be identified and targeted by autophagy machinery, leading to their inclusion within autophagosomes and subsequent degradation, not only within phagocites but also within non-immune cells (Nakagawa et al., 2004). Ubiquitinilation of intracellular pathogens can target them for inclusion within growing autophagosomes, via recruitment of receptor proteins such as p62 described above. Alternatively, mechanisms exist, which cause direct recruitment of autophagy machinery such as ATG16L1 to pathogens or pathogen-containing membrane compartments. For example, the C-terminal WD-repeat domain of ATG16L1 interacts via a conserved motif identified on the cytosolic tail of late endosome membrane protein TMEM59 (Boada-Romero et al., 2013), allowing TMEM59 to direct autophagic destruction of internalized bacteria. The same conserved motif binding the ATG16L1 WD-repeat domain was also identified on the cytosolic tail of TLR2 as well as on the cytosolic protein NOD2 (Boada-Romero et al., 2013), explaining the mechanism by
which these proteins can recruit ATG16L1 complexes to bacteria-containing phagosomes (Sanjuan et al., 2007) and to the site of bacterial entry into the cell (Travassos et al., 2010), respectively, to induce a non-canonical autophagic pathway. The destruction of intracellular pathogens by autophagy has a special term, xenophagy, and is of particular relevance to this review.

2 | CLINICAL TARGETING OF AUTOPHagy

The most commonly used clinical inhibitor of autophagy is hydroxychloroquine and its derivatives, which have been long used to treat malaria, but are now also used to treat rheumatoid arthritis, systemic lupus erythematosus and other inflammatory rheumatic diseases, due to their immunomodulatory properties (Schrezenmeier & Dorner, 2020). As such, these drugs have a well-understood safety profile and are widely available. Chloroquine is understood to target acidification of lysosomes and therefore prevents autophagosome maturation, possibly by interaction with palmitoyl protein thioesterase 1 (PPT1) (Rebecca et al., 2019), which is required for normal lysosomal function. Chloroquine was originally found to inhibit tumour cell growth in vitro, but development and testing by pharmaceutical industry did not initially find success (Dolgin, 2019), leading to a cooling of interest in anticancer targeting of autophagy. However, there have been case reports of individual treatment success stories in cancer of the brain (Levy et al., 2014) and pancreas (Kinsey et al., 2019). It is now emerging that autophagy targeting can be more successful in combination with targeting of MAPK pathways (Bryant et al., 2019; Lee et al., 2019), leading to re-emergent interest (Dolgin, 2019). With new autophagy-targeting inhibitors with improved selectivity becoming available (Honda et al., 2016), autophagy inhibition could be a future clinical goal for cancer therapy. In contrast to cancer treatment, where inhibition of autophagy seems to be advantageous, stimulation of autophagy is seen as desirable in other diseases, although not yet a target of clinical therapy. Many neurodegenerative diseases can be caused by accumulation of insoluble protein aggregates, which it is proposed can be cleared by increased levels of autophagy (Djajadikerta et al., 2020) and autophagy-stimulating compounds have had some positive effects in animal models of Alzheimer's disease and Huntington's disease (Pierzynowska et al., 2018). In type 2 diabetes, development of insulin resistance in peripheral tissues is compensated by an up-regulation of insulin expression by pancreatic beta cells, putting them under increased metabolic stress. In this situation, it has been found that autophagy is cytoprotective for pancreatic beta cells (Ebato et al., 2008), while autophagy-deficient mice have reduced beta-cell mass and therefore impaired insulin production (Jung et al., 2008). In addition, there is evidence that autophagy is dysregulated in beta cells from both diabetic humans and rodents (Fujitani, Kawamori, & Watada, 2009). Autophagy stimulation can therefore be advantageous for beta-cell survival by alleviating stress caused by high rates of protein production, leading to ER stress and protein misfolding (Quan et al., 2012). Indeed, mice with mutations leading to increased rates of insulin misfolding and aggregation showed improved symptoms of diabetes when treated with rapamycin, an autophagy-stimulating compound (Bachar-Wikstrom et al., 2013). In addition, the beneficial effects of the widely used diabetes treatment metformin may partially be due to autophagy stimulation (Jiang et al., 2014). Therefore, there is scope for future clinical intervention in cancer, neurodegeneration and type 2 diabetes by stimulation of autophagy pathways.

3 | AUTOPHagy ACTIVATION BY PATHOGEN-ASSOCIATED MOLECULAR PATTERNS

Before introducing the ways in which the complement system has been shown to influence autophagy, we will first describe how other known germline-encoded pattern recognition receptors (PRRs) can influence the same pathway. Pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) are recognized not only by activators of the complement system but also by PRRs such as toll-like receptors (TLRs) and NOD-like receptors, leading to autophagy induction (Oh & Lee, 2014). This has been recognized to be particularly important in infection, leading to killing of intracellular pathogens by targeted autophagy and LC3-associated phagocytosis.

Many species of pathogenic bacteria have the ability to escape from phagosomes into the cytosol or to inhibit the function of phagosomes, thereby enhancing their own survival after uptake by phagocytes. However, it was observed that bacteria such as Mycobacterium tuberculosis and Listeria monocytogenes can be found within autophagosomes (Gutierrez et al., 2004; Py, Lipinski, & Yuan, 2007), suggesting that alternative modes of killing led to their clearance after their escape into the cytosol. Just as receptor proteins such as p62 mediate linkage of ubiquitinylated targets to LC3-II-positive membranes, targeting them for recycling, it was suggested that similar cytosolic receptors would be able to specifically recognize pathogens and target them for destruction. Indeed, intracellular pathogen sensors NOD1 and NOD2 bind bacterial peptidoglycans (Chamaillard et al., 2003; Girardin et al., 2003; Inohara et al., 2003) and recruit ATG16L1 to sites of bacterial entry into the cytosol, leading to inclusion of these bacteria within LC3-decorated autophagosomes. The importance of the NOD2 interaction with ATG16L1 in controlling bacterial invasion by targeted autophagy is underlined by the association of mutations in NOD2 and ATG16L1 with Crohn's disease (Hugot et al., 2001; Rioux et al., 2007). It is therefore thought that defective clearance of gut bacteria from epithelial cells, due to defective targeted autophagy, contributes to the chronic inflammation that underlies the pathology of this disease.

Stimulation of TLRs also induces formation of autophagosomes and enhances clearing of intracellular bacteria such as M. tuberculosis (Delgado, Elmaoued, Davis, Kyel, & Deretic, 2008). However, unlike NOD-like receptors, TLRs are transmembrane proteins typically found at the cell surface or in the endosome–lysosomal compartment. Closer investigation has shown that TLRs often stimulate non-conventional autophagy, by recruiting autophagy machinery to
the phagosome, in a process termed LC3-associated phagocytosis. Some pathogens such as M. tuberculosis inhibit fusion of the phagosome with the lysosome, prolonging their survival, but this can be overcome by autophagy initiation via starvation, which leads to co-localization of the phagophore with autophagy markers such as LC3 (Gutierrez et al., 2004). Similarly, TLR-deficient mice are highly susceptible to the intracellular Leishmania parasite, but pharmacological stimulation of autophagy helped clear infection (Franco et al., 2017). TLR stimulation of autophagy can therefore accelerate phagosome maturation and lysosomal fusion (Blander & Medzhitov, 2004), resulting in increased killing of intracellular pathogens. This has been shown in a simplified model whereby latex beads coated with TLR ligands were taken up by macrophages. The presence of TLR ligands led to rapid association of LC3 with the phagosomal membrane, which did not occur in the absence of TLR stimulation and that occurred without formation of classical double-membranated autophagosomes (Sanjuan et al., 2007). This was also shown to be TLR and MyD88 dependent. Similarly, LC3 decorated phagosomes shortly after internalization of Escherichia coli and yeast particles and non-expressing cell lines transfected to express TLR2 gained the ability to deposit LC3 onto the cytosolic face of membranes of yeast-containing phagosomes (Sanjuan et al., 2007). Therefore, cytosolic NOD-like receptors can directly bind cytosolic pathogens while recruiting ATG16L1 and associated autophagy machinery, leading to inclusion of the pathogen within a double-membranated LC3-positive autophagosome, whereas TLRs can lead to similar decoration of phagosomal membranes with LC3, in both cases leading to lysosomal fusion and pathogen killing.

4 | THE COMPLEMENT SYSTEM

The complement system is made up of serum proteins that become activated in response to danger or infection, resulting in a cascade-like reaction that generates activatory signals to host cells (Kohl, 2006). Soluble PRRs of the complement system such as C1q, mannose-binding lectin and ficolins recognize PAMPs and DAMPs (Reis, Mastellos, Hajishengallis, & Lambris, 2019), triggering cleavage of complement components 4 and 2 (C4 and C2), the cleaved forms of which form together a C3 convertase, capable of cleaving the central complement component, C3, into C3b and C3a. While many copies of C3 can be cleaved by the C3 convertase before it decays, C3b can also bind to the C3 convertase and in turn form the C5 convertase, which then cleaves C5 into C5b and C5a. C5b in turn then binds to soluble proteins C6, C7 and C8, forming a complex that is then able to insert into lipid bilayers. This then catalyses recruitment of multiple copies of C9, which insert into the membrane, forming a pore known as the membrane attack complex (MAC), which is capable of lysing Gram-negative bacteria (Doorduijn, Rooijakkers, & Heesterbeek, 2019).

Complement proteins are synthesized in large amounts by liver hepatocytes but also other cell types throughout the body, which ensures that complement is present not only at high concentrations in blood but also locally in tissues. Once activated by PAMPs or DAMPs, complement has three major outcomes: transmitting danger signals to the host, directly attacking pathogens and covalently labelling pathogens or waste material for clearance (Kohl, 2006; Reis et al., 2019). When C3 or C5 are cleaved, the smaller split products, C3a and C5a, also known as the anaphylatoxins, can signal via specific receptors, C3a and C5a receptor, as well as a second C5a receptor, C5L2. These receptors often stimulate responses linked to inflammation, including chemotaxis, cell activation, vasodilation and smooth muscle contraction. The MAC is capable of directly lysing Gram-negative bacteria, causing rapid death on exposure to serum, unless the bacteria express resistance mechanisms to escape complement attack (Lambris, Ricklin, & Geissbrecht, 2008). Finally, C3 itself covalently labels target material once cleaved to C3b, marking it for clearance by professional phagocytes.

The role of C3 in clearing material from the extracellular environment is long established. C3 is well known as an opsonizing factor of extracellular material, involved in detection and clearance of pathogens as well as apoptotic cells and self-material (Ricklin, Reis, Mastellos, Gros, & Lambris, 2016). On cleavage to C3b, C3 undergoes a dramatic conformational change and reveals a reactive thioester group that forms a covalent bond with amine or carboxyl groups on target surfaces, leading to irreversible covalent binding of C3b. This activated C3b in turn is cleaved by serum factor I in combination with one of several cofactors to inactive C3b (iC3b), which is recognized by complement receptors 3 and 4 found on the surface of phagocytes and ligation of which stimulates phagocytosis of iC3b-opsonized material. Additionally, material coated with deposited C3b is transported away by red blood cells expressing complement receptor 1 and is taken either to the liver or to the secondary lymphatic organs for disposal by Kupffer cells or presentation to the adaptive immune system (Gonzalez et al., 2010) respectively. In this way, bacteria and virus particles are identified and removed, limiting infection and self-material such as apoptotic cells (Trouw, Blom, & Gasque, 2008) is also cleared, limiting immune autoreactivity. Further final cleavage of iC3b forms C3d, which remains covalently bound to the target via the thioester group and is the ligand for complement receptor 2 (CR2), found on the surface of B cells. CR2 ligation lowers the threshold for B-cell activation at least by a 1,000-fold (Dempsey, Allison, Akkaraju, Goodnow, & Fearon, 1996) and C3 opsonization of antigen is therefore an important molecular adjuvant for adaptive humoral immune responses. Complement therefore represents a sophisticated pathogen and danger detection system, able to mediate multiple responses in many host cell types due to production of a myriad of activation products. In particular, sequential C3 activation products have different conformations, with various specific receptors and interaction partners (Ricklin et al., 2016), allowing C3 to deliver context-specific stimuli depending on how far it has been processed (C3 → C3b → iC3b → C3d) and dependent on which receptors are present to interact with these different products. C3 is therefore involved in many effector functions within immune defence, as well as tissue homeostasis and development (Ricklin, Hajishengallis, Yang, & Lambris, 2010). Although an important part of innate
TABLE 1

| Component  | Action                                                                 | References |
|------------|-------------------------------------------------------------------------|------------|
| CD46       | Cyt1 splice variant of CD46 triggers autophagy upon pathogen sensing by recruiting the GOPC-VPS34–Beclin 1 complex | (Joubert et al., 2009) |
| CD46       | CD46 activates cytotoxic autophagy against oxidative stress in respiratory epithelium | (Tsai et al., 2018) |
| VSIG4/CR1g | Engagement of VSIG4 induces xenophagy in macrophage and epithelial cells, involving MyD88 and Beclin 1 signalling | (Kim et al., 2016) |
| VSIG4/CR1g | VSIG4/CR1g promotes acidification of bacteria-containing vesicles, possibly by LC3-associated autophagy | (Kim et al., 2013) |
| C3aR/C5aR  | C3aR and C5aR signalling suppresses lethal mitophagy, promoting dendritic cell survival and alleviating GVDH | (Nguyen et al., 2018) |
| C5aR       | C5aR-mediated degradation of Bcl-2 promotes autophagy in alveolar macrophages, contributing to increased apoptosis | (Hu et al., 2014) |
| MAC        | Podocytes utilize autophagy as a cytoprotective response against MAC-induced injuries | (Liu et al., 2017; Lv et al., 2016) |
| MAC        | MAC triggers membrane permeabilization of lysosomes suppressing lysosomal degradation of autophagosome contents | (Liu et al., 2017) |
| C3         | Directs xenophagy of bacteria by recruiting ATG16L1 to invasive C3-opsonized bacteria | (Sorbara et al., 2018) |
|            | Maintains homeostatic autophagy in beta cells via ATG16L1 interaction | (King, Kulak, et al., 2019) |

Immunity, complement can stimulate pathological inflammation when not properly regulated or triggered in the wrong context (Martin & Blom, 2016; Sjoberg, Trouw, & Blom, 2009) and contributes to pathology in autoinflammatory and rheumatic disease, meaning that it is increasingly becoming a target for clinical drug development (Mastellos, Ricklin, & Lambris, 2019).

For further details of complement activation in pathogen detection and homeostasis in the extracellular environment, readers are referred to more comprehensive reviews (Reis et al., 2019; Ricklin et al., 2010). This article will however focus on more recent discoveries that complement and C3 may also have roles in detection and disposal of pathogens and unwanted material via the process of autophagy, occurring within the intracellular environment. The next sections will therefore summarize the current evidence for involvement of complement in autophagy induction (summarized in Table 1), both by transducing signals across the cell membrane, as well as potential roles within the cellular environment.

5 | COMPLEMENT-DEPENDENT INDUCTION OF AUTOPHAGY VIA CELL SURFACE RECEPTORS

The complement system is activated by detection of PAMPs/DAMPs by the system's germline-encoded soluble PRRs, leading to production of complement protein cleavage products that signal to cells via specific receptors (Kohl, 2006). It is well recognized that this extracellular fluid-phase detection system can therefore trigger responses in cells, such as induction of phagocytosis, chemotaxis, exocytosis and muscle contraction, to name a few. The complement system can therefore be seen as a danger-sensing system able to induce and direct appropriate responses in host cells and one such response is autophagy, which can be triggered by the following cell surface complement receptors.

5.1 | CD46

CD46 is a cell surface transmembrane protein originally described as an inhibitor of complement activation (Liszewski, Post, & Atkinson, 1991). CD46 binds C3b and acts as a cofactor for factor I, a serum complement factor that cleaves deposited C3b into inactive C3b (iC3b), therefore preventing further activation of the complement cascade. Because of this cofactor activity, CD46 was originally named membrane cofactor protein (MCP). CD46 has several splice variants, with two potential cytosolic tails (Purcell et al., 1991). Cyt-1 and Cyt-2, which are capable of transducing signals across the cell membrane upon ligand binding. CD46 plays an important role as a potent co-stimulator of human CD4+ T-cell activation, leading to gene expression and metabolic changes (King et al., 2016; Kolev et al., 2015), which result in induction of an anti-inflammatory, IL-10-secreting regulatory T-cell phenotype (Kemper et al., 2003). Perhaps for this reason, CD46 is a "pathogen magnet" (Cattaneo, 2004), being the cell surface receptor for human pathogens such as adenoviruses B and D, measles virus, herpes virus 6, Neisseria and some strains of group A Streptococcus (GAS).

Intracellular GAS survival can be limited by xenophagy (Nakagawa et al., 2004) and Joubert et al. (2009) therefore tested whether this can be triggered by its binding to CD46. They found that antibody-mediated cross-linking of cell surface CD46 led to de novo formation of autophagosomes in HeLa cells, which was found to be dependent on the interaction of Golgi-associated PDZ and coiled-coil motif-containing protein (GOPC) with the CD46 Cyt-1, but not Cyt-2 tail. GOPC itself has been shown to interact with Beclin 1 (Yue
et al., 2002), a protein central to nucleation of the autophagosome phagophore. Further, GOPC can interact with both CD46 Cyt-1 and Beclin 1 via different domains, allowing formation of a heterotrimeric complex. Strains of measles virus and GAS that bound CD46 induced autophagy in human cells, in a manner dependent on GOPC and Beclin 1 and Cyt-1; isoforms of CD46 containing the Cyt-2 domain could not induce autophagy. CD46-binding GAS strains were also quickly degraded by autophagy within the cell, which was delayed by siRNA-mediated knockdown of the Cyt-1/GOPC pathway, demonstrating the functional importance of CD46-mediated uptake and xenophagy in restricting intracellular pathogen survival.

Antibody-mediated cross-linking of CD46 also induced autophagy in cultured primary human airway epithelial cells and A549 lung epithelial cells (Tsai et al., 2018). This resulted in increased protection against oxidative stress, which was reversed by the autophagy inhibitor 3-methyladenine. In addition, CD46 ligation enhanced GOPC expression and limited H$_2$O$_2$-induced increases in cellular pro-IL-1β and NLRP3 content, as well as IL-1β secretion, most likely due to the known role of autophagy in degrading intracellular inflammasome components (Harris et al., 2011; Shi et al., 2012). This shows a potential role for CD46 in protective autophagy in a non-infectious setting, although the physiological ligand for CD46 (C3b) was not investigated in this paper. Curiously, C3 expression has also been found to be protective for human epithelial cells undergoing oxidative stress (Kulkarni et al., 2019), but the direct mechanism for this was not investigated and autophagy was not implicated. Indeed, the role of C3 was suggested to be intracellular, away from the cell surface location of CD46.

These findings raise further questions as to whether C3b binding, or cross-linking of CD46 by material opsonized with C3b, also induces autophagy. The obvious case of this would be induced by the presence of C3b-opsonized pathogens, in which case xenophagy induction would be beneficial to the host by restriction of pathogen survival. However, some pathogens hijack autophagy for their own benefit (Romano, Gutierrez, Beron, Rabinovitch, & Colombo, 2007), which could also be a cause for selection of these pathogens to bind CD46 directly. Indeed, prolonged autophagy at a later stage of infection has been shown to contribute to the replication of measles virus particles (Richetta et al., 2013).

5.2 | V-set and immunoglobulin domain-containing 4

A second cell surface complement receptor implicated in autophagy induction is V-set and immunoglobulin domain-containing 4 (VSIG4), also known as complement receptor of the immunoglobulin superfamily (CRIg), which binds to both C3b and iC3b (Helmy et al., 2006). VSIG4 is expressed on macrophages and contributes to complement-dependent clearance of circulating particles by liver Kupffer cells. VSIG4 also contributes to phagocytosis and subsequent phagosome acidification, leading to enhanced killing of complement-opsonized bacteria (Kim et al., 2013). In addition to this, a role for VSIG4 has also been shown in the killing of intracellular bacteria via autophagy induction.

L. monocytogenes is an intracellular pathogen that is able to escape from phagosomes into the cytosol, therefore avoiding killing. Kim et al. (2016) found that once Listeria had escaped into the cytosol, cross-linking of cell surface VSIG4 by specific antibodies led to formation of LC3-II-positive autophagosomes, which contained labelled bacteria. As a result, fewer viable bacteria were isolated from cell lysates. In comparison, induction of autophagy in infected cells by serum starvation did not result in decreases in bacterial numbers compared with control cells, showing that VSIG4-triggered autophagy increased targeted capture and killing of intracellular bacteria via xenophagy. This was attributed to the increased polyubiquitinylation of intracellular bacteria found after VSIG4 stimulation, which recruits autophagy ubiquitin-binding receptor proteins such as p62. Although VSIG4 signalling is not fully understood, the adaptor protein MyD88 was implicated, which phosphorylates and activates Beclin 1 (Shi & Kehrl, 2008), therefore triggering autophasosome formation. Over-expression of VSIG4 in HeLa cells also conferred the same function, allowing them to kill intracellular bacteria by xenophagy, while conversely, macrophages from VSIG4$^{-/-}$ mice supported greater intracellular Listeria growth in the presence of VSIG4 receptor stimulation, compared with wild-type (WT) macrophages. While these results clearly show the ability of VSIG4 to trigger autophagy and targeted xenophagy, the use of cross-linking antibodies is not physiological. While complement-opsonized Listeria triggered autophagy in macrophages more efficiently than unopsonized bacteria, once bacteria have entered the intracellular environment, one would not expect continued surface VSIG4 signalling. However, it is likely that during an ongoing infection, the presence of extracellular bacteria or DAMPs would provide complement activation and VSIG4 stimulation, resulting in increased autophagy to clear potential intracellular pathogens.

5.3 | Anaphylatoxin (complement peptide) receptors

In addition to CD46 and VSIG4, the complement anaphylatoxin receptors for C3a and C5a, C3a and C5a$_r$, receptor, have also been implicated in regulating autophagy (Nguyen et al., 2018). Whereas CD46 appears to stimulate autophagy in tested cell types, C3a and C5a receptors seem to inhibit macroautophagy-dependent breakdown of damaged mitochondria, a process known as mitophagy. C3a and C5a production are thought to stimulate survival and activation of graft-derived dendritic cells (DCs) in graft versus host disease (Cravedi et al., 2013) and authors found that donor dendritic cells from C3a receptor$^{-/-}$/C5a receptor$^{-/-}$ mice were less able to mediate graft versus host disease in recipient mice (Nguyen et al., 2018). The knockout dendritic cells had a higher turnover of precursors of ceramides, lipids that cause mitochondrial membrane damage and induce mitophagy (Sentelle et al., 2012). Knockout dendritic cells had higher levels of mitochondrial proton leakage, a sign of mitochondrial membrane damage and higher colocalization of mitochondria with acidic lysosomal compartments, as well as higher levels of LC3-II. This
indicates increased autophagic turnover, with the implication that damaged mitochondria were being removed by autophagy, a process known as mitophagy. In addition, administration of C3a/C5a receptor antagonists replicated the protective outcome of C3a/C5a receptor knockout, whereas the protective effect of knockout dendritic cell transplantation was reversed by pre-incubation with the autophagy inhibitor chloroquine. Although the finding that C3a receptor$^{-/-}$/C5a receptor$^{-/-}$ immune cells are less able to mediate graft versus host disease is entirely consistent with the known role of these receptors in immune cell activation and inflammation, this paper highlights a downstream role of autophagy inhibition after anaphylatoxin exposure in dendritic cells. In this case, it seemed to be due to an alteration in lipid metabolism, which also fits into current understanding of the importance of metabolism (O’Neill, Kishton, & Rathmell, 2016) and in particular, the influence of complement signalling on metabolism (Kove1 & Kemper, 2017), on the outcomes of immune cell activation.

Contrasting results were however described for C5a receptor in a paper showing that C5a can induce, rather than inhibit, autophagy in lung alveolar macrophages (Hu et al., 2014). Acute lung injury can be induced by ischaemia–reperfusion (IR) injuries (de Perrot, Liu, Waddell, & Keshavjee, 2003), in which complement plays a pathological role (Keshavjee, Davis, Zamora, de Perrot, & Patterson, 2005; Naka, Marsh, Scesney, Oz, & Pinsky, 1997; Pierre et al., 1998). Ischaemia–reperfusion led to production of C5a in the lung bronchoalveolar lavage fluid and administration of neutralizing anti-C5a antibody before injury improved pathological outcome (Hu et al., 2014). Alveolar macrophages from ischaemia–reperfusion but not control mice had increased levels of LC3-II, indicating autophagy induction and this was reversed by C5a neutralization. Autophagy activation was linked to degradation of Bcl-2, a well-known regulator of apoptosis. However, Bcl-2 also interacts with Beclin 1, inhibiting Beclin 1-dependent macroautophagy (Pattingre et al., 2005). Consequently, a reduction in Bcl-2 in lung alveolar macrophages of mice with ischaemia–reperfusion injury resulted in activation of Beclin 1, demonstrating a crosstalk between complement signalling, apoptosis and autophagy pathways.

### 5.4 Activation of autophagy by the membrane attack complex (MAC)

The MAC forms a multimeric pore that breaches cell membranes, but while this mechanism evolved to attack microorganisms, MAC can be deposited on bystander host cells during infection and particularly in autoimmune or autoinflammatory disease, causing haemolysis and cell death. For example, MAC-induced cell death is a feature of complement-related nephropathy (Ma, Sandor, & Beck, 2013). Sublytic levels of MAC were found to trigger autophagy in podocytes in vitro, driving LC3-I/II conversion, while pharmacological autophagy inhibition increased levels of apoptosis and enhancing autophagy with rapamycin decreased resulting levels of cell death (Lv, Yang, Chen, & Zhang, 2016). Autophagy is therefore a cytoprotective response to cellular stress provoked by MAC disruption of membranes. These results were confirmed in a separate study of podocytes from human patients with idiopathic membranous nephropathy, which had increased MAC deposition and accumulations of LC3-positive puncta, increased levels of p62 and increased expression of Beclin 1, but no increase in autolysosomes (Liu et al., 2017). This suggests that autophagosome formation is induced by MAC, but that these do not fuse with functional lysosomes, resulting in “frustrated” autophagy. The MAC was internalized and triggered permeabilization of lysosomal membranes, preventing their adequate acidification and function, leading to accumulation of autophagosomes with undigested contents.

MAC therefore triggers initiation of autophagy, but internalization of the C5b-9 membrane pore by endocytosis may lead to defective lysosomal function, limiting the potential up-regulation of fully functional autophagy, although this may be augmented by application of autophagy-stimulating drugs such as rapamycin. It is unlikely that induction of autophagy by MAC is a specific response to complement, but rather a general cellular response to breaching of the surface membrane, as autophagy is also up-regulated by multiple membrane pore-forming toxins in both Caenorhabditis elegans (Chen et al., 2017) and mammalian cells (Kloft et al., 2010).

### 6 OUTSIDE IN: A ROLE FOR C3 IN INTRACELLULAR DETECTION OF PATHOGENS

As well as extracellular complement being able to induce autophagy via cell surface receptors and MAC, evidence exists for C3 being able to trigger autophagy from within the cell. This would not be the first case of a major serum protein inducing anti-pathogen responses when it is brought into the cytosolic environment; multiple papers have shown how antibody bound to pathogen surfaces triggers cell intrinsic immunity, cytokine production and pathogen restriction via proteasomal and autophagic mechanisms, via binding to the high-affinity cytosolic Fc receptor and E3 ubiquitin ligase, tripartite motif containing 21 (TRIM21) (Foss, Watkinson, Sandlie, James, & Andersen, 2015; Mallery et al., 2010; McEwan et al., 2013). TRIM21 is able to restrict both bacterial and viral infections in a strictly antibody-dependent fashion and is recruited to the surface of antibody-opsonized pathogens when they invade into the cytosol, by binding to Fc domains. TRIM21 not only ubiquitinylates the surface of the pathogen, targeting viral particles to degradation in the proteasome and bacteria to autophagosomes, but also triggers a signalling cascade leading to NF-κB and interferon response factor (IRF) signalling.

Similar to internalized antibody, C3 itself can be brought into cells on the surface of opsonized non-enveloped viruses and invading bacteria, triggering cell intrinsic immunity in a manner dependent on mitochondrial antiviral signalling protein (Tam, Bidgood, McEwan, & James, 2014); activation of which also leads to IRF and NF-κB signalling and expression of type 1 interferons (Vazquez & Horner, 2015). Serum opsonization of adenovirus before...
cellular invasion led to triggering of cell intrinsic immunity and restriction of adenovirus replication, via proteasomal function. This was only partially dependent on serum antibodies and TRIM21 (Tam et al., 2014). The remaining activity was heat labile, an important fact as antibodies are heat stable, while serum complement activity is easily destroyed by heating. C3 dependency was confirmed when viral particles were opsonized using purified complement proteins of the alternative pathway, leading to C3b deposition, which specifically triggered the same response. This pathway of intracellular C3 detection was highly conserved, with responses in human cells being triggered by adenovirus exposed to heat labile activity of serum from multiple mammalian species and was present in all tested non-immune cell types. Although complement-mediated adenoviral restriction was dependent on mitochondrial antiviral signalling protein, C3 and mitochondrial antiviral signalling protein were not found to interact directly, suggesting that there is still as-yet undiscovered intracellular C3 detection machinery. Of note, the autophagy receptor protein p62 was required for NF-κB induction by C3-opsonized particles.

7 | DIRECT TARGETING OF AUTOPHagy BY INTRACELLULAR C3

It is therefore established that C3 entering the cytosol on the surface of invading pathogens is capable of triggering cell intrinsic immune responses. It was also recently shown that this response can include direct induction of xenophagy, via a direct interaction of C3 with ATG16L1. A yeast two-hybrid assay was used to show that C3 is a ligand for ATG16L1 (Sorbara et al., 2018). ATG16L1 is involved in several stages of autophagosome biogenesis, including determining the site of autophagosome initiation (Fujita et al., 2008) and the lipidation of LC3-I into the autophagosome-associated form LC3-II (Fletcher et al., 2018). Considering its role in defining the site of LC3 lipidation, ATG16L1 is a key component of the autophagic response to invading pathogens that reach the cytosol (Mallery et al., 2010). Sorbara et al. (2018) went on to show that C3 opsonization of cytotoxic bacteria impacts the host response to infection through increased autophagy. Indeed, intracellular C3-opsonized L. monocytogenes and adherent-invasive E. coli (AIEC) were targeted by autophagy significantly more than unopsonized bacteria and autophagy induction restricted intracellular bacterial growth via the C3/ATG16L1 interaction; the C3-mediated reduction in recovered bacteria required expression of ATG16L1. C3 was also shown to play a role in vivo in restricting Listeria invasion in a murine model of intragastric infection. In this model, C3 expression was increased in caecal and colonic tissue in response to infection, as well as to dextran sodium sulfate-induced colitis, showing that mucosal inflammation could increase C3 expression, deposition of which can then protect mucosal barriers against infection. After 24 h of infection, C3−/− mice had higher numbers of Listeria colony-forming units per gram of caecal and colonic tissue, as well as lower levels of autophagic turnover as assessed by LC3-II conversion.

Listeria expresses several virulence factors that participate in autophagy escape:− ActA and InlK recruit host cell proteins—cytoskeletal proteins or major vault protein, respectively at the bacterial surface, to disguise themselves and escape autophagic recognition (Birmingham et al., 2007; Dortet et al., 2011), while phospholipases PlcA and PlcB reduce autophagic flux and PI3P levels (Tattoli et al., 2013). Despite these autophagy escape mechanisms, Listeria growth can be restricted by autophagy in the presence of C3, perhaps due to the C3-dependent recruitment of ATG16L1, which acts downstream of the PI3K complex. In contrast, Sorbara et al. showed that two other cytovirulence bacteria, Shigella flexneri and Salmonella enterica serovar Typhimurium, escaped C3-dependent autophagy-mediated growth restriction. They first noticed that intracellular Shigella rapidly shed C3 upon invasion of epithelial cells. The ompT proteases IscP in Shigella and PgtE in Salmonella are partly responsible for C3 cleavage, which enables these bacteria to escape from C3-dependent autophagic restriction. Unlike Listeria, these two bacteria have therefore evolved C3-specific defence mechanisms in addition to their more general autophagy escape mechanisms. Loss of IscP expression in Shigella, or PgtE in Salmonella, prevented C3 shedding and sensitized the bacteria to ATG16L1-dependent intracellular killing. This paper therefore not only established the existence of an intracellular C3 detection mechanism directly targeting intracellular pathogens for xenophagy but also revealed that some pathogenic bacteria have evolved mechanisms to resist it. Similarly, some viruses have also evolved anti-C3 defences to avoid intracellular detection: both human rhinovirus and poliovirus express cytosolic 3C proteases that cleave C3 and reduce intracellular C3-mediated mitochondrial antiviral signalling protein-dependent NF-κB induction (Tam et al., 2014). Pathogen mechanisms for preventing C3 opsonization therefore also protect against these “novel” intracellular detection mechanisms, including antimicrobial intracellular signalling and autophagy-mediated bacterial growth restriction.

8 | A ROLE FOR C3 IN HOMEOSTATIC AUTOPHagy

Besides targeting extracellularly opsonized bacteria for autophagy once they enter the cell, C3 has been found to be important in maintaining basal flux of autophagy in pancreatic beta cells. Autophagy is essential as an adaptive response during development of insulin resistance in peripheral tissues, a state of increased insulin demand that places extra stress on insulin-producing beta cells. If beta cells are unable to keep pace with the metabolic demand and die off, insulin production is halted and overt type 2 diabetes develops. Autophagy acts against the breakdown of pancreatic islet architecture and the failure of beta cells to sustain sufficient insulin secretion, therefore aids the maintenance of glucose tolerance (Watada & Fujitani, 2015) and it has been demonstrated to protect beta cells against apoptosis induced by ER stress (Bachar-Wikstrom et al., 2013) or lipotoxicity (Ebato et al., 2008; Kong, Wu, Sun, & Zhou, 2017). Autophagy is also required for homeostasis of pancreatic islets under
normal conditions (Jung et al., 2008). Identifying beta-cell-intrinsic triggers of autophagy therefore has considerable value for advancing strategies to limit beta cell loss during disease. The complement system has recently been shown to have a number of non-traditional roles in diabetes development (King & Blom, 2017). Recently, we reported a protective effect of C3 against apoptosis of beta cells, attributed to a role in maintenance of homeostatic autophagy (King, Kulak, et al., 2019). We found high C3 expression in isolated human pancreatic islets, which was significantly further up-regulated in islets from T2D donors, correlating with donor body mass index and glycated haemoglobin levels, a clinical marker of diabetes. This islet-specific C3 up-regulation was also identified in several rodent models of diabetes. Surprisingly, we found that as well as being secreted, C3 had a cytosolic distribution in human islets and clonal beta cells. We therefore probed for C3-interacting partners using protein microarrays and, in parallel with Sorbara et al. (2018), found an interaction between C3 and ATG16L1. To investigate this further, we explored the autophagy phenotype of CRISPR/Cas9-mediated C3 knockouts in insulinoma INS-1832/13 cells, a widely used beta-cell model cell line (Hohmeier et al., 2000). Studying the resulting phenotype of C3 knockouts revealed a dramatic arrest in the autophagy pathway. C3 knockouts displayed accumulation of LC3-II puncta as measured by immunoblotting, as well as observed by confocal microscopy. These puncta did not further accumulate in the presence of a lysosomal inhibitor, indicating an inhibited turnover of LC3-positive autophagosomes, rather than increased rate of autophagosome formation. Heterozygote knockout clones exhibited an intermediate level of autophagy inhibition, indicating a gene dose-dependent effect. An increased level of autophagic substrate p62 in C3 knockout clones confirmed autophagic dysfunction and electron microscopy displayed an accumulation of autophagosome-like structures. Pancreatic islets isolated from C3 knockout mice also demonstrated accumulated p62 and LC3-II levels, compared with WT mouse islets.

Autophagy-dependent targeting of insulin granules to lysosomes plays an important role in protein quality control and insulin turnover. Consistent with this, C3 knockout clones also had a significant increase in numbers of insulin granules, which translated into increased glucose-stimulated insulin secretion. However, it cannot yet be excluded that extracellular C3 might be recruited to damaged organelles for utilization in the autophagy pathway, in a mechanism similar to C3-targeted xenophagy. C3 can be internalized from the extracellular environment and forms insoluble toxic deposits in the pancreas of human diabetic patients (Jurgens et al., 2011), triggered up-regulation of C3 and autophagy and caused increased apoptosis in C3-knockout cells.

Having demonstrated a requirement for C3 for autophagic homeostasis in beta cells, it is therefore possible that a lack of C3 could lead to an increased loss of insulin secreting cells in the face of metabolic challenge, as has been observed for beta-cell-specific autophagy gene Atg7-deficient mice (Ebato et al., 2008; Jung et al., 2008). On the other hand, chronically up-regulated C3 expression observed in type 2 diabetes subjects may lead to hyperactivation of autophagy and a literal consumption of cellular insulin content. Further in vivo investigation of C3 involvement in maintaining insulin content and beta-cell mass is therefore required, including an assessment of contributions of beta-cell-derived C3, compared with serum-derived C3. The hypothetical existence of separate pools of C3 within the cell, both within the conventional secretory pathway and within the cytosol, also provides challenges to investigating these separately.

9 | INVOLVEMENT OF CYTOSOLIC C3 IN AUTOPHagy

Our study therefore identified C3 as a relevant effector in cytoprotective autophagy and also revealed possible derivation of C3 endogenously expressed within the cytosol. C3 is known primarily as a secreted protein, raising questions as to how it may interact with cytosolic ATG16L1 in the context of normal homeostasis. We found that an alternative in-frame translational start site could initiate C3 expression within the cytosol (King, Kulak, et al., 2019, and unpublished data). The C3 mRNA contains in-frame AUG codons immediately downstream of the encoded signal peptide, the utilization of which produces non-secreted, cytosolic C3. Indeed, site-directed mutagenesis of the first AUG codon of the C3 coding sequence did not affect translation of a non-secreted, cytosolic form C3 that was not associated with organelle/membrane structures, as opposed to WT C3 that is found abundantly in organelle/membrane fractions, which include the endoplasmic reticulum and Golgi components of the secretory pathway. The “scanning” model of translational initiation, in which the translation initiation complex uses the first available AUG codon (Kozak, 1978), could explain aberrant use of a non-physiological start site when the canonical site is removed, but cytosolic C3 was also expressed from the endogenous gene with an intact canonical AUG start site. Introduction of a frame shift within the signal peptide-coding sequence of C3 by genomic CRISPR/Cas9 gene editing of C3-expressing cells, without altering the canonical start codon, completely prevented expression of secreted C3, but did not affect detection of cytosolic C3 (King, Kulak, et al., 2019), supporting constitutive use of additional downstream translational start sites in C3-expressing cells.

Exogenous addition of C3 to cell culture medium did not rescue the inhibited autophagy phenotype in C3 knockout cells (King, Kulak, et al., 2019), emphasizing the importance of intrinsic intracellular C3 to preserve optimal autophagic function. However, it cannot yet be excluded that extracellular C3 might be recruited to damaged organelles for utilization in the autophagy pathway, in a mechanism similar to C3-targeted xenophagy. C3 can be internalized from the extracellular environment and forms insoluble toxic deposits in the pancreas of human diabetic patients (Jurgens et al., 2011), triggered up-regulation of C3 and autophagy and caused increased apoptosis in C3-knockout cells.

Having demonstrated a requirement for C3 for autophagic homeostasis in beta cells, it is therefore possible that a lack of C3 could lead to an increased loss of insulin secreting cells in the face of metabolic challenge, as has been observed for beta-cell-specific autophagy gene Atg7-deficient mice (Ebato et al., 2008; Jung et al., 2008). On the other hand, chronically up-regulated C3 expression observed in type 2 diabetes subjects may lead to hyperactivation of autophagy and a literal consumption of cellular insulin content. Further in vivo investigation of C3 involvement in maintaining insulin content and beta-cell mass is therefore required, including an assessment of contributions of beta-cell-derived C3, compared with serum-derived C3. The hypothetical existence of separate pools of C3 within the cell, both within the conventional secretory pathway and within the cytosol, also provides challenges to investigating these separately.
extracellular space (Elvington, Liszewski, Bertram, Kulkarni, & Atkinson, 2017; Kremlitzka et al., 2019), although a mechanism has not been demonstrated by which C3 within the lumen of internalized membrane-bound compartments should cross into the cytosol to access the autophagy machinery.

10 | DISCUSSION

Autophagy is a regulated cytoprotective cellular response to stress or infection. It should therefore not be surprising that the extracellular system of complement proteins, known to act as pathogen and danger sensors, should be able to induce autophagy as a reactive response within host cells, contributing to pathogen clearance and enhanced cellular survival. This occurs not only by signal transduction via cell surface receptors (summarized in Figure 1), such as in the case of CD46, VSIG4 and the anaphylatoxin receptors, but also by direct interaction of C3 with cytosolic proteins, once C3 activation products are carried into the cell on the surface of invading pathogens (Sorbara et al., 2018; Tam et al., 2014), in a manner similar to autophagy induction by transmembrane TLRs and cytosolic NOD-like receptors, as described above. Given the known interplay between complement receptors and TLRs (Hajiashengallis & Lambris, 2016), the interaction between these different pathways of innate immunity and the resultant effect on autophagy-dependent pathogen clearance would be of interest to investigate.

What is more surprising however is the evidence that C3 also exists within the cytosolic fraction of cells, separate from the secretory pathway (King, Renstrom, & Blom, 2019). In parallel to C3b being introduced into the cell on bacterial surfaces and interacting with ATG16L1, inducing xenophagy, we also see evidence that cytosolic C3 is also involved in homeostatic autophagy. Careful regulation of C3 activation is a key feature of the extracellular complement system (Sjoberg et al., 2009) and the same should also be true of intracellular, cytosolic C3. The mechanistic details of how autophagy is triggered by intracellular C3 or C3 cleavage products and how this is regulated, are currently under investigation, but potential interactions are presented in Figure 2. The opsonizing ability of C3 was derived from the presence of the thioester group that is exposed after C3 cleavage. We found that native C3 and its thioester-exposed (C3(H2O) and C3-methylamine) but not cleaved products (C3b, iC3b, C3d and C3c) have higher affinity to ATG16L1 (King, Kulak, et al., 2019), providing a possibility for intracellular reaction-driven conformational changes of C3 regulating binding to desired surfaces or ligands, in the same way in which C3 cleavage alters binding affinity to known ligands and receptors in the extracellular environment (Ricklin et al., 2016). The interaction of ATG16L1 with full-length C3 provides a possible mechanism of regulation of interaction, by cleavage of C3. Cytosolic C3 may also be involved in the tethering of cellular components required for progression of autophagosome maturation, thereby regulating ATG16L1 complex recruitment, as it known that proper localization of the ATG16L1 complex is essential for lipidation of LC3-I to LC3-II for phagophore membrane elongation. Maturation of the autophagosome is accompanied by the dissociation of ATG proteins (including ATG16L1), which occurs prior to fusion with lysosomes. Thus, cleavage of C3 may decrease binding affinity to ATG16L1, leading to dissociation of ATG16L1 from autophagosomes, allowing fusion with lysosomes. Alternatively or additionally, C3 fragments remaining on the autophagosome surface might be involved in dynamics of autophagosome and lysosome fusion, by
interaction with other factors. Although the scenarios of C3 involvement in autophagy pathway described here are all hypothetical, they are conceivable as based on the current understanding of C3 and diversity of its binding ligands, dependent on the state of C3 processing. Further experimental investigation is required.

The mechanism for the interaction domain of ATG16L1 with C3, or C3 cleavage products, must also be defined. Combining our findings (King, Kulak, et al., 2019) with that of Sorbara et al. (2018), we can deduce that C3 interacts with the central coiled-coil domain of ATG16L1, the only domain common to both the positive hits on protein microarrays and the positive result found by yeast two-hybrid assay (Figure 3). This domain is present in both mammal and yeast ATG16L1/ATG16 and is required for homeostatic autophagy (Rai et al., 2019) and is responsible for ATG16L1 homodimerization and lipid binding (Dudley et al., 2019), therefore mediating recruitment to the elongating isolation membrane of the forming autophagosome. ATG16L1 is recruited to the forming phagophore by WIPI2, which also binds to the coiled-coil domain and is also involved in both homeostatic autophagy and xenophagy (Dooley et al., 2014). It is possible that C3 functions in a similar manner, whereby C3 deposited on intracellular pathogens, or material to be recycled, recruits ATG16L1, which then, in complex with ATG5 and ATG12, lipidates LC3-II and
contributes to the growing phagophore. We have identified that the ATG16L1-recruiting amino acid motif found on TMEM59, TLR2 and NOD2 (Boada-Romero et al., 2013) is also present at C31206–1216 within the C3d fragment of C3 and overlapping with the known CR2 binding site. In the extracellular environment, this binding site is only revealed once C3 is cleaved by factor I and undergoes subsequent conformational changes, but this could be the mechanism by which ATG16L1 is recruited to C3-opsonized cytopathic pathogens. Investigation of these molecular interactions is ongoing.

What is clear, however, is that C3-opsonized bacteria are targeted for destruction within eukaryotic cells via autophagy-dependent xenophagy (Sorbara et al., 2018), should they invade cells before destruction by complement-mediated phagocytosis. It remains to be shown directly whether intracellular "self"-material can also be marked by intracellular C3 for autophagic clearance, mirroring the extracellular clearance of self-material such as apoptotic cells and immune complexes and whether familiar features of C3 so important to its extracellular function, such as the thioster group, are also required for this intracellular function. It should be noted, however, that thioster-containing proteins are conserved across species, mediating similar functions within innate immunity in insects, tunicates and mammals (Nonaka, 2014). A conserved connection between thioster-containing proteins and autophagy can be observed in the finding that the Drosophila thioster-containing protein and complement orthologue macroglubulin complement-related (MCR) play an essential role in development and inflammation, by mediating autophagy in macrophages, via an immune cell surface receptor (Lin et al., 2017). C3 has so many diverse functions, from opsonin, warning signal, phagocytosis inducer, mediator of clearance and even acting as a component of its own convertase, which it has been termed a "Swiss army knife" of a protein (Ricklin et al., 2016). Given its clearly understood function in the clearance of extracellular material for disposal, it may be unsurprising to find that it has parallel functions within the cell as well. Production of multiple protein variants from one gene is a fundamental process allowing proteome diversity and generates potential for evolutionary adaptivity, whereby one variant can take on new or non-overlapping functions (Conant & Wolfe, 2008). The discovery of a cytoprotective intracellular function of C3 supports an emerging concept of complement as a defender of the intracellular space (Elvington, Liszewski, & Atkinson, 2016). Now it remains to be seen how far, beyond beta cells, C3-regulated autophagy plays a significant role in determining cell fates.

10.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018) and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos et al., 2019; Alexander, Kelly et al., 2019).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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