Outside In: Roles of Complement in Autophagy

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Abstract

The complement system is a well-characterised cascade of extracellular serum proteins that is activated by pathogens and unwanted waste material. Products of activated complement signal to host cells via cell-surface receptors, illicting 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, upregulating protective homeostatic autophagy in response to perceived stress, but also intiating targeted anti-microbial autophagy in order to kill intracellular cyto-invasive pathogens. In particular, we will focus on recent discoveries 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.

Introduction:

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 pattern recognition receptors of the complement system such as C1q, mannose-binding lectin and ficolins recognize pathogen- or danger-associated molecular patterns (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 3 major outcomes: transmitting danger signals to the host, directly attacking pathogens, and covalently labeling 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, C3aR and C5aR, 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, & Geisbrecht, 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 changes conformation 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-opsonised material. Additionally, material coated with deposited C3b is transported away by red blood cells expressing complement receptor 1, and is taken to either the liver or to 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 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 1000-fold (Dempsey, Allison, Akkaraju, Goodnow, & Fearon, 1996), and C3 opsonisation 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, C3 activation products take several forms, with multiple specific receptors and interaction partners (Ricklin et al., 2016), and C3 is therefore involved in many effector functions within immune defence, as well as tissue homeostasis and development (Ricklin, Hajishengallis, Yang, & Lambris, 2010).

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 within the *intracellular*environment, via the process of autophagy. This review will therefore summarize the current evidence for involvement of complement in autophagy induction, both by transducing signals across the cell membrane, as well as potential roles within the cellular environment.

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 upregulated when cells face certain pressures. Three main pathways exist; microautophagy, chaperone-mediated autophagy, and macroautophagy. Microautophagy describes lysosomal membrane invagination and vesicle scission into the lumen, in a process similar to the formation of multi-vesicular bodies, which leads to the digestion of the vesicles and their contents. While this is a constitutive process of membrane homeostasis, it becomes upregulated 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) (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 sub-cellular 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 upregulated by hypoxia, oxidative or edoplasmatic reticulum (ER) 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 ER. Cellular stress causes activation of Unc-51-like kinase (ULK1) complex, which then phosphorylates the Class III phosphatidylinositol 3-kinase (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 homologs 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, that occurs via lipid interactions and binding to WIPI2. ATG16L1 itself binds to a conjugate of ATG12 and ATG5 (Otomo, Metlagel, Takaesu, & Otomo, 2013). The ATG12-5 complex has E3 ligase-like activity, and is involved in lipidation of the ubiquitin-like cytoplasmic protein LC3 (a homolog to yeast ATG8), by coupling it to phosphoethanolamine, thus relocalising 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 (T. N. 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 inhibit 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, lyosomal 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 fuse 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 phagocytes but also within non-immune cells (Nakagawa et al., 2004). Ubiquitinylation 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 internalised 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.

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 pattern recognition receptors, leading to production of complement protein cleavage products that signal to cells via specific receptors (Kohl, 2006). It is well-recognised 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.

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 co-factor 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, that 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), that 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 adenovirus 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 Joubet *et al* therefore tested whether this can be triggered by its binding to CD46 (Joubert et al., 2009). 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 crosslinking 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_2O_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-opsonised 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 virion particles (Richetta et al., 2013).

VSIG4

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-opsonised 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.

Listeria monocytogenes is an intracellular pathogen that is able to escape from phagosomes into the cytosol, therefore avoiding killing. Kim *et al* 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 labeled bacteria (Kim et al., 2016). 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 to 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[14]. Although VSIG4 signaling is not fully understood, the adaptor protein MyD88 was implicated, which phosphorylates and activates Beclin 1 (Shi & Kehrl, 2008), therefore triggering autophagosome formation. Overexpression 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 to 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-opsonised *Listeria* triggered autophagy in macrophages more efficiently than unopsonised bacteria, once bacteria have entered the intracellular environment, one would not expect continued surface VSIG4 signaling. 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.

Anaphylatoxin receptors

In addition to CD46 and VSIG4, the complement anaphylatoxin receptors for C3a and C5a, C3aR and C5aR, have also been implicated in regulating autophagy (H. Nguyen et al., 2018). Whereas CD46 appears to stimulate autophagy in tested cell types, C3aR and C5aR 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 (GVHD) (Cravedi et al., 2013), and authors found that donor DCs from C3aR$^{-/-}$/C5aR$^{-/-}$mice were less able to mediate

GVHD in recipient mice (H. Nguyen et al., 2018). The knockout DCs had a higher turnover of precursors of ceramides, lipids that cause mitochondrial membrane damage and induce mitophagy(Sentelle et al., 2012). Knockout DCs had higher levels of mitochondrial proton leakage, a sign of mitochondrial membrane damage, and higher colocalisation 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 C3aR/C5aR antagonists replicated the protective outcome of C3aR/C5aR knockout, whereas the protective effect of knockout DC transplantation was reversed by pre-incubation with the autophagy inhibitor chloroquine. Although the finding that $C3aR^{-/-}/C5aR^{-/-}$ immune cells are less able to mediate GVHD 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 DCs. 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 signaling on metabolism (Kolev & Kemper, 2017), on the outcomes of immune cell activation.

Contrasting results were however described for C5aR 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). IR 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 IR 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 IR injury resulted in activation of Beclin 1, demonstrating a cross-talk between complement signaling, apoptosis and autophagy pathways.

Activation of autophagy by the membrane attack complex

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). Sub-lytic 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 internalised and triggered permeabilisation of lysosomal membranes, preventing their adequate acidification and function, leading to accumulation of autophagosomes with undigested contents.

MAC therefore triggers initiation of autophagy, but internalisation of the C5b-9 membrane pore by endocytosis may lead to defective lysosomal function, limiting the potential upregulation 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 upregulated by multiple membrane pore-forming toxins in both *C. Elegans* (Chen et al., 2017) and mammalian cells (Kloft et al., 2010).

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 protein 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-opsonised 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 signaling cascade leading to NF-kB and interferon response factor (IRF) signaling.

Similar to internalised antibody, C3 itself can be brought into cells on the surface of opsonised non-enveloped viruses and invading bacteria, triggering cell intrinsic immunity in a manner dependent on mitochondrial anti-viral signaling protein (MAVS) (Tam, Bidgood, McEwan, & James, 2014), activation of which also leads to IRF and NF-kB signaling, and expression of type 1 interferons (Vazquez & Horner, 2015). Serum opsonisation 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 heat inactivation. 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 MAVS, C3 and MAVS 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-kB induction by C3-opsonised particles.

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 2-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[64]. Sorbara *et al.* went on to show that C3 opsonisation of cyto-invasive bacteria impacts the host response to infection through increased autophagy (Sorbara et al., 2018). Indeed, intracellular C3-opsonised*Listeria monocytogenes* and adherent-invasive *E. coli*(AIEC) were targeted by autophagy significantly more than unopsonised bacteria, and autophagy induction restricted intracellular bacterial growth via the C3/ATG16L1 interaction; the C3-mediated reduction in recovered viable bacteria required expression of ATG16L1. C3 was also shown to play a role *in vivo* in restricting *Listeria*invasion in a murine model of intra-gastric infection. In this model, C3 expression was increased in cecal 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 hrs of infection, $C3^{-/-}$ mice had higher numbers of*Listeria* colony forming units per gram of cecal 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 phosphatidylinositol 3-phosphate (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 cyto-invasive bacteria, *Shigella flexneri* and *Salmonellaenterica* serovar Typhimurium, escaped C3-dependent autophagy-mediated growth restriction. They first noticed that intracellular *Shigella* rapidly shed C3 upon invasion of epithelial cells. The omptin proteases IscP in *Shigella* and PgtE in *Salmonella* are partly responsible for C3 cleavage, that 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 sensitised 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 revealed that some pathogenic bacteria have evolved mechanisms to resist it. Similarly, some viruses have also evolved anti-C3 defenses to avoid intracellular detection: both human rhinovirus and poliovirus express cytosolic 3C proteases that cleave C3 and reduce intracellular C3-mediated MAVS-dependent NF-kB induction (Tam et al., 2014). Pathogen mechanisms for preventing C3 opsonization therefore also protect against these 'novel' intracellular detection mechanisms, including anti-microbial intracellular signaling and autophagy-mediated bacterial growth restriction.

A role for C3 in homeostatic autophagy

Besides targeting extracellularly opsonised bacteria for autophagy once they enter the cell, C3 has been found to be important in maintaining basal flux of autophagy in pancreatic β-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 β-cells. If β-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 β-cells to sustain sufficient insulin secretion, and therefore aids the maintenance of glucose tolerance (Watada & Fujitani, 2015), and has been demonstrated to protect β-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 homesotasis of pancreatic islets under normal conditions (Jung et al., 2008). Identifying β-cell-intrinsic triggers of autophagy therefore has considerable value for advancing strategies to limit β-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 β-cells, attributed to a role in maintenance of homeostatic autophagy (King et al., 2019). We found high C3 expression in isolated human pancreatic islets, that was significantly further upregulated in islets from T2D donors, correlating with donor body mass index and glycated haemaglobin levels, a clinical marker of diabetes. This islet-specific C3 upregulation 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 β-cells. We therefore probed for C3 interacting partners using protein microarrays and in parallel with Sorbara *et al* (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-1 832/13 cells, a widely used β-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 to WT mouse islets.

Autophagy-depended 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, that translated into increased glucose-stimulated insulin secretion. However, it is possible that some of this is secreted as biologically inactive proinsulin, since the ELISA assay does not discriminate between these two forms. Circulating plasma proinsulin levels are also increased in patients with type 2 diabetes mellitus, consistent with reports of diabetes-induced islet autophagy dysfunction (Ji et al., 2019; Masini et al., 2009).

Finally, the involvement of C3 in the cytoprotective function of autophagy in stressed β-cells was also confirmed in the C3 knockout β-cell clones. Exposure to free fatty acids, used to model *in vivo* glucolipotoxicity, led to increased apoptosis in β-cell clones lacking C3 expression. These same conditions increased both C3 expression and autophagic turnover in normal cells. Knockdown of ATG7, essential for homeostatic autophagy, also increased apoptosis in WT cells. Similarly, exposure to islet amyloid polypeptide, an amyloidogenic peptide hormone that forms insoluble toxic deposits in the pancreas of human diabetic patients (Jurgens et al., 2011), triggered upregulation of C3 and autophagy, and caused increased apoptosis in C3-knockout cells.

Having demonstrated a requirement for C3 for autophagic homeostasis in β-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 β-cell-specific autophagy gene*Atg7* -deficient mice (Ebato et al., 2008; Jung et al., 2008). On the other hand, chronically upregulated 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 β-cell mass is therefore required, including an assessment of contributions of β-cell-derived C3, compared to serum-derived C3. The hypothetical existence of separate pools of C3 within the cell, both within the conventional secretory pathway and also within the cytosol, also provides challenges to investigating these separately.

Involvement of intracellular C3 in autophagy

Our study therefore identified C3 as a relevant effector in cytoprotective autophagy, but 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 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 wild type C3 that is found abundantly in organelle/membrane fractions, which include the ER 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 et al., 2019), supporting constituitive 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 et al., 2019), emphasizing the importance of intrinsic intracellular C3 to preserve optimal autophagic function. However, it can not 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 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 internalised membrane-bound compartments should cross into the cytosol to access the autophagy machinery.

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 (Tam et al., 2014)(Sorbara et al., 2018). What is more surprising however is the evidence that C3 also exists within the cytosolic fraction of cells, separate from the secretory pathway (King et al., 2019). In parallel to C3(b) 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, Trouw, & Blom, 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 derives 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 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), that 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 et al., 2019) with that of Sorbara *et al* (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 2 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 homodimerisation 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 $C3_{1206\text{-}1216}$, 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 complement opsonised cyto-invasive pathogens.

Investigation of these molecular interactions is ongoing.

What is clear however, is that C3-opsonised 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 extracelluar function, such as the thioester group, are also required for this intracellular function. It should be noted however, that thioester containing proteins (TEPs) are conserved across species, mediating similar functions within innate immunity in insects, tunicates, and mammals (Nonaka, 2014). A conserved connection between TEPs and autophagy can be observed in the finding that the Drosophila TEP and complement orthologue Macroglobulin Complement-Related (MCR) plays 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, that 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 β-cells, C3-regulated autophagy plays a significant role in determining cell fates.

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Figure legends

Figure 1: Mechanisms of extracellular complement-mediated autophagy induction via surface receptors and MAC. For details, see text.

Figure 2: Potential mechanisms of intracellular C3 involvement in autophagy: (1) C3-coated bacteria have been shown to be targeted by autophagy through interaction with ATG16L1, leading to bacterial growth restriction. Similarly, intracellular C3 deposited on organelles could recruit the ATG16L1-ATG12-ATG5 complex that catalyzes the lipidation of LC3-I to LC3-II, driving the formation of autophagosomes. (2) C3 may deliver small portions of material for expansion of the autophagosome membrane. This may involve C3 activity upstream or downstream of ATG16L1. (3) Processing of C3 could liberate ATG16L1 complex from the autophagosomal membrane, allowing fusion of completed autophagosomes with lysosomes. (4) Cleaved forms of C3 anchored in the membrane of autophagosomes could facilitate autophagosome-lysosome fusion via interactions with yet unknown factors. These mechanisms are not mutually exclusive.

Figure 3: A scheme for the potential C3 binding domain on ATG16L1, based on experimental evidence. The recombinant proteins found on protein microarrays and the construct used in yeast 2-hybrid assay suggests that C3 binds to the coiled coil domain of ATG16L1, which is responsible for ATG16L1 recruitment to the phagophore membrane during homeostatic autophagy. ATG5BD: ATG5 binding domain.