Intracellular complement activation – an alarm raising mechanism?

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1. Abstract

It has become increasingly apparent that the complement system, being an ancient defense mechanism, is not operative only in the extracellular milieu but also intracellularly. In addition to the known synthetic machinery in the liver and by macrophages, many other cell types, including lymphocytes, adipocytes and epithelial cells produce selected complement components. Activation of e.g. C3 and C5 inside cells may have multiple effects ranging from direct antimicrobial defense to cell differentiation and possible influence on metabolism. Intracellular activation of C3 and C5 in T cells is involved in the maintenance of immunological tolerance and promotes differentiation of T helper cells into Th1-type cells that activate cell-mediated immune responses. Adipocytes are unique in producing many complement sensor proteins (like C1q) and factor D (adipsin), the key enzyme in promoting alternative pathway amplification. The effects of complement activation products are mediated by intracellular and cell membrane receptors, like C3aR, C5aR1, C5aR2 and the complement regulator MCP/CD46, often jointly with other receptors like the T cell receptor, Toll-like receptors and those of the inflammasomes. These recent observations link complement activation to cellular metabolic processes, intracellular defense reactions and to diverse adaptive immune responses. The complement components may thus be viewed as intracellular alarm molecules involved in the cellular danger response.

2. Introduction

From an evolutionary perspective, the complement system is a very ancient molecular cascade with homologs of C3 observed already in invertebrates, like the echinoderms (sea urchin) and mosquitoes [1, 2]. Although the study of the complement system for the past century has focused on its critical role in defense against microbial infections and regulation of immune homeostasis, the very ancient origin of these molecular pathways hints at a much broader palette of functions. With the evidence for local complement production and the more recently discovered intracellular complement activation, novel functions in regulating metabolism, inflammasome activation and cell survival have emerged.

The complement system consists of approximately 50 fluid-phase and cell membrane-associated molecules. Activation of complement leads to a number of outcomes relevant to human health. Complement is activated via three major pathways, the classical, lectin and the

alternative pathway. A sufficient density and/or appropriate orientation of complement activating IgG or IgM antibodies allows binding of C1q and activation of the classical pathway. In addition, many other structures like cellular components released from injured cells, can bind C1q. Analogously, the lectin pathway is activated after binding of mannose-binding lectin (MBL) or related ficolins to certain carbohydrates or acetylated structures. The alternative pathway does not require specific activators but gets activated by default by structures that lack complement inhibitors and fail to bind the soluble complement inhibitor factor H [3]. Cleavage of C3 to C3a and C3b is the central event in complement activation. C3 is activated by the classical/lectin pathway C3 convertase C4b2a, or by the alternative pathway (AP) convertase C3bBb, both made of a structural subunit that can bind covalently to targets (C4b and C3b) and of a serine esterase protease that is activated after proteolytic cleavage (C2a and Bb). The alternative pathway can amplify complement activation regardless of the initiating pathway. Amplification is based on the unique cyclic capacity of the AP convertase C3bBb to activate C3 to C3b molecules, which become subunits for new active C3 convertases. The C3 convertases also act as C5 convertases that generate the anaphylatoxin C5a and C5b to initiate the terminal complement pathway resulting in the formation of the membrane attack complex (MAC) composed of C5b, C6, C7, C8 and multiple C9 molecules [4].

To prevent excessive or inappropriate complement activation several inhibitors acting at different steps of complement activation exist. Soluble inhibitors include those in the classical pathway, C1 inhibitor (C1INH), to prevent C1r and C1s esterase-mediated activation of C2 and C4, and C4b binding protein (C4bp) that controls the C4b2a convertase. C1INH also inhibits activation of the lectin pathway associated serine proteases (MASPs). To control the key amplification step, C3 cleavage by the AP C3 convertase, soluble factor H and factor I can inactivate C3b to inactive iC3b that loses the ability to function in new C3 convertases. Factor H also has a C3bBb disassembling effect called “decay accelerating” activity. On autologous cell membranes, complement activity is controlled by the major membrane regulators CR1 (receptor for C3b/C4b, CD35), decay accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46) and the membrane attack complex inhibitor protectin (CD59). Of these, CD46 has been shown to have signaling activity through the cytoplasmic domain (cyt1 or cyt2) [4, 5].

3. Location specific production of complement

Liver hepatocytes remain the major source of systemic complement production. C1-C9, factors B, H and I, and as well C4BP and C1INH are all produced in the liver and delivered to the systemic blood circulation. However, it has long been known that local complement production by a vast array of cell types contributes substantially to the pool of complement molecules in the tissues, see table 1. As such, monocytes and macrophages, including tissue resident macrophages, have been shown to produce the full array of complement components [6]. Non-immune cells such as fibroblasts, epithelial cells, endothelial cells and even placental syncytiotrophoblasts have all been shown to produce many complement components, all contributing to the local extracellular pool of activation molecules [6-8]. Notable examples of proteins with major synthetic sites outside the liver are C1q (macrophages and epithelial cells), factor D (fat tissue), and properdin and C7 (neutrophils), respectively [9, 10]. Although little cell specific expression data has been identified for components of the lectin pathway, it is clear that MBL and MASP-2 are found expressed in extra-hepatic tissues such as testis and the small intestine. Likewise, MASP-1 and particularly MASP-3 are expressed through-out the GI-tract, in the kidneys, as well as in the heart, lungs, placenta and reproductive organs [11]. The negative regulator MAp44 is primarily expressed in the heart [12]. Adipocytes and fat tissue produce most complement components and, interestingly, the synthesis of components of all the early pathways is increased in obese individuals, while the production of terminal pathway components is decreased [13]. T and B lymphocytes have been shown to produce C3, C5 and an array of complement receptors. Together, both myeloid and non-myeloid blood cells contribute with a substantial part of serum C3 [6, 14, 15]. While functional levels of plasma complement components are high, up to 1.3 mg/ml for C3, the local tissue levels may be substantially lower [16]. With this in mind, the kinetics of complement activation and regulation should be viewed through a location-specific perspective. Thus, even in cases where local complement production is smaller compared to that of hepatocytes, substantial evidence for a functional relevance has been provided. In immunologically privileged sites, such as the brain, local production of complement is critical for synaptic remodeling and brain development. In rodents, C3 and C1q are expressed in the developing brain and localize to the synapses, where classical complement activation plays a crucial role in refining synaptic development and remodeling [17-19]. Furthermore, in the kidney local complement production by epithelial, endothelial and mesangial cells has been proposed to contribute to anti-microbial defense, as well to local inflammation [20]. This may be particularly relevant for transplant rejection, as has been demonstrated in animal models with hepatic complement deficiency [21, 22]. Complement C3 has also been shown to be synthesized e.g. by keratinocytes, synoviocytes

as well as by malignant skin tumor cells [23-25]. C3 synthesis is upregulated by various cytokines, like IL-1β, IFN-γ and TNF-α. Most interestingly, glucocorticoids suppress C3 synthesis but increase the synthesis of the major soluble complement inhibitor factor H [23, 26]. Thus, suppression of local complement activity could be one of the mechanisms behind the well-known anti-inflammatory activity of glucocorticoids.

[Table 1]

4. Complement inside cells

It has long been recognized that complement activation products, such as C3b/iC3b/C3d and C4b, as well as the anaphylatoxins C3a and C5a play a major role in immune regulation by binding to cell membrane complement receptors, such as CR1 (C3b/C4bR, CD35), CR2 (C3dR, CD21), CR3 (iC3bR, CD11b/18), C3aR, C5aR1 and C5aR2, respectively [5]. Also, the complement inhibitor CD46 has been proposed to act as a receptor for activated C3b [115]. The receptors mediate chemotactic and phlogistic signals to many different types of cells, especially to leukocytes, to attract and activate them and to induce release of further mediators of inflammation e.g. from mast cells (histamine, heparin, prostaglandin D2, leukotriene C4, thromboxane and various cytokines). However, current advances in the field of complement has shown that receptors such as C3aR, C5aR1 and C5aR2 are crucial in complement signaling from the intracellular as well as the extracellular compartment.

4.1 Intracellular C3

Invading microbes will under normal conditions be coated with C3-cleavage products in the extracellular compartment prior to uptake by immune cells. While this is recognized to be important for e.g. phagocytosis, a recent study demonstrated an intracellular-specific effect of internalized C3 activation products in non-phagocytic cells [116]. By incubating adenovirus particles with serum, the authors demonstrated surface-bound C3-cleavage products, and using confocal microscopy, the C3-coated viral particles were localized intracellularly in Hela and HEK293T cells [116]. Endocytosis of adeno-virus led to C3-dependent NF-κB activation, and this particular response was abolished by blocking endocytosis. This study thus demonstrates the presence of intracellular receptors and signaling events based on the presence of C3

cleavage fragments in non-immune cells [116]. Although the specific intracellular receptor was not identified in this study, C3-products were found to impact multiple signaling pathways. The study ruled out a specific effect on NF-κB signaling mediated by CD11b, CD11c, CD18, CD35, CD46 or CD55. C3-signaling was shown to affect mitochondrial antiviral signaling (MAVS), leading to virion degradation by the proteasome. The authors demonstrated that other unknown pathways are also active in these non-immune cells [116]. In line with these observations, a study of dendritic cell processing of apoptotic debris was shown to depend on the binding of C3 cleavage products in mice [117]. A lack of C3 deposition was shown to alter the trafficking of the apoptotic material from phagosomes to lysosomes. Interestingly, the main phagocytic receptor for iC3b-coated particles, CR3, did not appear to be involved in the cellular trafficking. The altered cellular trafficking is thus mediated by intracellular signaling events involving C3-cleavage products, or by extracellular signaling initiated through an unspecified extracellular receptor during phagocytosis [117].

With local complement production in a variety of cells, complement is naturally found intracellularly, even without the presence of microbes or apoptotic material. However, a landmark paper in 2013 showed that not only is complement present, it is also activated intracellularly [52]. Confocal microscopy and flow cytometry were used to show the intracellular presence of C3, as well as the C3a neoepitope occurring only after C3 activation. This was shown in freshly isolated monocytes, neutrophils, $CD8^+$ T-cells, B-cells, as well as in cultured epithelial cells (Me-180), endothelial cells (HUVEC) and fibroblasts [52]. The activation of C3 was studied in detail in T-cells, and activation products were found to engage intracellular receptors with different outcomes to ligand binding as compared to the same receptors on the cell surface. C3 was found in endosomal and lysosomal compartments of the resting T-cells, where part of the C3 pool was converted to biologically active C3a and C3b by the protease cathepsin L [52]. C3a generation increased upon T-cell activation by extracellular anti-CD3 and/or anti-CD46 stimulation. Although C3 activation products were identified in other cell types, the mechanisms of C3 activation appear to vary. The addition of a cell permeable cathepsin L specific inhibitor abrogated C3a generation in T-cells. However, treatment of human lung epithelial cells with the cathepsin L specific inhibitor at 1000-fold higher concentrations, did not affect the intracellular C3a generation [52].

Many recent reports have demonstrated intracellular complement activation-mediated signaling. However, a number of controversies still surround the origin and stimulation of the signaling. In contrast to studies showing cell-derived C3 as the main source of intracellular C3, a recent study showed specific uptake of extracellular hydrolyzed C3(H_2O) into B-lymphocytes, T-lymphocytes as well as to epithelial cells [118]. The authors identified a C3 band in Western blotting of cell lysates, only after direct contact with human serum. Utilizing confocal microscopy of T-lymphocytes and ARPE-19, a retinal pigment epithelial cell line, the authors demonstrated an intracellular localization of C3 into endosomes. The presence of C3 in the internalized endosomes was time-dependent, showing the effect of a processing-mechanism or excretion of the internal C3 [118]. In line with other observations, a "tonic" low level of C3 was observed throughout the experimental time course [52, 118].

Interestingly, studies with B-cells revealed that other complement components may function intracellularly as well. When C3(H_2O) was incubated with both factor H and factor I, an intracellular cleavage of C3(H_2O) to iC3(H_2O) was observed in resting B-cells [118]. This study also demonstrated that cleavage of intracellular C3 following extracellular uptake, and the generation of intracellular C3a, were increased after activation in both B-cells and T-cells [118]. Further in line with these observations, an intracellular role of factor H has been demonstrated in apoptotic cells [119]. Early apoptotic, but not late-apoptotic, Jurkat T-cells and RPE epithelial cells were shown to actively internalize factor H. Interestingly, the uptake of factor H was an active process of early apoptotic cells, leading to the appearance of factor H-coated internal endosomes, which accordingly led to anti-inflammatory processing of the apoptotic material by phagocytic cells [119]. Intracellular Factor H + I processing of C3 could thus influence the internal receptors engaged by C3 cleavage products.

While the study re-produced the observation of cathepsin L dependent cleavage of C3 to C3a and C3b, this was dependent on the presence of factor H. The authors demonstrated an interaction between cathepsin L and factor H. They also showed a dose-dependent increase in C3b generation, when increasing levels of factor H were incubated with a constant concentration of cathepsin L [119]. The results thus suggest that factor H can act as a cofactor for cathepsin L in the intracellular cleavage of C3, a function that could be involved in apoptosis-related alarm signaling events and clearance of e.g. released nucleosomes.

Many proteases have been shown to cleave C3, including kallikrein, trypsin, and some of the coagulation cascade enzymes [120-123]. Cathepsins B and G have been shown to degrade C3, whereas cathepsin L was demonstrated to produce the active C3a and C3b fragments [52, 119, 124]. The outcome of intracellular C3 cleavage and the proteases involved thus appear to be cell- and context-specific. Further defining the C3a and C3b generating proteases in non-lymphocytic cells will thus be of great importance.

Tonic generation of intracellular C3a was shown to be crucial for cell homeostasis and cell survival in T-cells [52]. This is an intriguing observation, given that C3-deficient patients do have surviving and proliferating T-cells in circulation. A closer investigation of these patients revealed that in a number of cases C3-serum deficiency did not correlate with complete C3 deficiency [125-129]. Thus C3-products can be observed in isolated cells from apparently C3-deficient patients, and gene expression profiling has revealed C3 mRNA expression in peripheral blood mononuclear cells [52, 116]. Furthermore, a normal level of the C3a fragment was detected in $CD4^+$ T-cells from a C3-deficient patient [52]. Despite the presence of intracellular C3 in the C3-deficient individuals, impaired function is still observed for B-cells, T-cells and dendritic cells [52, 129]. Sequence analysis of two of our C3-deficient siblings showed a deletion mutation in the C3 beta chain (unpublished). This and similar mutations could potentially lead to a failure in correctly folded C3, and thus inhibit secretion, while simultaneously producing a C3a-containing C3 remnant, which would fulfill the intracellular function [52, 127, 129]. Whether the C3a produced by the peripheral blood mononuclear cells from C3-deficient patients maintains the ability to stimulate neutrophil oxidative burst, as described for the cathepsin L generated C3a, remains to be determined [52].

The evidence from the studies described above suggests that the role of intracellular complement C3 is highly dynamic. This is to be expected with a reactive compound such as C3. Thus, it appears that low levels of C3 and C3-activation products are present in both immune and non-immune cells. Following activation or stimulation, the cells respond by increased intracellular activation of C3. This may either be brought about by an increase in C3 uptake, or by a stimulated co-localization of intracellular C3-stores with intracellular proteases, such as cathepsin L.

4.2 Intracellular C5

While several studies have described the presence and functional relevance of C3 in the intracellular compartment, fewer studies have dealt with the intracellular function of C5. Recent work demonstrated the presence of C5, as well as the activation product C5a in T-cells. However, so far most work have dealt with the function of the C5a receptors, C5aR1 and C5aR2 [53, 130-132]. Yet, the understanding of C5aR1 and C5aR2 signaling has primarily been investigated in the light of extracellular C5a generation. If C5a, like C3a, is generated intracellularly, this suggests that we should revisit the current understanding of C5a receptor signaling.

In humans, the observation of an intracellular localization of both C5aR1 and C5aR2 has been described in monocytes, neutrophils and T-cells [53, 130-132]. Furthermore, a functional role linked to the intracellular space has been presented for both C5a receptors. C5a and C5adesArg bind to both C5aR1 and C5aR2, although C5aR2 has a 10-fold higher affinity for the deactivated C5adesArg than for C5a [133]. While the ligation of C5aR1 induces a proinflammatory response, the outcome of C5aR2 ligand binding appears more controversial [132]. C5aR2 has been linked to anti-inflammatory regulation by two separate mechanisms. Unlike C5aR1, C5aR2 does not couple to G proteins, which has led to the interpretation that it may function as a decoy receptor. Scola *et al*. used C5aR2-transfected HEK, Chinese Hamster Ovary, and Rat Basophilic Leukemia cells, as well as differentiated HL-60 and Hela cells to show that the non-signaling C5aR2 internalizes C5a/C5adesArg from the cell surface, thus inhibiting C5aR1 ligation [134]. However, a study by Bamberg *et al*. showed an alternative anti-inflammatory function. While focusing on the intracellular location of C5aR2 in neutrophils and macrophages, the authors found both C5aR1 and C5aR2 to complex with β-arrestin, leading to a competitive effect on ERK1/2 phosphorylation in response to C5a stimulation [130, 135]. It is important here to note a strong dose-dependent variation in the signaling outcome [135-138]. Finally, in contrast to both anti-inflammatory models, evidence from sepsis models in C5aR2-knockout mice has shown that C5aR2 is essential for the pro-inflammatory response of neutrophils and macrophages, and for the release of a range of cytokines such as IL-6, TNF-α and the upregulation of CR3 [139, 140].

It is clear from the conflicting results obtained thus far, that our understanding of the functional role of C5aR2 is limited. While the above described models show the intracellular localization C5aR2, neither of them investigates the potential of direct ligation of C5aR1/2 by

C5a/C5adesArg following C5 activation in the intracellular space. However, this was recently demonstrated as a mode of action in T lymphocytes. In resting and activated $CD4^+$ T-cells C5aR1 was exclusively observed in the intracellular compartment, while C5aR2 was also observed on the cell surface [53]. Using a specific cell impermeable receptor antagonist, the authors were able to demonstrate that blocking the function of C5aR2 on the cell surface led to the induction of IFN-γ production, and thus a Th1-type response. Likewise, extracellular incubation with C5a/C5adesArg or a C5aR2 agonist lead to a reduced Th1-type response [53]. In contrast, transfecting T-cells with C5aR1 silencing siRNA, to block the intracellular receptor, reduced IFN-γ production. This data thus suggests that ligation of C5aR1 in the intracellular space induces a Th1-phenotype, while extracellular ligation of C5aR2 inhibits this effect [53]. Essential for this pathway is the presence of intracellular C5a or C5adesArg, either produced by the T-cell or internalized from the surrounding environment.

It thus appears that the interplay between the two C5a-receptors is cell-type dependent. The studies with neutrophils and monocytes/macrophages suggest that C5aR1 plays a role on the cell surface, which may be interfered by C5aR2. In contrast, C5aR1 may primarily function intracellularly in T-cells, while the presence of C5aR2 on the surface of the cells may be relevant for fine tuning the cellular response.

Although many observations suggest that complement components C3 and C5 can indeed become activated inside cells and that this may lead to physiological consequences, some questions are left unanswered. Further studies are needed to show that intracellular complement activation, and in particular cleavage of C3 and C5, actually lead to an alarm response inside the cells and a resulting cellular adaptation. One of the questions currently challenging our perception of this complicated intracellular complement signaling cascade, is which mutations may cause a functional locally produced intracellular molecule, yet not a liver-derived secreted molecule. In the case of both C3 and C5 upstream mutations leading to intact C3a/C5a domains, but interfering with the rest of the molecule has been suggested. Other explanations could incude lineage-specific mutations that only affect the liver cells, and not cells of the myeloid lineage. A final explanation could be deficiency in the C3/C5 secretion mechanisms.

5. Intracellular complement and metabolic stimulation

The link between innate immunity and basic metabolic regulation has been well established. Specifically, the effect of complement components has been shown to affect the metabolism of various tissues such as liver, pancreas and adipose tissue [141]. C3adesArg induces insulin secretion from pancreatic beta-cells, while factor H has been found to suppress this effect [142, 143]. Both C1q, MBL and C3 have been implicated in type 1 diabetes, and C3-deficient mice, as well as mice with hematopoetic specific C3 deficiency were observed to be protected from diabetes [144-146]. These findings suggest a role for locally produced complement components, and possible complement activation, in the regulation of metabolism. It is well worth noticing, however, that others have tried to replicate the protective effect of C3 deficiency, but failed [147].

At the cellular level, the complement-mediated effects on adipocytes have been studied. Adipocytes produce both C3, factor D and factor B, and thus are able to produce C3a and the C3a breakdown product C3adesArg [148, 149]. C3adesArg has been suggested to increase triacylglycerol production in the adipocytes by increasing the uptake of free fatty acids [149]. Our recent study compared genetically identical monozygotic twins, where the siblings had an average weight difference of 18 kg [13]. In obese individuals the genes for components of both the classical and the alternative pathway were upregulated in fat tissue and in the isolated adipocytes, while the terminal pathway genes were downregulated. The upregulated genes included also receptors for C3a and C5a (C5aR1), and the iC3b receptor (CR3). Gene upregulation correlated positively with adiposity and hyperinsulinemia and negatively with the expression of insulin signaling-related genes. Obesity thus correlates with an inflammatory alarm response in adipocytes and fat tissue. This response includes synthesis of complement components that may be involved e.g. in the removal of excess or modified lipid waste [13].

[Figure 1]

T-lymphocytes have been shown to produce C3 activation products, and ligand-C3aR interaction was found to play a role in T-lymphocytes undergoing metabolic reprogramming during activation. The complement components C4b and C3b bind to the complement regulator CD46 on the surface of T-cells, leading to signaling through the cytoplasmic tail. By comparing T-lymphocytes isolated from a CD46-deficient patient, T-lymphocytes from healthy donors treated with CD46 siRNA, and a Jurkat T-cell line overexpressing CD46, it was demonstrated that autocrine stimulation of CD46 following cell activation could be important for glycolysis

and oxidative phosphorylation [150]. T-cell activation in both mice and humans depends on increased nutrient uptake through glucose and amino acid transporters [151, 152]. However, in CD46-deficient T-cells, upregulation of the glucose transporter GLUT1 and the L-type amino acid transporter LAT1 were impaired, thus further pointing to a potential role of complement activation products in cellular metabolism [150]. While intriguing, however, these studies need to be confirmed and mechanistically explained.

In macrophages, the specific effect of complement on energy metabolism has not been studied in detail. However, the effect of metabolic inhibition of oxidative phosphorylation and glycolysis on complement mediated phagocytosis has been investigated. The study revealed that phagocytosis and internalization of complement coated zymosan was dependent on glycolysis and the availability of glucose [153]. In the future, it will be interesting to investigate if binding of opsonized particles to complement receptors directly regulates the level of glycolysis in macrophages as observed in T cells. Obviously, complement-opsonized particles and the anaphylatoxins have major effects on macrophages in their normal clearance functions. How and specifically by which mechanisms this leads to “silent” removal host breakdown products or to a more aggressive inflammatory response remains to be worked out.

Extracellular stimulation of complement receptors is apparently essential for activation of many different types of cells. However, resting T-cells require tonic metabolic stimulation signals for survival, as well. Mammalian target of rapamycin (mTOR) is one key component providing such homeostatic signaling [154-156]. Ligation of the C3aR and C5aR has been shown to induce mTOR activation. Recent work has proposed that the C3aR stimulation necessary for mTOR activity may primarily be provided by intracellularly derived C3a [52]. Culturing of resting and $CD4^+$ T-cells in the presence of increasing concentrations of the cathepsin L protease, leads to apoptosis. This suggest the importance of C3aR stimulation for the metabolic survival of T-cells. Interestingly, the apoptotic phenotype could not be rescued by exogenous (extracellular) addition of C3a to the culture. The stimulation of mTOR thus seems to be dependent on the intracellular production of C3a [52].

While extracellular C3a has no effect on the basic homeostatic mTOR stimulation, engaging extracellular complement receptors are crucial for T-cell activation. Whereas the C3aR is not found on the surface of resting T-cells, it translocates to the surface upon T-cell receptor stimulation [52]. C3a-receptor interaction, as well as C3b interaction with CD46, are crucial

components in IFN-γ secretion. This was shown by incubating activated T-cells in the presence of a non-lethal dose of cathepsin L inhibitor. This dramatically reduced IFN-γ secretion, however, in case of the activated T-cells the phenotype could be rescued by the addition extracellular addition of C3a and agonistic activation of CD46 [52]. As a net result, C3 activation and IFN-γ secretion lead to differentiation of CD4+ T cells into Th1-type cells, whereas the generation of $FoxP3^+$ regulatory T cells is favored in their absence [154, 156]. Our studies on C3-deficient mice have also shown that C3 is involved in T helper cell differentiation, and immune responses to ovalbumin were biased towards Th2 direction in the absence of C3 [157]. In subsequent studies, we further observed that human C3-deficient patients as well as C3 knock-out mice had an impaired intestinal tolerance, and therefore e.g. an increased tendency to develop antibodies against intestinal microbes [158]. At least in part, the impaired intestinal tolerance was caused by the absence of C3-mediated signaling in T cells.

6. Intracellular alarm signaling

With the novel perspectives in our understanding of the role of complement activation from extracellular danger sensing to also include intracellular activation, multiple functional avenues open up. Could this alarm response involve complement-mediated recognition of intracellular microbes, bacteria, protozoa and viruses and promote their destruction?

The complement system has already been linked to a number of cellular danger sensing systems, including the Toll-like receptors [159, 160]. A key component of the innate immune defense system, is the inflammasome mediated generation of IL-1β and IL-18 [161]. These cytokines enhance the antimicrobial response of phagocytic cells and induce the adaptive Th1 and Th17 response [162]. Finally, IL-1β induces the expression of pentraxin 3 (PTX-3) leading to complement activation, as well as tissue repair and regulation of the clotting cascade [163]. While several types of multiprotein inflammasome-complexes have been described, a number of studies have shown a link between complement signaling and the pyrin domains-containing protein 3 (NLRP3) inflammasome [164, 165].

Inflammasome activation and IL-1β/IL-18 production take place in both myeloid and non-myeloid cells. The pathways inducing NLRP3 activation appear to be cell-specific [166].

Generally, a priming signal is essential for NLRP3 and IL-1β/IL-18 gene transcription, followed by an activating signal driving the inflammasome complex assembly [167, 168]. C3 and C5, as well as their activation products, have a direct impact on both the priming (signal 1) and the activation (signal 2) of the inflammasome. Cytokine stimulation and pattern recognition receptor ligation are key priming signals. C5a combined with TNF was identified as a strong inducer of IL-1β mRNA transcription in monocytes [165]. Likewise, in T-cells, a gene set enrichment analysis revealed a number of inflammasome-related genes to be upregulated following TCR/CD46 co-stimulation, including IL1-beta and NLRP3 [53]. In mice complement was a crucial inducer of Il-1β expression, and $C3^{-/-}$ knock-out mice display a reduced inflammasome activation in microglial cells following brain inflammation [169].

Complement components can also directly influence inflammasome assembly (signal 2). C5 and C5a were observed at low levels in resting monocytes and T-cells by confocal microscopy and flow cytometry. Following cell activation by TCR and CD46 stimulation, C5 expression was increased and the generated C5a was able to stimulate the intracellular C5aR1. This directly induced the generation of reactive oxygen species (ROS) in the cell, leading to inflammasome assembly [53]. In addition, as highlighted above, C3a-stimulation alters the cellular metabolic programming, and has directly been shown to increase ATP efflux in monocytes [164]. The available ATP engages the receptor P2X7, which also leads to NLRP3 activation and increased IL-1β generation [164].

After activation, intracellularly generated C5a translocates to the cell surface, where it may act as a negative regulator. In T-cells inflammasome activation leads to IFN-γ production, a response which was exacerbated by blocking of the inhibiting C5aR2 [53]. Interestingly, C1q has also been identified as a driver of inflammasome activation following priming in murine epithelial cells [170]. In contrast to this, C1q was shown to exert a negative regulatory effect on macrophage inflammasome function [171]. The complement components therefore appear to be an essential regulatory part of the cellular alarm-system controlling inflammasome activation. For a more detailed understanding, please refer to the recent review by Arbore and Kemper [172].

7. Conclusions

The profile of complement activities has become considerably broader in recent years. In particular, the window into intracellular activities has been opened, and it seems clear that active complement products are produced inside cells where they may have their effect. Alternatively, they engage cell membrane receptors after excretion from cells, thus mediating their function in an autocrine or paracrine fashion. As usual, most of the reports still need to be confirmed to be able to establish the real significance of these intriguing observations. Activated complement components, like C3a and C5a, appear to raise an alarm that promotes cellular defense mechanisms both inside and outside cells. They also seem to be able to launch cellular differentiation programs aimed at coordinating the other arms of the immune system into an active response against invaders, including those digging into cells. In addition to this, some of the novel complement mechanisms may include directing physiological clearance mechanisms, metabolic control, immune suppression and tolerance maintenance.

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9. Figure legends

Figure 1. An example of location-specific gene regulation of complement component expression. Expression of complement system genes in subcutaneous adipose tissue and isolated adipocytes from obese individuals as compared to genetically identical monozygotic twin siblings. Genes upregulates are indicated in red and those down-regulated in blue. Adapted from Kaye et al, 2017 (ref 13).

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