

# C3-dependent effector functions of complement

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## Summary

C3 is the central effector molecule of the complement system, mediating its multiple functions through different binding sites and their corresponding receptors. We will introduce the C3 forms (native C3, C3 [H<sub>2</sub>O], and intracellular C3), the C3 fragments C3a, C3b, iC3b, and C3dg/C3d, and the C3 expression sites. To highlight the important role that C3 plays in human biological processes, we will give an overview of the diseases linked to C3 deficiency and to uncontrolled C3 activation. Next, we will present a structural description of C3 activation and of the C3 fragments generated by complement regulation. We will proceed by describing the C3a interaction with the anaphylatoxin receptor, followed by the interactions of opsonins (C3b, iC3b, and C3dg/C3d) with complement receptors, divided into two groups: receptors bearing complement regulatory functions and the effector receptors without complement regulatory activity. We outline the molecular architecture of the receptors, their binding sites on the C3 activation fragments, the cells expressing them, the diversity of their functions, and recent advances. With this review, we aim to give an up-to-date analysis of the processes triggered by C3 activation fragments on different cell types in health and disease contexts.

## KEYWORDS

anaphylatoxin, autoimmunity, cancer, complement C3, inflammation, opsonin

## 1 | INTRODUCTION

The growing interest in recent years has put the complement system, part of the innate immune response, in the spotlight. Many novel functions of the complement system have been discovered, exceeding the limits of its bactericidal activity and complementing the action of antibodies. Biochemical and biophysical studies are the foundations of research in the complement system field. Over the years, accumulating evidence has contributed to the precise knowledge of the molecular mechanisms governing the complement system available to us today. Complement component C3 is considered the “Swiss Army Knife” of innate immunity and host defense and for

a good reason.<sup>1</sup> In this review, we will outline the main C3 functions and how they are carried out through interactions with complement receptors.

### 1.1 | C3—What is it and where to find it

C3 is the central component of the complement system, present in the blood in concentrations of more than 1 mg/mL, which makes it one of the most represented proteins in circulation. Native C3 is considered biologically inactive, but its activation fragments have a multitude of biological functions. A plethora of structure–function

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studies helped to unveil its multiple biologically active sites important in the mediation of complement effector functions, recently reviewed in Geisbrecht et al.<sup>2</sup> C3 is mainly expressed by the liver and circulates in plasma, but most of the cell types in the human body express some amount of C3. It is abundant in circulation, tissues, and even intracellularly. In each of these locations, it can be present in different activation states, and it can have different functions (Figure 1).

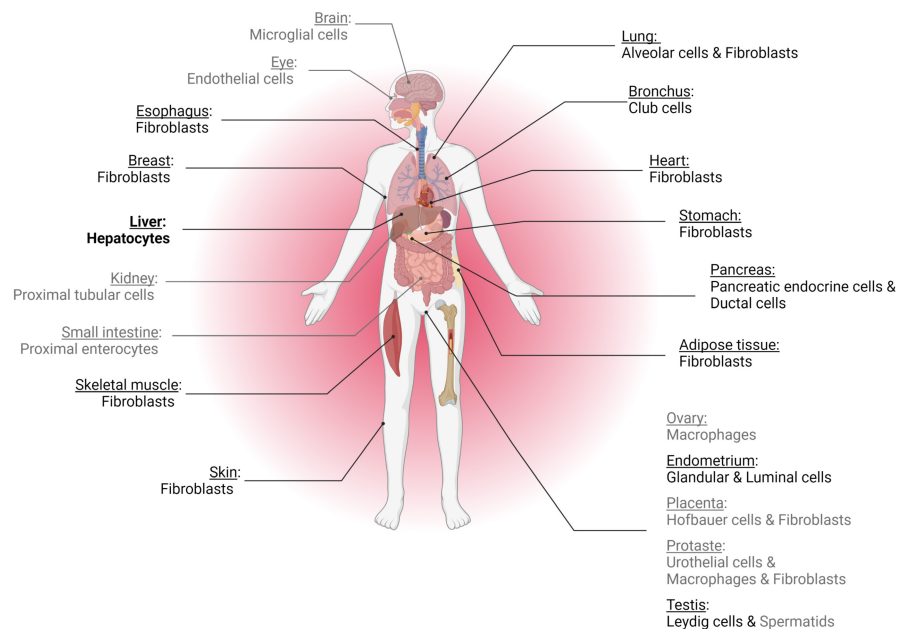
In the circulation and in tissues, C3 is activated by a series of enzymatic reactions upon pathogen infection or cell damage. Each cleavage gives biologically active fragments, indicated by lowercase letters, such as C3a, C3b, iC3b, C3d, and C3dg. For the last cleavage fragment, the C3dg form is rapidly transformed to C3d in tissues and on cell surfaces by proteases, and the available tools most often do not allow us to recognize whether C3dg or C3d is present. From this point onward, C3d is used to indicate this last fragment, which in some contexts could be C3dg. The C3 activation fragments serve as inflammatory modulators (the anaphylatoxin C3a) or opsonins (C3b, iC3b, and C3d) and have context- and/or receptor-dependent pro- and anti-inflammatory, destructive, or protective functions. The diversity of the functions is not restricted to the C3 cleavage fragments. The C3 conformation is also a source of diverse biological functions. Native C3 can undergo a conformational change upon binding of a water molecule to generate C3(H<sub>2</sub>O), a biologically active form resembling C3b in circulation. Intracellular forms of C3 were also recently discovered. This intracellular C3 could either (a) be internalized from the extracellular space in the form of C3(H<sub>2</sub>O),<sup>3</sup> (b) be expressed by the cell<sup>4–6</sup> from the same start site as the secreted form of C3, or (c) be from an alternative form, generating a shorter, cytoplasmic form lacking disulfide bridges and glycosylation.<sup>7</sup> The intracellular C3 forms regulate cell metabolism, autophagy and contribute to the intracellular detection of cytoinvasive pathogens, as described in detail in this special issue by Blom et al.<sup>8</sup>

Keeping in mind the diversity of the biologically active forms of C3, its widespread expression and its presence both in the extracellular and intracellular spaces, it is logical to expect that C3 is a key protein for the functioning of the human body.

## 2 | WHY IS C3 SO IMPORTANT—LESSONS FROM C3 DEFICIENCY

The pathological consequences of C3 deficiency in humans are illustrative of the key functional relevance of C3 and its activation fragments. C3 deficiency is extremely rare and results in recurrent pyogenic infections, mainly caused by *Streptococcus pneumoniae* and *Neisseria meningitidis*. In isolated cases, C3-deficient patients could suffer from immune complex (IC)-related diseases such as systemic lupus erythematosus (SLE)-like illness and renal diseases, although the deficiency of C3 is not considered a genuine SLE predisposing factor, in contrast to the deficiency of C1q and the other components of the classical pathway (CP).<sup>9,10</sup> Mechanistically, C3 deficiencies connected to a lack of opsonization are associated with impaired dendritic cell differentiation, memory B-cell responses, and regulatory T-cell development in humans.<sup>11,12</sup>

Mouse models are instrumental in further understanding the implication of C3 in physiology and in complement-mediated diseases. C3 knockout in vivo has been used to study several biological or pathological processes in the nervous, circulatory, skeletal, respiratory, digestive, urinary, and visual systems. From these results, the beneficial roles of complement C3, corresponding to the harmful effects of C3 deficiency, and detrimental ones, corresponding to the protective effects of C3 deficiency, can be deduced and will be reviewed in this section. The fact that C3 has been found to play a ubiquitous role underlines once again its importance in multiple contexts.



**FIGURE 1** Expression of C3 in different organs and cell types. Gray-colored items indicate expression lower than 200 transcripts per million (nTPM), in black expression is between 200 and 2500 nTPM, and in bold expression is above 2500 nTPM. Of note, hepatocytes reach 8000 nTPM. Data from protein atlas <https://www.proteinatlas.org/>

## 2.1 | Protective role of C3

The complement system, and in particular the CP, has an established role in maintaining homeostasis by removing pathogens and damage-associated molecular patterns (PAMPs and DAMPs, respectively) from the circulation in the case of infection but also in the case of tissue injury, autoimmune diseases, and cancer. Confirming its role in protection from infection in humans, *in vivo* studies in mice established a direct link between C3 deficiency and breakage of the immune defense in bacterial sepsis, acute influenza virus infection, Lyme borreliosis, and fungal infection.<sup>13–16</sup> Opsonophagocytosis, oxidative stress, and cell lysis are key functions of complement to ensure the rapid elimination of invading bacteria. Indeed, C3 deficiency was shown to increase bacterial load in a model of sepsis induced by cecal ligation and puncture. A decrease in inflammatory mediators was associated with failure to induce complement-specific functions (phagocytosis, oxidative burst, and cell lysis)<sup>15</sup> (see Section 4). C3 also controls acute influenza virus infection by increasing viral clearance through the regulation of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses. T-helper cell-dependent IgG responses are diminished in C3-deficient mice due to the failure to target viral antigens to lymphoid organs and to proceed to later stages of complement activation.<sup>14</sup> The *Borrelia burgdorferi* infection model elucidated that complement contribution is important to control infection dissemination in the early stages of the disease and to prevent the early development of arthritis, while other mechanisms come into play at later stages.<sup>13</sup> Moreover, the presence of C3 was shown to enhance B-cell responses to many antigens. Fungi are not susceptible to complement-mediated cell lysis, and the role of C3 in protecting against fungal infection is connected to fungal clearance. Interestingly, complement deficiency is protective when mice are challenged with high doses of fungi.<sup>16</sup>

The CP of the complement system has an important role in clearing ICs formed when the host is challenged by an invading pathogen but also in the context of autoimmunity and cancer by facilitating their transport to liver and spleen macrophages. Where circulating ICs are involved in a wide spectrum of pathology, the importance of C3 to prevent their deposition has mainly been evidenced in the kidney. *In vivo* studies have shown that C3 deficiency leads to the deposition of IgM and IgA ICs in the glomeruli of mice immunized with apoferritin.<sup>17</sup> In a model of lupus nephritis, C3-induced protection was attributed to IC clearance.<sup>18</sup>

Moreover, complement plays a role in tissue healing and repair, as recorded here in the aging central nervous system, in liver injury, in trauma, and in skin psoriatic injury. In the developing brain, complement contributes to synaptic circuit refinement by pruning away excess synaptic connections, a fundamental process for obtaining optimal cognitive ability in adulthood.<sup>19–21</sup> Conversely, complement is also involved in neurodevelopmental and neurodegenerative diseases, and the molecular mechanisms implicated are the subject of active investigation.<sup>22–25</sup> In Alzheimer's mouse models, C3 deficiency exacerbates the accumulation of amyloid beta plaques and neurodegeneration; therefore, C3 is critical in the clearance of these deposits<sup>26,27</sup> (see Section 8.2). Strikingly, recent evidence highlights

that constitutive loss of C3 in mice results in spontaneous locomotor deficits during the aging process, with decreased speed and gait ataxia, which can be observed in age-related locomotor deficits and in the absence of anatomical alterations linked to cerebellar function.<sup>28</sup>

The complement system is known to take part in other healing processes in organisms. Specifically, C3 has a fundamental role in liver regeneration and the removal of damaged tissue after toxic liver injury<sup>29–31</sup>; it also participates in bone development, hematopoietic stem cell homing and engraftment, and amphibians, such as newts and salamanders, limb regeneration.<sup>32</sup> Complement is known to mediate platelet activation, and platelets can activate complement. C3 is essential for physiological hemostasis, and its deficiency results in increased bleeding times, delayed thrombosis, and unstable thrombi.<sup>33</sup> Therefore, functional crosstalk of complement with the coagulation cascade is particularly important upon trauma, where an early clotting response coordinates with complement-mediated early inflammatory signals that eliminate DAMPs and invading pathogens.<sup>34</sup> In the context of inflammation, C3 was recently found to protect against psoriasis-like skin inflammation.<sup>35</sup> The absence of C3 exacerbated the inflammatory phenotype by upregulating IFN- $\gamma$ <sup>+</sup> T-cell responses, and the phenotype was reversed by caspase inhibition, indicating that C3 exerts its protective role by inhibiting apoptosis.<sup>35</sup>

Among the numerous physiological functions of C3 that remain unclear, C3 has been involved in apoptosis and angiogenesis regulation. In a model of retinopathy of prematurity, C3<sup>-/-</sup> mice displayed increased neovascularization,<sup>36</sup> and in a model of muscle ischemia, angiogenesis was associated with increased neutrophil and macrophage infiltration.<sup>37</sup>

C3 has a large number of direct and indirect downstream functions, the molecular details of which are discussed in the coming sections. Before that, to highlight the double-edged sword of C3 activation, we will give an overview of the deleterious effects of C3 activation.

## 2.2 | Deleterious effects related to C3 activation

While C3 functioning is key in normal physiology, excessive activation of C3 is a hallmark of many complement-mediated diseases. In this case, protective effects of C3 deficiency are observed in experiments *in vivo*. The excessive activation of C3, leading to the formation of active fragments C3b and C3a, has been implicated in many acute autoimmune or inflammatory disease models, including acute kidney inflammation, neurotrauma, anti-phospholipid thrombosis, asthma, and allogeneic transplantation. In the kidney, the iconic organ of complement activation, C3 knockout has been shown to be protective, not only in several models of acute kidney injury, such as ischemia-reperfusion injury,<sup>38</sup> in which it was associated with reduced neutrophil infiltration and NET formation<sup>39</sup> but also in rhabdomyolysis, where it was associated with inflammatory monocyte infiltration.<sup>40</sup> C3 deficiency decreases the risk

of rejection in renal transplantation, even if this deficiency is restricted to the kidney, by modulating T-cell responses.<sup>41</sup> Finally, C3 deficiency reduced the risk of pyelonephritis in a model of *Escherichia coli* pyelonephritis.<sup>42</sup> C3 usage was shown to be part of the *E. coli* mechanism to invade the renal epithelium. Strikingly, despite these protective effects of C3 knockout in ischemic and infectious mouse models in the kidney, in all mouse models of glomerulonephritis, such as lupus and IgA nephropathy, in which complement activation fragments are detected, C3 knockout was protective. This is a key paradox in C3 functions, which can at best be partially explained by the impaired protective IC clearance function of C3 opsonins.

C3 has a detrimental role in the acute assault, such as focal brain ischemia, spinal cord injury, and intracerebral hemorrhage.<sup>43–45</sup> Under these conditions, C3 deficiency protects against inflammatory tissue damage and promotes nerve fiber regeneration after spinal cord injury. In an induced intracerebral hemorrhage, behavioral tests show decreased asymmetry in limb use in C3-deficient mice, and brain water content measurements indicate lower levels of brain edema.<sup>45</sup> In anti-phospholipid-induced thrombosis and fetal loss, C3 deficiency protects against enhanced thrombosis, endothelial cell activation, and fetal injury.<sup>46,47</sup> Interestingly, C3 is a biomarker of non-alcoholic fatty liver disease in rheumatoid arthritis patients,<sup>48</sup> and C3 contributes to steatosis in fatty liver disease in mice.<sup>49</sup> The putative mechanism of action of C3 in this disease has recently been described: C3 upregulates the expression of glycine transfer RNA-derived fragments, which in turn downregulate the expression of Sirt1, a regulator of lipid metabolic pathways.<sup>50</sup> In the pathogenesis of asthma, C3 deficiency alters the allergic response by reducing the characteristic symptoms of airway hyperresponsiveness and IgE and IgG responses through the downregulation of IL-4 production, an important cytokine for Th2 function.<sup>51</sup> In pulmonary arterial hypertension, the absence of C3 attenuates prothrombotic and proangiogenic symptoms in a mouse model of the disease.<sup>52</sup> Regulated innate immune responses are critical for the success of allogeneic transplantation. The impact of excessive complement activation, particularly of C3, has been studied in an animal model for graft-versus-host disease (GVHD). C3<sup>-/-</sup> mice showed less mortality and GVHD than wild-type mice, associated with decreases in donor Th1- and Th17-cell differentiation, which are critical in the development of GVHD.<sup>53</sup>

If complement-mediated responses against self are intrinsic to several inflammatory and autoimmune diseases, the age-dependent increase in the expression of complement components suggests their implication in age-related diseases.<sup>54</sup> Indeed, in arthritis and atherosclerosis, inflammatory signals amplified by C3 are detrimental,<sup>55–57</sup> and C3 deficiency ameliorates age-related changes in mouse kidneys, manifesting as tissue sclerosis and fibrosis.<sup>58</sup> This finding may be linked to a detrimental role of C3 in renal fibrosis, exerted by polarizing macrophages toward a proinflammatory phenotype, increasing proinflammatory cytokine expression, and causing peritubular capillary rarefaction due to decreased angiogenesis.<sup>59</sup> Not only was C3 found to play a role

in acute neuropathologies, but C3-deficient mice are also protected from age-related hippocampal decline and neurodegeneration,<sup>60–63</sup> and in experimental autoimmune encephalomyelitis, a mouse model for multiple sclerosis, C3 is necessary for full disease development.<sup>64</sup> The absence of chemotactic activity toward inflammatory cells induced by C3 knockdown is beneficial in alleviating choroidal neovascularization, a common cause of age-related macular degeneration.<sup>65</sup>

Finally, C3 deficiency was found to protect against tumor growth in multiple mouse models.<sup>66,67</sup> Cutaneous squamous cell carcinoma was found to induce epidermal hyperplasia and drive tumorigenesis.<sup>68</sup> In a model of carcinogen-induced sarcoma, C3 and C3a receptors (C3aR) deficiency was associated with reduced tumor growth, reduced accumulation and functional skewing of tumor-associated macrophages, increased T-cell activation, and response to anti-PD-1 therapy.<sup>69</sup> Moreover, the local production of the proinflammatory anaphylatoxins C3a and C5a is crucial to the tumor response to radiotherapy and concomitant stimulation of tumor-specific immunity.<sup>70</sup> Many other examples highlight the role of C3 activation fragments in different types of cancers.<sup>71</sup> Overall, C3 contributes to the context-dependent functions of the complement system in cancer, where the outcome is defined by the site of complement activation, the composition of the tumor microenvironment, and the sensitivity of the tumor cell to complement attack, as recently reviewed by Roumenina et al.<sup>72</sup> Pancancer transcriptomic analysis based on the prognostic impact of complement revealed that the coregulated overexpression of complement genes, including C3, confers either poor or favorable prognosis depending on the cancer type. Interestingly, in a subgroup of tumors called the “Protective C3” group, C3 appeared to influence the patient prognosis independently from the other complement proteins.<sup>72</sup>

C3<sup>-/-</sup> mice are indeed susceptible to infections and have deficits in homeostatic processes, but they are protected in a multitude of inflammation-related disease models. These observations illustrate the “double-edged sword” of the functioning of the C3 activation fragments—their defensive action could turn offensive if misdirected in a pathological process. Not only is C3 involved in multiple biological processes, but this occurs in physiology, aging, allergy, and chronic and acute disease contexts, highlighting the importance and complexity of C3-mediated responses and the broad potential for therapeutic intervention. Compstatins are an ever-growing family of C3-targeting inhibitors<sup>73</sup> developed to address this ambitious goal. The original compound is a C3 inhibitor that can block all complement pathways and therefore all the C3 fragment-mediated functions.<sup>74</sup> Compstatins are derivatives of a 13-residue peptide macrocycle, and they bind C3 between the MG4 and MG5 domains<sup>75</sup> (see Section 3). In 2021, the compstatin pegcetacoplan was approved for the treatment of paroxysmal nocturnal hemoglobinuria (PNH), and several other compstatin-derived compounds are under investigation for periodontal disease, COVID-19, age-related macular degeneration, and neurodegenerative diseases.<sup>73</sup>

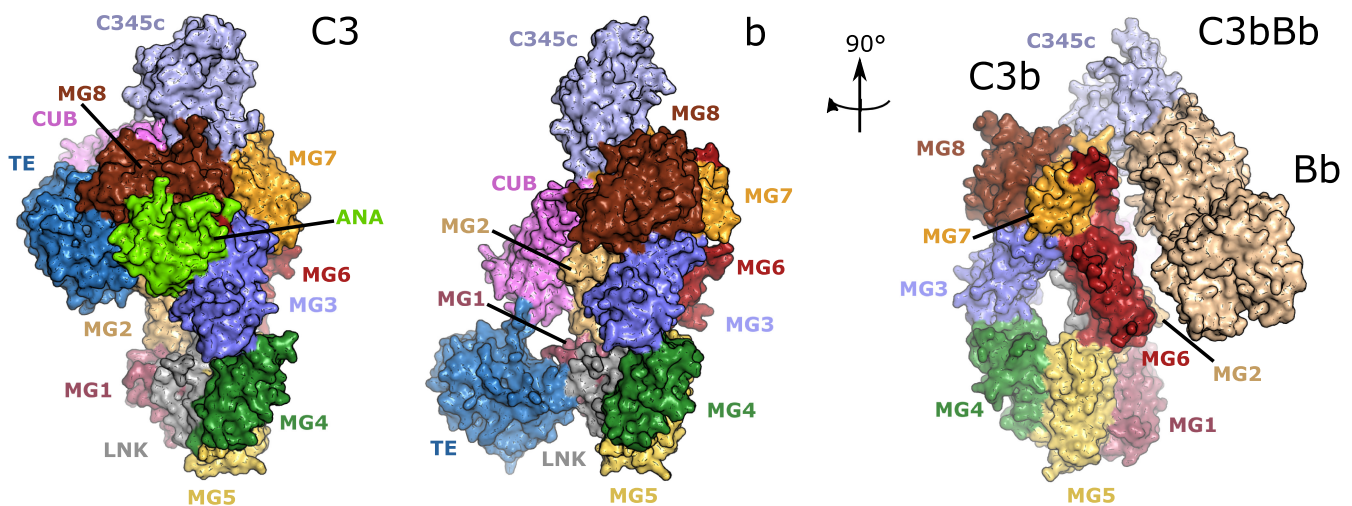
### 3 | FORMATION OF C3 AND C5 CONVERTASES—THE HEART OF THE COMPLEMENT CASCADE

C3 is the central molecule of the complement system and is the point of convergence of all its activation pathways. Structurally, C3 is part of the A2M protein family, with which it shares the domain architecture. Like all family members, it contains 8 macroglobulin-like (MG) domains, a linker region (LNK), an anaphylatoxin domain (ANA), a C1r/s, Uegf, B (CUB) domain, and a thioester (TE) domain. One last domain is present at the C-terminus of complement components C3, C4, and C5 called the C345c domain. Mature C3 is formed by an  $\alpha$ - and a  $\beta$ -chain, which are held together by disulfide bridges. The  $\alpha$ -chain encompasses domains MG1 to the first half of MG6 and the LNK, while the second half of MG6, ANA, MG7, CUB, TE, MG8, and C345c domains form the  $\beta$ -chain (Figure 2, left).<sup>76</sup> One characteristic structural unit of A2M family proteins is the “MG ring”, formed by the MG domains arranged in a ring-like fashion, visible in the right panel of Figure 2.

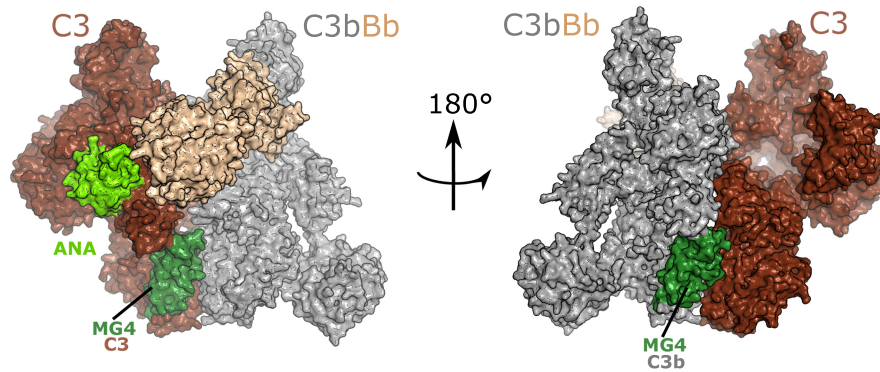
Complement can be activated by three pathways: the classical pathway (CP), lectin pathway (LP), and alternative pathway (AP).<sup>77</sup> C3 is cleaved by the CP and LP C3 convertase C4b2b (updated nomenclature from C4b2a according to the new guidelines<sup>78</sup>), while in the AP, it is cleaved by the AP C3 convertase C3bBb into the fragments C3a and C3b. Cleavage of C3 at an Arg-Ser bond releases the C3a anaphylatoxin in the fluid phase and triggers a dramatic conformational change in the nascent C3b molecule.<sup>79</sup> The TE domain is released, exposing the reactive glutamine acyl group of the internal thioester (Figure 2, center). Nucleophilic attack at this acyl group is predominantly by water hydroxyl groups, with C3b remaining in the fluid phase. However, a small proportion of the reactive C3 will

undergo nucleophilic attack by surface hydroxyl or amino groups, with consequent covalent attachment to that surface. This process is known as opsonization, and it can either trigger phagocytosis or proceed to the formation of the AP C3 convertase by binding to factor B (FB) and, following activation by FD, generating the AP C3bBb convertase (Figure 2, right).<sup>80</sup> The fact that C3b deposited by the CP can in turn form a C3 convertase, which will amplify C3b deposition on the activator surface, is known as the “amplification loop” of complement activation. AP C3 convertase is very short-lived and needs to be stabilized by properdin (FP) to have a fully functional impact *in vivo*. The binding of properdin leads to a 10-fold increase in the half-life of AP C3 convertase.<sup>81,82</sup>

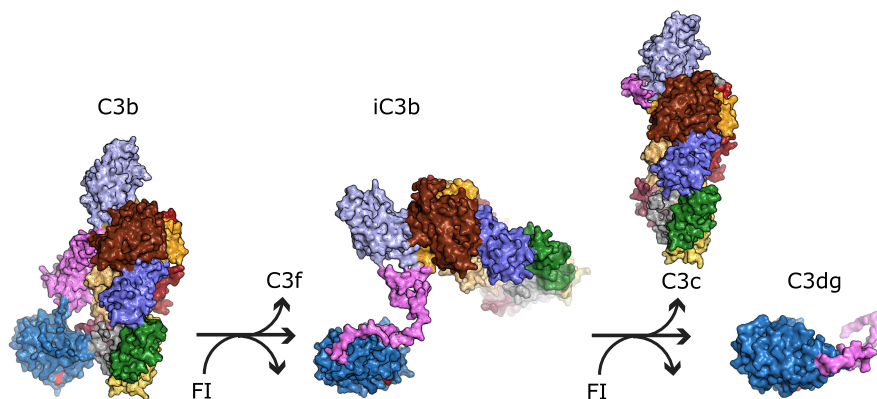
Properdin is the only known positive regulator of the complement system, and it will be described in detail in another contribution to this series. A model for C3 substrate binding to the AP C3 convertase is presented in Figure 3,<sup>83</sup> where it is evident that the C3/C3b MG4 mediates important enzyme-substrate contacts, making it a good target for complement inhibition at the C3 level. In the AP, inhibiting these contacts affects both the C3 substrate and the C3b molecule portion of the convertase enzyme (Figure 3). In the CP and LP, interfering with these interactions prevents the association of the C3 substrate molecule with the convertases (Figure 3, left). Another source of complement C3 activation is spontaneous hydrolysis of the labile thioester group. This nucleophilic attack by water to the C3 thioester is known as the “tick-over” mechanism, and it is accompanied by a conformational change in C3 resulting in a C3b-like conformation, generating a molecule called C3(H<sub>2</sub>O).<sup>84</sup> C3(H<sub>2</sub>O) is able to bind to FB and, after FB cleavage by FD, to form a fluid phase convertase. The role of this fluid phase convertase is still debated in the complement field. Some suggest that the labile thioester provides a



**FIGURE 2** The conformational change occurring upon C3 activation by a C3 convertase. When C3 (left, PDB ID 2A73) is cleaved by a C3 convertase (C4b2a or C3bBb), the anaphylatoxin domain is released to generate C3a in the fluid phase, and C3b which opsonizes the target surface, thanks to an extensive conformational change exposing the thioester in the TE domain, allowing it to react with a nucleophile on the surface (center, PDB ID 2I07). C3b can now form the alternative pathway C3 proconvertase by binding to FB. FB is cleaved by FD to give the alternative pathway C3 convertase C3bBb (right, PDB ID 2 WIN). All figures displaying structures were prepared in PyMOL 2.3.0 <https://pymol.org/2/> and edited in Inkscape.



**FIGURE 3** The substrate-bound alternative pathway C3 convertase. A model of substrate binding by the alternative pathway C3 convertase based on the crystal structure with PDB ID: 3PVM. C3b in the C3 convertase is shown in gray, Bb in roseate. The substrate C3 is shown in brown, with the highlighted ANA domain in lime green, and the MG4 domain in green. The figure underlines the importance of the MG4 domain interface for efficient convertase activity. In fact, residues in MG4 are part of the interface between the substrate C3 and the C3b in the convertase (left), but also between the C3b in the convertase and the substrate C3 (right). To generate the model, C3 was aligned to C5 in the CVF:C5 complex (PDB ID 3PVM), while C3b bound to Bb was aligned to CVF.



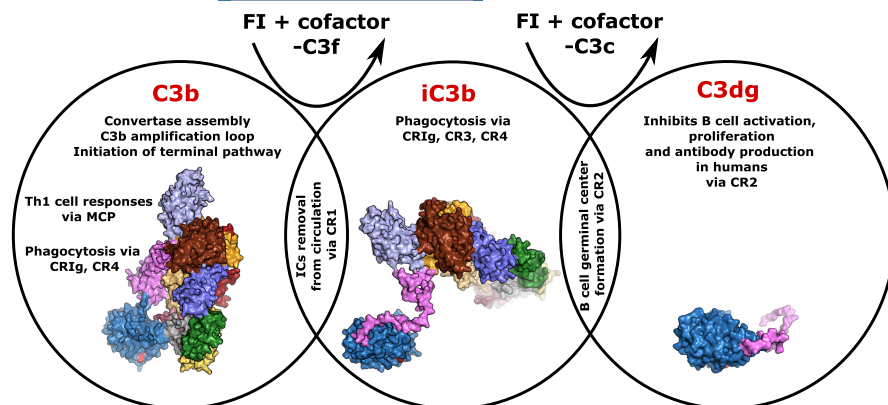
**FIGURE 4** The cleavage steps carried out by FI during C3b regulation. When C3b (left, PDB ID 2I07) is cleaved by FI in the CUB domain (pink), the C3f fragment is released in the fluid phase, and iC3b remains flexibly attached to the target surface (center, PDB ID 7AKK). A last FI cleavage in the CUB domain releases the C3c fragment and leaves C3dg bound to the surface (right, PDB ID 7AKK). The thioester glutamine is shown in red, anchoring the TE domain to the activator surface. The location of the remaining iC3b domains is putative, as they are flexibly attached to the TE domain through what remains of the CUB domain (pink).

constant low level of complement activation, which has the role of providing protection against invading pathogens and foreign substances such as toxins. However, this view is challenged by the absence of experimental evidence on the existence of a fluid phase convertase. The actual contribution of this pathway to complement activation requires further investigation.<sup>85–87</sup> Another burning question is whether and how a C3 convertase functions in the intracellular space. The intracellular cleavage of C3 and generation of a C3a-like molecule are now clearly evidenced,<sup>4,6,88–91</sup> and its biological relevance for cellular metabolism and functioning is undeniable, but the mechanism remains poorly understood. Experimental evidence suggests that this cleavage is made by cathepsin L in lysosomes<sup>4</sup> or by intracellular FB.<sup>89</sup> An intracellular C3bBb complex cleaving C5 was even detected in macrophages.<sup>92</sup> Whether a genuine C3/C5 convertase, similar to the extracellular convertase, exists intracellularly needs further investigation, as it would require fully folded intracellular C3 and FB. Describing the

molecular mechanisms of the generation of intracellular C3a and C5a will be a major breakthrough in the coming years.

#### 4 | TERMINAL PATHWAY OF COMPLEMENT—LYTIC CELL DEATH AS A FIRST LINE OF DEFENSE

Sustained C3b deposition at the activator surface results in a switch of substrate specificity of the convertases from C3 to C5. It is not yet known whether the C5 convertases form a trimeric complex by binding of a C3b molecule to the C3 convertases C4b2b and C3bBb or whether the additional C3b molecule at the surface is needed to prime C5 for cleavage by C4b2b/C3bBb.<sup>93,94</sup> However, recent data suggest that the conformational activation of C5 (or C5 priming) could be a mechanistic explanation for ongoing terminal pathway activity in the presence of C5 inhibitors.<sup>95</sup> The understanding



**FIGURE 5** Different opsonins can trigger specific effector functions. Each circle encloses the effector functions of the represented opsonins, and through which receptors they are implemented. Cell-type-specific effector functions were omitted for clarity, and they are presented in tables at the end of each section describing the corresponding receptor.

of the molecular mechanism and structural organization of the C5 convertases is a major challenge to be fully resolved in the upcoming years. The C5 convertases are denoted C4b2bC3b and C3bBbC3b, and they cleave C5 to release the anaphylatoxin C5a and the larger C5b, which is very unstable and can only associate with the membrane if it immediately binds to C6.<sup>96,97</sup> The resulting C5b6 complex can then bind successively to C7, C8, and multiple copies of C9. C8 starts membrane penetration, followed by C9, and forms a pore, named the membrane attack complex (MAC), leading to cell lysis.<sup>98,99</sup> However, MAC also has other nonlytic, proinflammatory functions, reviewed in ref. [100]. The steps from C5 cleavage to MAC assembly are known as the terminal pathway of the complement system.

## 5 | REGULATION OF C3 ACTIVATION

The activation of C3 is dangerous for host cells; therefore, C3 activation is tightly controlled by multiple regulators, part of the regulators of complement activation (RCA) protein family, which for C3 are complement receptor 1 (CR1), factor H (FH), factor H-like 1 (FHL1), decay-accelerating factor (DAF), and membrane cofactor protein (MCP).<sup>101</sup> These proteins are characterized by a shared structural unit called a complement control protein (CCP) domain (alternatively called SUSHI or short consensus repeat domain). The repetition of this domain forms the structure of these proteins. The RCA family members that bind to C3b for its degradation are FH, FHL1, CR1, and MCP.<sup>102</sup> FH is the most important regulator of the AP, and its detailed description is presented in another contribution to this series. The RCAs, except DAF, act as cofactors of the protease factor I (FI), in mediating C3b inactivation. Two cleavages by FI in the CUB domain of C3 release the fragment C3f and leave the iC3b fragment bound to the surface. A third and final cleavage releases the C3c fragment and leaves the C3dg fragment bound to the surface, which is further cleaved by proteases to yield C3d (Figure 4).<sup>103,104</sup>

While part of the binding site of FH and MCP is on the TE domain of C3b, CR1 does not have an affinity for this domain. Hence, FH and MCP can only catalyze FI cleavage up to iC3b formation under physiological conditions, as the third cleavage site is less available for FI cleavage, being between the TE and CUB domains, which are held together by the cofactor MCP or FH. When CR1 is the cofactor for

FI, the protease can proceed to the generation of opsonin C3d.<sup>104</sup> CR1 and FH also exert decay acceleration activity, a property shared with membrane-bound DAF, by binding the convertase and accelerating the decay of the AP C3 convertase. Mutations in *CFH*, *CFI*, and *C3* are risk factors for the genetic disease atypical hemolytic uremic syndrome (aHUS), and they are involved in the pathogenesis of a group of renal diseases called C3 glomerulopathies (C3G).<sup>105–108</sup> Autoantibodies against C3 are frequent in patients with lupus nephritis and correlate with disease activity.<sup>109–111</sup> The C3 nephritic factors are autoantibodies recognizing the C3 convertase, but not unbound C3 or C3b, found in C3G, and they impair the decay acceleration activity of the regulators.<sup>112,113</sup> The localization for their binding sites is unknown, but it involves neopeptides formed in the context of the AP C3 and C5 convertases complex.

Detection of the C3 activation fragments in *ex vivo* models of complement activation and in tissues is important for clinical practice and for understanding disease pathogenesis. Analysis of C3 activation fragments and C5b-9 on cultured endothelial cells incubated with serum from aHUS patients closely resembles the pathophysiological context of this disease.<sup>114</sup> It has shown elevated deposits from the sera of patients or model conditions of aHUS, malignant hypertension, elevated liver enzymes, low platelet syndrome (HELLP syndrome), sickle cell disease, preeclampsia, lupus nephritis, etc.<sup>109,115–118</sup> In one case, it was used to adjust the therapeutic regimen with a complement-blocking drug, as the test showed increased deposition of C5b-9 before the relapse of the disease, encouraging further studies.<sup>119</sup> To make specific diagnoses, tissue staining for C3 activation fragments is critical in clinical practice, especially on kidney biopsies. Anti-C3c (detecting C3b/iC3b but not C3d) immunostaining is performed as a routine work-up on kidney biopsies, which detect only ongoing complement activation. In lupus nephritis, C3c versus C3d (stains C3 and the activation fragments C3b, iC3b, and C3d) staining allows the differentiation between active and inactive lupus nephropathy.<sup>120</sup> Interestingly, rhabdomyolysis-induced acute kidney injury was long considered complement-independent because the anti-C3c staining on kidney biopsies was largely negative. A recent study indeed demonstrated negative anti-C3c but strongly positive anti-C3d,<sup>40</sup> which can be explained by the rapid cleavage of iC3b to C3c and then to C3d<sup>121</sup> because all biopsies were performed more than 24 hours after the initiation of rhabdomyolysis. This study

illustrates the importance of staining for C3d to understand the disease process when complement activation is expected, but the anti-C3c staining is negative, notably in chronic processes. Finally, tissue staining for C3c or C3d also detects local/intracellular C3 production in fixed and paraffin-embedded tissues.<sup>122</sup> This is clearly evidenced in renal cancer, where a fraction of the tumor cells produce C3 (and hence stain positive) and deposit C3 activation fragments on their surface due to complement activation.<sup>123</sup> The two patterns could be distinguished and quantified separately, as local production but not surface deposits correlate with poor prognosis in this type of cancer.<sup>124</sup>

Here, we have described the C3 cleavage fragments that elicit complement effector functions (Figure 5). In the following sections, we will explore these functions by detailing their interactions with complement receptors.

## 6 | THE C3A:C3AR AXIS: CONTEXT-DEPENDENT PRO- AND ANTI-INFLAMMATORY ACTIVITY, EXERTED IN THE CIRCULATION, TISSUE MICROENVIRONMENT, AND THE INTRACELLULAR SPACE

The only bioactive fluid phase fragment generated by C3 cleavage is anaphylatoxin C3a. C3a binding to its receptor (C3aR) elicits a response at the activation site involving histamine release from mast cells, smooth muscle contraction, augmented vascular permeability, and mast cell chemokine secretion<sup>125</sup> (Table 1). The biological activity of C3a is regulated by carboxypeptidase-N, which cleaves off the C-terminal arginine residue and generates the inactive molecule C3a-desArg.<sup>126,127</sup> C3aR is a transmembrane G-protein-coupled receptor distributed in peripheral tissue and the central nervous system and is expressed by all leukocytes.<sup>128</sup> Recently, a mouse atlas of C3aR1 mRNA levels in several organs has allowed a precise mapping of the expressing cells and tissues and a comparison with known human data.<sup>129,130</sup> Interestingly, C3aR was found to be stored intracellularly in eosinophils and macrophages, as reported for CD4<sup>+</sup> T cells, suggesting that it may function intracellularly in several contexts.<sup>4</sup> Indeed, a recent study showed that it

can downregulate mitochondrial metabolism in oxidatively stressed epithelial cells.<sup>131</sup> C3a has been reported to have bactericidal activity exerted by binding to the membrane and inducing cell lysis.<sup>132</sup> With increasing evidence over the years, research has pointed to a dual role of C3a depending on the context, and C3a is currently described as a modulator of inflammation.<sup>133</sup> In particular, C3a can act as an anti-inflammatory molecule on neutrophils, attenuating their mobilization from the bone marrow to the circulation after ischemia-reperfusion injury,<sup>134</sup> and it decreases the death rate in sepsis in mice.<sup>135</sup> It mediates the beneficial C3 function of tissue regeneration after liver injury (see Section 2.1) by stimulating cell proliferation, and it contributes to hematopoietic stem cell retention in the bone marrow, homing, and engraftment.<sup>136-138</sup> In the brain, the C3a:C3aR axis has an important role in the recovery after the acute phase of ischemic injury, modulating neurogenesis and axonal and synaptic plasticity.<sup>139</sup> In chronic inflammation, such as autoimmune disease, C3a acts as a proinflammatory mediator. High levels of C3aR expression are found in the glomeruli of lupus nephritis patients.<sup>140</sup> In cancer, C3a can have protumoral effects impacting immune system activation at the tumor site and favoring tumor progression. The C3a:C3aR interaction maintains an immunosuppressive environment in sarcoma and promotes tumor progression by skewing the phenotype of tumor-associated macrophages,<sup>69</sup> while in lung cancer, C3a:C3aR signaling acts on CD4<sup>+</sup> T cells and induces an inhibitory phenotype.<sup>141</sup> In pancreatic ductal adenocarcinoma, C3a:C3aR activates the extracellular-regulated kinase pathway, inducing epithelial-to-mesenchymal transition.<sup>142</sup> Hence, C3a has pleiotropic effects depending on the specific context in which it engages its receptor C3aR. Therapeutic intervention to inhibit or recover its activity should be carefully targeted to the desired location and time point.

## 7 | RECEPTORS BEARING COMPLEMENT REGULATORY FUNCTIONS: CR1, MCP, DAF, AND CRIG

### 7.1 | C3b interaction with CR1

C3b is tightly regulated by CR1, which is capable of dissociating C3/C5 convertases and serving as a cofactor for FI to degrade C3b into

TABLE 1 Cells expressing C3aR and the known functions triggered by C3a binding

| Expressing cell type     | Function  | References |
|--------------------------|---|------------|
| Mast cells               | Histamine release   | [125]      |
| Eosinophils, macrophages | Unknown, intracellular?   | [129,130]  |
| CD4 <sup>+</sup> T cells | Important for homeostasis, induction of inhibitory phenotype in lung cancer | [4,141]    |
| Epithelial cells         | Metabolism downregulation   | [131]      |
| Neutrophils              | Attenuates mobilization   | [134]      |
| Hematopoietic stem cells | Retention in the bone marrow, homing, engraftment                           | [136-138]  |
| Astrocytes, neurons      | Modulates neurogenesis, and axonal and synaptic plasticity                  | [139]      |



iC3b and C3d. CR1 is a membrane-bound complement receptor and a member of the RCA family. Similar to the other members, CR1 has a modular domain architecture formed by 30 CCP domains, a transmembrane domain, and a cytoplasmic tail.<sup>143</sup> The C3b binding interactions are mediated by two regions, each composed of three CCP domains, CCP8-10 and CCP15-17.<sup>144-146</sup> CR1 is one of the cofactors binding to C3b and aiding in FI degradation. CR1 also has decay-accelerating activity, exerted by binding to C3b in the C3 convertase and by dislodging the bound FB (Figure 2, right). CR1 is expressed on many different cell types, namely, monocytes, granulocytes, B and some T lymphocytes, kidney podocytes, and particularly cells of the circulatory system<sup>147</sup> (Table 2). CR1 expressed on erythrocytes represents 90% of the total circulating CR1.<sup>148</sup> In addition to its regulatory role in C3b convertase formation, CR1 is also important for the fixation of ICs and their removal from circulation by transporting them to the liver and spleen.<sup>149</sup> Due to its primary role in IC clearance, CR1 is an important receptor for the maintenance of homeostasis, and CR1 (and CR2; see Section 8.1) is abnormally expressed on B cells of lupus patients.<sup>150</sup> The role of CR1 in Alzheimer's pathogenesis is not yet clear. Studies have reported that impaired C3b regulation by CR1 due to overexpression of a subfunctional isoform is correlated with an increased risk of Alzheimer's disease development<sup>151,152</sup>; however, a recent review highlights that an isoform with the most active sites might be responsible for the increased disease risk.<sup>153</sup>

A secondary function of CR1, triggered by high C3b density, is the induction of IL-1 production and secretion of IL-1 $\beta$  on monocytes.<sup>154</sup> C3b binding to CR1 has been reported to modulate adaptive immunity, particularly by inhibiting B-cell differentiation and T-cell proliferation.<sup>155-159</sup> Finally, CR1 also facilitates human T-cell infection with C3 fragment-opsonized HIV by generating C3 fragments through its cofactor activity, which can in turn interact with CR2.<sup>160</sup> Due to its multiple functions and implications in disease, CR1-based therapeutics are being developed to control C3 deposition on tissues and complement activation in the circulation; one example is CSL040. CSL040 is a soluble version of CR1 truncated at residue 1392, and it is promising for preventing complement-mediated kidney damage.<sup>161,162</sup> Another CR1-based inhibitor consists of a monoclonal antibody targeting C3d covalently linked to CR1 CCP1-10 domains (C3d mAb-CR1<sub>1-10</sub>). CR1 CCP1-10 are important for decay-accelerating activity exerted on the CP and AP convertases. This inhibitory fusion protein could allow targeting the complement inhibitory activity of CR1 CCP1-10 to sites of ongoing complement activation, avoiding systemic complement inhibition and its side effects.<sup>163</sup>

## 7.2 | The multiple functions elicited by C3b binding to MCP

C3b can also be degraded by FI in the presence of membrane cofactor protein (MCP or CD46) as a cofactor. MCP is composed of 4 CCP domains, 1-3 serine/threonine-rich domains, a transmembrane domain, and a cytoplasmic tail,<sup>164,165</sup> and it is a member of the RCA protein family. MCP is expressed on all cells exposed to the circulatory

complement system, except erythrocytes (Table 3); MCP binds to C3b deposited by the AP on host cells and promotes its inactivation by FI.<sup>166,167</sup> In addition to its regulatory function of C3 deposits, MCP has an important signaling role in CD4<sup>+</sup> T cells.<sup>4</sup> Extracellular activation of C3aR and MCP by C3a and C3b, respectively, promotes effector functions, in agreement with the impaired Th1 responses in MCP- and C3-deficient patients. Moreover, the MCP cytoplasmic tail enables Treg cells to remain in an anti-inflammatory state. MCP in CD8<sup>+</sup> T cells transmits important signals for fatty acid metabolism for optimal effector functions.<sup>168</sup> In both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, MCP plays a role in cell metabolism, although through different signaling events.<sup>169</sup> CD8<sup>+</sup> T cells regulate amino acid influx and fatty acid metabolism, while CD4<sup>+</sup> T cells tune amino acid influx, oxidative phosphorylation, and glycolysis. Loss of function mutations on the MCP gene leads to the development of aHUS,<sup>170,171</sup> and elevated serum levels of soluble MCP have been reported in one study for SLE patients,<sup>172</sup> indicating how the lost ability to regulate complement by C3b binding at the host cell surface is detrimental for the host.

## 7.3 | Decay-accelerating factor of C3 and C5 convertases

The C3 and C5 convertases can be dissociated by a decay-accelerating factor (DAF or CD55). DAF is a GPI-anchored receptor part of the RCA protein family. The ectodomain is composed of four CCP domains arranged in a rod-like fashion.<sup>173</sup> It is expressed by all blood cells and cells in contact with blood and tissue fluid (Table 4). It exerts its function by binding to C3b in the C3 and C5 convertases, and it induces the decay of the complexes without acting as a cofactor for FI activity. The DAF inhibitory activity of the generation of C3a and C5a fragments by complement convertases is crucial in the response to vascular injury, preventing excessive leukocyte accumulation and thickening of the vascular lining by neovascularization.<sup>174</sup> In addition to its homeostatic regulatory functions, preventing undesired complement activation, DAF is a negative modulator of T-cell immunity, inhibiting the costimulatory activity exerted by the AP through inhibition of products of AP activation on antigen-presenting cells and on T cells.<sup>175</sup> New research found that DAF expression is downregulated during germinal center (GC) formation. In this context, augmented C3a:C3aR signaling is necessary for sustaining GC formation; concomitantly, the MAC inhibitor CD59 is upregulated so that B cells are protected from lysis by the terminal pathway of complement.<sup>176</sup> Paralleling its role in normal physiology, DAF is involved in the rare genetic disease PNH, where a mutation in the *PIGA* gene leads to loss of the GPI anchor, and DAF can no longer protect host cells from complement attack.<sup>177</sup>

## 7.4 | C3b and iC3b interact with the CRIg receptor on tissue-resident macrophages

The early complement opsonin C3b has a pivotal role in silent phagocytosis, and the key receptor for this process is the

TABLE 2 Cells expressing CR1 and the known functions triggered by C3b binding

| Expressing cell type | Function  | References |
|----------------------|---|------------|
| Erythrocytes         | Immune complex clearance  | [149]      |
| Monocytes            | Promotes IL1 $\beta$ secretion  | [154]      |
| B cells              | Inhibition of differentiation   | [156–158]  |
| T cells              | Inhibition of proliferation, infection with C3 fragment opsonized HIV | [159,160]  |

TABLE 3 Cells expressing MCP and the known functions triggered by C3b binding

| Expressing cell type     | Function  | References |
|--------------------------|---|------------|
| Nucleated cells          | Regulation of AP activation   | [166,167]  |
| CD4 <sup>+</sup> T cells | Promotes effector functions, restrains autoimmunity, Regulates metabolism: glycolysis | [4]        |
| CD8 <sup>+</sup> T cells | Regulates metabolism: fatty acid metabolism   | [168]      |

TABLE 4 Cells expressing DAF and the known functions triggered by C3b binding

| Expressing cell type                                   | Function   | References |
|--|--|------------|
| Blood cells and cells in contact with blood and tissue | Decay acceleration of C3 and C5 convertases          | [174]      |
| T cells  | Inhibitory   | [175]      |
| Germinal center B cells                                | Unwanted decay acceleration of C3 and C5 convertases | [176]      |

TABLE 5 Cells expressing CR1g and the known functions triggered by C3b and iC3b binding

| Expressing cell type | Function   | References |
|----------------------|--|------------|
| Kupffer cells        | Phagocytosis   | [178]      |
| T cells              | Restrains autoimmunity, may be mediated by C3b binding             | [182]      |
| Macrophages          | Inhibits proinflammatory phenotype, may be mediated by C3b binding | [183]      |

complement receptor of the immunoglobulin superfamily (CR1g or V-set and Ig domain-containing 4 [VSIg4]). The N-terminal C3b/iC3b-binding domain of CR1g belongs to the IgV family of immunoglobulin-like domains,<sup>178,179</sup> and the binding site is located between the MG3 and MG4 domains of C3b/iC3b.<sup>180</sup> As predicted by the location of its binding site and its structure, CR1g does not act as a cofactor for FI-mediated C3b degradation; however, it acts as a potent inhibitor of the AP C3 convertase by preventing the C3b molecule in the convertase from binding to the C3 substrate molecule (Figure 3). CR1g is expressed by tissue-resident macrophages, in particular Kupffer cells in the liver, and other macrophages in the digestive organs, as well as by neutrophils and DCs (Table 5). Subsets of these macrophages also express C3 and/or C1q, but the functional link is unknown.<sup>181</sup> CR1g participates in the phagocytosis of microbes, but it is also important to restrain T-cell responses, and it is upregulated in mouse autoimmune tissue.<sup>182</sup> Its expression also inhibits macrophage activation by

reprogramming mitochondrial pyruvate metabolism and inhibiting reactive oxygen species secretion.<sup>183</sup> The mechanisms for these processes have not yet been elucidated, and whether they are mediated by C3b binding is unknown. The binding of complement fragments triggers the recycling of CR1g on endosomes, depleting the inhibitory activity of the receptor at the surface and promoting inflammation. However, silent phagocytosis not only of pathogens but also of cell debris is important for host homeostasis, as it ensures continuous removal of potential complement activators from circulation.<sup>178</sup> Thus, CR1g is another way in which the innate immune system contributes to host homeostasis and fine-tunes adaptive immune responses. The binding site on C3b/iC3b of CR1g between MG3 and MG4 is shared by the inhibitory nanobody hC3Nb2.<sup>184,185</sup> The nanobody blocks all complement pathways at the C3 cleavage event, indicating how natural receptor binding sites are an inspiration for complement-targeting molecules and potential therapeutics.

## 8 | RECEPTORS MEDIATING COMPLEMENT EFFECTOR FUNCTIONS, WITHOUT COMPLEMENT REGULATORY ACTIVITY: CR2, CR3, AND CR4

### 8.1 | iC3b and C3d interaction with CR2

iC3b and C3d regulate immune cell activation via interaction with complement receptor 2 (CR2, CD21). CR2 has a modular architecture similar to that of CR1; however, it possesses 15 or 16 CCP domains. CR2 mediates the effector functions of iC3b and C3d on B cells, follicular dendritic cells in lymphoid organs, and epithelial cells (Table 6), and the binding site for C3d is at the first two CCP domains.<sup>186-188</sup> In mice, CR1 and CR2 are products of the same gene (*Cr2*), and alternative splicing leads to the shorter form of the receptor CR2. In humans, they are encoded by separate genes (*CR1* and *CR2*), giving rise to two different proteins with distinct functions.<sup>189</sup> While in mice CR2 acts as a coreceptor for the B-cell receptor, amplifying the B-cell response, in human B cells, the costimulatory effect of CR2 is much less pronounced. In mice, the opsonized antigen binds to the B-cell receptor, while the bound C3d simultaneously binds to CR2 expressed on the same B cell. This coligation lowers the threshold for B-cell activation by 10 000-fold.<sup>190</sup> These functions have been extensively studied in mice; however, in humans, C3d-mediated co-engagement of CR2 with the B-cell receptor has an inhibitory effect. In particular, it suppresses the expression of the activation marker CD69, IL-6 secretion, proliferation, and antibody production of B cells.<sup>191</sup> Another important function of CR2 is mediating antigen transport to follicular dendritic cells and antigen presentation during GC formation and the generation of high-affinity antibodies.<sup>192,193</sup> CP activation by IgG clustering leads to the decoration of the antigen with iC3b molecules and the formation of ICs. These molecules interact with CR3 (see Section 8.2) on the surface of subcapsular sinus macrophages, which carry ICs to the lymph nodes. Here, the ICs are relayed to B cells through CR2 interaction with C3d and are transported to follicular dendritic cells, where the antigen is retained through CR2:iC3b interaction during GC formation and B-cell affinity maturation.<sup>194</sup> Decreased CR2 expression leads to the development of SLE and rheumatoid arthritis.<sup>195,196</sup> In the context of lupus, CR2 binds to IFN $\alpha$  with the same CCP1-2 domains used for the iC3b, C3d interaction,<sup>197</sup> and this could be involved in lupus pathogenesis since the receptor would no longer be able to bind opsonized ICs and carry out its function.

In addition to its function in IC transport, C3d has a novel role in anti-tumor immunity, mediated by its interaction with CR2, as it

amplifies anti-tumor T-cell responses. In fact, C3d deposition on irradiated tumor cells, or its recombinant expression inside tumor cells, resulted in the recruitment of CD8<sup>+</sup> cytotoxic T cells, depletion of Tregs, and suppression of T-cell PD-1 expression in a mouse model.<sup>198</sup> Recent research has demonstrated that the C3d:CR2 interaction plays a role in marginal zone B cells (MZ B cells), which can acquire dendritic cell (DC) functions. Namely, CR2 on MZ B cells interacts with C3d deposited by tick-over of C3 on MHC II molecules on the DC surface, and MZ B cells acquire the antigen-presenting ability by trogocytosis.<sup>199</sup> This cooperation between DCs and MZ B cells allows a broader antigen repertoire presentation to CD4<sup>+</sup> T cells. From this discussion on the findings related to the CR2 functions, it emerges that careful consideration has to be given to results obtained only in mouse models, and the findings should be reproduced in human-derived material.

### 8.2 | iC3b and C3d trigger phagocytosis by interacting with their integrin-like receptors CR3 and CR4

The late opsonins iC3b and C3d have the specialized functions of inducing phagocytosis and clearance of targets through interactions with complement receptors 3 and 4 (CR3, CD11b/CD18, or  $\alpha_M\beta_2$  and CR4, CD11c/CD18, or  $\alpha_X\beta_2$ ). CR3 and CR4 belong to the  $\beta_2$  family of integrins and hence to a structurally different family of receptors. Integrins are heterodimeric adhesion molecules formed by  $\alpha$  and  $\beta$  subunits. They are organized into an extracellular ligand-binding region, a transmembrane domain, and a cytoplasmic tail. Ligand binding dependent on the chelation of an Mg<sup>2+</sup> ion triggers a conformational change that propagates a mechanical signal through the actin cytoskeleton.<sup>200,201</sup> In CR3 and CR4, the  $\alpha$  subunit is an alpha-I domain, which has the peculiarity of increased flexibility, allowing binding to more rigid and less accessible ligands on cell surfaces.<sup>202</sup> CR3 and CR4 are expressed on all leukocytes (Table 7), and their structures allow them to carry out specific functions. Generally, CR3 binds preferentially to positively charged ligands, while CR4 binds preferentially to negatively charged ligands.<sup>203</sup> iC3b is the main ligand for CR3 and CR4; however, the two receptors target different domains.<sup>204-207</sup> CR3 can bind to MG1-MG2, MG6-MG7, and the TE domain, while available evidence suggests that CR4 binds iC3b at the MG3-MG4 interface. The interaction of iC3b with CR3 on macrophages and granulocytes promotes the phagocytosis of ICs.<sup>208</sup> Moreover, CR3 is important for the intracellular degradation of the phagocytized target.<sup>209</sup> An

| Expressing cell type                   | Function   | References |
|--|--|------------|
| B cells and follicular dendritic cells | Inhibits B cell activation, proliferation, and antibody production in humans, sustains germinal center formation | [190-193]  |
| Tumor cells                            | Reactivates the immune response to tumor   | [198]      |
| Marginal zone B cells                  | MZ B cells acquire DC functions  | [199]      |

TABLE 6 Cells expressing CR2 and the known functions triggered by C3dg binding

TABLE 7 Cells expressing CR3 and CR4 and the known functions triggered by iC3b and C3dg binding

| Interaction        | Expressing cell type   | Function   | References   |
|--------------------|--|--|--------------|
| iC3b:CR3           | Macrophages and granulocytes                                 | Phagocytosis, degradation of phagocytized target     | [208,209]    |
| iC3b:CR4           | Monocytes, monocytes derived macrophages and dendritic cells | Adhesion   | [212,213]    |
| iC3b:CR3, iC3b:CR4 | Leukocytes   | Extravasation and migration to the inflammatory site | [214]        |
| iC3b:CR3           | Microglia  | Phagocytosis   | [20,210,211] |
| iC3b:CR3           | T cells  | Inhibition of T cell proliferation                   | [217]        |
| C3dg:CR3           | Immune cells in the tumor microenvironment                   | Activation of immune responses                       | [218]        |
| iC3b:CR3           | Immune cells in the tumor microenvironment                   | Inhibition of immune responses                       | [218]        |

established function of complement and the phagocytic receptor CR3 is at play during brain development and in neurodegenerative diseases. First, in the developing brain, complement contributes to the sculpting of synaptic circuits. Weak synapses, opsonized by iC3b, are eliminated through interaction with CR3 on the surface of microglia, the brain-resident macrophages.<sup>20,210</sup> Second, the iC3b:CR3 receptor interaction is important for the removal of amyloid plaques in brains affected by Alzheimer's disease.<sup>27</sup> Recent findings contributed to further differentiation of the functions of CR3 and CR4. While it was confirmed that the iC3b:CR3 interaction is prevalent for the phagocytic response, the iC3b:CR4 interaction is dominant in the adhesion of monocytes, monocyte-derived macrophages, and monocyte-derived dendritic cells to fibrinogen on podosomes, thus facilitating cell migration.<sup>211,212</sup> Notably, CR3 and CR4 deficiency results in life-threatening recurrent infections, as these receptors are important for leukocyte extravasation and migration to the inflammatory site.<sup>213</sup>

The iC3b:CR3 interaction contributes to both pathogen removal and maintenance of immune tolerance,<sup>214</sup> as apoptotic cells are also opsonized by iC3b. Their uptake by marginal zone dendritic cells is mediated in part via CR3 and to a lesser extent by CR4, and it causes decreased secretion of proinflammatory cytokines without any effect on the anti-inflammatory cytokines, thus contributing to the maintenance of peripheral T-cell tolerance.<sup>215</sup> In vitro studies suggest that the iC3b interaction with CR3 inhibits T-cell proliferation and IL-2 release, in line with the hypothesis of a complement-mediated regulatory function of the iC3b:CR3 interaction on T cells.<sup>216</sup> In the eye, the iC3b:CR3 interaction is key for the development of antigen-specific tolerance. CR3 stimulation of antigen-presenting cells results in the sequential production of transforming growth factor- $\beta$ 2 and interleukin-10, the latter being essential for the induction of tolerance.<sup>217</sup> At the same time, low-grade complement activation generates enough C3b to sustain the defense against pathogens in this immune-privileged site.

### 8.3 | iC3b and C3d as regulators of the immune response and promoters of immune tolerance

The interplay between the opsonins iC3b and C3d with CR2, CR3, and CR4 is key in the balance between triggering the immune

response and maintaining immune tolerance. Despite the progress in understanding this process described above, the exact mechanisms at play are still not fully understood and are likely context and organ-dependent. It has been suggested that iC3b and C3d may have opposite functions in immune regulation in the tumor microenvironment, as they can engage CR3 in two receptor conformations: upon iC3b binding, CR3 adopts the extended *trans* conformation, while it binds C3d in the closed *cis* conformation.<sup>218</sup> In this configuration, binding to C3d would occur on the surface of CR3-expressing cells, activating immune cells. iC3b binding would instead take place on a neighboring cell, extending up to 150 Å distance, thereby inhibiting immune responses. Competition between these two responses is actively taking place in the tumor microenvironment at the synapse between tumor and immune cells. Targeted delivery of C3d to the surface of tumor cells could boost the immune activation induced by C3d binding to CR3.<sup>218</sup> Moreover, C3d associated with tumor cells, or even free C3d, recruits, accelerates, and amplifies anti-tumor T-cell responses, allowing reactivation of immunity, up to the point of preventing tumor growth in mouse models.<sup>198</sup> C3d stimulates the increase in tumor-infiltrating CD8<sup>+</sup> T cells by depleting Tregs and suppressing the T-cell expression of PD-1 through its interaction with CR2. Therefore, modulating the iC3b/C3d balance in tissue could either help maintain tolerance or stimulate immune responses, a process that, if disrupted, could result in autoimmunity, which is now being explored in anti-cancer therapy.

## 9 | CONCLUSION

Herein, we have illustrated the central role of complement C3 in the realization of complement effector functions. In particular, C3 can be seen as a basket that is opened upon complement activation, releasing the multitasking mediators of complement functions. Early opsonin C3b contributes to further complement activation and phagocytosis, while anaphylatoxin C3a and the late opsonins iC3b and C3d have multiple immune-related functions. We have outlined, with structural explanations, how the different fragments carry out specific functions, mainly driven by three synergistic events: (a) release of a bioactive fragment in the fluid phase, (b) conformational changes brought by complement

activation that reveal new binding sites and add flexibility, and (c) the exposed binding sites bear differential specificity for the receptors belonging to the structural families of G-protein-coupled receptors, RCA, immunoglobulin superfamily, and  $\beta_2$  integrins, thus enabling a multitude of unrelated functions. Furthermore, the expression patterns of the receptors on different cell types add a layer of complexity to the differentiation of the responses triggered by the binding of the C3 fragments.

We can now understand how C3 activation has elegantly regulated context-dependent functions and how a tip of this balance is a hallmark of many diseases. Thanks to the precise knowledge of C3-dependent effector functions, an open avenue is available for the development of C3-targeting therapeutics tailored to the specific disease context.

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## CONFLICT OF INTEREST

The authors do not declare any conflict of interest to this review.

## DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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