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Application of the C3 inhibitor compstatin in a human whole blood model designed for complement research – 20 years of experience and future perspectives

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ABSTRACT

The complex molecular and cellular biological systems that maintain host homeostasis undergo continuous crosstalk. Complement, a component of innate immunity, is one such system. Initially regarded as a system to protect the host from infection, complement has more recently been shown to have numerous other functions, including involvement in embryonic development, tissue modeling, and repair. Furthermore, the complement system plays a major role in the pathophysiology of many diseases. Through interactions with other plasma cascades, including hemostasis, complement activation leads to the broad host-protective response known as thromboinflammation. Most complement research has been limited to reductionistic models of purified components and cells and their interactions in vitro. However, to study the pathophysiology of complement-driven diseases, including the interaction between the complement system and other inflammatory systems, holistic models demonstrating only minimal interference with complement activity are needed. Here we describe two such models; whole blood anticoagulated with either the thrombin inhibitor lepirudin or the fibrin polymerization peptide blocker GPRP, both of which retain complement activity and preserve the ability of complement to be mutually reactive with other inflammatory systems. For instance, to examine the relative roles of C3 and C5 in complement activation, it is possible to compare the effects of the C3 inhibitor compstatin effects to those of inhibitors of C5 and C5aR1. We also discuss how complement is activated by both pathogen-associated molecular patterns, inducing infectious inflammation caused by organisms such as Gram-negative and Gram-positive bacteria, and by sterile damage-associated molecular patterns, including cholesterol crystals and artificial materials used in clinical medicine. When C3 is inhibited, it is important to determine the mechanism by which inflammation is attenuated, i.e., whether the attenuation derives directly from C3 activation products or via downstream activation of C5, since the mechanism involved may determine the appropriate choice of inhibitor under various conditions. With some exceptions, most inflammatory responses are dependent on C5 and C5aR1; one exception is venous air embolism, in which air bubbles enter the blood circulation and trigger a mainly C3-dependent thromboembolism, with the formation of an active C3 convertase, without a corresponding C5 activation. Under such conditions, an inhibitor of C3 is needed to attenuate the inflammation. Our holistic blood models will be useful for further studies of the inhibition of any complement target, not just C3 or C5. The focus here will be on targeting the critical complement component, activation product, or receptor that is important for the pathophysiology in a variety of disease conditions.

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1. Introduction

Methods in complement research range from genetic studies, protein structural characterization, and biomarker quantification to functional analyses of single components, individual pathways, and the whole system [1]. Research in recent years has revealed that complement has biological functions far beyond the protection of the host against microbes. Complement is a player in an orchestra of systems that contribute to homeostasis and engages in mutual crosstalk with a huge number of molecular and cellular systems [2]. This interplay challenges the methodological repertoire available for studying the role of complement in complex physiological and pathophysiological conditions. Animal models, particular various knockout and knock-in strains of mice, have proven useful for studying the complement system. However, mice are not man [3], and complex *ex vivo* human models to study the role of complement are still highly desirable.

2. A human whole blood model for complement research

Complement can be measured in serum or plasma derived from whole blood. Complement activity is typically studied in serum, whereas the measurement of activation products formed in vivo requires EDTA plasma to block further in vitro activation during sampling and processing. However, neither serum nor EDTA blood can be used to study *ex vivo* activation of complement and its interaction with other blood components. An anticoagulant is needed when working with fresh whole blood as a model system. Calcium chelators such as EDTA and citrate cannot be used because they partially or completely inhibit complement activation. Heparin allosterically activates antithrombin, interferes with several coagulation factors, and has effects on platelets and leukocytes. In low concentrations it can enhance complement activation, and at higher concentrations it is an effective complement inhibitor [4]. Heparin should not be used as an anticoagulant in complement models because of its potential influence on the system; however, there is a "window" in the range of 1–2 IU heparin/mL within which complement is neither activated nor inhibited [4]. Thus, we have instead investigated the effect of the highly specific thrombin inhibitor lepirudin (recombinant hirudin) as an anticoagulant to study the role of complement in the inflammatory reactions in human whole blood and have found that it has no influence on the complement system [4].

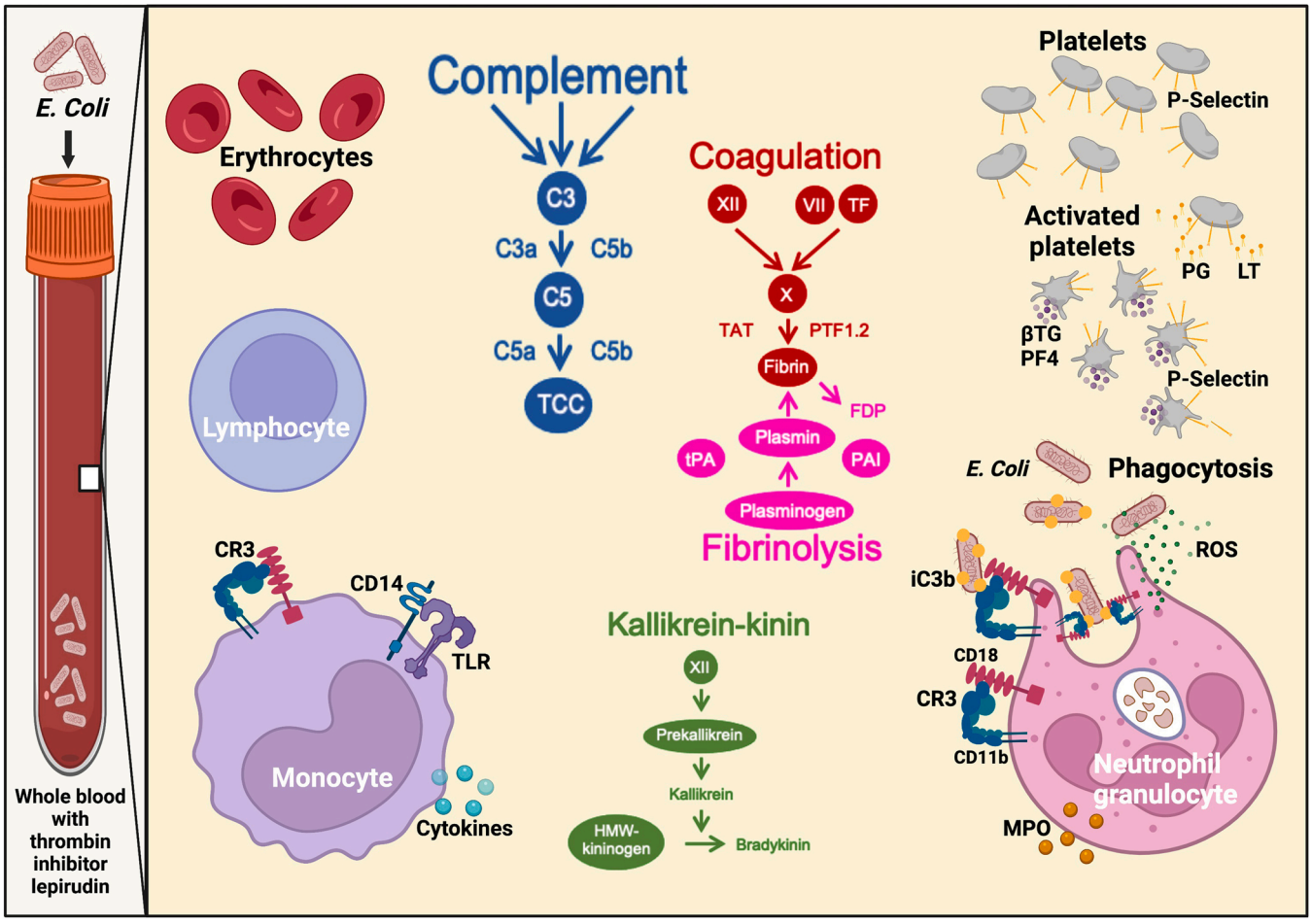

Fig. 1. The lepirudin-based human whole blood model. The whole blood model is based on the highly specific anticoagulant lepirudin, which does not interfere with complement activation or any other thrombin-independent reactions (for details, see Chapter 2.1 in the text). Whole blood is drawn directly into vacuum tubes containing lepirudin. The experiment starts immediately to avoid background activation. Here, the model for incubation with *E. coli* bacteria is illustrated. Any possible activator of complement can be studied in this model. Readouts include complement activation, activation of the other plasma cascades, and activation of platelets and leukocytes. The role of complement in inducing the inflammatory reaction can be studied in detail by adding different complement inhibitors to the system and evaluating their role on the various thromboinflammatory mediators released by the activator. The crosstalk of the whole blood inflammatory network can also be investigated in this model. Abbreviations: BTG, β-thromboglobulin; CR3, complement rector 3; FDP, fibrin degradation products; LT, leukotrienes; MPO, myeloperoxidase; PG, prostaglandins; PF4, platelet factor 4; ROS, reactive oxygen species; TAT, thrombin-antithrombin; PTF1.2, prothrombin fragment 1.2; TLR, Toll-like receptor; tPA, tissue plasminogen activator; PAI, plasminogen activator inhibitor.

2.1. The lepirudin-based model specifically inhibits thrombin

The model has been described in detail previously [4]. The principle of the model is to draw blood from a donor directly into a vacuum tube containing lepirudin (Fig. 1). Lepirudin over a wide concentration range has no effect on complement activation. The experiment should start immediately after the blood is drawn to keep the background activation as low as possible and thereby increase the signal-to-noise ratio when the blood is incubated with an activator. To correct for the background activation occurring in blood that has not been incubated with a specific activator, a separate background control sample must be incubated under the same conditions as the samples, typically on a tube roller at 37 °C. The optimal time for analysis of complement activation is approximately 30 min. The experiments may last for up to 6 h without any significant changes occurring in either blood gases or metabolism. Incubations of 2–4 h are optimal for analyzing inflammatory markers that are not pre-formed and therefore require gene transcription, including many cytokines.

2.1.1. A holistic versus reductionistic approach to studying biological systems

Throughout history, most complement studies have been performed using a reductionistic approach, i.e., studying complex systems by reducing them to simpler components. Typically, the various components are purified, and the interaction between the components is studied in isolation from the other proteins and cells normally present. From a mechanistic view, such studies are essential but only examine a fraction of the system; that is, they yield only limited insights. Our aim was instead to study complement using a holistic approach, i.e., an approach based on the idea that the whole is more than the sum of its parts. Examination of the interaction of complex systems, such as the role of complement in the inflammatory reaction, requires a holistic model.

2.1.2. Advantages of the model

The main advantage of the whole blood model is that it enables us to study the interaction of complement with other biological systems and cells, since they are mutually able to interact in this system (Fig. 1). We can pinpoint where in the cascade complement activation occurs by targeting the various complement proteins or receptors by using highly specific inhibitors, and then measure the multiple mediators released from the cascade. This approach may aid the identification of suitable inhibitors for specific therapeutic applications. The converse is also true: by inhibiting other systems such as coagulation or key cytokines, we can examine their influence on complement activation, thus allowing us to answer the question of what comes first – the chicken or the egg. After years of experience with this model, we have found that, most frequently, complement and another central pattern recognition system of innate immunity, the Toll-like receptors (TLR), are together the initial triggers of a broad inflammatory response.

2.1.3. Application of the model to study crosstalk in complex biological systems

Taking advantage of the opportunity for inflammatory crosstalk in the whole blood model, we initiated studies involving combined inhibition of complement and the TLRs. The very first observation of the synergistic effect of a combined inhibition of these systems was documented by Brekke et al. [5]. Here we found that combined inhibition of the potent complement C5a fragment and the CD14 molecule, which is part of the TLR system and serves as a cofactor for several TLRs, including TLR4 and TLR2, completely abolishes *E. coli*-induced leukocyte activation. These effects could not be obtained by using the single inhibitors alone. Further studies confirmed that a combination of upstream inhibition of complement at the level of C2 and factor D with inhibition of CD14 by using the mAb r18D11 [6] abolishes the cytokine storm in the same model [7]. Extensive investigation of whole blood from a C5-deficient individual examined without and with C5 reconstitution, together with inhibition of C3 by compstatin and of CD14 by r18D11 has enabled us to dissect the relative role of these molecules in a global inflammatory reaction [8]. Finally, a gene array study in the same model has shown that > 2000 genes are regulated by *E. coli* incubation in whole blood, and the regulation of 70% of these genes can be reversed with a mean 80% fold-change by a combined inhibition of C3 using compstatin and CD14 using the mAb r18D11 [9].

2.1.4. Limitations and strengths of the model

The main limitation of the lepirudin anticoagulated whole blood model is the associated blocking of thrombin, the penultimate step of the coagulation cascade, which therefore precludes the study of thrombin's crosstalk with complement and its function as an amplification loop in the coagulation system. However, because anticoagulation is a prerequisite for the model, this is an unavoidable limitation, which we have taken into account in all the studies we have performed using this model.

In addition to revealing several thrombin-independent inflammatory responses, the lepirudin model has also helped us determine what thrombin can do in the whole blood system, which is indeed an advantage of this model. After developing a novel second model, in which the four-aminoacid Gly-Pro-Arg-Pro (GPRP) inhibits blood clotting (see item 2.2 below) but thrombin activity is maintained, [10] we were able to study the effects of thrombin in whole blood by comparing the results we obtained using the lepirudin and GPRP models. Employing the holistic GPRP model, we were able to confirm the important conclusion that thrombin does not cleave C5, as had been postulated in several papers [11–13] on the basis of C5 studies performed in reductionistic models. Unfortunately, reductionistic models do not reflect the complete physiology of the blood. Thus, we found that thrombin cannot cleave C5 under physiological conditions; but only after C5 has changed conformation by processes such as purification or plasma acidification [10].

2.2. The GPRP-based model specifically inhibits fibrin polymerization

The desire to study the impact of thrombin in complement activation and acute inflammation in human whole blood led us to employ our second model discussed here, the fibrin polymerization-inhibiting peptide GPRP. Synthetic GPRP, first described by Laudano and Doolittle in 1978 [14], corresponds to the Gly-Pro-Arg A-knob sequence of the fibrinogen α-chain, which is exposed after thrombin-mediated cleavage and release of fibrinopeptides A and B [15]. GPRP acts as a competitive inhibitor and prevents the knob-to-hole interaction of fibrin monomers, thereby blocking fibrin's polymerization into protofibrils [16]. We found that GPRP at 8 mg/mL completely blocks fibrin polymerization in whole blood and thus prevented clotting for as much as eight hours [10].

2.2.1. Model advantages and limitations

A unique feature of the GPRP model is that this inhibitor targets the coagulation cascade at the very last step of fibrin polymerization; thus, every upstream component, including thrombin, is left intact to interact with its natural substrate(s) and receptor(s). In contrast, in the lepirudin-based model, thrombin is immediately inhibited once prothrombin is cleaved [17]. When whole blood is added to tubes containing GPRP, surface contact between the blood and the tube wall instantly triggers coagulation [18], regardless of whether the blood collection was performed into tubes made of polypropylene, generally considered relatively inert with regard to thrombin generation, or into tubes made of a less- inert material such as glass [19]. Small amounts of thrombin immediately exert positive feedback on the activation of upstream coagulation factors V, VIII, and XI [19,20] and activate the thrombin-dependent protease-activated receptors (PAR)− 1 and − 4 on platelets [21,22]. Platelet activation then amplifies the thrombin generation by releasing coagulation factors [23], including factor V and phosphatidylserine exposure, ensuring that the factor Xa-Va

prothrombinase complex is assembled on activated platelets for further prothrombin cleavage [24]. We have found that prothrombin is fully consumed within 20 min after addition of whole blood to polypropylene tubes containing only GPRP [10]. This consumption of prothrombin is associated with elevated thrombin-antithrombin complexes and complete release of platelet β-thromboglobulin [10]. As compared to uncoagulated blood, free thrombin levels are even higher in GPRP-anticoagulated blood [25] because of the lack of a fibrin clot, which traps free thrombin [26]. The massive thrombotic response in GPRP-anticoagulated blood is an advantage of the model, but it can also be a limitation under certain circumstances. In this model the thrombotic response is spontaneously initiated and uncontrolled but can be counteracted by supplementing GPRP with a low level of citrate (final concentration, 0.7% [w/v]) (*H.Q.Quach* et al., *Front Immunol, In press*). Supplementation with citrate prevents the activation of thrombin and platelet activation during blood collection but can later be counterbalanced by adding 6.25 mM $CaCl_2$ at the start of an experiment.

2.2.2. Thromboinflammation – Crosstalk between the complement and hemostatic systems

Thromboinflammation connects the highly integrated hemostatic and inflammatory systems in its propagation of thrombotic and inflammatory manifestations. A number of plasma proteins have pleiotropic effects on thromboinflammatory events that involve blood cell interactions. Thrombin and thrombin-dependent activation of platelets are central hubs in thrombosis and inflammatory events. The major advantage of the GPRP model is that it allows the study of inflammation under pro-thrombotic conditions. We have found no direct effect of thrombin on complement activation markers at the C3 or C5 level [10] in human whole blood. However, thrombin can cleave complement C5 if the C5 has first undergone a conformational change induced by stress [10]. Platelets undergo a dramatic phenotypic change upon activation that is linked to an increased interaction with complement proteins. Platelet activation has been shown to mediate fluid-phase complement activation [27]; complement activation on the surface of platelets is less definite. Many complement proteins are found on the surface of activated platelets, but a tight complement regulation accomplished through efficient recruitment of fluid-phase complement regulators such as factor H and C4b-binding protein [27,28] tends to prevent the activation of that complement. Nevertheless, studies have reported the occurrence of both classical and alternative pathway-dependent complement activation [29,30]. Platelets bind C3(H_2O) to their surfaces upon activation [28]. The C3(H_2O) then serves as a ligand for complement receptors 1 [28] and 3 [31], promoting the formation of platelet-leukocyte aggregates that may also involve properdin [32]. Thrombin acts as a major determinant of the release of inflammatory cytokines during the acute inflammatory response to bacterial stimulation (*H.Q.Quach* et al., *Front Immunol, In press*) via direct thrombin-mediated activation of PAR on monocytes [33] and platelets [34] as well as indirect platelet-dependent activation of monocytes via granule-stored mediators [35] and platelet-leukocyte aggregates [36]. Inhibition of C5aR1 largely attenuates the expression of inflammatory cytokines in GPRP-anticoagulated blood, pointing to complement C3 and C5 as suitable targets for therapeutics to reduce acute inflammation [37].

2.3. Extending the model to include endothelial cells

Vascular endothelial cells in vivo maintain a sophisticated level of anti-thrombotic and anti-inflammatory control, sensing and responding to pathogenic stress or trauma. Combining whole blood with endothelial cells in experimental models introduces a further dimension in thromboinflammation. When human whole blood is incubated with endothelial cells, the impact of complement activation fragments, cytokines, other inflammatory mediators, and thrombin on the vascular endothelial cells can be studied. A variety of models combining whole blood with endothelial cells have been described, including endothelial cells under static and flow conditions, on flat-bedded solid and transpermeable surfaces, and in round-shaped vessels [38]. Also, models in which the endothelial cells are cultured on beads have been described [39]. In general, endothelial cells cultured under flow conditions are preferable, although shear stress induces a morphology accommodation that resembles the in vivo phenotype, with elongated cells aligned with the direction of flow [38,40] and abundant tight junction-formation [41]. The flow rate is a major determinant of endothelial cell-mediated uptake of nanoparticles, with nonspecific binding and uptake of nanoparticles being prevented under flow conditions but not static conditions [41]. In contrast, nanoparticles specifically targeted to bind endothelial cell fucose can be taken up under both static and flow conditions. Even if the flow-based models are more physiologically relevant, endothelial cells cultured under static conditions have the advantage that they permit a higher throughput evaluation of multiple conditions at one time. Endothelial cell cultures under static conditions can also be combined with a transpermeable membrane to allow the assessment of transendothelial leakage.

2.3.1. Interactions between whole blood and endothelial cells

The endothelium is a gatekeeper, exerting tight control over plasma and cell extravasation. Endothelial cells express pattern recognition receptors for the direct recognition of pathogens and alarmins, and they sense the presence of inflammatory mediators, including TNF, IL-1β [42], thrombin [43], complement C4a [44], C5a [45] and bradykinin [46]. We have shown that endothelial cells in vitro can strongly respond to bacterial incubation with *Staphylococcus aureus (S. aureus)* in whole blood, but not in plasma [47]. The underlying mechanism here is a *S. aureus*-dependent complement activation leading to the generation of C5a that produces leukocyte activation and release of TNF and IL-1β, which activate the endothelial cells. Endothelial cell activation can be prevented by targeting either C5aR1 alone or TNF and IL-1β in combination [47].

3. The compstatin family of C3 inhibitors

The compstatin family consists of highly selective peptidic C3 inhibitors that represent a novel class of complement inhibitors with a distinctive mechanism of action, acting on native C3 to prevent its activation. Discovered in 1996, the original compstatin is a cyclic peptide that blocks the convertase-mediated activation of C3 by all three complement pathways [48]. The molecular and structure-guided characterization, lead optimization, preclinical evolution, and clinical development of the compstatin family of C3 inhibitors have been extensively reviewed in [49–53]. In addition to inhibiting all three initial pathways at the C3 level, compstatin also inhibits the alternative pathway-mediated amplification of the complement response and prevents the downstream formation of complement effectors (i.e., C3a, C5a, C5b-9/MAC).

3.1. Structure and function

Structural investigations have corroborated earlier biochemical studies, revealing that compstatin binds to a shallow pocket formed by the macroglobulin ring of the β-chain of C3/C3b and C3c from human and non-human primates exclusively. The binding of the compstatins occurs between the MG4 and MG5 domains of C3 and selectively blocks convertase-mediated cleavage of C3 in any of the three initiation pathways (Fig. 2) [52,54]. The binding region of compstatin appears to be critical for the binding of native C3 to the convertase [55]. It should be noted that compstatin and its analogs neither prevent direct cleavage of C3 by proteases such as thrombin nor the initial activation of the alternative pathway through spontaneous hydrolysis of C3 into C3 (H_2O); also, deposition of C4b via the upstream activation of the classical and lectin pathway remains intact [49]. Therefore, compstatin

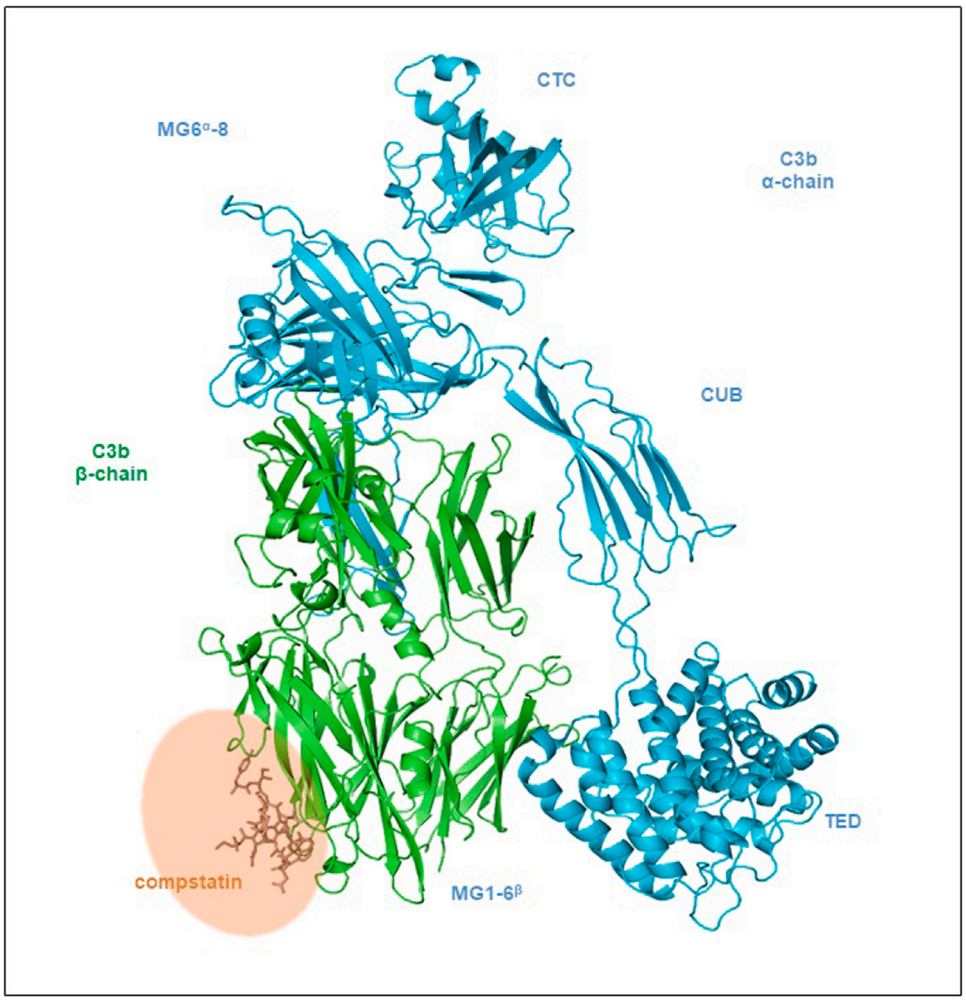

Fig. 2. *Structure of the C3b-Cp40 complex at 2-Å resolution.* In green: Chain B. In blue: Chain A. In brown: Cp40. Adapted from C Lamers et al., 2022 [52], with permission from CellPress.

treatment may still allow residual opsonic activity in serum and complement-mediated immune surveillance for pathogens. Since its discovery in 1996, compstatin and its various analogs have been extensively studied in terms of their physicochemical properties; binding and inhibitory mode; in vitro, *ex vivo,* and in vivo inhibitory potency; anti-inflammatory activity; and therapeutic efficacy. More than 150 publications reported in the scientific literature have endorsed the compstatin technology.

3.2. Development of compstatin to a high-affinity C3 inhibitor

The newly approved compstatin-based C3 inhibitor, Empaveli (pegcetacoplan/APL-2) is a PEGylated C3 therapeutic comprising two copies of the second-generation compstatin analog APL-1/POT-4 joined by a 40-kDa PEG spacer added to improve plasma residence [49,53]. The pharmacologically active moiety of this drug is the second-generation compstatin analog 4(1MeW)7 W (Cp05), which was discovered through targeted peptide modification and backbone N-methylation of the compstatin scaffold [52,53,56]. The clinical approval of Empaveli marks a milestone in the history of complement therapeutics that has changed the course of complement therapeutic discovery, validating in the clinical setting both the safety and therapeutic potential of proximal complement inhibition. Almost 15 years after the approval of the first complement-specific drug for paroxysmal nocturnal hemoglobinuria (PNH), compstatins finally entered the clinic. This major development in the complement drug space broadens the spectrum of available complement therapeutics, offering patients with unmet clinical needs or insufficient response to C5 inhibition therapy an alternative treatment option with a broad activity profile. A brief historical account of the discovery and clinical evolution of the compstatin family of C3-targeted complement inhibitors, emphasizing the most promising drug candidates advancing in priority indications, can be found in [52] and [53].

The currently most advanced clinical candidate in the compstatin family of C3 inhibitors is AMY-101. AMY-101, referred to as Cp40 in the scientific literature, is a third-generation, non-PEGylated C3-targeted inhibitor from the compstatin family [52,53]. AMY-101 is currently being developed by Amyndas Pharmaceuticals as a local or systemic treatment for diverse clinical indications associated with deregulated C3 activation, including COVID-associated ARDS, periodontal inflammation and bone loss, ischemic stroke, cancer immunotherapy, hemodialysis-induced thromboinflammation, and ABOi-organ transplantation [49]. AMY-101 has shown clinical efficacy in ameliorating clinical indices of gingival inflammation and reducing markers of periodontal tissue destruction in a Phase IIa study in adults with gingivitis [57]. AMY-101 features a unique set of pharmacologic properties and PK/PD improvements over earlier compstatin analogs, including a 6,000-fold greater binding affinity for its target C3 than the original compstatin, enhanced inhibitory potency, and a prolonged circulatory half-life of > 60 h, which exceeds the typical plasma residence of most peptide-based drugs [49]. AMY-101 was the first compstatin-based C3 inhibitor to receive orphan designation by the FDA/EMA for the treatment of paroxysmal nocturnal hemoglobinuria and C3 glomerulopathy

[49]. AMY-101 has provided proof of concept for the therapeutic efficacy of C3 inhibition in several preclinical disease models, including polytrauma-induced hemorrhagic shock [58], an RPE-based genetic model of age-related macular degeneration [59], malaria-induced inflammation [60], autoimmune hemolytic anemia, and xenotransplantation, as reviewed in Mastellos et al. [49].

Notably, fourth-generation compstatin analogs have been developed through targeted peptide modifications, including the conjugation of small-sized PEG moieties to the N-terminus of the Cp40 analog or the extension of its C-terminus with a charged tail of 2–3 residues of lysine [61]. These new analogs display enhanced solubility at physiological pH, increased binding affinity for C3b, and improved pharmacokinetic profiles with a prolonged intraocular residence after intravitreal administration in non-human primates [62]. These new compstatin analogs are expected to broaden the spectrum of administration routes, likely reducing dosing frequency during chronic treatment and offering a more tailored solution for therapeutic C3 modulation in retinal inflammatory diseases in which C3 dysregulation plays a pathogenic role.

4. Application of compstatin to the whole blood model

4.1. Initial experiments in an ex vivo perfusion model of xenotransplantation

Several years before developing the lepirudin model, we performed *ex vivo* perfusion of pig kidneys using human whole blood (AB blood group) in a clinically used oxygenator and pump system [63]. In this model, heparinized blood was used; thus, the system was not optimal when compared to lepirudin, but within the window of heparin concentrations used, there was no effect on the complement system. The addition of C1-inhibitor markedly reduced the inflammatory response and substantially prolonged the kidney survival, whereas the albumin control group kidneys demonstrated hyperacute rejection within minutes [64]. C1-inhibitor is often in the list of complement inhibitors, but while it controls classical pathway activation, it is not an efficient therapeutic complement inhibitor. On the other hand, it very efficiently inhibits the kallikrein-kinin system, preventing the release of bradykinin. It also has regulatory roles in the coagulation and fibrinolytic systems. Thus, to investigate in a specific manner to what extent the complement system was responsible for the favorable effects we saw with C1-inhibitor, we used compstatin to specifically inhibit C3 in our xenotransplantation model [65]. Compstatin completely blocked C3 and C5 activation and abolished the activation of leukocytes and platelets. As compared to the controls, which underwent immediate hyperacute rejection, i.e. anuria and cessation of blood blow, the compstatin-treated kidneys survived the 6 h of observation with normal urine production and blood circulation, confirming that complement activation was responsible for the hyperacute rejection.

4.2. Compstatin as a complement inhibitor in the lepirudin-based whole blood model

Establishing a lepirudin-anticoagulated blood model enabled us to safely study the complement system without any direct influence from the anticoagulant. With this system we used a number of specific complement inhibitors, including anti-C2 (classical and lectin pathway), anti-factor D (alternative pathway), compstatin (blocking C3, the first bottleneck molecule where all initial pathways merge), eculizumab and RA101295 (blocking C5 cleavage), anti-C5a clone 137–26 (C5a inhibitor; binding to the C5a moiety of the whole C5 molecule, but without preventing its cleavage), and PMX53 (a selective C5aR1 antagonist). This panel of inhibitors enabled us to study the mechanism of complement activation and the role of the various molecules in inducing the secondary inflammatory response, including cytokines, reactive oxygen species, arachidonic acid metabolites, platelet and leukocyte activation, as well as opsonisation and phagocytosis (Fig. 1). This model can easily be used to study any potential activator of complement. We have focused on non-sterile inflammation, including the inflammatory response to Gram-negative bacteria (*E. coli* and *Neisseria meningitidis (N. meningitidis)*) and Gram-positive bacteria (*S. aureus*), as well as sterile inflammation induced by artificial surfaces, cholesterol crystals, and air bubbles, as described in detail below.

4.3. Dissecting the relative roles of C3, C5, the C5a-C5aR1 axis, and CD14 in various inflammatory conditions

4.3.1. Infectious inflammation induced by Gram-negative and Gram-positive bacteria

We initially studied the effect of selective and combined inhibition of complement and the TLR co-receptor CD14 on *E. coli*-induced leukocyte activation and cytokine release [4]. Compstatin and selective inhibition of C5, C5a, and C5aR1 efficiently reduced the *E. coli*-induced oxidative burst in granulocytes and monocytes. Complement inhibition by compstatin alone reduced only interleukin (IL)− 8 release and had no major effect on cytokines IL-6 and TNF release. In comparison, blockage of CD14 using $F(ab`)_2$ fragments of the anti-CD14 antibody 18D11 efficiently reduced IL-6, IL-8, IL-10, and TNF release, indicating a crucial role for the TLR co-receptor CD14. The combined inhibition of complement C5a and CD14 efficiently blocked the *E. coli*-induced CD11b (CR3) up-regulation on leukocytes [66]. Later, Egge and coworkers showed that the addition of compstatin and anti-CD14 with a delay of up to 30 min post-challenge, significantly reduces the *E. coli*-induced inflammation [67]. Notably, the combined inhibition of complement and CD14 was more efficient in attenuating bacterially induced inflammation than was complement inhibition with the specific TLR-4-MD2 complex antagonist Eritoran [68]. We have developed a humanized chimeric anti-CD14 $IgG_{2/4}$ hybrid antibody (r18D11) that efficiently blocks CD14 without adverse effector activity and could potentially be used in therapy [69]. It appears to be possible to delay the combined inhibition application to within a narrow therapeutic window after the onset of the bacteria-induced inflammation. These findings indicate that combined upstream inhibition of complement and CD14 has the potential to efficiently inhibit the inflammatory responses induced by bacteria and other complement activators.

Knock-out models are generally accepted as an effective means of generating data to provide experimental proof of concepts. We therefore used blood from a genetically C5-deficient patient (i.e., a human "C5 knock-out") to confirm the results from experiments using reconstitution experiments with C5 and specific complement inhibitors. The *E. coli*-induced upregulation of monocyte tissue factor (TF) surface expression was highly C5-dependent in experiments performed using blood from the C5-deficient patient [8]. Combined inhibition using compstatin and anti-CD14 efficiently blocked *E. coli*-induced TF surface expression on monocytes, TF activity on microparticles, and coagulation [70]. Compstatin alone efficiently reduced the *E. coli*-induced TF mRNA, while anti-CD14 had no effect. Anti-CD14 alone modestly reduced the *E. coli*-induced TF surface expression on whole blood monocytes, whereas inhibition combining anti-CD14 with either compstatin or C5aR1 inhibition, almost completely blocked TF expression [70,71]. Anti-CD14 efficiently reduced the LPS-induced TF, but compstatin had only minor effects [71]. Furthermore, selective or combined inhibition of complement C3, C5, and CD14 revealed that a range of *E. coli*-induced responses are either highly C5-dependent or highly dependent on CD14 or complement C3. Selective inhibition with compstatin showed that the *E. coli*-induced release of lactoferrin, elastase, and myeloperoxidase enzymes is highly dependent on complement C3 [8]. The beneficial effect of compstatin on *E. coli*-induced TF release, coagulopathy, thrombocytopenia, and organ damage was confirmed in an in vivo study in baboons by Silasi-Mansat et al. [72]. Thus, early complement inhibition may prevent *E. coli*-induced thromboinflammation and organ damage in sepsis.

Both *E. coli* and *N. meningitidis* are protected from phagocytosis in

leukocytes through binding to complement receptor 1 (CR1) on erythrocytes in whole blood [73]. Most bacteria added to human whole blood in vitro are initially bound to erythrocytes, and this binding can be efficiently blocked by a neutralizing anti-CR1 mAb [73]. Compstatin efficiently reduces the phagocytosis of bacteria in human whole blood, and moderately reduces the binding of *E. coli* and *N. meningitidis* to erythrocytes [73]. Compstatin efficiently reduces the complement C3b opsonisation on the *E. coli* surface, but increases the C4b opsonization, indicating that C4b may be an alternative ligand for binding bacteria to CR1 on human erythrocytes in the presence of compstatin. In another study of the involvement of complement C3 in the binding of properdin, compstatin efficiently reduced the C3b opsonisation and binding of properdin to *E. coli, N. meningitidis,* and zymosan in human serum [74]. These results indicate that the binding of properdin to bacteria is not a primary event but rather secondary to C3b opsonization. Similarly, compstatin efficiently reduces the binding of properdin to *Aspergillus fumigatus* in serum, indicating that the C3b opsonization occurs first [75]. However, when soluble collectin-12 first binds to *Aspergillus fumigatus*, properdin binds in a C3-independent fashion. This result indicates that although compstatin efficiently reduces C3b opsonization, C4b opsonization may increase, and other opsonins such as soluble collectin-12 can then bind in a C3 independent manner.

Gram-positive bacteria such as *S. aureus* are a common cause of human sepsis. Three different *S. aureus* strains have been shown to efficiently enhance the levels of many inflammatory biomarkers, including cytokines, in the lepirudin-based model of inflammation [76]. Combined inhibition by compstatin and anti-CD14 efficiently reduces most of the *S. aureus*-induced biomarkers, whereas compstatin and anti-CD14 alone are both less efficient. However, most inflammatory biomarkers induced by *S. aureus* are dependent on the C5a-C5aR1 axis. Interestingly, *S. aureus* also efficiently enhances the TF surface expression on whole blood monocytes as well as TF on microparticles and coagulation [77]. The *S. aureus*-induced TF upregulation on monocytes and coagulopathy are most efficiently reduced by the combined inhibition of C5 or C5aR1 with CD14 or TLR-2, whereas compstatin alone is less efficient. The results indicate that the *S. aureus*-induced thromboinflammation shows a highly complement-dependence that is mediated via the C5a-C5R1 axis. Combined upstream inhibition of complement and anti-CD14 may have beneficial effects in *S. aureus* sepsis if treatment is rapidly initiated.

4.3.2. Sterile inflammation induced by cholesterol crystals, alginate, and artificial surfaces

The role of complement C3 and its compstatin-mediated inhibition in sterile inflammation have been examined in several studies utilizing the lepirudin-based human whole blood model. Cholesterol crystals (CC) are typically found in atheromatous plaques, and low-grade inflammation is a common finding in atherosclerosis. The CC-induced activation of endothelial cells and up-regulation of adhesion molecules has been shown to be complement C3- and C5-dependent and mediated by the cytokine TNF [78]. Importantly, CC induce up-regulation of TF surface expression on human whole blood monocytes and coagulation activation, as indicated by increased PTF1.2 in plasma [79]. The CC-induced TF surface expression on monocytes and PTF1.2 increase can be blocked by compstatin, eculizumab, or C5aR1 inhibition. Interestingly, complement activation, CC, and TF have also been detected in a human intracranial brain thrombus from a patient with advanced carotid atherosclerosis [79]. Thus, compstatin may have beneficial effects on CC-induced inflammation and coagulation in atherosclerosis, although the effect may occur mainly via inhibition of downstream C5 activation.

Alginates are salts of alginic acid extracted from seaweed that are candidates for use in alginate microbeads in future cell-encapsulation therapies [80]. However, the host inflammatory responses, including complement activation and cytokine release, that occur in response to the surface of the transplanted alginate microcapsules remain a major challenge. Different alginate microspheres activate the complement system and cytokine release in human whole blood to varying degrees, but the calcium/barium alginate microbeads have a low activation potential [80]. Compstatin efficiently abolishes the C3 deposition on complement-activating microspheres as well as leukocyte adhesion and cytokine release [80,81]. Furthermore, compstatin efficiently reduces the TF mRNA expression and coagulation induced by the alginate poly-L-lysine APA and AP microcapsules [82]. The results of these experiments indicate that compstatin efficiently inhibits alginate microsphere-induced complement activation and cytokine release. Compstatin also inhibits TF and coagulation induction by some alginate microcapsules [82]. These findings indicate that compstatin may be effective in preventing these inflammatory reactions in future cell-based therapies employing alginate microspheres.

Many artificial surfaces in contact with blood activate complement and induce sterile inflammation. The effect of compstatin has been examined in several models, including plastic surface-induced activation of complement and release of eicosanoids. Compstatin reduces the polyvinyl chloride tubing-induced release of the eicosanoid leukotriene B4, but not the release of thromboxane B_2 and prostaglandin E_2 in human whole blood [83]. However, compstatin can efficiently reduce most of the polyvinyl chloride-induced release of cytokines, chemokines, and growth factors [84]. Similarly, compstatin and C5 inhibition with eculizumab have been shown to efficiently reduce iron oxide nanoparticle-induced cytokine release in human blood [85]. Sensory implants such as glucose sensors need to be biocompatible, but they are covered with various materials or membranes that may activate complement [86]. Compstatin efficiently reduces the leukocyte activation induced by three of these types of membranes, indicating that C3 plays an important role in sterile inflammation induced by plastic devices, foreign surfaces, and certain membranes. As mentioned previously, the effects of C3 inhibition may be explained by inhibition of downstream C5. Thus, a comparison of C3 and C5 inhibition is crucial to discriminating the selective effects of C3 activation from the effects of downstream C5 activation.

5. Selective activation of C3 induced by air bubbles

5.1. Vascular air embolism

Vascular air embolism, a condition in which air bubbles enter the blood circulation, can occur as a complication of many surgical and medical procedures [87] as well as in scuba diving and high-altitude aviation [88]. It is estimated that iatrogenic vascular air embolism occurs in 0.03–1.3‰ of hospitalized patients [87]. During some high-risk procedures such as hysteroscopy, the incidence may be as high as 85% [89]. Vascular air embolism often causes transient and self-resolving symptoms such as decreased pulmonary gas exchange or a drop in blood pressure. However, it may also lead to hemodynamic collapse, organ infarction, or death [90,91]. Animal studies and human case reports have shown that air embolism triggers inflammation [90,92,93], and in vitro studies have demonstrated that air emboli activate the complement system through the alternative pathway via the hydrolysis of C3 [94,95]. To date, no specific treatment for air-induced thromboinflammation is in routine use.

5.2. Inflammatory response to air bubbles in the ex vivo human whole blood model

Ex vivo human studies, in vivo animal studies, and human case reports have shown that air emboli trigger a complex thromboinflammatory cascade involving both the complement system and leukocytes [92,96], platelets [97,98], and coagulation [94]. We have examined the effect of air emboli on thromboinflammation in the lepirudin anticoagulated whole blood model [99]. Consistent with the findings of Ekdahl et al. [94], we found that air emboli activate complement through the alternative pathway. We also made the important

and not previously described observation that C3 convertase (C3bBbP) formation occurs without concomitant C5 convertase ($C3b_2BbP$) formation (Fig. 3). Furthermore, air emboli trigger the release of 25 cytokines, including interleukins, chemokines, and growth factors, and activated platelets, and they also trigger coagulation [99]. It is noteworthy that not only air emboli but also ambient air often present in test tubes during in vitro incubations can activate complement, albeit to a lesser extent than do air emboli [95].

5.3. *Effect of complement inhibition on air bubble-induced inflammation*

In the aforementioned study [99], we examined the effects of inhibiting C3 with compstatin, C5 with eculizumab, C5aR1 with PMX53, and CD14 with r18D11. C3 blockade attenuated the activation of complement and coagulation as well as the cytokine response of 25 of the 27 measured cytokines. Combined C3 and CD14 inhibition further reduced some cytokines slightly. In contrast, C5 inhibition reduced only two cytokines, and inhibition of CD14 alone reduced only three cytokines, suggesting that the activation of leukocytes and other cytokine-producing cells during air embolism is primarily C3-driven. Neither C3 inhibitors nor C5 inhibitors reduced β-thromboglobulin, suggesting that platelets were activated through a non-complement-dependent mechanism. C3, C5, and C5aR1 inhibition reduced TF mRNA production and PTF1.2 equally, but only C3 inhibition reduced microparticle tissue factor, suggesting that coagulation is activated differentially through C3 and C5-C5aR1-dependent mechanisms. Our findings are in contrast to the mechanisms described for most other complement-driven conditions, in which the C5-C5aR1 axis plays a major role [100].

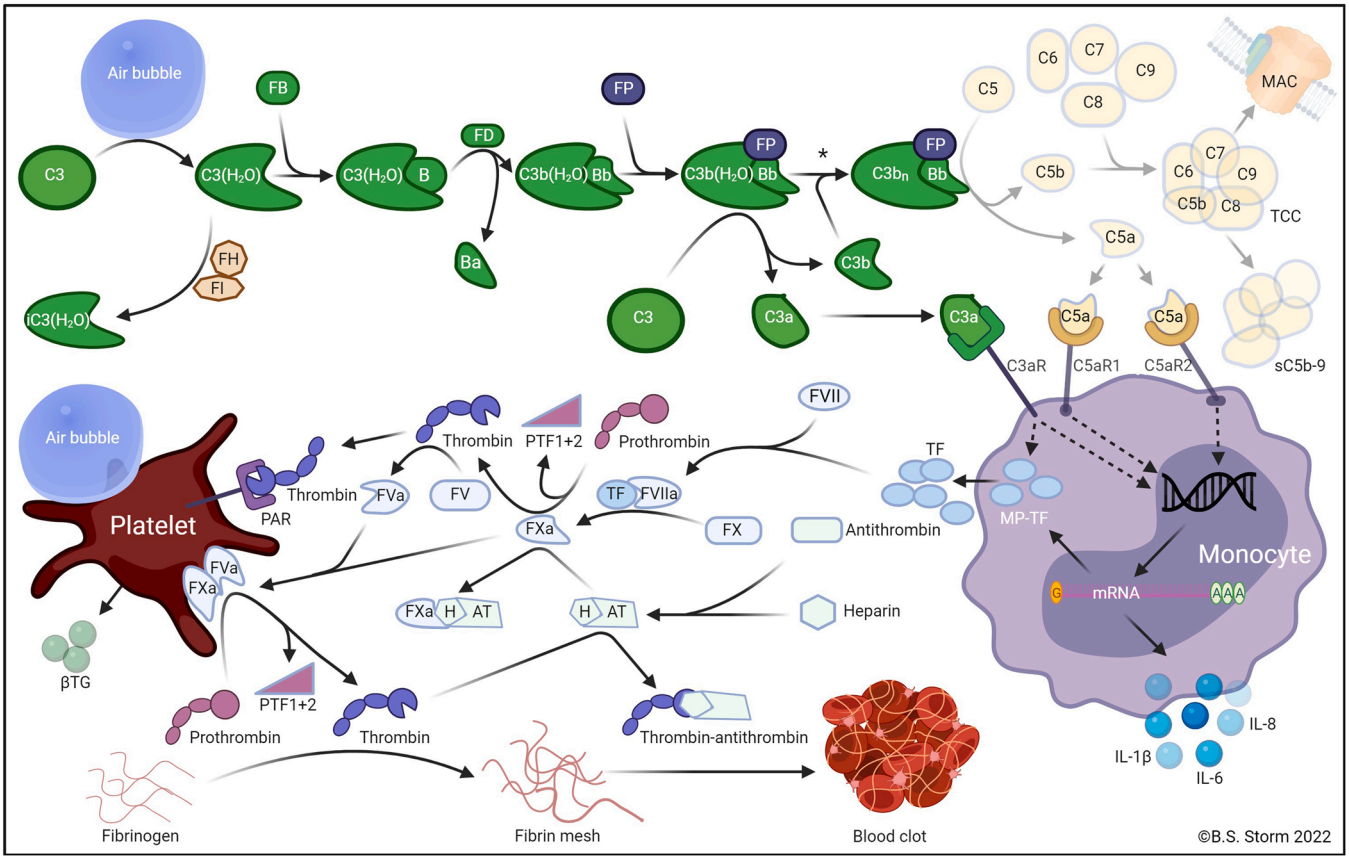

Fig. 3. Air emboli activate the alternative pathway and trigger a C3-dependent thromboinflammation. In plasma, C3 undergoes spontaneous slow-rate hydrolysis of the internal thioester to form $C3(H_2O)$. The C3 hydrolysis is accelerated by contact with foreign surfaces, e.g., an air embolus. $C3(H_2O)$ is inactivated to $iC3(H_2O)$ by factor I (FI) in the presence of a cofactor such as factor H (FH). $C3(H_2O)$ may bind FB (FB) to form $C3(H_2O)B$. Catalyzed by factor D (FD), the small Ba fragment is cleaved from $C3(H_2O)B$ to form the fluid-phase C3 convertase $C3(H_2O)Bb$. The C3 convertase is stabilized by properdin (FP) binding to form $C3b(H_2O)BbP$. The stabilized C3 convertase cleaves additional C3 molecules to C3b and C3a or forms the C5 convertase $C3b_nBbP$ by binding to one or more C3b fragments deposited on foreign surfaces. The C5 convertase then cleaves C5 into C5a and C5b. C5b combines with C6, C7, C8, and C9 to form C5b-9, the terminal complement complex (TCC). TCC may become anchored in cell or bacterial membranes, forming a pore termed the membrane attack complex (MAC), which can cause the lysis of sensitive cells. Alternatively, the TCC may form a soluble complex (sC5b-9) in plasma. The C3a and C5a anaphylatoxins bind receptors for C3a and C5a (C3aR and C5aR1 or C5aR2, respectively) on various cells, including monocytes and granulocytes. The activation of anaphylatoxin receptors on monocytes stimulates de novo synthesis and surface expression of tissue factor (TF), extracellular release of microparticles expressing TF (MP-TF), and various inflammatory cytokines, including IL-1β, IL-6, and IL-8. TF binds to coagulation factor VIIa (FVIIa), subsequently activating FX to FXa. FXa catalyzes the cleavage of prothrombin to PTF1 + 2 and thrombin. Thrombin then catalyzes the cleavage of FV to FVa. FVa and FXa combine to form the prothrombinase complex on the surface of activated platelets. The prothrombinase complex cleaves prothrombin into prothrombin fragment 1 + 2 (PTF1 +2) and thrombin. Platelets can be activated by several mechanisms, including thrombin binding to protease-activated receptors (PAR) 1 and 4, and possibly by direct contact with air emboli, whereby β-thromboglobulin (βTG) and many other mediators are released. Thrombin cleaves fibrinogen to fibrin, which crosslinks and forms a fibrin mesh leading to the formation of a blood clot. The affinity of antithrombin for thrombin is enhanced by binding heparin to form the heparin-antithrombin complex (HAT), which binds to and inactivates FXa.
Footnote: The asterisk (*) indicated between the two C3 convertases (upper right) indicates that air emboli-activated C3 is less likely to form an active C5 convertase than is C3 activated by solid substances when C3b is bound to the surface, and the C5a-C5aR axis thus plays only a minor role in air-induced C3-driven thromboinflammation. *Note*: only key components relevant to our model are included in the figure. The figure was created with BioRender.com.

5.3.1. Compstatin research reveals the major importance of C3 in the inflammatory response

The pivotal role of C3 in air-induced thromboinflammation provides the rationale for complement inhibition as a therapeutic approach in patients with vascular air embolism. It seems clear that the cytokine response is predominantly C3-driven, and neither C5 nor CD14 plays an important role. This is an interesting finding, given that most complement-driven diseases are perceived to be C5-driven and thus are treated using the C5 inhibitor eculizumab. However, treating the thromboinflammation resulting from air embolism with a C5 inhibitor would have only minimal or no effect. In this respect, the C3 inhibition obtained by using compstatin was extremely important for delineating this unique pathophysiological mechanism. If these findings transfer to in vivo conditions, compstatin (Cp40, AMY-101) or another specific C3 inhibitor should be used to reduce thromboinflammation in patients with air embolism. Finally, these findings underscore the importance of comparing C3 and C5 inhibition when trying to dissect the effect of C3 alone, as compared to the secondary effects of subsequent C5 activation.

6. Conclusions and future perspectives

In the present review, we have underscored the importance of working in holistic models when studying the role of complement in thromboinflammation and in the inflammatory response in general. Such models enable the complement system to be activated under experimental conditions, allowing crosstalk and mutual interaction with other molecular and cellular systems. Here we have described two whole blood models using either the thrombin-specific inhibitor lepirudin or the fibrin-polymerization-inhibiting peptide GPRP in fresh human whole blood to investigate inhibitors targeting different levels of the complement cascade. In particular, we have studied the inhibition of C3, the most potent inhibition of the complement system, using compstatin (Cp40, AMY-101) to block all three initial complement pathways at level of C3 conversion as well as the internal amplification loop in the alternative pathway, preventing further downstream activation. Such studies are pivotal for investigating the mechanism of various activators and pathophysiological conditions with respect to future therapeutic potential. It is crucial to differentiate conditions mediated by C3a activation itself from those mediated by downstream activation and the release of C5a and C5b-9. Using inhibitors of C5 cleavage and C5aR1 blockers, we have frequently found that the sole or main effect is mediated through the C5aR1. In these cases, using C5 inhibitors or C5aR1 blockers to preserve the upstream functions of complement, including opsonization and phagocytosis, may be desirable. However, we also describe venous air embolism, a condition that is mainly C3-driven and minimally accompanied by terminal pathway activation. Under such conditions, when C3 activation selectively induces the inflammation, C3 is the optimal target for inhibition. Thus, careful investigation of the basic mechanism of complement activation is crucial for the design of further studies in the field of therapeutic complement inhibition [100].

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Authorship

All authors have made substantial contributions to all of the following: (1) the conception and design of the study (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Conflict of interest

J.D.L. is the founder of Amyndas Pharmaceuticals, which is developing complement inhibitors for therapeutic purposes; is the inventor of patents or patent applications that describe the use of complement inhibitors for therapeutic purposes, some of which are being developed by Amyndas Pharmaceuticals; is the inventor of the compstatin technology licensed to Apellis Pharmaceuticals (Cp05/POT-4/APL-1 and PEGylated derivatives such as APL-2/pegcetacoplan and APL-9).

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