Clinical and Developmental Immunology

Complement in Human Disease

Guest Editors: Michael A. Flierl, Daniel Rittirsch, Markus S. Huber-Lang, and Philip F. Stahel

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Editorial Board

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Editorial

Complement in Human Disease

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As part of innate immunity, the complement system embodies the "first line of defense" to various initial insults, including trauma, infection, hemorrhage, ischemia, and autoimmunity. While of beneficial intention, excessive complement activation can inadvertently damage healthy host tissues and thereby exacerbate the initial pathological events by causing an "innocent bystander effect."

"Complementology" represents a rapidly evolving field. Recent scientific efforts have identified novel complement activation pathways and select complement-inhibiting compounds, which have been shown to ameliorate myriad autoimmune or inflammatory conditions. These findings have renewed the enthusiasm for novel pharmacological strategies targeting numerous inflammatory conditions, which currently elude successful transfer "from bench to bedside," such as acute lung injury, sepsis, burn, ischemia-reperfusion injury, traumatic brain injury, and kidney disease.

The abundant involvement of the complement system in human health and disease is reflected by the wide range of topics covered in this special issue of *Clinical and Developmental Immunology*.

B. Nilsson and N. Ekdahl provide a summarizing article on current indications, techniques, sampling, and interpretations for clinical complement analyses. In addition, an easy-to-follow algorithm is provided for routine laboratory operations.

G. Cazander et al. illustrate the success of targeted complement inhibition of complement in wound healing and demonstrate how revesal of complement hyperactivity using several complement inhibitors has resulted in novel and innovative wound care strategies, which may soon be subject of clinical trials.

K. J. Welsh et al. challenge recent evidence that complement plays a critical role in the host defense against *Mycobacterium tuberculosis*. Following aerosol challenge with *Mycobacterium tuberculosis*, the authors demonstrate that "C7-knockout" mice had markedly reduced liver colony forming units and lung occlusion in conjunction with significantly increased total lymphocytes, decreased macrophages, and increased numbers of CD4+ cells. In line, expression of lung IFN-γ and TNF-α was increased in these animals, underscoring a crucial role of C7 in the disease manifestation of *Mycobacterium tuberculosis*.

S. R. Stowell et al.'s manuscript reviews the role of complement in transfusion-related mortality. This is of particular interest, as excessive complement activation during transfusion represents one of the most common features associated with fatality. The treating physician is furthermore provided with several strategies aiming to immunomodulate the complement system during incompatible red blood cell transfusions.

In two reviews, F. Bu et al. and S. F. Heeringa and colleagues review complement involvement in renal disease. Bu reviews the pathogenetic role of genetic variations in complement genes and complement dysregulation at the cell surface results in different forms of atypical hemolytic uremic syndrome, and how this understanding may result in

novel complement-modulating treatment approaches. In S. F. Heeringa's paper, the pathogenesis of complement associated glomerulopathies is reviewed. Heeringa describes how defective complement control via the alternative pathway results in glomerular C3 deposition and how this understanding has generated a complement pan-blockade or plasma substitution as potential treatment strategies, which have been proven successful.

H. Nishiura et al. shed further light on the importance of the C5a receptor (C5aR) during erythropoiesis. Strikingly, expression and regulation of RP S19 oligomer, which binds to C5aR, may regulate C5aR function as a connector for the erythroblast-macrophage island or for erythroblast differentiation in the bone marrow.

D. Rittirsch et al. and J. Charchaflieh et al. review the highly elaborate immune response following trauma and sepsis, which is—at least in part—driven by excessive complement activation and may result in a deadly downward spiral of SIRS, sepsis, septic shock, and multiorgan failure. Recent clinical concepts, such as "damage control surgery," transition such findings into the clinical setting in an attempt to improve outcome of a highly vulnerable patient population.

These manuscripts represent an exciting and insightful snapshot of current complement research. State-of-the-art, existing challenges, and emerging future topics are highlighted in this special issue, which may inspire the reader and help advance the present complement research. We would like to thank all authors, reviewers, and the Editor-in-Chief for making this special issue in *Clinical and Developmental Immunology* possible.

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Review Article

Complement Activation and Inhibition in Wound Healing

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Complement activation is needed to restore tissue injury; however, inappropriate activation of complement, as seen in chronic wounds can cause cell death and enhance inflammation, thus contributing to further injury and impaired wound healing. Therefore, attenuation of complement activation by specific inhibitors is considered as an innovative wound care strategy. Currently, the effects of several complement inhibitors, for example, the C3 inhibitor compstatin and several C1 and C5 inhibitors, are under investigation in patients with complement-mediated diseases. Although (pre)clinical research into the effects of these complement inhibitors on wound healing is limited, available data indicate that reduction of complement activation can improve wound healing. Moreover, medicine may take advantage of safe and effective agents that are produced by various microorganisms, symbionts, for example, medicinal maggots, and plants to attenuate complement activation. To conclude, for the development of new wound care strategies, (pre)clinical studies into the roles of complement and the effects of application of complement inhibitors in wound healing are required.

1. Introduction

1.1. Wound Healing. Wound healing is often completed within two weeks after injury, although tissue remodeling may take several months up to two years. The process of wound healing consists of three, overlapping phases, that is, inflammation, tissue proliferation and tissue remodeling [1–3]. During the different phases, a complex series of sequential cellular and biochemical responses, which are described in some detail in Section 1.2, restores the injured tissue.

Chronic wounds occur in individuals having defects that either prevent the healing process or allow healing to continue without leading to a proper anatomical and functional result. Risk factors for the development of chronic wounds include vascular diseases, diabetes mellitus, pressure (necrosis), alcohol and nicotins abuse, and old age [2]. Current therapies for chronic wounds include debridement, reduction of bacterial load, pressure offloading, topical negative pressure, a variety of wound dressings, skin grafting, and reconstructive tissue flaps [4, 5]. However, the outcome of these therapies is unsatisfactory in up to 50% of chronic (present for one year) wounds [6], resulting in significant morbidity and mortality to patients. Development of new therapies that promote the healing of chronic wounds is therefore an important area of current research. A potential new treatment could be cellular therapy with bone marrow-derived mesenchymal stem cells [6, 7]. Other promising strategies involve the application of anti-inflammatory agents, for example, complement inhibitors, as persistent inflammation is often key to impaired wound healing [2, 8, 9].

1.2. Cellular and Molecular Processes Restore Injured Tissues. Tissue injury immediately initiates an array of physiological processes that lead to wound repair and regeneration. Although the exact underlying mechanisms of action are unclear, it is known that the immune systems play an essential role in the regulation of these processes [1–3]. Instantly after tissue injury, damage-associated molecules, such as S100 and the high mobility group box 1 (HBGM1) proteins, defensins, lectins, cardiolipin, cellular DNA and

dsRNA, and even intact mitochondria, occur in the extracellular microenvironment. Interaction of these molecules with multiligand receptors, such as toll-like receptors (TLRs) and C-type lectins, on surfaces of tissue and immune cells activate the cellular and molecular effector mechanisms of the innate immune system, including activation of the clotting and complement system, acute phase protein and pentraxin production, and the cellular inflammatory responses [10].

Following blood capillary vessel injury, an immediate reflex promotes vasoconstriction, slowdown of blood flow, and the local formation of a platelet clot. In addition, injured tissue cells release factors that stimulate the formation of a fibrin clot (containing a.o. fibronectin and vitronectin), that traps blood cells including platelets and red blood cells. This provisional extracellular matrix allows tissue cells to migrate to the wound area. The activated kallikrein-kinin system provides vasoactive kinins that mediate vasodilation and increased vascular permeability. The complement system is activated by distinct carbohydrate and lipid residues on altered self-molecules and injured cells and the cellular inflammatory response is subsequently initiated. Neutrophils are the first inflammatory cells that migrate into wounds to debride necrotic and apoptotic cells and eliminate infectious agents from the wound bed [3]. Gradually neutrophils are replaced by monocytes that exert the same scavenging activities. Monocytes at the wound site will also develop into macrophages that produce an array of inflammatory molecules, including chemokines, anti-inflammatory mediators, enzymes (proteolytic enzymes, metalloproteases), reactive oxygen species, and growth factors. A major drawback of infiltration of activated phagocytes is their ability to produce and release reactive oxygen species and proteolytic proteases that exert detrimental effects on healthy tissue cells [3]. In addition, immature dendritic cells collect antigens, for example, altered self-antigens, at the site of the wound and transport them to the draining lymph nodes where the dendritic cells mature and instruct T cells become effector cells.

The chemotactic mediators and growth factors produced by macrophages and healthy bystander cells stimulate angiogenesis and attract endothelial cells and fibroblasts that contribute to the proliferative phase of wound healing [3]. Simultaneously, effector T lymphocytes migrate to the wound and play a regulatory role in wound healing and collagen levels [3]. During the remodeling phase of the healing process, redundant cells die by apoptosis and collagen is remodeled and realigned. While the functions of the cells involved in the healing processes have been reported in much more detail than that described above, the biochemical responses leading to the activation of these cells at the site of injury are not widely investigated. However, it is well known that activation of the complement system is crucial in regulating the cellular responses in innate immunity.

1.3. Aims of This Paper. As described above, the first response to tissue injury is characterized by activation of the cellular and molecular effectors of the innate immune system, including the complement system. However, inappropriate complement activation, for example, in chronic wounds, will result in detrimental effects due to its ability to induce cell death and promote prolonged inflammation [10, 11]. Experiments in animals with deficiencies in complement components indicate that attenuation of complement activation promotes wound healing [12–19]. Therefore, complement inhibitors are considered as candidates for development of novel therapeutic agents for chronic nonhealing wounds.

Based on these considerations, this paper focuses on (1) the current understanding of the dual roles of complement activation in wound healing and (2) the present and novel complement inhibitors to be considered for treatment of chronic wounds.

2. Overview of the Complement Pathways and Their Functions in Wounds

2.1. The Complement System. The activated complement system is a crucial effector mechanism of the innate immune response to tissue injury. In general, the complement system can be activated by a number of pathways: the classical pathway (by immune complexes), the lectin pathway (by mannose residues and ficolins), and the alternative pathway (by spontaneous activation and microbial structures) and by properdin and thrombin [20]. The result of activation of any of these pathways is cleavage of the central factor C3 into C3a and C3b by C3 convertase (except thrombin, which activates the cleavage of C5 by C5 convertase) [21]. Thereafter, the terminal pathway of the complement system with factors C5b to C9 is completed (Figure 1). These latter factors form the membrane attack complex (MAC), which creates pores in the microbial cell wall resulting in cell lysis. C3a and C5a are the most important chemoattractants that are produced as part of the activation of the complement system. In addition, recognition of necrotic and apoptotic cells by activated complement components leads to the deposition of complement components, such as C3-fragments, on their membrane, which promotes phagocytosis and elimination of the damaged cells by phagocytic cells and also results in the generation of the MAC on these damaged cells. The major drawback of complement activation is that the tolerance against self-molecules can be broken, leading to responses to these self-molecules and, as a consequence, to further tissue injury and impairment of wound healing (Figure 1). Fortunately, host cells are protected from complement-mediated injury by fluid phase and membrane-bound regulators of complement activation, such as factor B, factor D, factor I, CD35, CD46, CD55, and CD59 [22, 23]. However, during tissue injury, the expression of these complement regulators may be decreased, resulting in reduced protection of the cells and increased tissue damage. Together, while complement activation is needed to restore tissue injury, inappropriate complement activation can cause injury and contribute to further tissue damage [11].

2.2. Roles of Complement in Wound Healing. There are a few studies that report beneficial effects of complement-activating components on wound healing. First, Strey et al.

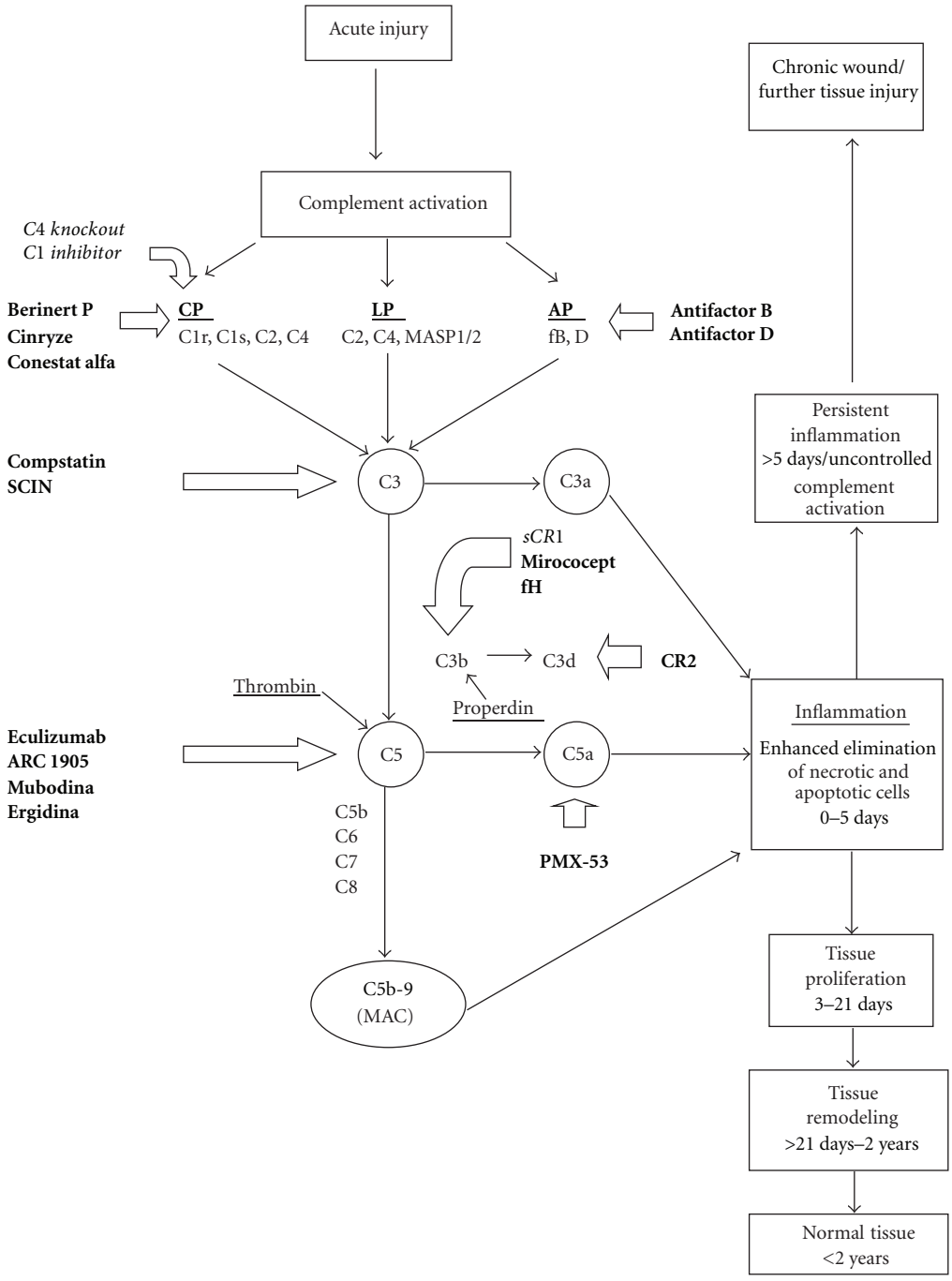

Figure 1: A simplified overview of the complement activation cascade after injury leading to wound healing. Three major pathways of complement activation, that is, the classical pathway (CP), the alternative pathway (LP), and the lectin pathway (LP), and two minor pathways initiated by properdin and thrombin are known. C is a complement component, MASP is mannan-binding serine peptidase, fB and D are factors B and D, SCIN is staphylococcal complement inhibitor, sCR1 is soluble complement receptor 1, fH is factor H, CR2 is complement receptor 2 and MAC is membrane attack complex. For simplicity, not all of the natural regulators of complement activation are shown in this diagram. The (pre)clinical complement inhibitors are denoted in bold and the complement factors that have been investigated in burn wound models in italic. C1 inhibitor affects C1r, C1s from the CP, and MASP 1 and MASP 2 from the LP. C4 knockout also affects both CP and LP.

reported that complement C3a and C5a are absolutely required for liver repair in a mouse model of liver injury [24]. Second, Bossi et al. topically applied C1q, vascular endothelial growth factor, or saline on wounds in rats and after 2 weeks vessel formation was examined [25]. Results revealed that animals treated with C1q and vascular endothelial growth factor exhibited increased numbers of new vessels as compared to control animals. In addition, application of C1q resulted in increased permeability, proliferation, and chemotaxis of endothelial cells, indicating that C1q has proangiogenic activity and thus can promote wound healing [25]. Third, topical application of C3 (100 nM) on a rat wound model resulted in a 74% increase in maximum wound strength as compared to control rats [26]. Also, inflammatory cells, fibroblast migration and collagen deposition in the wounds were enhanced in the C3-treated mice as compared to control animals. Despite the positive effects of C1q or C3 application on wound healing in these models of acute injury, the possibility that complement components exert an entirely different, that is, detrimental, effect on chronic wounds is likely. In agreement, in the majority of chronic wounds, MAC deposition is found at the ulcer margin, but not in the intact skin [27]. It has also been shown that patients with chronic leg ulcers have increased serum levels of C3 [28, 29].

While enhanced levels of complement activating factors are found in chronic wounds, it is interesting to study the outcomes of wounds in which complement activation is attenuated. It has been shown that animals with a genetic complement deficiency or individuals treated with a complement inhibitor are protected from the symptoms resulting from chronic inflammatory processes [12–17]. Interestingly, Wahl et al. published a study regarding the effect of complement depletion by cobra venom factor (CVF) on healing of acute wounds in guinea pigs [13]. CVF forms a stable complex with Bb resulting in continuously activated C3/C5 convertase [14], resulting in depletion of complement activity, while it is resistant to complement regulatory factors, such as factor H and I. CVF was administered intraperitoneally to guinea pigs over a 24-hour period while control animals received the diluent of CVF. After 24 hours, the wound exudates from the complement-depleted pigs showed a 50% reduction in infiltrating neutrophils and four times more erythrocytes than exudates from control animals. Wound debridement, fibroblast proliferation, connective tissue formation, and capillary regeneration did not differ between CVF-treated and control, wounded animals. It should be realized that only acute wound healing was investigated and that CVF could have had other systemic effects that affected wound healing in the guinea pigs. In this connection, it has been described that additional injections of CVF were administered and that these guinea pigs developed lethargy, leucopenia, and loss of weight. Unfortunately, no definitive conclusion as to the role of complement in wound healing can be drawn from these data. Furthermore, CVF initially is a complement activator, which can induce tissue damage instead of repair. Together, complement components play opposite roles in acute and chronic wounds.

2.3. Roles of Complement in Burn Wounds. Studies by Van de Goot et al. into the roles of complement in burn wounds showed enhanced levels of complement degradation factor C3d, indicative of complement activation, in the wound [30]. C3d remains elevated in the wound until 46 days after the burn injury. The amount of the acute phase reactant C-reactive protein and the influx of neutrophils and macrophages were also higher in the wounds during this period and indicate the persisting inflammation. Machens et al. compared the amount of C3a in wound fluids from a group of patients younger than 60 years and from a group older than 60 years with deep second-degree burn wounds [31]. Results revealed elevated C3a levels in both groups during the first 24 hours after thermal injury. However, thereafter the C3a levels in the wound fluid decreased in the young group, but not in the group with the older patients, indicating that persistent complement activation is associated with the delayed wound healing in the older patients. In agreement, others reported elevated serum levels of C3 and C3d in patients with burn wounds and these levels correlated with the severity of the trauma and the clinical outcome [32]. Furthermore, Mulligan et al. found that intravenous injection of soluble human recombinant complement receptor type 1 (sCR1) at 5 and 15 minutes and at 1 and 4 hours after thermal injury into rats resulted in decreased dermal vascular permeability and water content and reduced recruitment and activation of neutrophils in wound biopsies as compared to the biopsies from control rats [15]. The sCR1-treated rats were protected against complement-dependent tissue injury. In another study, the effects of a C1 inhibitor intravenously administrated immediately after thermal injury on progression of the depth of fresh burn wounds in pigs were assessed [16]. In contrast to the control group, the lower dermal vascular network was not altered in the C1 inhibitor treatment group and there was only activation of endothelial cells in the subepidermal and mid-dermal layer. Whereas in the control group there was necrosis of the lower dermal zones, these zones were normal in the C1 inhibitor group. As most studies focused on the short-term effects of complement inhibitors on wound healing, Begieneman et al. determined the effects of 14 daily intravenous administrations of C1 esterase inhibitor on wound progression in dorsal full-thickness burn wounds in rats [17]. Results revealed that the C1 inhibitor reduced the amount of granulation tissue and macrophage infiltration in these animals. The amounts of complement factors C3 and C4 in the wounds were lower (although not significant) in the C1 inhibitor-treated group than in the control group. Furthermore, the C1 inhibitor did enhance reepithelialization. The data from this study show that systemic administration with C1 inhibitor improves healing in burn wounds. In addition, Radke et al. demonstrated in a pig burn wound model that inhibition of C1 is beneficial for the clinical outcome, as indicated by vital signs and reduced edema formation, and C1 inhibitor diminished bacterial translocation [33]. Finally, Suber et al. found reduced burn wound depth and neutrophil migration in C4 knockout mice as compared to wild type animals [18]. Burn wounds in C4-deficient mice healed without

contracture, scar formation, or hair loss in contrast to the wild type mice. Moreover, the severity of the burn wound was significantly less in C4 knockout mice than in wild type animals. Together, both in preclinical and animal studies, attenuation of complement activation stimulates the wound healing process. Therefore, the various potential complement-inhibiting agents and their therapeutic effects are discussed in the next section.

3. Exogenous Complement Inhibitors

3.1. Current (Pre)Clinical Complement Inhibitors. In clinical practice, only a few complement inhibitors are currently available (Table 1). Plasma-derived human C1 inhibitors berinert P and cinryze and the recombinant human C1 inhibitor conestat alfa are currently applied in patients suffering from hereditary angioedema (HAE) [34, 35]. Furthermore, C5 inhibitor eculizumab is used in patients with paroxysmal nocturnal hematuria (PNH) [36]. An overview of these and other (pre)clinical complement inhibitors and their interaction with the complement system is given in Table 1 and Figure 1.

Recently, the C5 inhibitor pexelizumab failed in a Phase III study as it did not reduce infarction and mortality in patients after coronary intervention [37]. Pexelizumab inhibited both C5a and MAC formation *in vitro*, while *in vivo* only C5a was reduced with minimal effects on inflammation and risk biomarkers. Compstatin (POT-4), isolated from a phage-displayed random peptide library, is the only C3 inhibitor under investigation in Phase II studies for the treatment of acute macular degeneration (AMD) [38]. Compstatin is also tested in preclinical experiments for possible applications in PNH, sepsis, transplantation, and cancer. Furthermore, Mirococept (APT070), a membrane-targeted myristoylated peptidyl construct derived from soluble complement receptor 1, is currently examined in a multicenter, double-blind, randomized, case-control study for prevention of ischemia-reperfusion injury in cadaveric kidneys for transplantations [39, 40]. Anticomplement factor D is analyzed in a Phase II study in patients with AMD [36]. However, the Phase II study with C5a-inhibitor PMX-53 in AMD patients was discontinued because of lack of success. Nevertheless, this inhibitor is still under investigation for the use in osteoarthritis.

Phase I studies are performed with targeted factor H (TT30), that is, factor H coupled to CR2, for AMD and PNH [41]. This targeted inhibitor binds to C3b/C3d coated cells and blocks assembly of C3 and C5 convertases. Various other complement inhibitors coupled to CR2 were tested in patients with chronic glomerulonephritis [42]. In addition, the C5 inhibitor eculizumab, which is already approved by the FDA for PNH, was also tested as treatment for several other diseases, including kidney transplants and haemolytic uraemic syndrome (HUS) [36]. The anti-C5 aptamer ARC 1905 is investigated for its potential use in AMD [36]. Finally, the effects of plasma-derived factor H concentrate, anti-complement factor B (TA106) and C5 inhibitors, such as mubodina and ergidina, in complement-mediated diseases were evaluated in preclinical studies [36].

3.2. Medicinal Maggots Produce Complement Inhibitors. Larvae of medicinal maggots (*Lucilia sericata)* are successfully used to heal severe, infected acute and chronic wounds in the clinical practice [43–46], and in 2004, Maggot Debridement Therapy (MDT) was approved by the US Food and Drug Administration (510[k] no. 33391) [47]. Our current research focuses on the mechanisms underlying the beneficial actions of maggots on wound healing. So far, maggot excretions/secretions (ES) in therapeutic concentration ranges lack direct antibacterial properties [48] but inhibit biofilm formation and multiple proinflammatory responses [49, 50], which could explain part of the mechanism of action of maggots in wound healing. Others reported beneficial effects of maggot ES on the modulation of extracellular matrix components leading to enhanced tissue formation and accelerated healing [51, 52].

Recently, we found that maggot ES efficiently reduced complement activation in normal and immune-activated sera in a dose-dependent fashion with maximal inhibition of 99.9% (Figure 2) [53]. Most likely, ES degrade individual complement components, at least C3 and C4, in a cation-independent manner. Consumption of complement components via ES-mediated initiation of the complement cascade has been ruled out. The complement inhibitory molecule(s) in maggot ES proved to be temperature- and protease-resistant. Together, attenuation of complement activation by ES may contribute to the improved wound healing that is observed during MDT in the clinical practice [43–46]. As maggots and their ES are well tolerated by patients, it can be envisaged that the complement inhibitory molecules within ES are potential candidates for the development of novel complement inhibitors.

3.3. Complement Inhibitors Produced by Other Symbionts. As the complement system is a rapid and effective defense system, practically each successful microorganism has developed strategies and molecules to evade the actions of complement [54, 55]. Therefore, it is virtually impossible to give a brief, complete overview of all complement inhibitors produced by infectious agents described in the literature, but we will show some examples. *Staphylococcus aureus* is one of the pathogens that produces at least seven molecules with complement inhibitory molecules, including C3 inhibiting molecule staphylococcal complement inhibitor (SCIN), which prevents the conversion of C3 by convertases (C3b/Bb and C4b2a) and staphylococcal superantigen-like protein 7 that prevents C5 cleavage [54, 56]. Another example pertains to the herring worm *Anisakis simplex* [57]. Consumption of raw herring can cause intestinal infections by this herring worm, which possesses complement-inhibiting properties to evade the human immune defense. *Anisakis simplex* also excretes biochemical substances that harm the intestines. Therefore, the human immune system evolutionary developed (undefined) strategies against this parasitic infection resulting in death of the herring worm in all immunocompetent patients. Borreliaespecies, causing borreliosis (Lyme disease), also produce complement inhibitors to evade the innate immune system [58, 59]. Binding of a borrelial surface protein to complement factor H limits

Table 1: An overview of (pre)clinical complement inhibitors.

Complement inhibitor	Medicine	Diseases	Study phase
Recombinant C1 inhibitor	Conestat alfa (Ruconest in Europe/Rhucin in USA)	HAE Side effects: headache and allergy.	In clinical use, EU approved.
Plasma-derived C1 inhibitors	Berinert P/cinryze	HAE	In clinical use, FDA approved.
C3 inhibitors	Compstatin (POT-4)	AMD	Phase II
	Staphylococcal complement inhibitor (SCIN)		Preclinical
Myristoylated peptidyl derived from soluble CR1	Mirococept (APT070)	Delayed graft function of cadaveric kidney after transplantation.	Phase II
Factor H	Plasma-derived factor H concentrate	HUS, AMD	Preclinical
	TT30/targeted alternative pathway inhibitor/factor H	PNH, AMD	Phase I
Factor D inhibitor	Anticomplement factor D	AMD	Phase II
Factor B inhibitor	TA106/anti-complement factor B	AMD	Preclinical
C5 inhibitors	Eculizumab	PNH Side effects: headache, thrombocytopenia, gastrointestinal complaints and infections. Before use: vaccination against meningococcal infection.	In clinical use, FDA approved.
		Various other diseases, for example, kidney transplants, HUS, AMD.	Phase I
	Pexelizumab		Phase III study failed
	Mubodina	HUS	Preclinical
	Ergidina	Ischemia/reperfusion injury	Preclinical
	ARC 1905	AMD	Phase I
C5a inhibitor	PMX 53 and several other compounds	AMD	Phase II study discontinued
		Osteoarthritis	Phase I
Targeted complement inhibitors	Targeted (CR2 mediated) complement inhibitors	Chronic glomerulonephritis	Phase I

HAE: hereditary angioedema; AMD: acute macular degeneration; HUS: haemolytic uraemic syndrome; PNH: paroxysmal nocturnal haematuria.

AP activation and binding to complement inhibitor C4b-binding protein avoids CP activation. However, *Borreliae* appear to have specific effects on the complement cascade which finally do not result in a decrease of the inflammatory response. Adversely, aggravated inflammation is observed during borrelial infection. The scabies mite *Sarcoptes scabiei*, which can cause a parasitic infestation of the skin, expresses serine protease inhibitors in their gut and faeces that interfere with all three complement activation pathways leading to an overall complement inhibition [60]. Probably, the scabies protect themselves by excreting complement inhibitors.

3.4. Complement Inhibitors in Medicinal Plant Extracts. Although plants lack genes encoding complement molecules, complement inhibitors have been found in extracts from various species of plants and trees (Table 2). Here, we will only mention some interesting examples from plants used in traditional medicine all over the world to treat (inflammatory) diseases and wounds. Deharo et al. studied complement inhibiting properties of plant extracts used by the Tacana ethnic group in Bolivia and found six new species that produced molecules that inhibited the classical and alternative pathway [71]. Fernández et al. showed complement reducing effects in extracts of five different plants that are traditionally used in Argentina [61]. Hawaiian medicinal plants were investigated by Locher et al. and *Eugenia malaccensis* was found to produce molecules that inhibit the classical pathway, which could explain (in part) its activity against inflammatory diseases, including wound healing [80]. Other examples of plants producing complement inhibitors in Mali are the extracts of the root of *Entada africana*, leaves of *Trichilia emetica* and

Table 2: An overview of complement inhibitors in extracts from plant species.

Plant L.	Part of plant (extract)	Mode of action	Beneficial effects	References
Achyrocline flaccida (Yellow Marcela)	Aerial parts	CP inhibition. IC_{50} (CP) = 23.5–88.9 μg/mL	Antispasmodic, antipyretic, antihelmintic, antibacterial, antiviral. Stimulant, emmenagogue, excitant.	[61]
Aloe vera	Leaves	AP activation, resulting in consumption of C3.	Antibacterial, antifungal, antiparasitic, antitumor, laxative. Used for seborrheic dermatitis, radiation dermatitis, psoriasis vulgaris, genital herpes, burn wounds, diabetes, HIV infection, ulcerative colitis, pressure ulcers, mucositis, aphthous stomatitis, acne vulgaris, lichen planus, frostbite, alopecia, systemic lupus erythematosus, arthritis, tic douloureux.	[62, 63]
Apeiba tibourbou (Monkey comb)	Leaves	CP and AP inhibition.	Antispasmodic, mucilaginous, and pectoral properties. Used for rheumatism.	[64]
Artemisia species (A. dracunculus, A. montana, A. princeps, A. rubripes, A. tripartita)	Leaves	CP inhibition. IC_{50} (CP) = 54.3–64.2 μg/mL	Used for colic pain, vomiting, diarrhea, dysmenorrhea.	[65–68]
Ascophyllum nodosum (Brown seaweed)	Leaves	CP inhibition. Fucoidan binds C1q and prevents the formation of active C1. It forms a complex with C4	Anti-inflammatory, antiangiogenic, anticoagulant, antiadhesive.	[69, 70]
Astronium urundeuva	Stem bark	CP and AP inhibition. IC_{50} (CP) = 64 μg/mL IC_{50} (AP) = 111 μg/mL	Used for wound healing, bone healing, inflamed sores, gastric ulcers, uterine hemorrhages, metrorragias, cervicitis.	[71]
Avicennia marina (Evergreen shrub)	Stem bark	CP inhibition. IC_{50} (CP) = 23–248 μg/mL	Antitumor, anti-inflammatory, antiviral. Used for skin diseases, wound healing, rheumatism, smallpox, ulcers, malaria.	[72]
Biophytum petersianum Klotsch	Aerial parts	CP inhibition. IC_{50} (CP) $\leq$2–86 μg/mL	Used for wound healing, inflammation.	[65, 73]
Boswellia serrata (Frankincense)	Oleogum resin	CP inhibition, it inhibits C3 convertase	Antihelminthic, antiseptic, haemostatic, analgesic, cardiotonic, diuretic, aphrodisiac, laxative. Used for Crohn's disease, ulcerative colitis, bronchial asthma, rheumatoid arthritis, osteoarthritis, wound cleaning, reducing fat, diarrhea, improving menstruation.	[74, 75]
Bridelia ferruginea	Stem bark	CP and AP inhibition. Inhibition of C1 and terminal complex.	Used for rheumatism.	[76]
Cochlospermum vitifolium (silk cotton tree)	Stem bark	CP and AP inhibition. IC_{50} (CP) = 104 μg/mL IC_{50} (AP) = 135 μg/mL	Used for diabetes, hepatobiliary and cardiovascular diseases, hypertension, pain, kidney diseases, ulcers.	[71, 77]
Croton draco	Latex	CP and AP inhibition. IC_{50} (CP) = 430–590 μg/mL IC_{50} (AP) = 680–930 μg/mL	Antibacterial, antitumor, antiviral. Used for wound healing, inflammation.	[78]
Entada africana	Roots	CP inhibition. IC_{50} (CP) = 75–370 μg/mL	Hepatoprotective, haemostatic, antipyretic, antiseptic, diuretic, antigonococci, antisyphilitic, antiparasitic, abortifacient. Used for wound healing, malaria, respiratory diseases, psoriasis, rheumatism, cataract, dysentery.	[79]

Table 2: Continued.

Plant L.	Part of plant (extract)	Mode of action	Beneficial effects	References
Eugenia malaccensis (Malay rose apple)	Stem bark	CP inhibition: IC_{50} (CP) = 12 μg/mL AP was activated: 50 % activation at 6 μg/mL	Used for general debility, sore throat, wound healing, candidiasis, venereal diseases, tuberculosis, digestive tract disorders.	[80]
Eupatorium arnottianum	Aerial parts	CP and AP inhibition. IC_{50} (CP) = 5.0–155.9 μg/mL IC_{50} (AP) = 101.3 μg/mL	Antimicrobial, antiviral, antinociceptive. Used for gastric pain.	[61]
Eupatorium buniifolium	Aerial parts	CP inhibition. IC_{50} (CP) = 44.1–66.7 μg/mL	Hepatoprotective, antiviral, antiseptic.	[61]
Euterpe precatoria (Açai)	Roots	CP and AP inhibition. IC_{50} (CP) = 105 μg/mL IC_{50} (AP) = 147 μg/mL	Antioxidant. Used for muscular pain, sciatic pain, liver and kidney diseases, wound healing, skin ulcers, edema, inflammatory diseases.	[71]
Glycine max (Soyabean)	Seeds	*In vitro* it inhibits synthesis and secretion of C2 and C4 by guinea pig peritoneal macrophages	Antioxidant, anti-inflammatory, antitumor, antioestrogenic, antifungal, insulinotropic. Used for atherosclerosis, skin whitening,	[81, 82]
Glycyrrhiza glabra (Licorice)	Roots and rhizomes	Glycyrrhizin binds to C3a and C3. It induces conformational changes in C3 and it inhibits CP at the level of C2.	Anti-inflammatory, antiviral, antimicrobial, antioxidative, antitumor, immunomodulatory, hepatoprotective, cardioprotective, diuretic, anabolic, laxative, contraceptive. Used for wound healing, cystitis, diabetes, cough, stomachache, tuberculosis, nefrolitiasis, lung ailment, Addison's disease, gastric ulcers, improvement of voice, improvement of male sexual function.	[83, 84]
Isopyrum thalictroides	Roots and aerial parts	CP inhibition. Ca^{2+} and Mg^{2+} dependent complement inhibition. It inhibits C1 formation.	Rheumatism, neuralgia, silicosis, malaria.	[81]
Jatropha multifida/Jatropha curcas (Coral plant)	Latex	CP inhibition, mediated by Ca^{2+} depletion	Used for infected wounds.	[81, 85]
Lithraea molleoides	Leaves	CP inhibition. IC_{50} (CP) = 59.0–86.1 μg/mL	Anti-arthritic, haemostatic, diuretic, tonic. Used for respiratory diseases. It causes allergic contact dermatitis.	[61]
Opilia celtidifolia	Leaves	CP inhibition. IC_{50} (CP) = 0.5–29 μg/mL	Haemostatic. Used for wound healing.	[73]
Piper kadsura (Japanese pepper)	Stem bark	It inhibits C5a-induced chemotaxis and decreased the stimulated production of TNF-α and IL-1-β	Asthma, rheumatic arthritis	[86]
Phyllanthus sellowianus	Leaves and stems	CP and AP inhibition. IC_{50} (CP) = 11.2–22.0 μg/mL IC_{50} (AP) = 280.6 μg/mL	Hypoglycemic, diuretic, laxative, antiseptic, antinociceptic.	[61, 87]
Rosmarinus officinalis/Melissa officinalis (Rosemary)	Leaves	CP and AP inhibition. It binds C3 and inhibits C5 convertase. C5a generation is decreased. IC_{50} (CP) = 2 μg/mL	Antispasmodic, choleretic, hepatoprotective, anti-inflammatory, antitumor, antioxidant. Used for renal colic pain, dysmenorrhea, respiratory disorder (bronchial asthma), stimulation of hair growth, relaxation of smooth muscles of trachea and intestine, peptic ulcers, atherosclerosis, ischaemic heart disease, cataract, improvement of sperm motility.	[81, 88, 89]

Table 2: Continued.

Plant L.	Part of plant (extract)	Mode of action	Beneficial effects	References
Trichilia emetica (Natal mahogany)	Leaves	CP inhibition. IC_{50} (CP) ≤15–62.5 μg/mL	Antipyretic, antiepileptic, antigonococci, antisyphilitic, anti-parasitic. Used for wound healing, dysmenorrhea, asthma, vomiting, hepatitis, improvement of fertility (women), gastric diseases, malaria, hypertension, rheumatism, lumbago.	[90]
Triplaris americana (Ant tree)	Stem bark	CP and AP inhibition. IC_{50} (CP) = 74 μg/mL IC_{50} (AP) = 89 μg/mL	Antioxidant, parturifacient. Used for metrorragias, diarrhea, stomachache, intestinal worms, leishmaniasis, skin ulcers.	[71]
Ulex europaeus (Common gorse)	Seeds	It attenuates MBL binding on human endothelial cells and inhibited C3 deposition. The dcreased LP activation resulted in less complement-dependent neutrophil chemotaxis. IC_{50} = 10 pmol/L	None.	[91]
Uncaria tomentosa (Cat's claw)	Stem bark	CP and AP inhibition. IC_{50} (CP) = 124 μg/mL IC_{50} (AP) = 151 μg/mL	Anti-inflammatory, antiviral, immunostimulating, antimutagenic, antioxidant. Used for gastritis, dermic and urogenital inflammations, asthma, rheumatism, irregular menstruation, digestive, liver and kidney diseases, adjuvant therapy for breast cancer.	[71, 92]

CP: classical pathway; AP: alternative pathway; LP: Lectin Pathway; IC_{50}: concentration required for 50% complement inhibition. Most of these complement inhibition tests were performed using complement haemolytic activity assays. Compounds in these plant species inhibiting the complement system are; for example; flavonoids, glucosides, polysaccharides, terpenes, iridoids, polymers, peptides, alkaloids, and oils [81]. Other complement inhibitors from plants are found in *Acanthus ilicifolius*[72], *Atractylodes lancea* [73], *Angelica acutiloba* [73, 81, 93], *Azadirachta indica* [81], *Bupleurum falcatum* [94], *Cedrela lilloi* [81], *Centaurium spicatum* [81], *Cochlospermum tinctorium* [95], *Crataegus sinaica* [81], *Crataeva nurvala* [81], *Curcuma longa* [96], *Dendropanax morbifera Leveille* [97], *Glinus oppositifolius* [79], *Juglans mandshurica* [98], *Ligularia taquetii* [99], *Litsea japonica* [100], *Ligustrum vulgare* [81], *Lithospermum euchromum* [81], *Magnolia fargesii* [101], *Melothria maderaspatana* [102], *Morinda morindoides* [81], *Olea europaea* [81], *Osbeckia octandra* [102], *Ocimum basilicum* [66], *Osbeckia aspera* [81], *Panax ginseng* [103], *Paulownia tomentosa var. tomentosa* [104], *Persicaria lapathifolia* [81], *Petasites hybridus* [81], *Phillyrea latifolia* [81], *Phyllanthus debilis* [102], *Picria fel-terrae* [105], *Plantago major* [81], *Sorghum bicolor* [106], *Terminalia amazonia* [71], *Thymus vulgaris* [66], *Tinospora cordifolia* [81], *Trichilia elegans* [90], *Trichilia glabra* [81, 90], *Vernonia Kotschyana* [72, 73, 95], *Wedelia chinensis* [107], and *Woodfoidra fruticosa* [81].

Opilia celtidifolia, and water extract of the aerial parts of *Biophytum petersianum Klotsch,* which are traditionally used in Mali to cure wounds and to reduce fever [65, 73, 79, 90]. Natural latex from rubber trees also has wound healing properties and extracts of *Jatropha multifida* and *Croton Draco* were able to inhibit the classical pathway of complement activation [78, 85]. Plant extracts interfere with the complement system at different stages of the cascade (Table 2). *Bridelia ferruginea*, *Isopyrum thalictroides,* and *Ascophyllum nodosum* inhibit C1 formation and the latter one also forms a complex with C4 [69, 70, 76, 81]. *Glycyrrhiza glabra* reduces C2 [83, 84] and *Glycine max* inhibits synthesis of C2 and C4 [81, 82]. C3 is affected by *Aloe vera*, *Boswellia serrata, Glycyrrhiza glabra, Rosmarinus officinalis,* and *Ulex europaeus* [62, 63, 74, 75, 81, 83, 84, 88, 89, 91]. Production of anaphylatoxin C5a is decreased by *Piper kadsura* and *Rosmarinus officinalis* [81, 86, 88, 89]. Future research should focus on the purification and characterization of the effective substances in plants and the specificity and exact mechanisms of action of these compounds.

4. Discussion and Future Research

Complement serves as a rapid and efficient immune surveillance system to control infection and tissue injury. The complement system regulates the clearance of necrotic and apoptotic cells, inflammation, and tissue regeneration. However, elevated levels of C3, C3a, C3d, and MAC have been reported in chronic wounds and burn or traumatic wounds [27–32], indicating that uncontrolled complement activation occurs in such wounds. In addition, studies in animals with deficiencies in complement components and in patients treated with complement inhibiting agents confirmed the importance of controlling the complement system in wound healing and fibrosis [12–18, 108]. Specific inhibitors can balance the functional activities of the complement system and progress the healing process, as shown in patients with burn wounds treated with a C1 inhibitor or a soluble human recombinant complement receptor type 1 as well as in C4-deficient mice [15–18]. Thus, attenuation of complement activation by therapeutic agents may improve the healing process in chronic wounds.

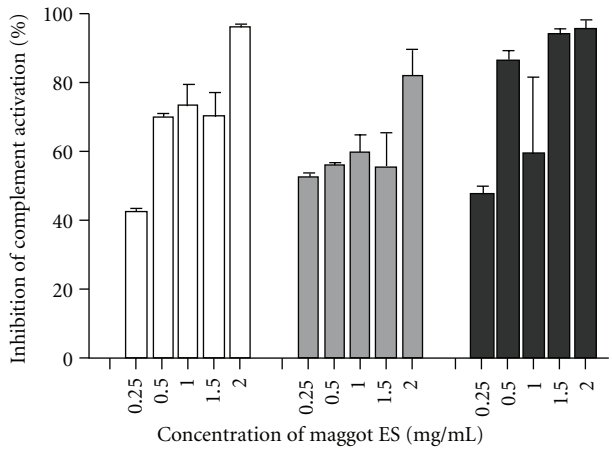

Figure 2: Dose-dependent effect of fresh collected maggot ES on activation of the classical pathway (white bars), the alternative pathway (grey bars), and the lectin pathway (black bars) in normal human sera. The complement activation in four different sera was determined with the enzyme immunoassays from Wieslab (EuroDiagnostica BV, Arnhem, The Netherlands) according to manufacturer's instructions. The percentages inhibition was calculated using the values in the sera without maggot ES as 0%. The results are means and SD of four independent experiments.

However, several challenges have to be overcome before complement inhibitors can be included in the therapeutic arsenal for wound care. For example, complement inhibitors should act locally at the site of inflammation or injury, thus avoiding the adverse effects of a systemic complement blockade, that is, infection and impaired wound healing [109]. For this purpose, current research focuses on the development of strategies to target the complement inhibitor to the sites of complement activation, regardless of the location. In this connection, a Phase I study has recently been performed in which various complement inhibitors were linked to a targeting moiety consisting of complement receptor 2 (CR2) [110]. CR2 binds long-lived C3 fragments and thereby acts to target the attached complement inhibitor to the site of inflammation/injury. In agreement, experiments in mice showed an increased potency and prolonged local presence of such complement inhibitors, while leaving the systemic complement activation intact [42]. No increased risk of infection or sepsis was observed in these animals. Another example is perfusion of cadaveric kidneys during the transfer from the donor to the recipient with mirococept which is a peptidyl derivative of sCR1 engineered to stick to the organ during this process [40].

One more issue pertains to the contribution of local production and functional activities of complement components and their regulators. Although the liver is the main source of complement components, the production of several complement components, for example, properdin, C1, C3, and C7, at sites of inflammation/injury should be studied in more detail. Furthermore, good affinity of the complement inhibitors for the target and selectivity are important factors to consider in anti-complement therapies. Moreover, the complement inhibitor must have a long half-life.

The choice of the complement inhibitor depends on the role the complement has in the disease. C5 inhibitors are preferred for the treatment of diseases in which C5a and MAC play a major role, for example, in HUS and in patients suffering from an infection with the EHEC bacterium [111]. Cleavage of C5 generates C5a, a major inflammatory mediator, and C5b initiating the formation of MAC. These two factors are the key effectors of the complement system responsible for both wound repair and persistent inflammation [112]. Obviously, the effects of complement inhibitors also depend on the stage of the disease in the patients. In this context, it is interesting to see that the C5-inhibitor eculizumab is efficacious for PNH and HUS [36], while pexelizumab having the same mode of action as eculizumab was ineffective in patients undergoing percutaneous coronary intervention after myocardial infarction [37]. This failure of pexelizumab could be due to late administration of the antibody after ischemia-reperfusion and/or differences in their half-lifes, that is, eculizumab has an average half-life of 272 hours and pexelizumab of 7–14 hours. In agreement, administration of pexelizumab before coronary arterial bypass grafting did have a beneficial outcome [37]. Of note, it was found that *in vitro* both C5a and MAC were both blocked by these antibodies directed against C5 while *in vivo* C5a activity (but not MAC) was blocked. Finally, there are concerns about the clinical use of nonspecific complement inhibiting agents as these agents may have adverse consequences for patients, such as (recurrent) infections [109].

Although there are a lot of challenges to overcome, there are some promising complement inhibitors. For example, the pathway-independent inhibitor compstatin is extensively tested in clinical studies in patients suffering from acute and chronic inflammatory conditions. The results up to date are successful [36]. Furthermore, a novel complement inhibitor could be based on the active component(s) in ES of *Lucilia sericata* larvae as ES reduce all three complement activating pathways in normal and immune-activated human sera in a dose-dependent manner [53]. Moreover, it should be kept in mind that these maggots are already in clinical use for many years without any side effects reported in the literature nor in our own clinical experience with this therapy over the past ten years [44, 45].

Another important question that remains unanswered is how much the complement system can be attenuated without the risk of loss of protection. Based on our finding that a single maggot produces approximately 2 μg of ES per hour [53, 113] and assuming that 125 maggots are applied on a wound surface (of about 25 square centimeter), the amount of ES in the wound (per hour) is 250 μg, which correlates with a 50% complement reduction (Figure 2). Thus, we believe that reduction of the local complement activity of about 50% is safe and effective. However, further research is required before a definitive conclusion can be drawn.

To conclude, well-designed (pre)clinical studies aimed at understanding the roles of complement in the pathology of chronic wounds, with the hope of innovative drugs and their

clinical implementation to promote healing in patients with chronic wounds, are urgently needed.

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Research Article

Dual Functions of the C5a Receptor as a Connector for the K562 Erythroblast-Like Cell-THP-1 Macrophage-Like Cell Island and as a Sensor for the Differentiation of the K562 Erythroblast-Like Cell during Haemin-Induced Erythropoiesis

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The transcriptional nuclear factor binding to the Y box of human leukocyte antigen genes (NF-Y) for the *C5a receptor* (*C5aR*) gene is active in erythroblasts. However, the roles of the *C5aR* in erythropoiesis are unclear. We have previously demonstrated that apoptotic cell-derived ribosomal protein *S19* (*RP S19*) oligomers exhibit extraribosomal functions in promoting monocyte chemotaxis and proapoptosis via the *C5aR* without receptor internalisation. In contrast to the extraribosomal functions of the *RP S19*, a proapoptotic signal in pro-EBs, which is caused by mutations in the *RP S19* gene, is associated with the inherited erythroblastopenia, Diamond-Blackfan anaemia. In this study, we detected *C5aR* expression and *RP S19* oligomer generation in human erythroleukemia K562 cells during haemin-induced erythropoiesis. Under monocell culture conditions, the differentiation into K562 erythrocyte-like cells was enhanced following the overexpression of Wild-type *RP S19*. Conversely, the differentiation was repressed following the overexpression of mutant *RP S19*. An *RP S19* oligomer inhibitor and a *C5aR* inhibitor blocked the association of the K562 basophilic EB-like cells and the THP-1 macrophage-like cells under coculture conditions. When bound to *RP S19* oligomers, the *C5aR* may exhibit dual functions as a connector for the EB-macrophage island and as a sensor for EB differentiation in the bone marrow.

1. Introduction

The homeostasis of haematopoietic stem cells is partially maintained by the differentiation stage-specific activation of transcription factors [1]. In human leukocyte antigen genes, the binding of NF-Y to the CCAAT motif upstream of the promoter at the terminal differentiation stage of myeloid cells is crucial for the expression of the C5aR protein, a member of the Gi protein-coupled receptor (GiPCR) family [2]. In leukocytes, when bound to complement C5-derived anaphylatoxin C5a at the acute inflammatory phase, the C5aR functions as a proinflammatory factor and exhibits receptor internalisation [3]. The G$\beta\gamma$ subsets transmit an extracellular signal-regulated kinase 1/2 (ERK1/2) signal via phospholipase C. Therefore, the activation of the C5aR is limited by the binding of arrestin to its C-terminal intracellular regions, which are phosphorylated sequentially by protein kinase C and G protein-coupled receptor kinase 2, downstream of ERK1/2 [4]. Recently, the neutrophil C5aR was shown to function briefly as an antiapoptotic factor that phosphorylates the pro-apoptotic Bax on the mitochondrial membrane, inducing the translocation of Bax to the 14-3-3 protein for degradation by the 26S proteasome [5]. Therefore, it has been suggested that C5a attracts neutrophils via the neutrophil C5aR, and the antiapoptotic signal is briefly transmitted in neutrophils to prolong of the lifespan of the cell.

We have previously demonstrated that NF-Y can be activated in any apoptotic cell and that RP S19 is cross-linked at Lys122 and Gln137 by the activated type II tissue

transglutaminase (TGII) [6]. The activation site in the C5aR bound to the Leu_{131} AspArg moiety of RP S19 oligomers functions as a pro-apoptotic factor for apoptotic cells and as a chemotactic factor for monocytes/macrophages in the absence of receptor internalisation. The $G\beta\gamma$ subsets of the monocyte C5aR transmit the p38 mitogen-activated protein kinase (p38MAPK) signals, indicating that the C5aR C-terminus is not phosphorylated. When RP S19 oligomers bind to the C5aR on apoptotic cells (including neutrophils), the additional binding of the RP S19 C-terminus to an inhibitory molecule inhibits p38MAPK signalling. However, the *de novo* synthesis of the regulator of G protein signalling 3 (RGS3) is initiated to inhibit the microenvironment factor-dependent ERK1/2 signalling mediated by the constitutively activated GPCRs. Therefore, we suggest that the RP S19 oligomers released from apoptotic cells attract macrophages for the connection between the *de novo* synthesised C5aR on apoptotic cells and the monocyte C5aR on macrophages without receptor internalisation. The pro-apoptotic signal is transmitted continuously in apoptotic cells for the execution of apoptosis.

Erythropoiesis is maintained primarily by transcription factors via the differentiation stage-specific activation of growth factor receptors [7]. Early erythroid progenitors (burst-forming unit-erythroid, BFU-E) are sensitive to GPCR 48 or the receptor-type tyrosine kinases C-kit and FLT3, which function as transcription factors for the *antiapoptotic protein* genes [8, 9]. In contrast, late erythroid progenitors (colony-forming unit-erythroid, CFU-E) and morphological erythroid precursors (proerythroblast, pro-EB) are sensitive to the activities of the Fas ligand receptor, which functions as an activator of pro-apoptotic caspase-3 [10–12]. Moreover, monocyte chemoattractant protein-2/4, released from the CFU-E-derived EBs, was recently suggested to contribute partially to erythropoiesis through the formation of the EB-macrophage islands [13]. However, an inherited erythroblastopenia in a case of Diamond-Blackfan anaemia was recently reported to be associated with mutations in at least *8 different ribosomal protein* genes [14]. The number of peripheral blood erythrocytes in FVB/N mice is decreased by the dominant negative effect of overexpressing the Arg62Trp mutant RP S19. These data confirm a role for the constitutive pro-apoptotic signal through a defect in the ribosome formation mediated by the mutant RP S19 at the BFU-E stage [15]. However, the roles of the differentiation stage-specific activation of pro-apoptotic signals and the formation of the EB-macrophage islands under normal conditions are not clearly understood.

A number of interesting studies report the erythroid-specific transcriptional activation of the *growth factor-independence-1B* and the *nucleolar spindle-associated protein* genes that contribute to the functional cooperation between GATA-1 and NF-Y in immature human erythroleukemia K562 cells and mature erythroleukemia MEL cells [16, 17]. If the C5aR is expressed during erythropoiesis, the RP S19 oligomer-induced extraribosomal functions will regulate the intracellular pro-apoptotic signal via the C5aR on EBs and the interaction of basophilic-EBs with the macrophages for a long period in the absence of receptor internalisation, as shown previously in apoptotic cells.

In this study, to confirm the similarity between the morphological changes in K562 cells after the induction of apoptosis using $MnCl_2$ and the differentiation using iron-containing porphyrin (haemin), we first transfected the K562 cells with the enhanced-green-fluorescent-protein-(EGFP-) fused ANXA3 cDNA as a marker for apoptosis [18, 19]. The haemin-induced differentiation of the K562 cells was limited to the basophilic-EB-like cell stage by the constitutively active ERK1/2 signalling downstream of a *Bcr-Abl* chimeric gene. Therefore, a blockade of the constitutively activated ERK1/2 signalling pathway would allow further cell differentiation. Next, we investigated the effects of RP S19 and RGS3 overexpression under monocell culture conditions and the EB-macrophage islands under coculture conditions on overcoming the haemin-induced differentiation defect in the K562 cells.

2. Materials and Methods

2.1. Antibodies and Recombinant C5a. The phosphorylated and unphosphorylated anti-ERK1/2 and anti-p38MAPK rabbit IgGs were from Cell Signaling Technology (Boston, USA). The anti-ventricular myosin light chain 3 (LC3) rabbit IgG was from Sigma (Tokyo, Japan). The anti-C5aR, anti-TGII, anti-RGS3 and anti-actin rabbit IgGs, the fluorescein isothiocyanate- (FITC-) conjugated anti-C5aR mouse IgG, and the FITC- and HRP-conjugated anti-rabbit IgG goat IgGs were from Santa Cruz Biotechnology (CA, USA). The FITC-conjugated anti-transferrin receptor (CD71) mouse IgG and the phycoerythrin- (PE-) conjugated anti-glycophorin A (CD235a) mouse IgG were from BD (Tokyo, Japan).

The recombinant C5a was prepared using the pET32a-Rosetta gami(B) Lys-S system to generate anti-C5a rabbit IgGs [20]. Briefly, human C27R C5a cDNA, prepared using the polymerase chain reaction, was inserted into the pET32a vector between the BamH1 and EcoR1 sites. The expression host, *E. coli* Rosetta-gami(B) Lys-S cells transformed with pET32a-C27R-C5a cDNA, was cultured for 6 hr and reincubated for another 5 hr in the presence of 1 mM isopropyl 1-thio-β-D-galactoside. The Trx-His-S-Tag C27R-C5a protein recovered from the cultured *E. coli* cells was purified using the Hi-Trap chelating HP column (GE, Tokyo, Japan) preloaded with 1 mL 100 mM $NiSO_4$. The S-Tag C27R-C5a protein dialysed against thrombin was purified using the Hi-Trap benzamidine FF column and the Hi-Trap heparin HP column (GE). The purity of the protein was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting using anti-RP S19 rabbit IgG. The anti-C5a rabbit IgG was raised in a male New Zealand White rabbit (Kyudo, Kumamoto, Japan). The blood was collected for 2 months after injection of 500 μL of 1 mg/mL Trx-His-S-Tag C27R-C5a protein in PBS mixed with 500 μL of Freund's complete or incomplete adjuvant (Sigma), and the serum was prepared using standard protocols. The IgG was separated from the

sera using a Hi-Trap protein-G HP column (GE) and was stored at a concentration of 4 mg/mL in PBS at −80°C.

2.2. Cells. The human leukaemia K562 and monocytic THP-1 cells were obtained from the RIKEN BioResource Center in Japan. The K562 cells were differentiated into K562 basophilic-EB-like cells using 30 μM haemin, and the monocytic THP-1 cells were differentiated into THP-1 macrophage-like cells using 160 nM phorbol-12-myristate-13-acetate (PMA) (Sigma) [21]. To select for apoptosis-resistant clones (EGFP-ANXA3 K562^R cells) using limiting dilution methods, the EGFP-ANXA3 K562 cells were cultured for 3 days in the presence of 1 mM $MnCl_2$ [18, 22]. To examine the haemin-induced differentiation of the K562 cells using fluorescence-activated cell sorting (FACS), the cells were stained with FITC-conjugated anti-CD71 and PE-conjugated anti-CD235a mouse IgGs for 30 min at 4°C.

2.3. Vectors and K562 Transformations. The ANXA3 cDNA, prepared from human hepatoma HepG2 cells using RT-PCR, was inserted into the pENTR4-H1 vector. The H1-ANXA3 cassette was placed within the RfA region of the pCS-RfA-EG vector by LR recombination (Life, Tokyo, Japan). The 293T cells (5×10^6 cells/mL) were maintained in DMEM medium containing 10% FBS in poly-lysine-coated 10-cm tissue culture plates for 24 hr before adding fresh medium. To produce the HIV-1-based lentivirus packaged with the CSIIE-ANXA3 cDNA, a plasmid DNA solution (450 μL) containing 17 μg of the packaging plasmid and 10 μg of the VSV-G and Rev-expression plasmids was mixed with 50 μL 2.5 M $CaCl_2$ and 500 μL 2x HEBS (1.5 mM Na_2HPO_4, 280 mM NaCl, and 50 mM HEPES) and was used to transfect the 293T cells. The HIV-1-based lentivirus was recovered by ultracentrifugation and resuspended in 50 μL of HBSS medium. To establish the stably transformed K562 cells harbouring the CSIIE-ANXA3 cDNA, we infected 100 μL of the cells (1×10^6 cells/mL) with the lentivirus at a multiplicity rate of infection of 10, and the stably transfected clones were screened using 1 mM Zeocin.

We constructed the pCAGGS-IRES neomycin-resistant vectors containing the Wild-type RP S19, Gln137Asn mutant RP S19, and RGS3 cDNAs [23]. In addition, the pDsRed vector was used to label the nucleus (Takara, Kyoto, Japan). To establish the transformed K562 cells, the cells were suspended in DMEM medium at a density of 5×10^6 cells/mL and were electroporated with 40 μg of linearised plasmid DNA using a Gene Pulsar II electroporator (Bio-Rad, CA, USA) in a 4 mm gap cuvette at 200 V and 950 μF. Of the 24 positive clones that were screened using 250 μM Geneticin, the best clone was selected based on the cell growth rate (data not shown).

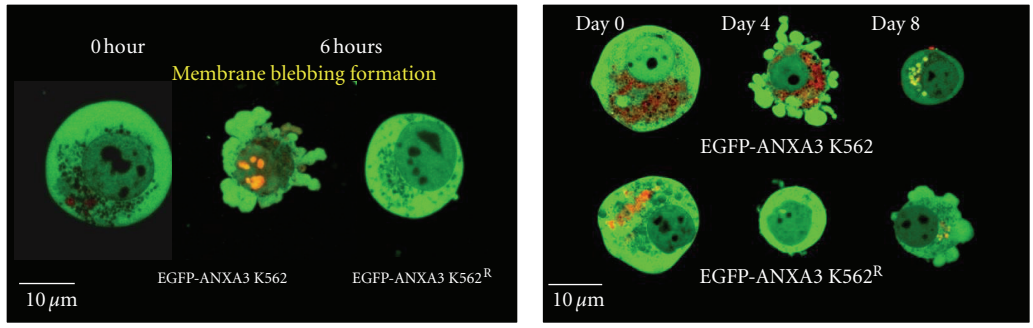
2.4. SDS-PAGE and Western Blotting. The ectosome fractionation was performed in accordance with the isolation protocol described previously [24]. After precipitation with cold acetone, the samples were resolved by SDS-PAGE and were silver-stained. Following the SDS-PAGE, the K562 proteins were electrophoretically transferred from the gel onto an Immobilon Transfer Membrane (Millipore, MA, USA) using a Semi-Dry Electroblotter (Sartorius, Göttingen, Germany) at 15 V for 90 min. The membrane was treated with 4% Block Ace (Dainippon, Osaka, Japan) for 30 min at 22°C. The first immunoblotting reaction was performed using rabbit IgG antibodies (200 ng/mL) in PBS containing 0.03% Tween-20 for 1 hr at 22°C. After washing, the second immunoblotting reaction was performed using the HRP-conjugated goat anti-rabbit IgGs (20 ng/mL) for 30 min at 22°C. The enhanced chemiluminescence reaction was performed using the ECL Plus Western Blotting Detection System (GE).

2.5. Statistical Analysis. The results from representative experiments were confirmed by performing multiple experiments using a minimum of triplicate samples. The statistical significance was calculated using the nonparametric or parametric tests with two-way analysis of variance. The values were expressed as the means ± SD. A P value of <0.05 was considered statistically significant and is shown as $^*P < 0.05$; a P value of <0.01 is labelled $^{**}P < 0.01$.

3. Results

3.1. Common Morphological Changes in K562 Cells during $MnCl_2$-Induced Apoptosis and Haemin-Induced Erythroid Differentiation. To demonstrate the morphological changes that occur during the $MnCl_2$-induced apoptosis and haemin-induced differentiation of the K562 cells, we prepared EGFP-ANXA3 K562 cells and EGFP-ANXA3 K562^R cells. The markers characteristics of programmed cell death, cell shrinkage, and membrane blebbing were detected in the EGFP-ANXA3 K562 cells using confocal laser microscopy (CLSM) (FLUOVIEW FV300, Olympus, Tokyo, Japan) at 6 hr after $MnCl_2$ induction under monocell culture conditions (DMEM medium containing 10% FBS and 1 g/mL glucose (the base medium)) (Figure 1(a)). These changes were absent in the EGFP-ANXA3 K562^R cells [25, 26]. When the EGFP-ANXA3 K562 cells were examined under CLSM at several time points after the haemin induction under monocell culture conditions, the EGFP-ANXA3 was translocated to the cell surface to form membrane blebbing-like vesicles, similar to those detected in apoptotic cells, and on day 8 after the haemin induction, the cell was decreased in size (Figure 1(b)). Conversely, these morphological changes were initiated in the EGFP-ANXA3 K562^R cells for 8 days after the haemin induction, indicating that programmed cell death plays a role in promoting the haemin-induced differentiation of the K562 cells. When the K562 cells were cultured for 20 days under basic cell culture conditions, almost all the K562 cells exhibited the morphological features of necrosis (data not shown).

3.2. Roles of the C5aR in the Haemin-Induced Differentiation of K562 Cells under Monocell Culture Conditions. To overcome the haemin-induced cell differentiation defect of the K562 cells, the composition of the culture medium was modified by adding 50 mM HEPES, 3 g/L glucose and 1 nM



(a) (b)

Figure 1: Common morphology between K562 cells after $MnCl_2$ and haemin treatments. ((a) and (b)) K562 and K562^R cells expressing EGFP-ANXA3 were observed under CLSM at several time points after $MnCl_2$ or haemin induction ($n = 6$).

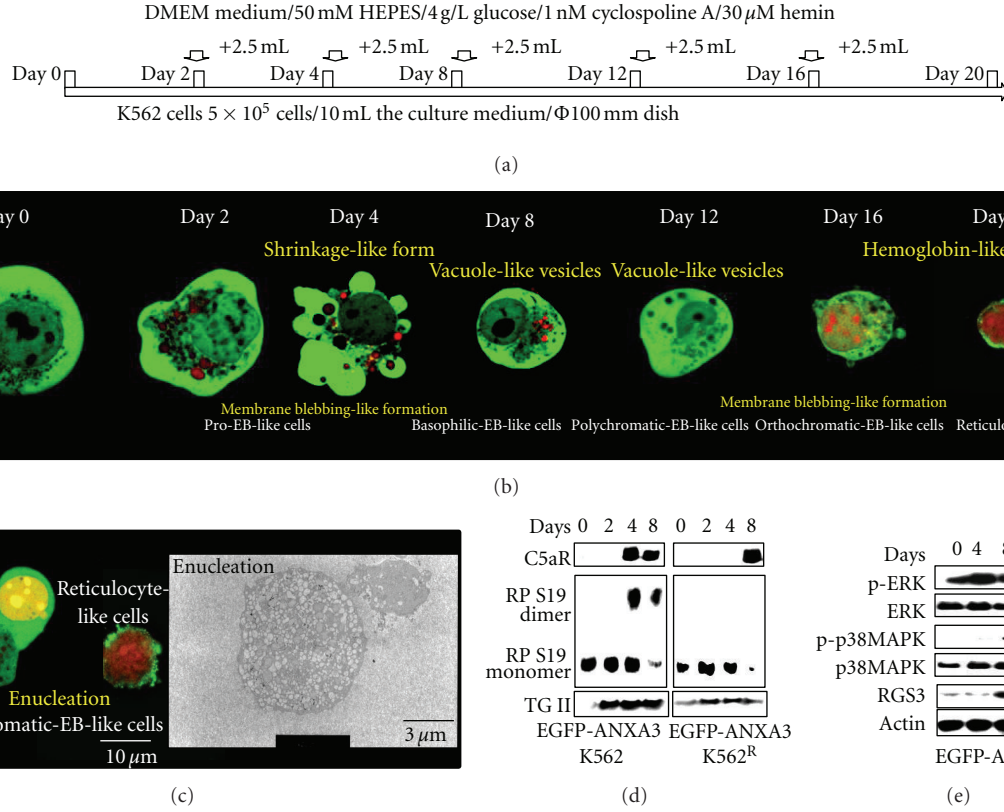


Figure 2: Contribution of the C5aR-RP S19 oligomer system to the haemin-induced differentiation of K562 cells under monocell culture conditions (a) Time schedule for the K562 monocell culture. ((b) and (c)) K562 cells expressing EGFP-ANXA3 were observed under CLSM or TEM at several time points after the haemin induction ($n = 6$). ((d) and (e)) EGFP-ANXA3-expressing K562 and K562^R cells were harvested at several time points after the haemin induction. After transferring the proteins from the SDS gel onto the membrane, the first immunoblotting was performed using the rabbit IgGs against C5aR, RP S19, and TGII (d) or ERK1/2, p38MAPK, RGS3, and actin (e) ($n = 3$).

cyclosporine A to the base culture medium to stabilise the pH and to promote the glycolysis and calcineurin-dependent homeostasis of the cells [27] (Figure 2(a)).

Using CLSM, the morphological changes in the membrane blebbing-like formations were observed on days 4 and 16, the vacuole-like vesicles [28, 29] on days 12 and 16, and the haemoglobulin-like molecules on day 20 after the haemin induction in the differentiated EGFP-ANXA3 K562 cells (Figure 2(b)). In addition, we occasionally observed a large number of the vacuole-like vesicles using CLSM and transmission electron microscopy (TEM, Hitachi H-300, Hitachi, Tokyo, Japan) on day 12 after haemin induction, and

nuclei in the vacuole-like vesicles were observed 16 days after the haemin induction (see Figures 1(a) and 1(b) in Supplemental Material available at doi:10.1155/2012/187080). Moreover, an increase in the LC3-II expression in the differentiated EGFP-ANXA3 K562 cells was detected by western blotting (see Figure 1(c) in Supplemental Material). These data indicated that the cells were differentiated into EGFP-ANXA3 K562 polychromatic-EB-like cells at 12 days after the haemin induction under the monocell culture conditions.

Typically, the EGFP-ANXA3 was translocated to the perinuclear in the differentiated EGFP-ANXA3 K562 cells during the 20 days after haemin induction, indicating that the K562 orthochromatic-EB-like cells were differentiated into reticulocyte-like cells and pyrenocyte-like cells. In the reticulocyte-like cells, the EGFP-ANXA3 had disappeared and haemoglobin-like molecules were generated, and in the pyrenocyte-like cells, the membrane blebbing-like formations were induced to allow for phagocytic clearance (Figure 2(c)) [30]. These morphological findings indicated a transition into the EGFP-ANXA3 K562 orthochromatic EB-like cell stage by day 18 after the haemin induction under monocell culture conditions. However, 99.9% of the differentiated EGFP-ANXA3 K562 cells underwent necrosis even under the modified base culture conditions. Only 0.1% of the EGFP-ANXA3 K562 erythrocyte-like cells (data not shown) were recovered.

The morphological changes in the K562 cells after the $MnCl_2$ treatment were similar to the changes associated with the haemin induction (Figure 1), indicating that NF-Y binds to *C5aR* and that RP S19 oligomers were generated because of cross-linking by the activated TGII. To confirm the activities of NF-Y and RP S19, the differentiated EGFP-ANXA3 K562 cells were harvested at several time points after the haemin induction and were analysed by western blotting. The expression of the C5aR, RP S19 dimer, and TGII was increased on day 8 after the haemin induction (Figure 2(d)); the expression of the proteins was delayed in the EGFP-ANXA3 $K562^R$ cells.

We previously reported that RGS3 promotes pro-apoptosis by downregulating ERK1/2 phosphorylation [23]. In addition to the C5aR, we recently discovered the expression of RGS3 on apoptotic cells. MAPK phosphorylation was switched from the ERK1/2 pathway to the p38MAPK pathway in the EGFP-ANXA3 K562 prochromatic-EB-like cells following the expression of RGS3 (Figure 2(e)). These data indicated that the *de novo* synthesised C5aRs that are bound to the RP S19 dimer are released in the K562 basophilic-EB-like cells to promote haemin-induced erythropoiesis byswitching the MAPK signalling from the ERK1/2 pathway to the p38MAPK pathway through the expression of RGS3.

3.3. Roles of RP S19 Oligomers in the Haemin-Induced Differentiation of K562 Cells under Monocell Culture Conditions. Recently, we demonstrated that the RP S19 oligomers are distinguishable from the monomers by immunoblotting with anti-human C5a rabbit IgG [20]. Under our current experimental conditions, the monocyte chemotactic activity of the K562 basophilic-EB-like cell supernatant was neutralised not only by anti-RP S19 rabbit IgG but also by anti-C5a rabbit IgG (data not shown). When the supernatant of the K562 basophilic-EB-like cells was analysed using SDS-PAGE and western blotting with anti-RP S19 rabbit IgG and anti-C5a rabbit IgG, a 34-kDa protein band was shown to cross-react with both rabbit IgGs (data not shown). The RP S19 oligomers in the cytoplasmic and extraplasmic plasma membranes of the K562 orthochromatic EB-like cells were detected under CLSM and by FACS using anti-C5a rabbit IgG (Figure 3). Conversely, using FACS, the C5aR was detected on the extraplasmic plasma membranes of the K562 cells from the basophilic-EB-like cell stage to the orthochromatic-EB-like cell stage because the C-terminus of the C5aR was not phosphorylated by downstream signals when the C5aR was bound to RP S19 oligomers, as previously shown in HL-60 cells [6].

Next, to investigate the dominant negative effects of the nonfunctional Q137N RP S19 oligomers on the haemin-induced differentiation of K562 cells by the neutrophil-like C5aR without receptor internalisation, we transfected the EGFP-ANXA3 K562 cells or the nonlabelled K562 cells with the Wild-type RP S19 and Gln137Asn mutant RP S19 cDNAs (Mock, Wild RP S19 and Q137N RP S19 K562 cells). As shown in Figure 4, almost all the EGFP-ANXA3/Mock K562 erythrocyte-like cells were necrotic and did not demonstrate clear plasma membranes when grown under monocell culture conditions on day 20 after the haemin induction.

To confirm these pathological findings, the Mock K562 erythrocyte-like cells not expressing EGFP-ANXA3 were collected for the FACS analysis of CD235a and/or CD71 expression (Figure 4). Using FACS, we detected $0.77 \pm 0.12\%$ of $CD235a^+/CD71^-$ cells (5.9 cells) in $0.1 \pm 0.2\%$ of the cells exhibiting viability rates of $15.3 \pm 6.4\%$ (7.7×10^2 cells) recovered from the starting Mock K562 cells (5×10^7 cells), indicating that the necrotic cells resembled the haemin-induced K562 erythrocyte-like cells.

In contrast to the EGFP-ANXA3/Mock K562 cells, the EGFP-ANXA3/Wild RP S19 K562 erythrocyte-like cells occasionally exhibited red spots on their clear plasma membranes that were caused by the production of haemoglobin-like molecules. Conversely, the differentiated EGFP-ANXA3/Q137N RP S19 K562 cells exhibited green spots on their clear plasma membranes, indicating that haemoglobin-like molecules were not produced. Using FACS, we detected $26.66 \pm 6.43\%$ of $CD235a^+/CD71^-$ cells (4.7×10^2 cells) in $1.0 \pm 0.4\%$ of the cells exhibiting viability rates of $35.1 \pm 14.7\%$ (1.8×10^3 cells) recovered from the starting Wild RP S19 K562 cells. In contrast, we detected $4.41 \pm 2.14\%$ of $CD235a^+/CD71^-$ cells (8.0×10^2 cells) in $8.0 \pm 4.1\%$ of the cells exhibiting viability rates of $45.6 \pm 4.7\%$ (1.8×10^4 cells) recovered from the starting Q137N RP S19 K562 cells (5×10^7 cells). These data indicated that the effect of the RP S19 oligomers on the pro-apoptotic signal is crucial for the haemin-induced K562 cell differentiation.

To examine the direct effects of RGS3, induced by the RP S19 oligomer-C5aR binding, on the haemin-induced differentiation of K562 cells, we transfected the EGFP-ANXA3 K562 cells or non-labelled K562 cells with the RGS3

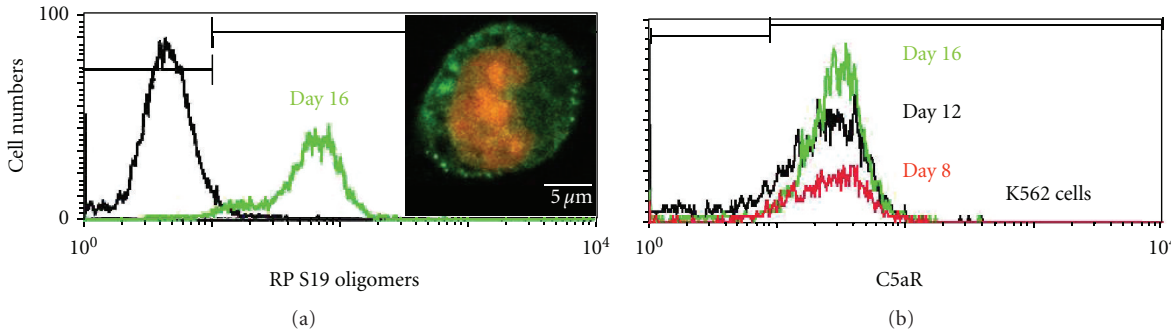


Figure 3: Generation of RP S19 oligomers and expression of the C5aR during the haemin-induced differentiation of K562 cells under monocell culture conditions K562 cells were harvested at several time points after the haemin induction and were stained with anti-C5a rabbit IgG or anti-C5aR mouse IgG for the FACS and CLSM analyses ($n = 3$).

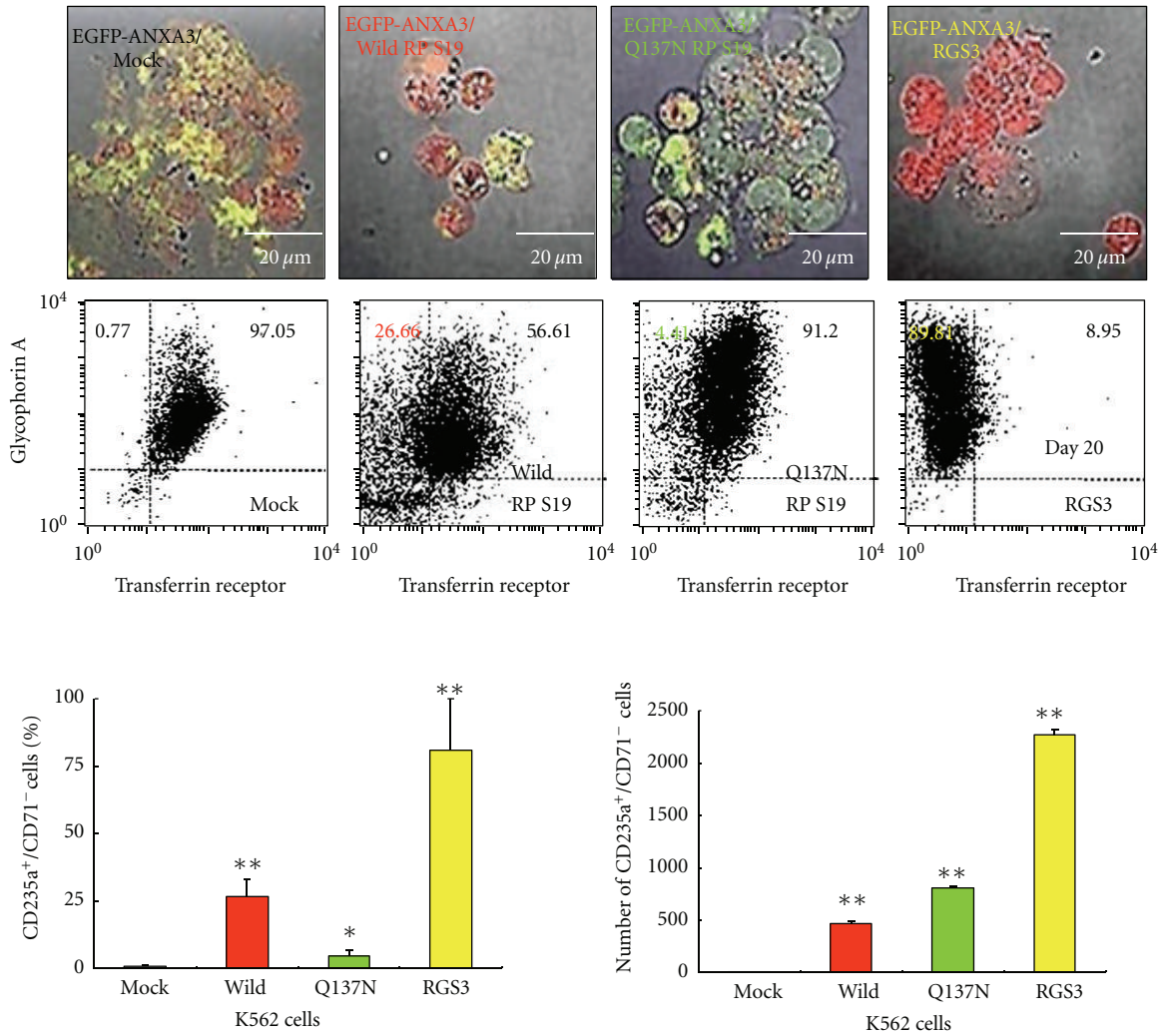


Figure 4: Contribution of the C5aR-RP S19 oligomer system to the haemin-induced terminal differentiation of K562 cells under monocell culture conditions. K562 cells expressing EGFP-ANXA3/Mock, EGFP-ANXA3/Wild-type RP S19, EGFP-ANXA3/Q137N mutant RP S19, and EGFP-ANXA3/RGS3 were harvested at several time points after the haemin induction and analysed under CLSM ($n = 3$). The mock (black), Wild-type RP S19 (red), Q137N mutant RP S19 (green), and RGS3 (yellow) K562 cells were harvested at several time points after the haemin induction and stained with FITC-conjugated anti-CD71 and APC-conjugated anti-CD235a mouse IgGs for FACS analyses ($n = 3$). The averages of the percentage and number of CD235a$^+$/CD71$^-$ erythrocyte-like cells were calculated on day 20 of the monocell culturing. P values of less than 0.05 were considered statistically significant ($^*P < 0.05$ or $^{**}P < 0.01$).

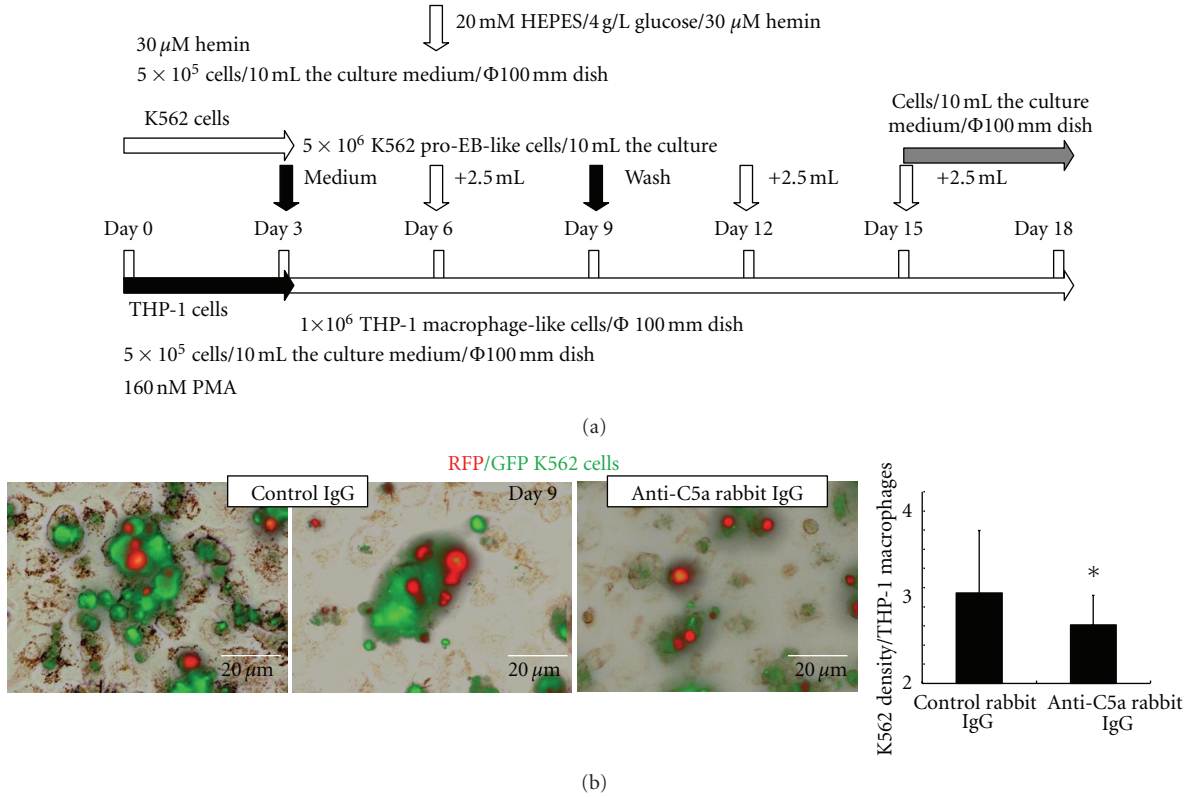


Figure 5: Contribution of the RP S19 oligomers to the K562-THP-1 cell interaction (a) Time schedule for the K562 cell that were cocultured with the THP-1 cells. (b) K562 cells expressing RFP-Nucleus/EGFP-ANXA3 were cultured for 9 days after the haemin induction and were examined under fluorescence microscopy. The microscopic images (4 pairs) were used to count the number of K562 cells on each THP-1 macrophage-like cell using the Image-J software ($n = 3$). P values of less than 0.05 were considered statistically significant ($^*P < 0.05$).

cDNA (RGS3 K562 cells). The EGFP-ANXA3/RGS3 K562 erythrocyte-like cells with clear plasma membranes exhibited red spots caused by the production of haemoglobin-like molecules (Figure 2(b)). Using FACS, we routinely detected 80.81 ± 19.28% of $CD235a^+/CD71^-$ cells (2.3×10^4 cells) in 1.0 ± 0.4% of the cells exhibiting viability rates of 56.2 ± 12.1% (2.8×10^2 cells) recovered from the starting RGS3 K562 cells.

These data indicated that the apoptotic C5aR was expressed and the RP S19 oligomers were generated in the K562 EB-like cells. The C5aR bound by RP S19 oligomers, which were released from the K562 basophilic-EB-like cells in an autocrine manner, induced the initiation of the transcription of *RGS3* without receptor internalisation. The RGS3 decreased the ERK1/2 signalling and shifted the pro-apoptotic signal towards apoptosis. We only showed the modulation of one signalling pathway during the haemin-induced differentiation of the K562 cells.

3.4. Effects of Coculturing with THP-1 Macrophage-Like Cells on the Haemin-Induced Differentiation of K562 Cells. To examine the effects of coculturing with macrophages on the haemin-induced differentiation of K562 cells, we used K562 cells expressing the RFP nuclear entry signal-Tag and EGFP-ANXA3 cDNAs (RFP/EGFP K562 cells) and unlabelled THP-1 cells. The RFP/EGFP K562 cells differentiated in the culture medium within 3 days after the haemin induction. The differentiated RFP/EGFP K562 cells were washed 3 times with PBS, and 5×10^6 cells were resuspended in 10 mL of the culture medium containing the control rabbit IgG or the anti-C5a rabbit IgG. The cells were mixed with the THP-1 macrophage-like cells (5×10^5 cells) that were treated with PMA for 3 days (Figure 5(a)). The K562 cells that were not bound to the THP-1 macrophage-like cells were removed using 3 PBS washes on day 6 of the coculturing, and the remaining RFP/EGFP K562 basophilic EB-like cells that were bound to the THP-1 macrophage-like cells were examined under fluorescent microscopy (IX70, Olympus) (Figure 5(b)). The fluorescent images were analysed using the NIH Image-J software to determine the number of bound K562 basophilic EB-like cells, which were visible as solid red circles. In the presence of the control rabbit IgG, 3.2 ± 0.35 RFP/EGFP K562 basophilic EB-like cells were bound to one THP-1 macrophage-like cell. However, the number of bound RFP/EGFP K562 basophilic EB-like cells to each THP-1 macrophage-like cell was reduced significantly to 2.6 ± 0.32 in the presence of the anti-C5a rabbit IgG.

To examine the effects of coculturing on further differentiation of the cells, the K562 cells that were not bound to the THP-1 macrophage-like cells were harvested from the culture medium on days 9 and 15 after the haemin induction and were analysed using FACS and CLSM. Using FACS, the K562 orthochromatic-EB-like cells (R2 in Figure 2(a) in Supplemental Material) and the relatively small K562 reticulocyte-like cells without nuclei (R3 in Figure 2(a) in Supplemental Material) were clearly distinguishable as RFP^{high}/GFP^{high} cells and $RFP^{middle}/GFP^{middle}$ cells, respectively. In addition, we prepared K562 cells or THP-1 cells bearing the RFP cDNA or the EGFP-ANXA3 cDNA, and the RFP K562 pyrenocyte-like cells were observed in the EGFP-ANXA3 THP-1 macrophage-like cells under CLSM on day 15 after the haemin induction (see Figure 2(b) in Supplemental Material).

The EGFP-ANXA3 K562 reticulocyte-like cells that were disassociated from the unlabelled THP-1 macrophage-like cells were harvested from the culture medium on day 15 after the haemin induction and were recultured in the culture medium in the absence of haemin for additional 3 days. Using fluorescence microscopy (IX70), the EGFP-ANXA3 K562 basophilic-like cells containing the RFP-labelled nuclei were shown to colocalise with the unlabelled THP-1 macrophage-like cells (see Figure 3(a) in Supplemental Material). Only the cells containing the RFP-labelled nuclei colocalised with the unlabelled THP-1 macrophage-like cells (see Figure 3(b) in Supplemental Material). Conversely, we detected the EGFP-ANXA3 K562 reticulocyte-like cells with no RFP-labelled nuclei in the culture supernatants using CLSM (see Figure 3(c) in Supplemental Material). Interestingly, the EGFP-ANXA3 disappeared in the K562 reticulocyte-like cells and induced the production of haemoglobin-like molecules when the cells were cultured for additional 3 days in the absence of haemin (see Figure 3(d) in Supplemental Material).

3.5. Direct Observation of the Dominant Negative Effect of Gln137Asn Mutant RP S19 Overexpression on the Interaction of K562 Basophilic-EB-Like Cells with the THP-1 Macrophage-Like Cells Using Time-Lapse Microscopy. To confirm the data using time-lapse microscopy, we used the EGFP-ANXA3/Wild RP S19 K562 cells, the EGFP-ANXA3/Q137N RP S19 K562 cells, and the unlabelled THP-1 cells. The K562 cells that differentiated in the culture medium within 5 days of the haemin induction were washed 3 times with 3 mL of PBS, and 1×10^5 cells were resuspended in 2 mL of fresh culture medium in the absence of haemin (Figure 6(a)). In contrast to the K562 cells, the THP-1 cells were differentiated into macrophage-like cells within 2 days of PMA treatment in a glass-bottom dish. The cells were cocultured and were examined under the fluorescence microscope LCV110 (Olympas) for 1–3 days.

The EGFP-ANXA3/Wild RP S19 K562 basophilic-EB-like cells bound to the THP-1 macrophage-like cells within 12 hr of coculturing and started to move about on the THP-1 macrophage-like cells (Figure 6(b)). The haemoglobin-like molecules appeared in a time-dependent manner. However, the EGFP-ANXA3/Q137N RP S19 K562 pro-EB-like cells did not bind to the THP-1 macrophage-like cells during the 48 hr coculturing (Figure 6(c)).

3.6. Direct Observation of Effects of RP S19 Oligomer Inhibitor and the C5aR Inhibitor on the Interaction of the K562 Basophilic-EB-Like Cells with the THP-1 Macrophage-Like Cells Using Time-Lapse Microscopy. The haemin-induced differentiation of the EGFP-ANXA3 K562 cells on the THP-1 macrophage-like cells within 72 hours of coculturing was reproducible. This cellular differentiation was not modified by the presence of either the control rabbit IgG (5 μg/mL) or the C3aR antagonistic peptide (10^{-6} M SB 290157) (Figures 7(a) and 7(e)). However, the interaction between the K562 basophilic-EB-like cells and the THP-1 macrophage-like cells was inhibited when the RP S19 oligomers were neutralised using anti-C5a rabbit IgG (5 μg/mL) or when the ligand-binding site of the C5aR was blocked using the C5aR antagonistic peptides (10^{-6} M PMX-53 and W-54011) (Figures 7(b)–7(d)), preventing the haemin-induced differentiation of the K562 cells. The average maximal number of cells containing haemoglobin-like molecules from 4 random fields at 72 hr after coculturing was 15.00 ± 2.16 and 15.25 ± 2.06 in the presence of the control rabbit IgG and SB 290157, respectively. However, the number of red cells decreased to 4.00 ± 3.65, 6.75 ± 0.96, and 8.25 ± 6.13 in the presence of the anti-C5a rabbit IgG, PMX-53, and W-54011, respectively.

4. Discussion

4.1. The RP S19 Oligomer-C5aR System in Apoptotic Cells. We have demonstrated that the RP S19 monomer translocates to the cytoplasmic plasma membrane in almost all apoptotic cells and interacts with the Lys_{33}–Lys_{39} moiety of the negatively charged phosphatidylserine [31, 32]. After the cross-linking of residues Lys122 and Gln137 by an activated TGII, RP S19 oligomers are released by the flip/flop system and function as monocyte C5aR ligands to induce migrating cells into apoptosis [33]. Interestingly, we have previously reported that the NF-Y transcription factor binds to the CCAAT box in the promoter region of *C5aR* even though the cells do not constantly express *C5aR* [23]. When the RP S19 oligomers bind to the apoptotic C5aR, *RGS3* is expressed and the programmed cell death process is induced with the decrease in the ERK1/2 signalling. Phosphatidylserine exposure on the extraplasmic plasma membrane is crucial for recognition by macrophages [34]. In addition, microparticles, such as ectosomes and exosomes, bud directly from the cell surface of apoptotic cells accompanied by the loss of cell organelles [35, 36]. The RP S19 oligomer-C5aR system appears to synchronise the execution of apoptosis with the recruitment of macrophages. This mechanism may explain the maintenance of homeostasis without inflammatory cues.

4.2. C5aR on the Haemin-Induced Differentiation of K562 Cells. The *C5aR* is expressed not only in myeloid cells but also in various nonmyeloid cells including microvascular

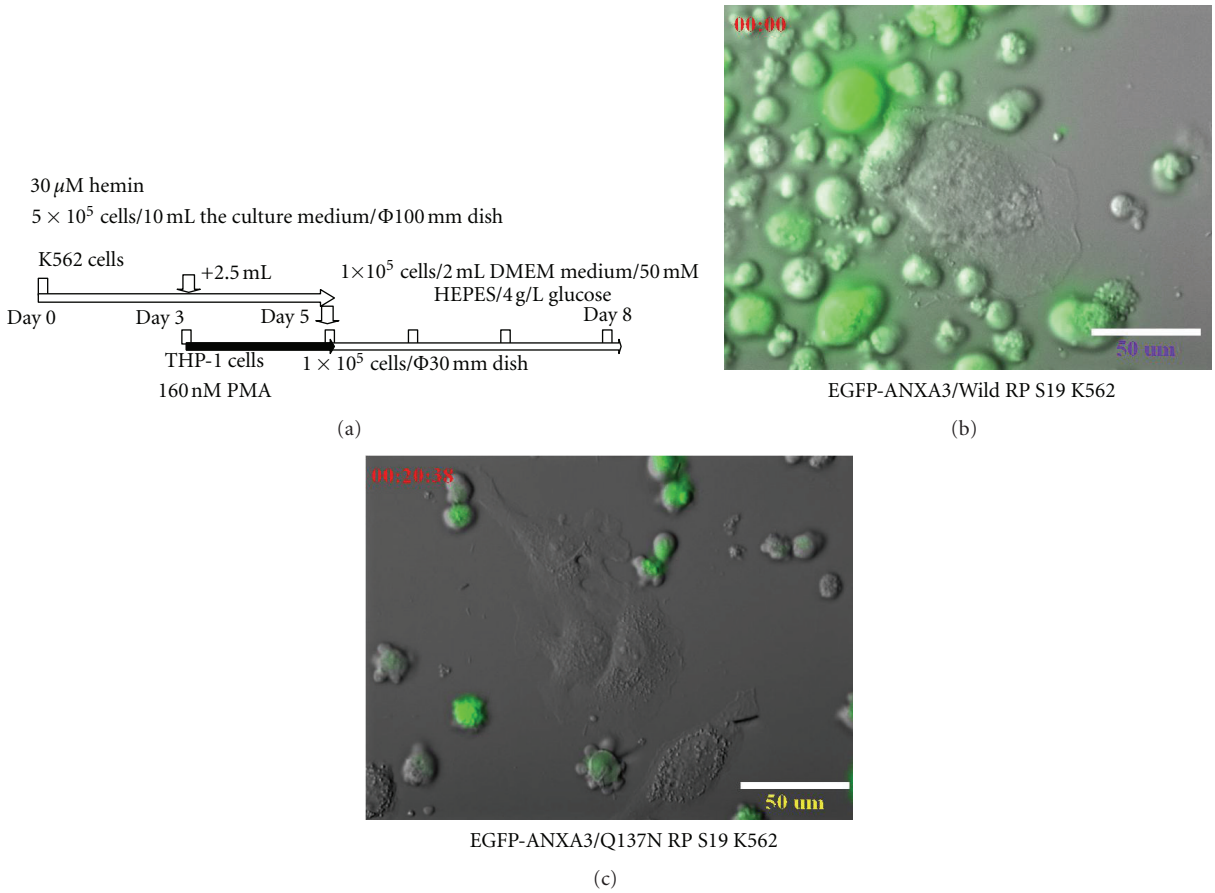


FIGURE 6: Detection of the contribution of the K562-THP-1 cell interaction to the haemin-induced terminal differentiation of K562 cells using time-lapse microscopy. (a) Time schedule for the K562 cells that were cocultured with the THP-1 cells. K562 cells expressing EGFP-ANXA3/Wild-type RP S19 (b) and EGFP-ANXA3/Gln137Asn mutant RP S19 (c) were cultured with the THP-1 macrophage-like cells for 1–3 days and monitored using the LCV110 microscope ($n = 3$).

endothelial cells, articular chondrocytes, and stimulated hepatocytes [3]. Interesting studies have reported that the erythroid-specific transcriptional activation of *growth factor-independence-1B* and *nucleolar spindle-associated protein* genes contributes to the functional cooperation between GATA-1 and NF-Y in immature K562 cells and mature erythroleukemia MEL cells [16, 17]. This study may be the first report the generation of the C5aR protein on the cell surface of K562 cells during the pro-EB-like cell stage to the orthochromatic EB-like cell stage (Figure 3).

4.3. The RP S19 Oligomer-C5aR System during the Haemin-Induced Differentiation of K562 Cells. In this study, we generated the EGFP-ANXA3 K562 cells to examine the RP S19 oligomer-C5aR system using microscopy. ANXA3 binds to the phosphatidylserine of dying cells using its calcium-binding and lipid-interacting moieties as markers for macrophages [19]. Interestingly, we observed membrane blebbing-like formations composed of vacuole-like vesicles that contained high amounts of EGFP-ANXA3 in the K562 pro-EB-like cells, similar to those detected in apoptotic K562 cells after 6 hr of $MnCl_2$ treatment (Figure 1). Similar morphological features were reported during erythropoiesis to allow the clearance of the transferrin receptor and the loss of cell organelles in apoptotic cells [36]. We propose that the RP S19 monomer in the K562 EB-like cells moves to the phosphatidylserine on the cytoplasmic plasma membrane and is cross-linked by TGII, similar to the events that occur during apoptosis [4]. Interestingly, we detected fluorescence spots that were relatively large in size and cross-reacted with anti-C5a rabbit IgG not only on the cytoplasmic plasma membrane but also in the cytoplasm (inside in Figure 3). Recently, vesicle curvature has been shown to change the optimal binding of lactadherin to phosphatidylserine in apoptotic cells [37]. These data suggested that the RP S19 monomer in the K562 EB-like cells translocates to the phosphatidylserine on the cytoplasmic plasma membrane and on vacuole-like vesicles.

4.4. Effects of the Interactions of the THP-1 Macrophage-Like Cells with the K562 Basophilic-EB-Like Cells during Erythropoiesis. Bone marrow macrophages play an important role

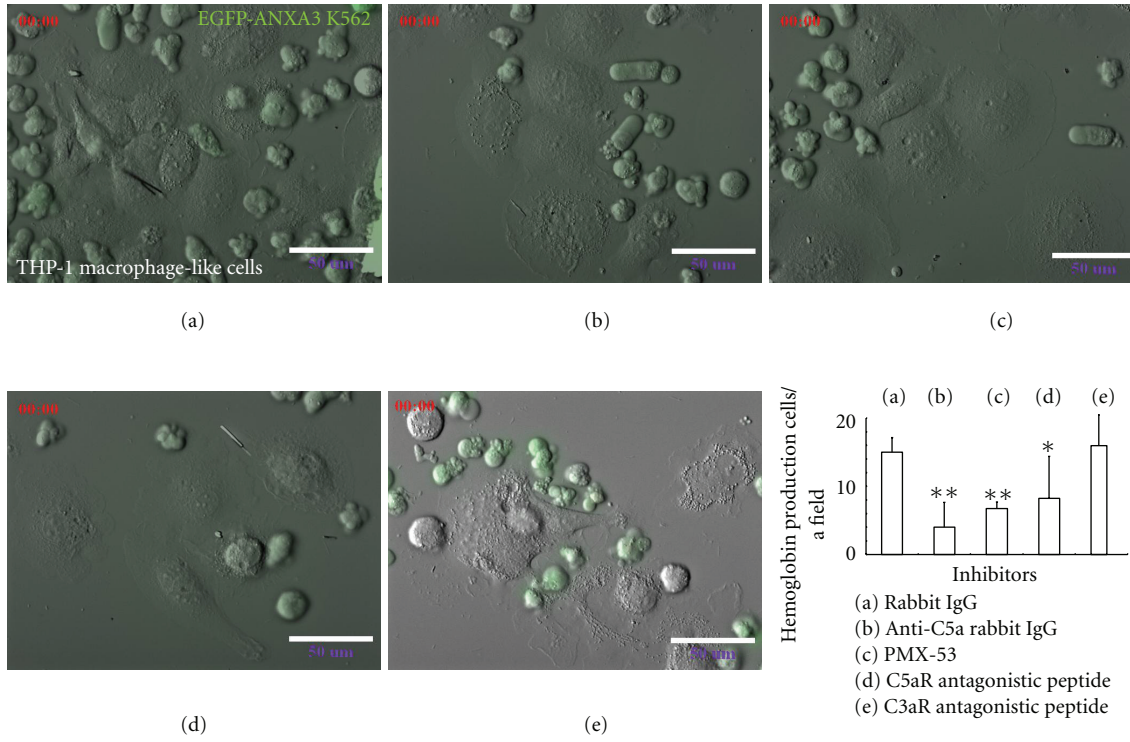


Figure 7: Detection of the inhibition of the K562-THP-1 cell interactions by the inhibitors of the C5aR-RP S19 oligomer system using time-lapse microscopy. ((a)–(e)) EGFP-ANXA3-overexpressing K562 cells were cultured with the THP-1 macrophage-like cells in the presence of control rabbit IgG, anti-C5a rabbit IgG, PMX-53, W-54011, or SB 290157 for 1–3 days and were recorded using the LCV110 microscope ($n = 3$). The maximal number of cells containing haemoglobin-like molecules at 72 hr of coculturing was counted automatically from 4 random fields, and the averages were calculated. P values of less than 0.05 were considered statistically significant ($^{*}P < 0.05$ or $^{**}P < 0.01$).

in creating a special microenvironment for EBs [38]. For example, the interaction between the erythroblast intercellular adhesion molecule-4 with the macrophage αV integrin is crucial for the formation of the EB-macrophage island [39]. In this study, we demonstrated that the interaction of the K562 basophilic-EB-like cells with the THP-1 macrophage-like cells was inhibited by overexpressing the Gln137Asn mutant RP S19, by neutralising the RP S19 oligomers using anti-human C5a rabbit IgG or by blocking C5aR with the C5aR antagonistic peptides (Figures 5–7). Our data indicated that the K562 basophilic-EB-like cells express the C5aR and generate RP S19 oligomers. The released RP S19 oligomers attract the THP-1 macrophage-like cells, and both cells are connected by RP S19 oligomers via their C5aRs without the internalisation of the receptor. The K562 basophilic EB-like cells move about on the THP-1 macrophage-like cells through the interaction of erythroblast-associated molecules with the macrophage integrins. However, there are no reports of erythroblastopenia in *C5aR* knockout mice; it is likely that redundant mechanisms involving other chemotactic receptors exist in these knockout mice [40].

The morphological changes associated with the maturation of haemin-induced K562 cells *in vitro* under monocell culture conditions take four times longer than the process of erythroid maturation in the bone marrow (Figure 2(b)); however, the maturation process was shortened by the *in vitro* coculture conditions used (Figures 5(a) and 6(a)). Only 5.9 K562 erythrocyte-like cells were recovered from the 5×10^7 K562 cells under monocell culture conditions (Figure 4); however, a relatively larger number of K562 erythrocyte-like cells were recovered following coculture with the THP-1 macrophage-like cells. These data highlight the roles of the macrophages during the haemin-induced differentiation of K562 cells. The macrophages of the EB-macrophage islands in the bone marrow negatively and positively modulate the balance between the survival and apoptotic signals of EB by releasing various cytokines, including interleukin-1, granulocyte-macrophage colony-stimulating factor, transforming growth factor-β, and activin, to maintain and promote erythropoiesis [39].

In contrast, the RP S19 oligomers released from the K562 basophilic-EB-like cells activated both the K562 erythrocyte-like cells and the THP-1 macrophage-like cells via the C5aR. However, when the K562 basophilic-EB-like cells and the THP-1 macrophage-like cells were cultured in trans-well chambers, we observed low numbers of the K562 erythrocyte-like cells (see Figure 4 in Supplemental Material). Interestingly, we occasionally observed the EGFP-ANXA3 from the K562 cells as green spots in the THP-1 macrophage-like cells (see Figure 3(b) in Supplemental

Material). Therefore, we need to examine further the direct transfer of intracellular proteins between the basophilic-EB-like cells and the bone marrow macrophages.

Acknowledgment

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Review Article

Role of Complement in Multiorgan Failure

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Multiorgan failure (MOF) represents the leading cause of death in patients with sepsis and systemic inflammatory response syndrome (SIRS) following severe trauma. The underlying immune response is highly complex and involves activation of the complement system as a crucial entity of innate immunity. Uncontrolled activation of the complement system during sepsis and SIRS with in excessive generation of complement activation products contributes to an ensuing dysfunction of various organ systems. In the present review, mechanisms of the inflammatory response in the development of MOF in sepsis and SIRS with particular focus on the complement system are discussed.

1. Introduction

In the 1970s, a syndrome of progressive, sequentially dysfunctional organ systems has been firstly characterized, eventually referred to as multiorgan failure (MOF) [1, 2]. As a predominant underlying condition, sepsis and sepsis-associated MOF represent one of the leading causes of death of hospitalized patients with reported morality rates ranging from 28% to 56% [3, 4]. Likewise, severe trauma and trauma-related multiorgan failure remain the leading cause of death in people below the age of 40 [5, 6].

The conception of organ failure has changed over the years and various scoring systems for the classification and diagnosis of MOF exist all of which attempt to quantify the degree of organ failure [7–9]. Currently, MOF is regarded as a continuous process of varying levels of organ failure rather than an all-or-none event [10]. To characterize MOF, six different organ systems are regarded as "key organs": lungs, cardiovascular system, kidneys, liver, coagulation system, and central nervous system.

Depending on the severity and various predisposing conditions, the initial insult (tissue trauma, infection) can induce a systemic host response that is characterized by the release of pro- and anti-inflammatory cytokines and metabolites (e.g., reactive oxygen (ROS) and nitrogen species (NOS)), activation of plasmatic cascade systems, such as the complement and the coagulation systems, and the appearance of acute phase proteins as well as hormonal and neuronal mediators [11–13]. Imbalanced systemic immune responses can ultimately lead to accumulation of leukocytes, disseminated intravascular coagulation (DIC), and microcirculatory dysfunction with subsequent apoptosis and necrosis of parenchymal cells, finally resulting in the development of MOF [12, 14, 15].

As a central entity of innate immunity, the complement system is immediately activated after trauma or infection in order to control the replication of intruding pathogens. In humans, the plasma levels of complement activation products rise early, are persistently elevated in patients after thermal injury, trauma, and sepsis, and correlate with the severity of injury and inversely with the outcome [16–22]. It is well established that activation of the complement cascade alters functional responses of neutrophils (PMN) in the course of systemic inflammation and contributes to the development of organ failure [15, 23]. In experimental sepsis, the blockade of complement anaphylatoxin C5a virtually prevented the appearance of MOF and improved the outcome [24–26]. Previous studies strongly suggest a mutual

crosstalk between the complement and the coagulation system [27–30]. Due to the complex nature of plasmatic cascades and their interconnections, the role and regulations of the complement system, especially in states of disease, are still inadequately understood.

This article is sought to provide insights into the pathogenesis of multiorgan failure associated with systemic inflammation with particular focus on the role of the complement system. Furthermore, potential therapeutic strategies targeting the complement cascade to prevent the development of MOF as well as possible future research directions are addressed.

2. Pathways of Complement Activation

The complement system can be activated via four different pathways, the classical, the alternative, and the lectin pathway [31–33]. All three pathways lead to the assembly of the C3 convertase which cleaves C3 into C3a and C3b [31, 32]. Incorporation of C3b into the C3 convertase results in formation of the C5 convertase, which cleaves C5 into C5a and C5b. The split products C3a and C5a act as potent anaphylatoxins. C3b is an important opsonic factor, while C5b initiates the formation the membrane attack complex (C5b-9). In addition, various non-complement serine proteases seem to cleave complement components into biologically active complement products with variable efficacy [34]. In particular, thrombin has been found to function as a C5-convertase that does not require the presence of C3 or C3b [28]. Moreover, proteases from PMN and macrophages can cleave C5 as well [35, 36].

There is evidence that all three complement activation pathways are activated in SIRS and sepsis. Interestingly, it has been demonstrated that during the course of sepsis alternative pathway activation occurs earlier than activation of the classical pathway [37]. Based on their distinct mechanisms and kinetics of activation, it has been hypothesized that classical pathway activation in sepsis plays a crucial role in the clearance of pathogenic factors, while the alternative pathway is thought to be essential for fighting against infections by invading microorganisms [38]. Although the knowledge about the underlying mechanisms is limited, recent reports suggest a particular role of mannose-binding lectin (MBL) and the lectin pathway in the development of MOF. In sterile systemic inflammation (systemic inflammatory response syndrome, SIRS), patients with functional MBL deficiency due to MBL consumption did not develop MOF unless MBL was reconstituted by transfusion of fresh frozen plasma [39]. In contrast, septic patients with MBL depletion showed significantly higher sequential organ failure assessment (SOFA) scores, whereas functional MBL levels and activity in sepsis were associated with moderate SOFA scores and better prognosis [40], suggesting that MBL might be essential for defence against infections on the one hand, but might also harm the host and contribute to the development of MOF on the other hand. Therefore, as indicated by this dual function of the lectin pathway, the role of the complement system in systemic inflammation sometimes is referred to as a double-edged sword.

3. Dysfunction of the Central Nervous System

Historically, the central nervous system (CNS) was defined as an "immunological privileged organ" because of its separation from peripheral circulation by the blood-brain barrier (BBB). However, it became evident that the CNS is a rich source of inflammatory mediators and complement proteins can be produced by neurons, astrocytes, microglia, and oligodendroglia [41–43]. Severe trauma and sepsis are associated with systemic inflammation that can lead to blood-brain barrier (BBB) dysfunction and cerebral edema, regardless of the presence of traumatic brain injury (TBI) [44]. The breakdown of the BBB is considered to be a key event in the development of septic encephalopathy, while the cellular and molecular mechanisms of sepsis-induced brain damage are still vastly unknown [45]. Interestingly, the direct contact between blood and cerebrospinal fluid leads to complement activation, and the extent of intrathecal complement activation is associated with BBB dysfunction [46]. In addition, intracerebral complement levels increase under pathological conditions due to leakage of serum-derived complement proteins into the subarachnoidal space after breach of the BBB as well as increased complement biosynthesis in the CNS [47]. C1q, C3a, and C5a contribute to intracranial inflammation by induction of BBB damage and increase in vascular permeability [47, 48]. Blood-derived leukocytes, predominantly PMN, are then able to transmigrate into the CNS and release proteases and free radicals resulting in tissue damage (Figure 1) [47, 49]. In line with this, in experimental sepsis blockade of C5a attenuated pathophysiological changes that are typically associated with septic encephalopathy [50]. C3 and its derivates seem to play a central role in the pathogenesis of CNS dysfunction. Accumulation of C3 fragments is related to neuronal cell death and intracerebral PMN infiltration [51]. Previous studies suggested that the alternative pathway activation is a leading mechanism for neuronal cell death after closed head injury [52, 53]. C5a can induce neuronal apoptosis via the interaction with its receptor (C5aR), which is abundantly expressed on various cell types in the CNS [54, 55]. Finally, inactivation of the complement regulatory proteins on neurons during inflammation pave the road for complement-mediated lysis of homologous cells by the membrane attack complex [56]. Despite the unambiguous involvement in various pathological mechanisms, the role of the complement system in the pathogenesis of CNS dysfunction appears to be a double-edged sword since it has been reported that C3a as well as C5a also may mediate neuroprotective and neuroregenerative effects [57, 58].

4. Respiratory Failure

Respiratory failure or acute respiratory distress syndrome (ARDS) represents a frequent complication after burn injury, multisystem trauma, shock, and systemic inflammation [59–61]. Although the liver represents the main source for the production of complement proteins, virtually all complement proteins can be locally produced in the lung by type II alveolar pneumocytes, alveolar macrophages,

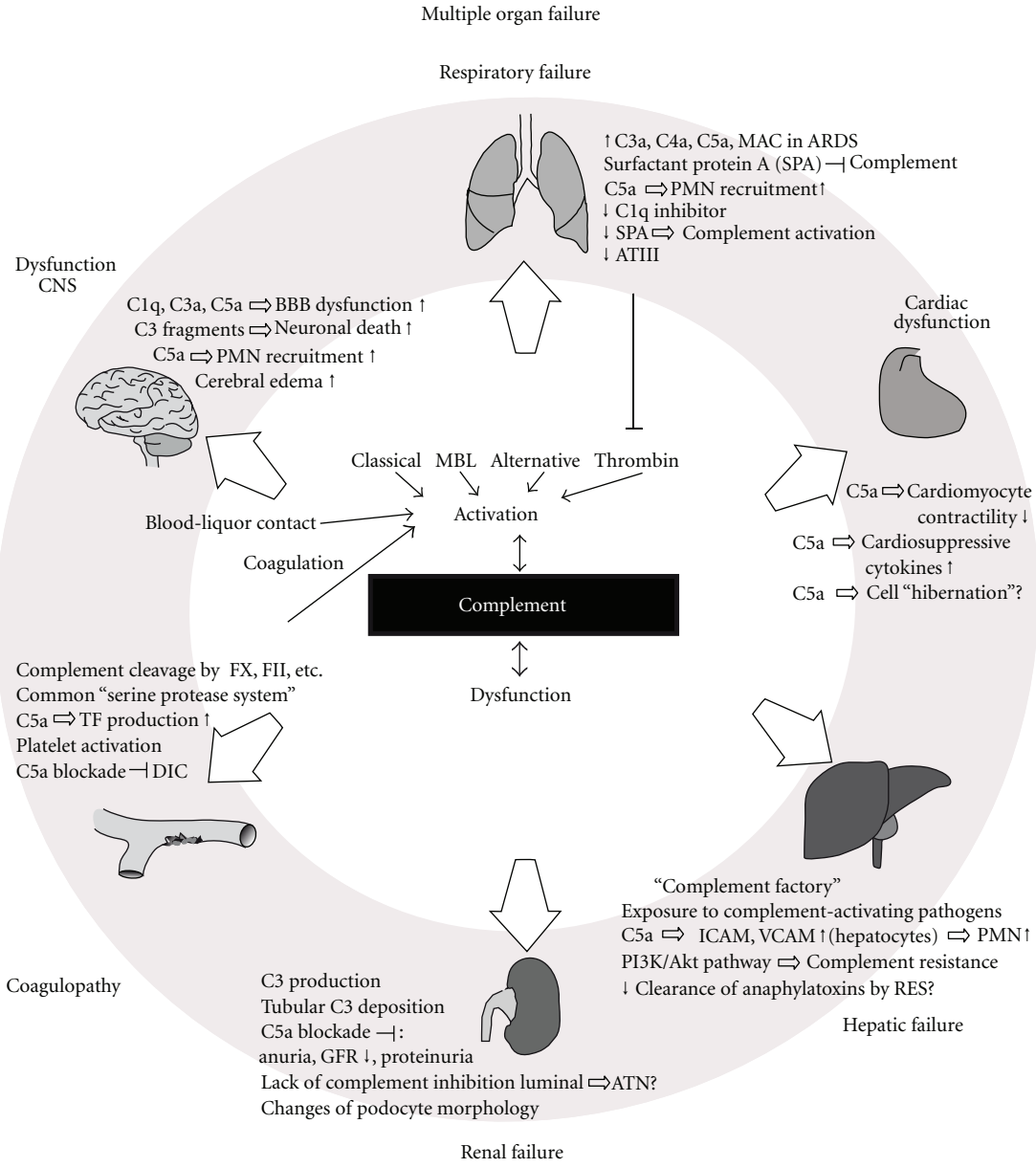

Figure 1: Summarizing illustration on the effects of excessive complement activation on various organ systems and the development of organ failure. For details see text. MBL: mannose-binding lectin, CNS: central nervous system, BBB: blood brain barrier, PMN: polymorphonuclear neutrophils, ARDS: acute respiratory distress syndrome, ATIII: antithrombin III, RES: reticuloendothelial system, GFR: glomerular filtration rate, ATN: acute tubular necrosis, FX: coagulation factor X, FII: coagulation factor II, TF: tissue factor, DIC: disseminated intravascular coagulation.

and lung fibroblasts [62–64]. While the total pulmonary complement protein concentration is at comparable levels as found in serum, its activity in normal lung is markedly reduced which is attributed to the ability of surfactant protein A (SPA) to inhibit complement [65, 66]. In various studies, patients with ARDS showed evidence for robust complement activation, the extent of which correlated with the degree and outcome of ARDS [67, 68]. In particular, the complement anaphylatoxin C5a and the MAC are in the focus of ARDS pathophysiology, but also elevated levels of C3a and C4a have been linked to the development of ARDS [68–72]. C5a promotes inflammation by causing extensive influx of activated PMN into lung tissue and the alveolar space and by enhancement of the early cytokine response (reviewed in [72, 73]). However, only little is known about the local regulation of complement activation. Besides, the complement inhibitory function of SPA, C1 inhibitor, which inhibits classical pathway activation, has been detected in human bronchoalveolar lavage fluids [65, 66]. Lung activity of both, surfactant protein and C1 inhibitor, is significantly reduced in patients with trauma-related ARDS [74, 75]. Beside complement activation, ARDS is accompanied by

tissue factor generation and widespread pulmonary fibrin deposition [76, 77]. Here, antithrombin III (ATIII), which inhibits activated proteases including thrombin, seems to play a central role since ATIII levels inversely correlate with the outcome in the setting of sepsis, and ATIII has been shown to block the thrombin pathway of complement activation in a murine model of acute lung injury (Figure 1) [28, 78, 79]. In conclusion, systemic inflammation provokes local imbalances of the complement and the coagulation cascade shifting the lung equilibrium to a proinflammatory and procoagulant state, which then stimulates accumulated leukocytes to locally release cytokines, enzymes, and radicals that promote the classical features of ARDS.

5. Cardiac Dysfunction

Heart dysfunction during inflammatory states shows a biphasic process with an early hyperdynamic phase followed by a pivotal hypodynamic phase [80]. Hallmarks of the hypodynamic phase are decreased cardiac output, reduced microvascular flow, and increased peripheral vascular resistance with rising plasma levels of catecholamines. It has been suggested that these changes initiate the vicious circle of multiorgan failure due to compromised organ perfusion, decreased oxygen and nutrient supply, and ischemia [81]. Various myocardial depressant factors that collectively trigger cardiac contractility deficits in systemic inflammation have been described, but no single agent responsible for myocardial dysfunction could be identified [81–87]. In previous reports, complement activation has been linked to hemodynamic depression, but the mechanisms by which complement activation products might cause dysfunction of cardiomyocytes remain to be defined in detail [81, 88, 89]. In experimental studies, C5a has been demonstrated to induce cardiac dysfunction with impaired cardiomyocyte contractility, which could be restored by blockade of C5a [90, 91]. But it is far from certain if C5a-C5aR interaction directly causes cellular alterations in cardiomyocytes that lead to impaired calcium handling, oxygen and ATP depletion, and loss of mitochondria with energy deficit [92, 93]. Recent research suggests that C5a causes the local release of cardiosuppressive cytokines and chemokines in cardiomyocytes eventually leading to cardiac dysfunction [94]. But it is also conceivable that complement anaphylatoxins contribute to induce "hibernation" in cardiomyocytes as it occurs in the response of the myocardium to ischemia [95]. In the ischemic heart, it is a common observation that the induction of contractile dysfunction by C5a is not a direct effect but rather involves secondary production of mediators (e.g., arachidonic acid metabolites), which then act on target cells (Figure 1) [96]. Further, predominantly the classical and the alternative pathway are activated upon myocardial ischemia. Treatment with C1 inhibitor or soluble complement receptor 1 has cardioprotective effects by suppression of adhesion molecule expression (p-selectin, ICAM-1), blockade of C3 deposition and its activity on cardiomyocytes, and by antiapoptotic activity [97–100]. However, it remains to be evaluated whether similar events de facto occur in cardiac dysfunction during systemic inflammation.

6. Hepatic Failure

The liver represents the "major production facility" for most complement proteins found in the blood compartment except C1q, factor D, and properdin [101]. Because of its integral role in metabolism and host defense, the liver plays a key role in the initiation of MODS [102, 103]. Enhanced interaction of leukocytes with hepatic endothelial cells and hepatic microperfusion disorders are fundamental contributors to liver failure during sepsis [104]. Like in other organs, complement activation products are generated among other inflammatory mediators during systemic inflammation, which initiate a cascade of intracellular events in target cells leading to upregulation of adhesion molecules (ICAM-1, VCAM-1) on hepatic epithelial cells, increase of vascular permeability, and priming and influx of leukocytes [104, 105]. Treatment with C1 inhibitor reduced VCAM expression and hepatic leukocyte adhesion in experimental acute hepatic failure, even after delayed injection [104]. Besides this mechanism, PMN mediate parenchymal damage after accumulation in sinusoids, which does not depend on cellular adhesion molecules [106]. The liver is not only the main source of complement proteins but is also constantly exposed to complement-activating pathogens via the portal venous system [107, 108]. Immune complexes, anaphylatoxins, and activated complement components are cleared from circulation by the reticuloendothelial system lining the sinusoids without being detriment to hepatic function [101]. However, the efficiency of the reticuloendothelial system does not suffice to protect the liver. Therefore, hepatocytes are endowed with a unique mechanism to protect themselves from complement-induced cytotoxicity [107]. It is intriguing that this protection is not dependent on the complement regulatory proteins on the cell surface [107]. Instead, the inurement of hepatocytes to complement and its activated products requires the integrity of the PI3K/Akt pathway [107]. In turn, the PI3K/Akt pathway supposedly controls C5a-mediated effects in PMN and monocytes [109]. In experimental sepsis, anti-C5a treatment circumvented the development of MOF and attenuated markers of acute hepatic failure (e.g., bilirubin, ALT, AST, LDH) (Figure 1) [24]. Thus, it is tempting to speculate that under conditions, in which C5a is systemically generated, impairment of the PI3K/Akt pathway may lead to increased susceptibility for complement-mediated cytotoxicity of hepatocytes and subsequent organ failure. On the other hand, a potential role for C5a in tissue repair has been suggested [73].

7. Renal Failure

Acute renal failure (ARF) is hallmarked by abrupt decline in glomerular filtration and acute tubular necrosis in association with the appearance of multiple inflammatory mediators [110–112]. In sepsis, ARF occurs already at modest levels of hypotension suggesting that other mechanisms than ischemia are involved [110]. Like in parenchymal cells of lung and brain, complement proteins can be locally produced by renal cells, such as proximal tubular cells, *in vitro* and *in vivo* [113, 114]. In the case of C3, there is evidence that

its renal production even contributes to the circulating C3 pool [115]. Proximal tubular cells are capable of activating the alternative pathway, terminating in the binding of MAC to the cell surface [116]. In this context, it is of particular interest that the luminal brush border lacks complement regulatory proteins on the cell surface [117]. Under certain circumstances, paucity of protection against complement-mediated cell lysis predisposes to tubular damage due to the luminal deposition of filtered complement components [118]. The deposition of C3 and C4 is well established in glomerular disease, but only C3 deposition, and no evidence for C4 deposition, along tubules could be found in acute tubular necrosis after renal ischemia/reperfusion injury, indicating that the alternative pathway is the predominant complement activation pathway for the development of acute tubular necrosis [118]. However, suppression of C3 activation failed to affect the degree of ARF in a murine model of systemic inflammation, although C3 synthesis was upregulated, resulting in basolateral tubular C3 deposition [110]. In disagreement with these authors' conclusion, this does not necessarily mean that complement is not responsible for ARF in the setting of systemic inflammation since it is now known that the downstream complement cascade can be activated despite the absence of C3 [28]. In contrast, the occurrence of ARF could be clearly linked to the generation of C5a during experimental sepsis, and parameters of ARF (creatinine, urine output, glomerular filtration rate, proteinuria) as well as morphological changes of podocytes were greatly attenuated by anti-C5a treatment [24]. Beyond their local inflammatory and chemotactic features, C3a and C5a have vascular effects that contribute to changes in renal hemodynamics in ARF (Figure 1) [119]. Taken together, the complement system represents a key effector of ARF by a variety of mechanisms, which affect renal perfusion and glomerular filtration as well as tubular function.

8. Dysregulation of the Coagulation System

The coagulation system and the complement system are both proteolytic cascades composed of serine proteases that share structural characteristics. As descendants of a common ancestor, both systems can be basically activated by similar stimuli [120, 121]. Trauma and tissue injury often cause damage of the vasculature and subsequent bleeding, which is also associated with the risk of infection by intruding microorganisms [11]. Activation of both cascades is intended to occur locally under thorough regulations, but under certain circumstances, loss of control can lead to systemic activation with harmful consequences for the host [29]. Disseminated intravascular coagulation (DIC) represents a frequent complication after trauma, systemic inflammation, and sepsis [122, 123]. After the initial phase of hypercoagulability with intra- and extravascular fibrin clots, consumption of coagulation factors and dysfunction of thrombocytes can lead to hemorrhagic diasthesis and diffuse bleeding [79, 122, 123]. Intravascular fibrin clots are finally responsible for impaired microcirculation and hypoxic cellular damage [79]. Trauma, thermal injury, and infection predispose to thrombosis and the development of DIC and trigger the inflammatory response including complement activation, which, in turn, can trigger coagulation and vice versa [121, 123]. As mentioned above, thrombin is capable of cleaving C5, resulting in the generation of C5a. This concept of a direct crosstalk between central components of the complement and coagulation cascades is corroborated by the findings of elevated thrombin-antithrombin (TAT) complexes in the clinical and experimental setting of multiple injury [34]. Beside the C5-convertase activity of thrombin, various factors of the coagulation and fibrinolysis system, including FXa, FXIIa, plasmin, and kallikrein, can cleave complement components or their fragments [28, 124–126]. On the other hand, the inflammatory response and the complement system in particular amplify coagulation by modification of phospholipid membranes required for the initiation of the tissue factor (TF) pathway, activation of platelets, and upregulation of TF expression [121]. Specifically, activation of C5 can increase TF expression on leukocytes and blockade of C5a-ameliorated DIC in a rodent model of sepsis [27, 127]. The procoagulant activities of complement are aggravated by inhibition of anticoagulant mechanisms, such as complex formation of C4b-binding protein with protein S (PS), which results in a loss of PS cofactor activity for activated protein C (APC) [128]. In turn, the protein C anticoagulant pathway does not only function as a regulator of the coagulation cascade by degradation of FVa and FVIIIa, but also dampens the inflammatory response [121, 129]. Traditionally, complement and coagulation were described as separate cascades, only linked by the ability of FXIIa to activate the classical complement pathway [124]. However, it becomes now more and more evident that the convergence between both systems extends beyond the biochemical nature of serine proteases, and multiple mutual interconnections form a highly complex network (Figure 1) [29, 30, 34]. Understanding the interplay is important to breach the vicious circle of systemic inflammation in order to be able prevent life-threatening complications.

9. Conclusions

Based upon the current understanding, the general role of complement in the pathogenesis of MOF can be conceptualized as follows: After trauma, burn, or severe tissue injury, systemic intravascular activation of the complement system with apparent loss over the control mechanisms occurs. Complement activation products trigger a cascade of cellular events in endothelial cells resulting in upregulation of adhesion molecules, release of proinflammatory mediators, and increased vascular permeability. Leukocytes are attracted by complement anaphylatoxins to transmigrate into parenchyma of various organs after adhesion to endothelial cells and extravasation. Activated leukocytes release inflammatory mediators, enzymes, and free radicals that harm parenchymal cells. Local production and activation of complement proteins in combination with loss of protection against complement-mediated lysis aggravate the degree of tissue injury. Interaction with the coagulation cascade causes disseminated intravascular coagulation and

compromised microcirculation, which then augments organ dysfunction by ischemia. All events of this vicious circle finally merge into apoptosis and necrosis of parenchymal cells with the development of multiple organ dysfunction syndrome. The complement anaphylatoxins C5a and C3a not only trigger the inflammatory response but also directly alter cellular functions of parenchymal cells as well as leukocytes by interaction with their specific receptors, which are abundantly expressed on numerous cell types. However, the organ-specific mechanisms and intracellular events that follow receptor binding, such as mitogen-activated protein kinase (MAPK) pathways, remain to be evaluated in future studies. As outlined above in the description of cardiac dysfunction, organ failure might reflect a cellular resting state, also described as hibernation, as a response to a proinflammatory environment with uncoupling of the respiratory chain and mitochondrial dysfunction. However, it is not clear yet if and to which extent complement activation contributes to the pathophysiology of hibernation in human cells.

Since complement activation occurs as a rapid event after the initial insult, it appears auspicious to use intervention in the complement system as a therapeutic approach in order to prevent the development of MOF. Strategies to inhibit complement include (i) the application of endogenous complement inhibitors (C1 inhibitor, soluble complement receptor-1) [130], (ii) administration of antibodies or antagonists which block key proteins (C3, C5) of the complement cascade or neutralize complement-derived anaphylatoxins (C3a, C5a) [25, 131], and (iii) interference of C5a, C3a interaction with their receptors by receptor-specific antagonists [26]. In addition, upregulation or incorporation of membrane-bound complement-regulatory proteins could protect organs from complement-mediated cytotoxicity. Protection against complement-mediated inflammatory tissue damage could be achieved in various experimental settings. However, total blockade of the complement cascade might impair the capability to clear invaded pathogens and increase the risk of infection. Therefore, targeting the complement system in inflammation should rather aim to balance or control its activation with suppression of the harmful effects, but without detriment of the protective and reparative complement functions.

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Review Article

Complement Diagnostics: Concepts, Indications, and Practical Guidelines

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Aberrations in the complement system have been shown to be direct or indirect pathophysiological mechanisms in a number of diseases and pathological conditions such as autoimmune disease, infections, cancer, allogeneic and xenogeneic transplantation, and inflammation. Complement analyses have been performed on these conditions in both prospective and retrospective studies and significant differences have been found between groups of patients, but in many diseases, it has not been possible to make predictions for individual patients because of the lack of sensitivity and specificity of many of the assays used. The basic indications for serological diagnostic complement analysis today may be divided into three major categories: (a) acquired and inherited complement deficiencies; (b) disorders with complement activation; (c) inherited and acquired C1INH deficiencies. Here, we summarize indications, techniques, and interpretations for basic complement analyses and present an algorithm, which we follow in our routine laboratory.

1. Introduction

The complement system is involved in numerous diseases and pathological conditions such as autoimmune disease, infections, cancer, allogeneic and xenogeneic transplantation, and inflammation [1]. The concentrations of various complement components and activation products have been measured in both prospective and retrospective studies of pathologic conditions, and significant differences have been found between groups of patients. However, in many diseases, it has not been possible to make predictions for individual patients because of the lack of sensitivity and specificity of many of the assays used. Basically, the indications for diagnostic complement analysis today can be divided into three major categories: (a) acquired and inherited complement deficiencies; (b) disorders with complement activation; (c) inherited and acquired C1INH deficiencies. Here, we give a personal view of how we perform basic complement investigations in our routine diagnostic laboratory.

2. The Complement System

2.1. Complement System Physiology. The complement system has a primary function in host defense and clears the body of foreign cells, microorganisms, and cell debris, either by direct lysis or by recruitment of leukocytes that promote phagocytosis and cytotoxicity (recently reviewed in [2]). It consists of more than 40 plasma and cellular proteins (receptors and regulators). The central complement reaction is the cleavage of C3 into C3b and C3a, which is promoted by two multimolecular enzyme complexes, the C3 convertases, which are assembled by three different recognition and activation pathways. The classical pathway (CP) is triggered by the formation of antigen-antibody complexes (immune complexes), which bind the C1 complex (C1q, $C1r_2$, $C1s_2$), and the lectin pathway (LP) by the binding of mannan-binding lectin (MBL) or ficolins to carbohydrates and other pathogen-associated molecular patterns. Both these events lead to the assembly of the CP/LP C3 convertase, C4b2a. The alternative pathway (AP) may be triggered directly by foreign

surfaces, for example, by microorganisms or man-made biomaterials, which do not provide adequate downregulation of the AP C3 convertase, C3bBb. This convertase is stabilized by properdin, which also recently has been reported to act as a recognition molecule that is able to form a nucleus for convertase assembly [3].

The nascent C3b molecule has the specific property of binding to proteins and carbohydrates via free hydroxyl or amino groups, resulting in covalent ester and amide bonds, respectively. The AP serves as a major amplification loop, so an initial weak stimulus mediated by any of the pathways may be markedly enhanced. The activation pathways converge in a common pathway to form the membrane attack complex (C5b-9), which elicits cell lysis by insertion itself into the lipid bilayer of cell membranes. The anaphylatoxins C3a and C5a activate and recruit leukocytes, while target-bound C3 fragments (C3b, iC3b, C3d,g) facilitate binding to and activation of the recruited cells (Figure 1).

2.2. Complement System Regulation. *In vivo*, the complement system is controlled by multiple soluble and membrane-bound regulators [2]. Most of the regulators are members of the "regulators of complement activation" (RCA) superfamily, which mainly regulate the convertases. The plasma proteins factor H and C4b-binding protein (C4BP), the membrane proteins complement receptor 1 CR1 (CD35), decay acceleration factor, DAF (CD55), and membrane cofactor protein MCP (CD46) all belong to this family and exert their action by functioning as cofactors for plasma protease factor I and/or by accelerating the decay of the convertases. In addition, CD59 is a regulator of the C5b-9 complex, and C1 inhibitor (C1INH) regulates the proteases of the C1 complex (C1r and C1s) and MASP-1, -2, and -3 of the LP (Figure 1).

2.3. Complement System Pathology. Excessive complement activation is part of the pathogenesis of a large number of inflammatory diseases. The pathologic effect may be due either to an increased and persistent activation, for example, caused by the presence of immune complexes (such as in systemic lupus erythematosus, SLE, and related disorders), or to a decreased expression or function of various complement inhibitors (see examples below), or to a combination of the two, as discussed in [4] and quoted references.

Ischemia, followed by reperfusion of an organ or blood vessel, occurs in a number of conditions, such as during heart infarction or stroke. It can also occur during medical treatment modalities such as cardiovascular surgery facilitated by cardiopulmonary bypass as well as after transplantation, in both allogeneic and xenogeneic settings, and can often be accompanied by ischemia/reperfusion (IR) injury. Complement activation and insufficient regulation play important roles in IR injury, and activation by all three pathways of complement has been implicated in the damage. The result is an multifunctional inflammatory process, involving generation of anaphylatoxins, upregulation of adhesion proteins and tissue factor on endothelial cells, and recruitment and extravasation of PMNs, as summarized in [5] and cited references.

The net result of this dysregulation between initiators and inhibitors of complement activation in all these diverse conditions is a prolonged complement activation, which ultimately results in tissue damage.

3. Examples of Indications for Complement Analysis

3.1. Inherited and Acquired Complement Component Deficiency

3.1.1. Complement Factor Deficiencies (General). Complement deficiencies are associated with an increased risk of infections and, in some cases, autoimmunity [6]. A deficiency of one component within the CP (the subunits of the C1 complex, C2, and C4) not only predisposes an individual to infections but also to immune-complex diseases, that is, SLE-like conditions; deficiencies in other components are mainly associated with infections [4]. The point in the complement cascade at which a deficiency occurs determines the specificity of the infection (in almost all cases bacterial) that affects a patient, the most common being caused by *Neisseria*, *Haemophilus*, and *Pneumococci* [7]. A deficiency of MBL in the lectin pathway increases the risk of any type of infection: bacterial, viral, or fungal, particularly in various immunosuppressive states, such as during the neonatal period, or during immunosuppressive treatments [6].

3.1.2. Monitoring of Complement Regulatory Drugs. Eculizumab is the first approved complement inhibitor in clinical use. It is a humanized monoclonal antibody that binds to complement component C5, hindering its proteolytic activation, and thereby inhibiting the generation of the anaphylatoxin C5a as well as the initiation of the lytic C5b-9 complex. The main indication for this complement inhibitor is for the treatment of paroxysmal nocturnal hemoglobinuria, PNH [8], and atypical hemolytic uremic syndrome, aHUS [9], for which it has orphan drug designation. In addition, successful off-label use has been reported in shiga toxin-induced hemolytic uremic syndrome [10] and refractive membranoproliferative glomerulonephritis, MPGN [11], as well as for reversing ongoing rejection in ABO-incompatible transplantation [12]. The functional effect of this treatment, and of other future complement inhibitors, *in vivo* may be monitored by complement analysis (see below) [12].

3.1.3. Paroxysmal Nocturnal Hemoglobinuria (PNH). Paroxysmal nocturnal hemoglobinuria (PNH) is a rare hematologic disorder in which the afflicted patients suffer from hemolysis with acute exacerbations that lead to anemia, as well as from an increased risk of venous thrombosis. The disease is caused by acquired somatic mutations of the X-chromosomal gene *PIG-A* in a limited number of hematopoietic stem/progenitor cells [13]. The afflicted cells lack the enzyme encoded by *PIG-A*, which is essential for synthesizing the GPI (glycosylphosphatidylinositol) anchor;

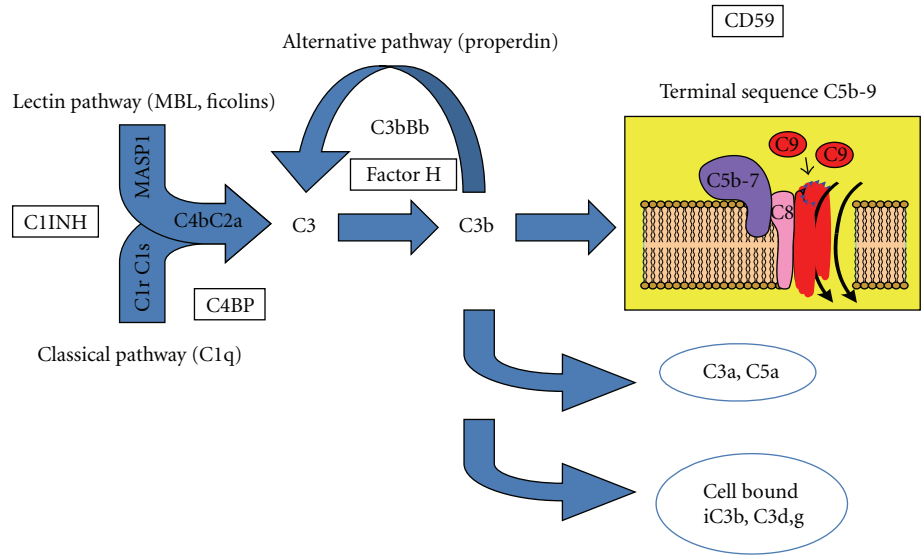

Figure 1: Overview of the complement system. Recognition by the lectin and classical pathways leads to the assembly of the C4b2a convertase, which cleaves C3. This reaction is greatly amplified by the alternative pathway, generating more C3b, and ultimately initiating the terminal sequence. The fluid phase anaphylatoxins, C3a and C5a, together with the cell-bound opsonins iC3b and C3d,g, facilitate phagocytosis. The main inhibitor of each step in the cascade is indicated in boxes: C1INH for initiation, C4BP for the classical pathway, factor H for the alternative pathway, and CD59 for the terminal sequence.

these cells (leukocytes, erythrocyte, or platelets) are therefore unable to bind GPI-anchored proteins, including the complement regulators DAF and CD59. Consequently, they are susceptible to attack by autologous complement ([14] and references therein). PNH will not be further mentioned in the text but the current state of the art regarding the diagnosis and management of PNH is described in detail in [15].

3.2. Disorders with Complement Activation

3.2.1. SLE and Urticarial Vasculitides. SLE and urticarial vasculitides belong to the group of autoimmune immune complex diseases [16, 17]. Cryoglobulinemia, rheumatoid arthritis with vasculitis, and rare cases of Wegner's and Henoch Schönlein disease also belong to this group [18, 19]. Complement analyses (see below), most notably the assessment of CP function, and the concentration of individual complement components, for example, C1q, C4, and C3 can be used for differential diagnosis and to follow disease activity. Detection of autoantibodies against C1q and C3 may corroborate diagnosis [20–22]. Hypocomplementemic urticarial vasculitis syndrome (HUVS) presents with severe complement consumption via the CP and anti-C1q antibodies [17].

3.2.2. Membranoproliferative Glomerulonephritis. Membranoproliferative glomerulonephritis (MPGN) types II are associated with C3 nephritic factors (C3Nef) [23]. C3Nef is an autoantibody directed against a convertase. In MPGN type II, the antibody is directed against the AP convertase, resulting in a dramatically extended half-life of the convertase. The consequence of this antibody association is a profound C3 consumption, leading to a functional deficiency of C3. The C3 consumption is accompanied by the generation of C3d,g, indicating a prominent activation of C3. In some cases of C3Nef (type II), the stabilized convertase also cleaves C5. Detection of C3Nef supports the diagnosis of MPGN. The resulting severe C3 deficiency may theoretically increase the risk of bacterial infections.

3.2.3. Poststreptococcal Glomerulonephritis (PSGN). In individuals suffering from poststreptococcal glomerulonephritis (PSGN), C3 and C5 may be consumed and sC5b-9 generated, in the rehabilitation period, up to 6 months after the infection [23]. The levels may be very low and, as in the case of C3Nef, there is a theoretical risk of other bacterial infections. C3d,g levels are elevated and, in particular, the ratio C3d,g/C3 is high. The mechanism of C3 consumption is not known, but the major difference in complement activation compared to C3Nef is that PSGN is associated with a concomitant consumption of properdin [23].

3.2.4. Atypical Hemolytic Uremic Syndrome (aHUS). Atypical hemolytic uremic syndrome (aHUS) is a disease that appears in the childhood and is characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure resulting from membranoproliferative glomerulonephritis. The cause of this disease is dysregulated complement activation following a mutation in factor H, factor I, MCP, or factor H-related proteins (FHR) 1, 3, or 5, that impairs the functioning of these inhibitors. In addition, mutations in C3 and factor B that lead to dysregulated activation have also been described [24] and referenced therein [25].

The cells that are affected are erythrocytes, platelets, and endothelial cells, including those of the mesangium of the kidney. Mutations within the factor H gene are the most common cause of aHUS. The majority of these mutations are localized in the C-terminally located short consensus repeats (SCRs) 19-20, which are involved in the binding of factor H to the cell surface. Factor H binds to carbohydrates, and heparan sulfate and sialic acid are common ligands for the protein. As in the case of factor H deficiency, engagement of the AP leading to consumption of C3 and generation of C3d,g (or other C3 fragments) and C5b-9 may be seen. In rare cases, the mobility in SDS/PAGE followed by western blotting may differ from that of normal factor H. Patients with suspected aHUS should be handled by laboratories specialized in determining mutations in all activators and soluble and cell bound regulators of the AP.

3.3. Inherited and Acquired C1INH Deficiency. Hereditary angioedema (HAE) and acquired angioedema (AEE) are rare disorders that are caused by a C1INH deficiency and, in rare cases, by mutations of the contact system proteins [26]. These diseases are caused by an unregulated formation of bradykinin of the contact system, and hence they are not primarily diseases of the complement system. However, the diagnosis is based on complement analyses. HAE is subdivided into three types (I–III), which can be identified only by laboratory analysis. The type I form of HAE is characterized by a low concentration and function of C1INH, and type II by a normal concentration of a dysfunctional C1INH. The third form, type III, which is not due to low C1INH function, is in many cases estrogen dependent. This is a heterogeneous group, which is less well characterized, than the other two forms. Some of the patients with type III have mutations in the contact system *F12* gene, coding forms of FXII with gain of function [26].

Acquired deficiencies of C1INH may occur in lymphoproliferative and autoimmune diseases, due to formation of paraproteins, for example, M-components and autoantibodies against C1INH, respectively, which result in consumption of the protein [27, 28].

4. Analytical Methods

Available complement assays have recently been comprehensively reviewed [1]. Here, we present analytical methods, which are suitable for routine diagnostics.

4.1. Quantification of Individual Complement Components. The concentration of individual proteins is determined by various types of immunoassays. The most common approach in clinical practice is to use immunoprecipitation assays, today mainly nephelometry and turbidimetry. In the latter techniques, polyclonal antibodies against the protein of choice (e.g., C1q, C1INH, C4, C3, or factor B) are added to the sample, forming complexes that will distort a detecting light beam that is passed through the sample. These techniques, which use polyclonal antibodies to detect the total amount of the antigen in question, are relatively robust with regard to the effects of suboptimal sample handling, such as proteolytic cleavage or denaturation of the target proteins. For example, the polyclonal antibodies raised against C3c used in such assays will recognize C3c-fragment containing intact, nonactivated C3 as well as its inactive proteolytic fragments C3b, iC3b, and C3c, on an equimolar basis. Similarly, anti-C4c antibodies will detect the corresponding forms of C4. If the sample is poorly treated, resulting in fragmentation of the intact protein, determination of the c-fragment (C3c or C4c) ensures that the determined concentration is similar to that *in vivo* (Figures 2(a) and 2(c)). However, this assay gives no information about the conformation or activation state of the protein and it is used mainly to determine the protein's *in vivo* concentration (i.e, to monitor consumption) (see Figure 2). Consequently, to give a measure of complement activation *in vivo*, it is necessary to measure an activation fragment/product, for example, C3d,g (Section 4.2).

4.2. Quantification of Activation Products. A number of complement proteins are activated and inactivated by sequential proteolytic cleavages that are accompanied by conformational alterations. These reactions have been studied most extensively for C3. Therefore, the strategy used to demonstrate that complement activation has occurred *in vivo* relies on detecting complement activation products (with altered size and conformation or composition) in the sample. Activation of C3 may be monitored either by identifying the protein fragment C3a, which is generated in the first proteolytic cleavage step, or the C3d,g fragment, which is the end product (together with C3c) (Figure 2). These peptides vary greatly with regard to their half-lives ($T_{1/2}$) *in vivo*: approximately 0.5 hr for C3a [29] and 4 hr for C3d,g [30]. In addition, there is a great risk that C3a will be generated *in vitro* during improper handling of samples. Consequently, since C3d,g is a more robust marker, it is more suitable for diagnostic use, while the generation of C3a is more suitable for *in vitro* analysis in experimental settings.

Complement activation gives rise to products with different properties than those of the zymogen molecules. Therefore, assays for the determination of complement activation products generally work according to one of two principles: either (1) the zymogen molecules and products are fractionated according to size before being detected by polyclonal antibodies, as described above for C3d,g (below), or (2) monoclonal antibodies specific for amino acid sequences that are hidden in the native zymogen molecule but exposed upon activation (so-called neoepitopes) are used. Since only the activation product and not the zymogen molecule will be detected in this assay, it is not necessary to include a precipitation step. Most available assays for C3a, C3b/iC3b/C3c, and C5b-9 (below) are based on neoepitope monoclonal antibodies [31–33].

C3d,g is detected by nephelometry/turbidimetry or enzyme immunoassays (EIA) using polyclonal antibodies (see Section 4.1). However, since these antibodies also recognize intact C3, C3b, and iC3b in addition to C3d,g, it is necessary to remove these larger molecules by polyethylene glycol

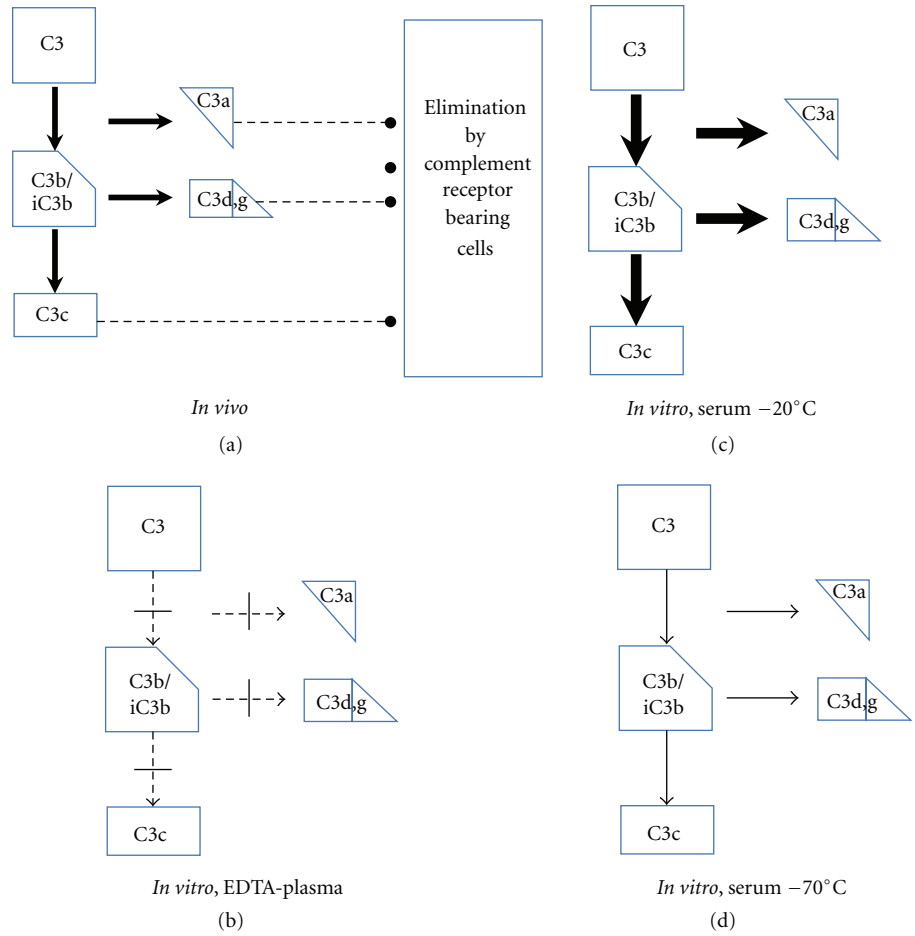

Figure 2: Activation and consumption of complement *in vivo* and *in vitro*. *In vivo*, complement is activated, and C3 gives rise to the activation products C3a, C3b/iC3b, C3d,g, and C3c (indicated by arrows). *In vivo*, a fraction of these complement products are bound to and eliminated by different complement receptor-bearing cells in contact with plasma (a). When blood is drawn in the presence of EDTA, all further complement activation is inhibited (b). The complement system is active in serum and may be activated to a substantial degree *in vitro* in maltreated samples (c), but it can be kept essentially intact in properly handled samples (d). The thickness of the arrows in each panel indicates the degree of C3 cleavage.

(PEG) precipitation prior to analysis. C3d,g is continuously generated *in vivo* under normal conditions, presumably as part of the physiological turnover of C3. Therefore, it is useful to determine the ratio between the C3d,g level and the total level of C3 in order to monitor ongoing complement activation (C3d,g/C3), for example, during a flare in SLE [34].

The final step in the complement cascade is the formation of the C5b-9 complex, which is inserted into cell membranes, thereby causing cell damage and/or lysis. sC5b-9, the soluble form of this high molecular weight complex, can be quantified in the fluid phase as a marker of full complement activation, by using an EIA with a monoclonal antibody specific for a neoepitope in C9, which is exposed in complex-bound but not intact C9. Detection of the formed complexes is performed by using polyclonal antibodies against C5 or C6 (i.e., another protein present in the same macromolecular complex) [33].

Since all these activation markers can be rapidly produced by complement activation *in vitro*, these assays are sensitive to preanalytical factors, so it is of critical importance that the samples are collected and handled properly (see Section 4.5).

4.3. Quantification of Complement Function. The function of each of the complement activation pathways is dependent on the integrity of each of the participating components, and therefore a deficiency in a single component will affect the activity of the whole cascade. One major advantage of functional tests that monitor a whole activation pathway from initiation to the effector phase (lysis) is that they will detect both deficiencies in complement components and consumption-related decrease of complement activity, thereby combining information obtained using the types of assays described above.

Complement activation by the CP is studied in hemolytic assays utilizing sheep erythrocytes coated with rabbit antibodies (IgM with or without IgG). When serum is added, the C1 complex will bind, leading to formation of the CP convertase, which activates C3. Activation of C3 then initiates

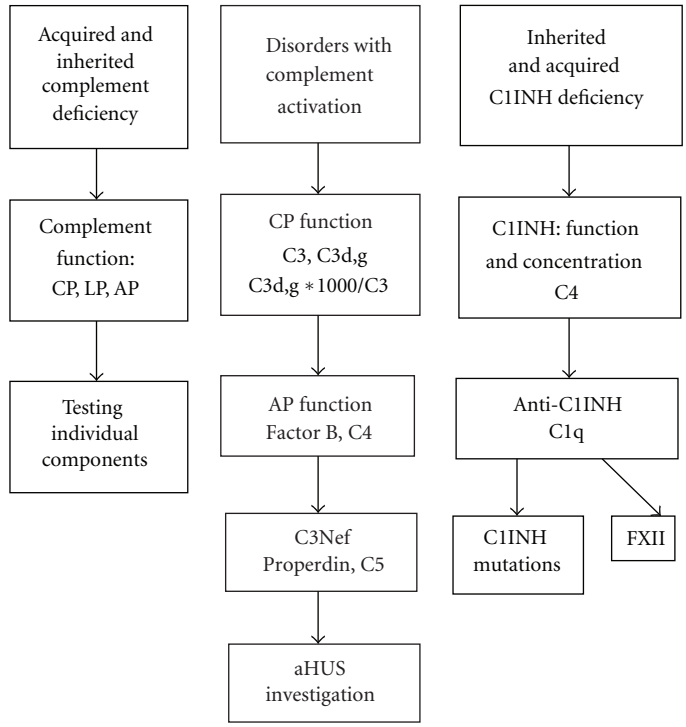

Figure 3: Algorithm for complement analyses. The aims are to diagnose complement deficiency in patients with recurrent bacterial infections (a), diagnose the cause of their persistent complement activation (b), and to dissect the cause of C1INH deficiency (c). See text (Section 5.1) for details.

the assembly of the C5b-9 complex, which ultimately results in erythrocyte lysis [35, 36].

Complement activation by the AP is studied by using rabbit or guinea pig erythrocytes, which are spontaneous activators of the human AP. When the cells are incubated in serum with the addition of EGTA to chelate Ca^{2+} (to inhibit activation by the CP and LP), the AP convertase is formed, resulting in the activation of C3 and subsequent lysis of the erythrocytes [36, 37].

Hemolytic assays can be performed in different ways; the original assays, the so-called CH_{50} and AH_{50}, are based on titration of the amount of serum necessary to lyse 50% of specified amount of cells [35, 37]. The considerably less laborious and faster one-tube assays, which only necessitate the use of one serum concentration, give corresponding results [36]. The commonly used hemolysis in gel technique is performed with erythrocytes cast in an agarose gel. The serum is added to punched holes and diffuses into the gel whereby the erythrocytes are lysed. This technique is excellent for screening for complement deficiencies but does not provide a quantitative measurement [38].

More recently, a method comprising three separate EIAs which for the first time enables the simultaneous determination of all three activation pathways (including the LP) has been reported. The assay can best be described as a solid-phase functional test, since it comprises recognition molecules specific for each pathway (IgM for the CP, mannan or acetylated bovine serum albumin, BSA, for the LP, and LPS for the AP). These molecules are coated onto ELISA plate wells, and then serum is incubated under conditions in which only one pathway is operative at a given time and the other two pathways are blocked. The final step in each EIA is the detection of the resulting C5b-9 complex by a monoclonal antibody against a neoepitope in complex-bound C9 [39]. This assay is commercially available (Wielisa, Wieslab, Lund, Sweden). The correlation between this assay and conventional hemolytic assays is linear for the CP and for the AP at high activity but not at lower levels.

These functional techniques are particularly useful for (1) identifying congenital deficiencies and (2) monitoring fluctuations in complement function, for example, in SLE patients during exacerbations. A tentatively identified deficiency can be confirmed by concentration determination using a protein-specific assay and by experiments in which the patient sample is reconstituted with the relevant protein. (Most plasma complement components are commercially available). These analyses will provide information whether it is a functional deficiency or a total lack of the protein (Figure 3(a)).

4.4. Quantification of Autoantibodies to Complement Components. C3Nef are autoantibodies that bind to components of

the AP convertase, thereby prolonging its functional $T_{1/2}$ and leading to increased complement activation. There are two basic assays for the detection of C3Nef: an AP-dependent hemolytic assay employing noncoated sheep erythrocytes [40] (in contrast to the CP hemolytic assay described above) and an assay to assess fluid-phase C3 cleavage, detected by, for example, crossed immunoelectrophoresis [41]. C3Nef are designated as C3Nef type I or II, based on the pattern of reactivity in these two assays. Over all, C3Nef type I predominately stabilizes the C3 AP convertase, while C3Nef type II also results in C5 cleavage [42].

Recent efforts to improve detection of C3Nef include construction of ELISA-based functional assays using nickel-stabilized C3bBb, and real-time monitoring of the formation and decay of C3-convertase formation using surface plasmon resonance, SPR [43, 44].

Anti-C1q autoantibodies are found in several autoimmune conditions and also in healthy controls. The assay is performed as follows: coating ELISA plates with purified C1q, incubation of patient serum and binding of true anti-C1q autoantibodies to the collagen part of C1q, and detection of bound IgG antibodies using antihuman IgG antibodies. In order to avoid that IgG-containing immune complexes in the samples bind to the coated C1q it is necessary to perform the assay in the presence of high concentrations of NaCl, typically 0.5–1.0 M, which dissociates the binding of C1q to IgG-containing immune complexes. The role of anti-C1q autoantibodies and methodological considerations are discussed in detail in [22].

Immunoconglutinins (IKs) are autoantibodies against fragments of C3 or C4 that affect the functioning of these components and are found in inflammatory states and autoimmune diseases, including SLE. IKs can be detected by EIA, using C3-coated wells for capture and polyclonal anti-human IgG, IgA, or IgM antibodies for detection [20, 21, 45].

4.5. Collection and Storage of Samples. EDTA is the only anticoagulant that completely inhibits any complement activation *ex vivo*, and EDTA-plasma should be used for the quantification of complement components and their activation products. Heparin and citrate are insufficient inhibitors of complement activation and are thus unsuitable anticoagulants for complement analysis. Serum or plasma anticoagulated with a specific thrombin inhibitor, such as lepirudin, is used for the quantification of complement function and autoantibodies. Alternatively, EDTA-plasma can be used for the functional assays, provided that the samples are transferred to Veronal buffer with Ca^{2+} and Mg^{2+} (to enable complement activation) and lepirudin (to inhibit coagulation) [34]. Plasma and serum should be separated within 2 hr of collection and frozen at −70°C. Storage at −20°C should be avoided. If samples need to be transported, they should be sent in packages containing dry ice (with samples pre-frozen at −70°C). Prior to analysis, the samples should be thawed rapidly, preferably in a 37°C water bath, and then kept on ice.

5. Concept and Interpretations

5.1. Algorithm for Complement Analyses. In this section we summarize the indications for complement analyses and present an algorithm, which we follow in our laboratory when we perform complement diagnostics. The algorithm is shown in Figure 3. Complement analysis is generally undertaken for three different indications.

(A) Acquired and Inherited Complement Deficiency. Complement deficiencies may be due to treatment with complement-inhibiting drugs such as the newly introduced eculizumab. Quantitative functional assays can be used to test the effect of the drug *in vivo*. This indication will probably increase with the introduction of new complement regulatory drugs and may also include, for example, measurement of sC5b-9 to monitor administration of eculizumab for the different indications mentioned in Section 3.1.2. Another important indication for complement analysis is recurrent severe invasive bacterial infections that may be due to an inherited complement deficiency. The first step in ascertaining an inherited complement deficiency (Figure 3(a)) is to determine complement function by all three pathways, either by hemolytic tests or by Wielisa. No hemolytic test has been described for the LP. Therefore, CP and AP hemolytic assays may be complemented with at least determination of the concentration of MBL as a surrogate marker for LP function. If a defect is found, it can be verified by a specific concentration assay for the lacking component, and by reconstituting the functional assay with the specific deficient protein. If relevant, genetic analysis may be performed.

(B) Disorders with Complement Activation. The initial step in our algorithm to determine and assess the cause of complement activation (Figure 3(b)) is to determine CP function and the concentrations of C3 and C3d,g. Computer analyses have shown that if these three parameters are within the reference values, the sample is normal with regard to complement activation, with 95% certainty (Nilsson, UR and Groth, T, unpublished data). Samples with values outside the reference intervals are then tested for AP function and determination of factor B and C4 concentrations. Combined, all these assays represent functional tests and markers for the CP, AP, and terminal pathways (Figure 1). If no plausible explanation for a solitary high C3d,g/C3 ratio is found and if the clinical condition necessitates further investigation, the next step is to determine the presence of C3Nef and the concentrations of properdin and C5. Cases where aHUS is suspected should be transferred to a laboratory specialized in aHUS diagnostics.

(C) Inherited and Acquired C1INH Deficiency. The cause of a C1INH deficiency (Figure 3(c)) is dissected by analyzing the concentration and function of C1INH and the concentration of C4. In type I deficiencies both the C1INH concentration and the function are low. In type II only the function is deficient. In both deficiencies, C4 is often low as

a result of remaining C1s activity. Samples, in which an acquired deficiency is suspected, are analyzed to determine the concentration of C1q and the presence of autoantibodies against C1INH. In acquired C1INH deficiency due to lymphoproliferative disease, paraproteins may lead to C1q consumption and in autoimmune rheumatic disease there may be anti-C1INH antibodies. The final step in the investigation of HAE includes determination of mutations in C1INH by specialized laboratories. If no aberrations are found in C1INH function despite clinically typical angioedema, then analyses of factor XII function and genetic determination may be performed. The current state of the art regarding the diagnosis and management of HAE and AAE is described in detail in [46, 47].

5.2. Differentiation between In Vivo and Ex Vivo Activation. Activation of the complement system both *in vivo* and *in vitro* leads to the generation of activation products, for example, the anaphylatoxins C3a and C5a and the fragments of C3 and C4 produced by sequential proteolysis, as described in Section 2.1 above. *In vivo*, most of these products interact with receptors on various cells in contact with plasma (erythrocytes, leukocytes, endothelial cells, and fixed macrophages, etc.) and are rapidly cleared from the circulation, leading to consumption of the components (Figure 2(a)). In contrast, complement activation *ex vivo*, in the collected serum or plasma samples, will generate the same products, but in this situation there are no cells present, so the activation products will remain in solution (Figures 2(b), 2(c), and 2(d)). Functional assays will in both cases be low but only samples where activation has occurred *in vivo* will show consumption of individual complement components.

5.3. Interpretation of Laboratory Results, with SLE as an Example. Use of the commonly employed combination of C3 and C4 concentrations to monitor complement in immune complex disease should be avoided, since both the sensitivity and specificity of these measurements are low. For example, SLE patients may have inherently low concentrations of C4 as a result of a low gene copy number [48]. Immune complex diseases are characterized by a moderate-to-severe CP activation and consumption. This activation reflects the activity of the disease, and many times the complement consumption precedes an exacerbation of the disease. In order to determine the first and initial complement status of the patient, a functional assay (e.g., hemolytic or EIA-based), combined with an assay to determine complement activation products (e.g., C3d,g, C5b-9), is necessary. By using this combination of tests, the laboratory can determine whether a low complement function measured by the functional test is really the result of a consumption/activation or is caused by a deficiency/dysfunction. For monitoring of the patient, a single test can be used. For SLE, the C1q concentration or a hemolytic assay, such as the single-tube CP assay or CH_{50}, can be used. Certain cryoglobulinemias can easily be detected by functional complement assays. These patients

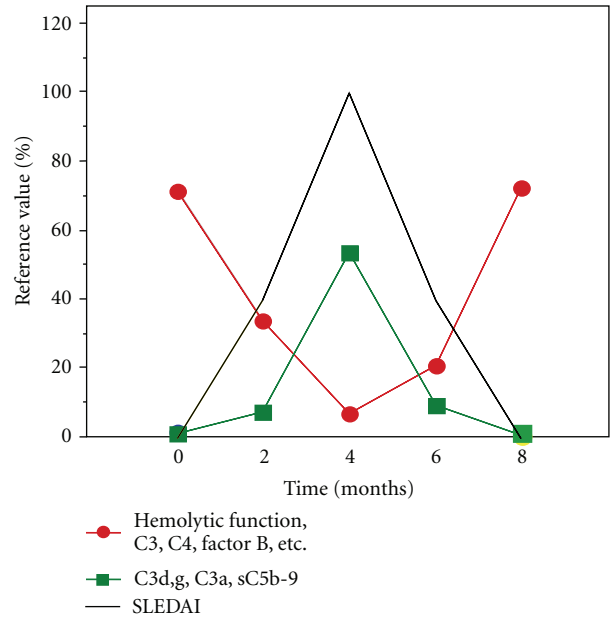

Figure 4: Complement activation and hemolytic function during an SLE exacerbation. C3, C4, factor B, and other components are consumed, leading to a depression in hemolytic function (red line). The resulting activation products, C3d,g, C3a, and sC5b-9 (green line), peak concomitantly with the SLE disease index (SLEDAI).

may consume complement via the CP already *in vivo*, but this activation is often amplified *in vitro* as a result of the handing of the sample, leading to a very low CP function (if serum is used) without a corresponding consumption of CP components, as determined by immunochemical assays (if EDTA is used). These samples may be misdiagnosed as deficiencies (Figure 4).

5.4. Interpretation of Laboratory Results, General. As an illustration of what was discussed in the previous section we have constructed Table 1. The first patient is an SLE with complement activation triggered by the CP. This patient has consumed components *in vivo* via the classical and terminal pathways. Thus, the function via CP is low. We also see that there is consumption of C4, C3 but not of factor B. There is also generation of C3d,g and the ratio C3d,g/C3 is above the reference value. In the second SLE patient, the function via CP is also low but there is no sign of consumption of complement components or C3d,g generation. This patient has a C2 deficiency. This illustrates that assessment of complement function in order to detect complement deficiencies is not sufficient without further analyses of complement consumption/activation. Another confusing condition is cryoglobulinemia, which will present an identical profile as in the C2 deficient patient, mimicking a deficiency. Here, all determinations of individual components and of complement fragments are measured on the EDTA-plasma in which no further complement activation occurs after withdrawal of blood. By contrast, the serum sample may be activated by cryoglobulins (immune complexes) and therefore the CP function will be affected.

Table 1: Complement function of the CP and AP, plasma concentrations of C3, C4, factor B, and C3d,g, and the C3d,g∗1000/C3 ratio in one patient with SLE and one patient with a C2 deficiency.

	CP (%)	AP (%)	C3 (g/L)	C4 (g/L)	Factor B (g/L)	C3d,g (mg/L)	C3d,g∗1000/C3
SLE	15	45	0.53	0.07	0.18	7.0	13.2
C2 def	5	97	0.80	0.14	0.30	3.5	4.4
Reference interval	80–120	50–150	0.67–1.29	0.13–0.32	0.16–0.44	<5.3	<5.3

Table 2: Complement pathology, differential diagnostics.

Analyses	HAE/AAE	SLE, urticarial vasculitis	PSGN	MPGN II and III	Complement deficiencies
CP	N	L	L	L	*
AP	N	N (L)	L	L	*
C1INH (conc)	L	N	N	N	*
C1INH (funct)	L	N	N	N	*
C1q	(L)	L	N	N	*
C4	L	L	N	N	*
C3	N	L	L	L	*
C5	N	—	L	(L)	*
Factor B	N	N	L	N	*
Properdin	N	—	L	N	*
C3d	N	H	H	H	N
C3d,g/C3	N	H	H	H	N

* Variable (dependent on which component is defective); N: normal; L: low; H: high.

Such mistakes can be avoided if the samples are drawn, centrifuged at 37°C, and CP function is analyzed without freezing of the sample or if the CP function is analyzed on the EDTA-plasma (see above).

6. Conclusions

In summary, complement analyses for individual patients are useful in a relatively limited number of conditions, which are summarized in Table 2. The profile for the typical case of each condition is presented in the Table. Indications for complement analyses will increase with introduction of new regulatory drugs of complement and with new assays for example, it is likely that assays of the LP will generate new indication for complement investigations.

Abbreviations

AEE: Acquired angioedema
aHUS: Atypical hemolytic uremic syndrome
AP: Alternative pathway of complement
BSA: Bovine serum albumin
C1INH: C1 inhibitor
C3Nef: C3 nephritic factor
C4BP: C4b-binding protein
CP: Classical pathway of complement
CR1: Complement receptor 1 (CD35)
DAF: Decay acceleration factor (CD55)
EIA: Enzyme immunoassay
FHR: Factor H-related protein
GPI: Glycosylphosphatidylinositol
HAE: Hereditary angioedema
HUVS: Hypocomplementemic urticarial vasculitis syndrome
IKs: Immunoconglutinins
IR: Ischemia/reperfusion
LP: Lectin pathway of complement
LPS: Lipopolysaccharide
MBL: Mannan-binding lectin
MCP: Membrane cofactor protein (CD46)
MPGN: Membranoproliferative glomerulonephritis
PEG: Polyethylene glycol
PNH: paroxysmal nocturnal hemoglobinuria
PSGN: poststreptococcal glomerulonephritis
RCA: regulator of complement activation
SLE: systemic lupus erythematosus
SLEDAI: SLE disease index
$T_{1/2}$: half-life.

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Review Article

Kidney Diseases Caused by Complement Dysregulation: Acquired, Inherited, and Still More to Come

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Inherited and acquired dysregulation of the complement alternative pathway plays an important role in multiple renal diseases. In recent years, the identification of disease-causing mutations and genetic variants in complement regulatory proteins has contributed significantly to our knowledge of the pathogenesis of complement associated glomerulopathies. In these diseases defective complement control leading to the deposition of activated complement products plays a key role. Consequently, complement-related glomerulopathies characterized by glomerular complement component 3 (C3) deposition in the absence of local immunoglobulin deposits are now collectively described by the term "C3 glomerulopathies." Therapeutic strategies for reestablishing complement regulation by either complement blockade with the anti-C5 monoclonal antibody eculizumab or plasma substitution have been successful in several cases of C3 glomerulopathies. However, further elucidation of the underlying defects in the alternative complement pathway is awaited to develop pathogenesis-specific therapies.

1. Introduction

The central function of the kidney for whole body homeostasis is based on adequate blood flow and pressure, sufficient glomerular capillary surface for selective filtration, and subsequent secretion and reabsorption of solutes in the tubular system. The essential role of the glomerulus as a filtration unit can be estimated by the fact that most diseases leading to chronic kidney disease and end-stage renal disease with the need for dialysis or transplantation are caused by glomerulopathies. The glomerulus as a specialized capillary convolute is prone to any vascular damage and is affected as part of a generalized microangiopathy in common diseases such as diabetes mellitus or arterial hypertension. However, the glomerulus can also be affected by specific circulating factors, including antibodies against glomerular antigens, circulating immune complexes, or activated factors of a dysregulated complement system.

The complement system as an essential component of the innate immune system plays an indispensable role in the elimination of invading microorganisms as a first line of defense [1, 2]. Furthermore, the complement system bridges innate and adaptive immunity. The cross-talk between toll-like receptors—as another key component of the innate immune system—and the complement system has been a key aspect of research as of their synergistic interaction to increase activation of inflammatory responses [3]. Complement activation runs through three major pathways (classic, alternative, and mannose-binding lectin) that all generate the enzyme complex C3-convertase which cleaves C3 into C3a and C3b. Herein, four main activation steps are distinguished: initiation of activation, activation and amplification of C3-convertase, activation of C5-convertase, and activation of the terminal pathway activity which is characterized by the assembly of the membrane attack complex (MAC) [4]. Importantly, the alternative pathway is constantly activated at low levels. Cascade progression and activation, however, is strictly controlled by complement regulating proteins such as complement factor H (CFH) and complement factor I (CFI): the two most important inhibitory proteins of the alternative pathway.

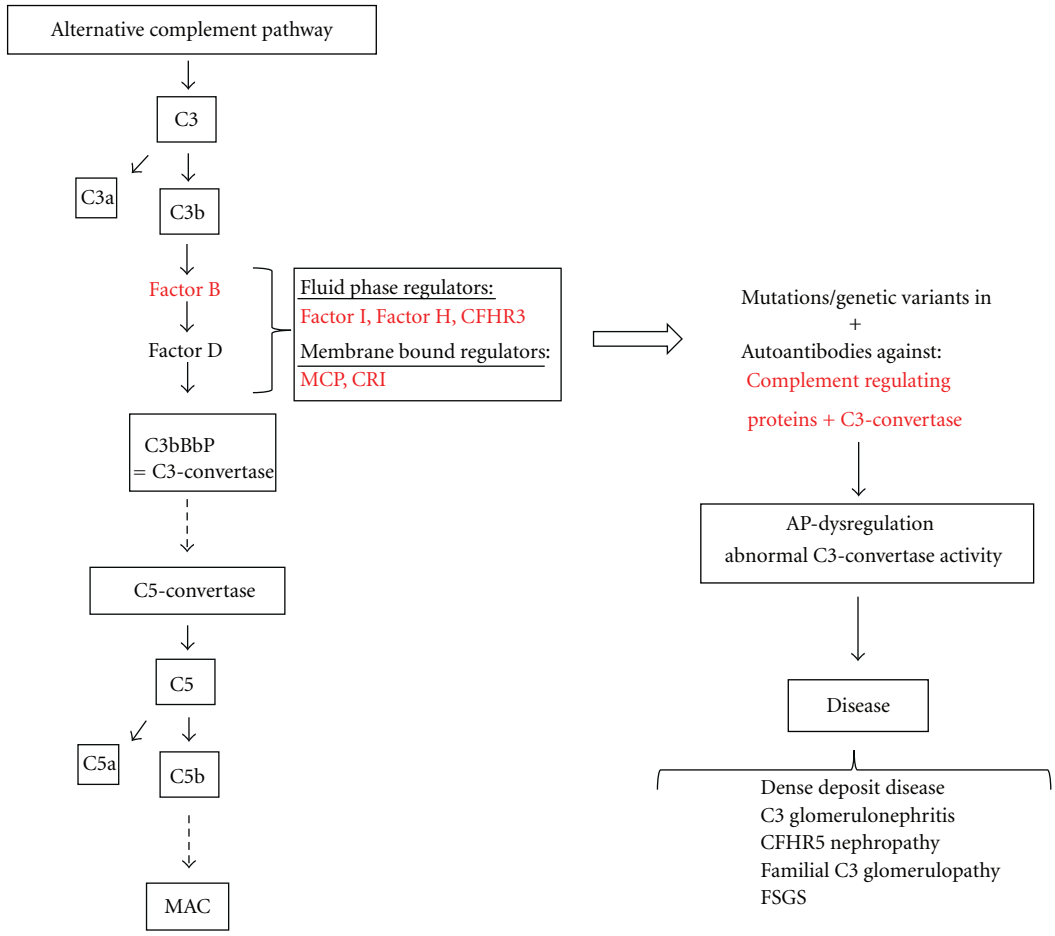

Figure 1: Dysregulation of the alternative complement cascade due to acquired or genetic factors leads to defective complement control causing a range of complement-associated glomerulopathies. C3 is cleaved to generate C3a and C3b. After binding of C3b to factor B, the complex is cleaved by factor D to form C3-convertase. The initial convertase constantly cleaves C3 at a low rate (referred to as "tick-over" of the alternative pathway). Binding of another C3b-fragment to C3-convertase creates a C5-convertase after which the pathway proceeds in the same manner as the classical pathway recruiting additional complement factors to ultimately form the membrane attack complex (MAC). The alternative pathway is strictly regulated by complement regulating proteins (listed in red). Mutations, genetic variations, or antibodies against complement regulating proteins or C3-convertase lead to abnormal C3-convertase activity. The subsequent deposition of activated complement products causes a range of complement-associated glomerulopathies. Abbreviations: C3; complement component 3, CFHR3; complement factor H related protein, AP; alternative pathway.

Complement dysregulation has been early recognized to be a central event in many nephropathies, and peripheral markers for complement activation (especially serum levels of C3 and C4) are tested routinely for different acquired renal diseases, for example, postinfectious glomerulopathy and proliferative lupus nephritis, glomerular capillaritis due to cryoglobulinemia or cholesterol embolism. Moreover, an increasing number of inherited renal diseases and renal diseases due to acquired factors with genetic predisposition for complement dysregulation are discovered such as atypical hemolytic uremic syndrome (aHUS) and membranoproliferative glomerulonephritis (MPGN) forms including dense deposit (DDD), C3 glomerulonephritis (C3GN) and CFHR5 nephropathy (Figure 1) [5, 6]. Mutations in *CFH* leading to CFH dysfunction and subsequently aHUS are the best known disease-causing mutations, but mutations in several other genes coding for complement factors and regulatory proteins have been identified in complement-related glomerulopathies (e.g., *C3, CFI, CFHR1-5, MCP* (membrane cofactor protein)). Genome-wide linkage analysis recently added novel polymorphisms and disease-causing mutations in complement genes to the list of hereditary complement-related nephropathies [7, 8]. In the following minireview we give an overview of complement-related glomerulopathies. Atypical HUS, a syndrome with prominent nonrenal, that is hematological and neurological manifestations, will not be discussed.

2. C3 Glomerulopathy

Patients with MPGN due to complement dysregulation manifest with proteinuria, (micro)hematuria and a variable degree of renal insufficiency. Kidney biopsy results show an altered glomerular basement membrane with double

Table 1: C3 glomerulopathies.

Diseases	EM-findings	Alternative pathway abnormalities	Disease specific treatment options
Dense deposit disease	(i) Osmophilic wavy dense deposits within GBM, mesangial matrix, tubular BM	(i) Autoantibodies (C3Nef, FHAA, FBAA, C3-convertase AA) (ii) Mutations/genetic variations (*CFH, CFI, CFB, MCP*)	(i) Infusion of fresh frozen plasma (ii) Plasmapheresis (iii) Eculizumab (iv) Immunosuppressive treatment (in case of autoantibodies)
C3 glomerulonephritis	(i) Mesangial, subendothelial, subepithelial and intramembranous deposits	(i) Mutations/genetic variations (*CFH, CFI, MCP*) (ii) Autoantibodies (C3Nef, FHAA)	(i) Eculizumab (ii) Immunosuppressive treatment (in case of autoantibodies)
CFHR5 nephropathy	(i) Mesangial, subendothelial, subepithelial deposits	(i) *CFHR5*-mutation	(i) No treatment of proven efficacy (ii) Plasma exchange associated with good outcome
Familial C3 glomerulopathy	(i) MPGN type III (ii) Subendothelial, subepithelial deposits	(i) Familial hybrid *CFHR3-1* gene autosomal dominant inheritance	(i) No treatment of proven efficacy

Abbreviations: C3: complement component 3, CFHR5: complement factor H related protein 5, CFH: complement factor H, CFI: complement factor I, MCP: membrane cofactor protein, FHAA: factor H autoantibody, FBAA: factor B autoantibody, (G) BM; (glomerular) basement membrane, MCP: membrane cofactor protein.

contours mostly due to subendothelial deposits, besides a variety of additional alterations such as hypercellularity and additional deposits [6]. As MPGN can be immune-complex-mediated, specific immunofluorescence has to be employed when evaluating the renal biopsy to differentiate between immunglobulin-mediated MPGN and complement-mediated MPGN.

Based on the localization of deposits in electron microscopy, MPGN has been classified into three different types: type I (subendothelial deposits), type II (intramembranous deposits), and type III (subendothelial and subepithelial deposits) [9]. Type I and III typically are immunglobulin-mediated diseases caused by the deposition of immune complexes as a result of for example circulating immune complexes, monoclonal gammopathies or chronic infections. MPGN II which is also called dense deposit disease, is characterized by complement component 3 (C3) containing dense deposits in the glomerular basement membrane that are a result of a dysregulation of the complement alternative pathway. As the inflammation is caused directly by the deposition of complement products, immunoglobulins are not involved and therefore not observed in immunofluorescence studies [6].

The subgrouping of MPGN has led to some confusion as all types of MPGN stain positive for C3 but immunglobulin staining can be negative even in some cases of MPGN I and III. Sethi et al. therefore proposed a classification driven by the findings on immunofluorescence, classifying MPGN as either immunoglobulin positive or negative [10]. Hence, immunoglobulin-negative but C3-positive MPGN is newly referred to as C3 glomerulopathy. Examples of C3 glomerulopathies are C3GN and DDD that can be distinguished by electron microscopical findings (see Table 1).

C3GN appears to be a key example of a dysregulated alternative and terminal complement pathway in which the deposition of complement is triggered despite the absence of antibody deposition [5, 10, 11]. Besides the identification of several disease-causing mutations in alternative pathway inhibitors, some autoantibodies leading to the activation or blockage of alternative pathway proteins have also been identified as a cause of C3GN. In a recent study by Servais et al., patients with C3GN (and additional patients with other forms of MPGN) were screened for mutations and rare variants in *CFH*, *CFI,* and *MCP* [12]. Although genetic abnormalities found in patients with C3GN were similar to the ones reported in individuals affected by aHUS, the genetic background predisposes specifically for the respective clinical and histological phenotype [12]. A rare, recently described variant of C3GN is CFHR5 nephropathy, a monogenic disease caused by mutations in the gene encoding complement factor-related protein 5 (CFHR5) [7]. CFHR5 is structurally related to CFH and possibly acts as a cofactor inhibiting C3-convertase [13]. In a cohort of patients with familial CFHR5 nephropathy sharing the same founder mutation, it was shown that the phenotype-spectrum among family members is broad [14]. As of this phenotypic heterogeneity, it is assumed that other factors like predisposing modifier genes and environmental factors as complement-activating infections also play a role in the development and phenotype of disease. CFHR5 is a member of the CFH related protein family, consisting of 5 proteins; CFHR1-5. Little is known about the function of these proteins, but there is increasing evidence that these protein families may either be involved in disease development or protection from complement dysregulation, respectively. CFHR1 inhibits C5-convertase activity and the formation of the terminal complex. CFHR3 also has complement regulatory activity as it inhibits C3-convertase [15]. Interestingly, a complete absence of both genes (Δ*CFHR3-1)* is not uncommon in the normal population. The deletion of *CFHR3-1* has even been associated with protection from both complement- and age-related macular degeneration as well as IgA nephropathy,

the most common mesangioproliferative glomerulonephritis with prominent mesangial IgA and secondary local complement activation [16]. In a recent study, a hybrid *CFHR3-1* gene was shown to cause familial C3 glomerulopathy [17]. The authors suggested a possible dominant mechanism of this genetic alteration leading to an increased expression of both proteins, interfering with complement processing and leading to accumulation of C3 [17].

Dense deposit disease (DDD) is closely related to C3GN and recent data suggest that both may represent extremes in a continuous spectrum of complement-related MPGNs [6, 12]. Both diseases show similar features in light and immunofluorescence microscopy and they are distinguished by electron microscopy. Here, C3GN is characterized by mesangial, subendothelial, and intramembranous deposits, whereas DDD is characterized by osmophilic dense deposits along the glomerular and tubular basement membranes [18, 19]. By an advanced mass spectrometry approach on glomerular isolates, Sethi et al. detected activated components of the alternative pathway and of the terminal complement pathway in patients with DDD [20]. Because of a very high recurrence rate after kidney transplantation, a systemic cause of DDD has been early suggested. Hence, the identification of the first C3 nephritic factor (C3Nef) as an autoantibody that stabilizes C3-convertase, was a major achievement [21]. The presence of C3Nefs is the most common association with alternative pathway dysregulation in DDD [22]. Binding of C3Nef to the alternative C3-convertase increases its half-life leading to uncontrolled alternative pathway activation and a massive consumption of C3. However, C3Nef activity is not always associated with low C3 levels in plasma and C3Nef is not specific for DDD as it can be found in other diseases as well as in healthy individuals [23]. Less common causes for DDD are inhibitory CFH autoantibodies, CFH deficiency, or functional CFH-defects, the latter both due to genetic mutations leading to reduced CFH activity. Mutations that lead to a complete CFH deficiency are rare though, and most functional CFH defects go along with normal CFH levels in plasma [24, 25]. Interestingly, not all patients with a functional CFH defect develop DDD, as CFH deficiency can also lead to aHUS and not all individuals with similar genetic variants develop the same phenotype [8].

In order to analyze the causes of alternative pathway dysregulation, several groups studied DDD cohorts employing functional and genetic tests [12, 22]. In these studies, a probable cause for complement dysregulation could be identified in about 80% of DDD patients [12, 22]. Detection of C3Nef was the most frequent single finding, but in some patients also autoantibodies against CFH or CFB were detected [22]. Gene variants in *CFH* were detected in 10–17% of DDD patients [12, 22]. Notably, a functional CFH defect frequently coexisted with the presence of C3Nef. Similarly, in C3GN the detection of C3Nef was the most common complement abnormality found in 45% patients with C3GN that were screened by Servais et al. [12]. Besides C3Nef, additional autoantibodies against CFH, CFB and to the individual components of C3-convertase (C3b and factor B) have been described in C3 glomerulopathies [12, 18, 26–28]. Anti-factor B autoantibody for example was found in a patient with DDD that was able to bind and thereby stabilize C3-convertase leading to an increased consumption of C3 [28]. This again indicates that DDD and C3GN have many pathogenetic and histological aspects in common and may represent extremes of a continuum.

3. Treatment

In order to decrease proteinuria and improve blood pressure control, nonspecific treatment with angiotensin converting enzyme (ACE) inhibitors or angiotensin type II receptor blockers is recommended in all patients. With improved understanding of the pathogenesis of C3 glomerulopathies more specific therapies could be applied. In patients with C3Nef or autoantibodies to CFH or CFB immunosuppressive therapies including rituximab or plasma exchange have been reported to slow disease progression or to help to avoid recurrence after transplantation [19, 26].

With the increasing knowledge about the underlying mechanisms and the specific complement defects in C3 glomerulopathies, specific complement-targeting therapies have been successfully applied in several cases of DDD and C3GN. As can be expected, large-scale clinical studies are missing in these rare diseases. Especially eculizumab, a humanized anti-C5 monoclonal antibody, represents a promising agent as it blocks C5b-9 formation, the terminal event in the complement cascade. The antibody has been approved by both the U.S. Food and Drug Administration as well as the European Commission for the treatment of paroxysmal nocturnal hemoglobinuria and, more recently, atypical HUS. Eculizumab was even suggested to be an effective agent in children with enterohemorrhagic *Escherichia coli* (EHEC) infection-caused HUS [29]. However, this was not evident in a case-control study reporting a mainly middle-aged population of a recent outbreak of EHEC induced HUS in northern Germany [30]. But for DDD and MPGN with CFHR1 deficiency, recent anecdotic reports suggest a treatment effect of eculizumab with stabilization of kidney function, decrease in proteinuria [31, 32], and even improvement in histopathological findings [33]. In a recent study reporting of three subjects with DDD and three subjects with C3GN who were treated with eculizumab for one year, response to treatment was seen in some but not all patients. Elevated serum membrane attack complex normalized on therapy, serum creatinine improved and proteinuria was reduced [34]. A very recent pathology report on C3GN patients treated with eculizumab showed de-novo monoclonal staining for IgG-kappa in the same distribution as C3 and C5b-9 in all posttreatment protocol biopsies, indicating binding to C5 and glomerular deposition of eculizumab [35]. As the authors state, the long-term clinical significance of these therapy-induced immune deposits together with apparent drug-tissue interactions is not known. Beside eculizumab as target-specific but costly treatment option fresh frozen plasma (FFP) infusions were given to several cases with functional CFH deficits and resulted in the prevention

of further disease progression [36, 37]. In a recent case report of two unrelated patients with MPGN and MPGN II with combined autoantibodies for factor B and C3, one patient received immunosuppressive treatment leading to a significant decrease of both autoantibodies [26].

In order to guide such disease specific treatment, it may become important to evaluate the alternative complement pathway more comprehensively in patients that have a renal biopsy consistent with C3 glomerulopathy. Complement proteins (CFH, CFI, CFB), complement degradation products (C3c, C3d), soluble serum membrane attack complex (sMAC), and disease associated autoantibodies (C3 nephritic factor, anti-factor H, anti-factor B, anti-C3b [26]) might expand the diagnostic arsenal of complement specific markers in the future.

4. Conclusions

In sum, the spectrum of renal disease phenotypes due to complement dysregulation is diverse. And it is still increasing: in a recent case report, Sethi et al. reported of single nucleotide polymorphisms in genes encoding CFH and C3 to be linked to the development of focal segmental glomerulosclerosis (FSGS), potentially extending the involvement of complement dysregulation to "podocytopathies" [38, 39]. Interestingly, this finding is in line with data from the European Renal cDNA Bank indicating alteration of intraglomerular transcript levels of complement-related gene products in FSGS (*own unpublished observation*).

As outlined above, several independent approaches such as Mendelian genetics, genome-wide association studies, transcriptomic and proteomic approaches as well as histopathology and functional studies underline the relevance of complement dysregulation in several kidney diseases, which currently undergo a redefinition. With the increasing insight into the pathophysiology, more specific complement targeting therapies may become available for the treatment of both ultrarare and more frequent complement-associated renal diseases.

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Clinical Study

The Effect of Weight Loss on Serum Mannose-Binding Lectin Levels

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Background. Serum levels of the mannose-binding lectin (MBL), which is an activator of the complement system, have been considered as a pathogenic factor in a broad range of diseases, and means of modulating MBL are therefore being evaluated. In this study we examine the effects of weight loss on MBL levels, and in continuation of this if MBL is synthesized in human adipose tissue. *Methods*. 36 nondiabetic obese subjects received a very low-calorie diet (VLCD) of 800 kcal/day for 8 weeks. Blood samples were collected at baseline and after VLCD. Furthermore, we measured MBL mRNA levels by the real-time RT-PCR on human adipose tissue compared to liver tissue. *Results*. The mean body weight was reduced from 106.3 ± 2.6 kg to 92.8 ± 2.4 kg, $P < 0.0001$. Median MBL at baseline was 746 μg/L (IQR 316–1190) versus 892 μg/L (IQR 336–1511) after 8 weeks, $P = 0.23$. No correlations were found between weight loss and changes in MBL ($r = -0.098$, $P = 0.57$). MBL real-time RT-PCR showed no expression of mRNA in adipose tissue, but as expected a good expression in liver tissue was seen. *Conclusions*. MBL levels are not affected by weight loss and MBL is not synthesized in human adipose tissue.

1. Introduction

Mannose-binding lectin (MBL) is a serum protein known to be synthesized by hepatocytes.

MBL exerts an important role in the innate immune system, where it upon binding to carbohydrate patterns of microorganisms activates the lectin pathway of the complement system through MBL-associated serine protease 1, 2, and 3 [1, 2]. This leads to recruitment of inflammatory cells, opsonization, and formation of the membrane attack complex [3], which causes destruction of the microorganisms.

Serum MBL level is dependent on genotype and, because of commonly occurring polymorphisms in the MBL encoding gene *MBL2*, the interindividually expressed serum level of MBL ranges from a few μg/L to several thousand μg/L. In healthy subjects approximately 56% have a high MBL encoding genotype (homozygote wild type), 40% have an intermediate MBL encoding genotype (mutation in one allele), and 4% have a low MBL encoding genotype (mutations in both alleles), the latter being functionally deficient in MBL [4], the most common immunodeficiency described.

Several clinical studies have shown low MBL levels to be associated with increased susceptibility or to poor outcome of infections. This lack of defense against invading microorganisms may be important especially in certain situations, for example, in infants with an immature immune system [5–7] and in adults with a compromised immune system [8, 9], as the immune system normally features a degree of redundancy.

Under disease circumstances other than an infection, activation of the complement system can be adverse and low MBL level may here confer an advantage. Keller et al. found that healthy men with low MBL levels are protected from cardiovascular disease [10].

Normally, MBL does not bind to the body's own cells, but may react with altered self-structures, for example, cells exposed to ischaemia/reperfusion injury [11, 12] or to apoptotic cells [13]. Also in autoimmune disease, as rheumatoid arthritis, MBL might bind to altered self-structures [14].

In a prospective study with 15 years of followup, our group documented an increased mortality among type 2 diabetic patients with high MBL levels compared with those with low MBL levels [15]. Furthermore, patients with a history of cardiovascular disease had significantly higher MBL levels than patients without prior cardiovascular disease [15]. Moreover the association between a history of cardiovascular disease and high MBL levels has been shown in type 1 diabetic patients and also that patients with nephropathy had significantly higher MBL levels than patients with normoalbuminuria [16]. A plausible explanation for the association between high MBL levels and diabetic vascular complications could be MBL binding to glucosylated cells in the endothelium and promoting low-grade inflammation through complement activation, resulting in accelerated atherosclerosis.

Because of MBL's possible pathogenic involvement in a broad range of diseases it might comprise a potential target of treatment. Studies of direct inhibition of human MBL in vitro [17] the rat MBL in vivo [12] and downstream regulation of complement activation with anti-C5 complement antibody in humans [18] are being conducted.

MBL level is, as mentioned above, mostly dependent on genotype and though it is known to be affected by hormonal stimuli [19] and is as such a modifiable parameter, intraindividual variation in MBL is small over time [20, 21].

So far the effects of weight loss and changes in insulin resistance on MBL levels have only been poorly investigated and with conflicting results. Regarding weight loss induced by bariatric surgery in obese subjects, both increased levels [22] and unchanged levels [23] have been reported. In the present study we aimed to investigate whether weight loss caused by dietary intervention influences MBL levels and in addition to investigate whether adipose tissue might be able to produce MBL.

2. Research Design and Methods

We investigated the effect of weight loss and change in insulin resistance on MBL levels in an intervention study including 36 healthy and nondiabetic obese subjects (18 women and 18 men). The subjects received a very low-calorie diet (VLCD) of ~800 kcal/day for 8 weeks.

Anthropometric data was obtained at baseline and after the 8 weeks of VLCD. Body composition (fat mass) and the percentage of body fat (fat mass/weight × 100%) were estimated by bioelectrical impedance using a multifrequency bioimpedance spectroscopy analyzer (SGB3; UniQuest Limited, Brisbane, Australia) as described by Heitmann [24].

We obtained fasting blood samples from the antecubital vein at baseline and after the 8 weeks of VLCD. Serum was separated and frozen at −80°C until the time of the analysis.

HOMA-IR was calculated as the product of the fasting plasma insulin level (microU/mL) and the fasting plasma glucose level (mmol/L), divided by 22.5.

Furthermore, to investigate if MBL is synthesized in adipose tissue real-time RT-PCR for MBL mRNA expression was performed on human adipose tissue obtained from fat biopsies from a subset of the participants and compared to commercial available human liver RNA.

The study was conducted according to the Declaration of Helsinki and was approved by the local Ethical Committee. Study participants gave a written informed consent before entering the study.

3. Blood Sample Assays

Serum glucose was measured by standard methods at the Clinical Biochemical Department, Aarhus University Hospital.

Serum insulin was measured by AutoDELFIA Insulin kit B080-101 (Perkin Elmer, Turku, Finland).

Serum MBL levels were measured by an immunoassay, as described previously [25], using an in-house time-resolved immunoflurometric assay with a lower detection level of 10 μg/L. In brief, microtiter wells were coated with monoclonal anti-MBL antibody followed by incubation with samples diluted 200-fold. After washing, monoclonal anti-MBL antibody labeled with europium was added, and after incubation and washing, the amount of bound, labeled antibody was assessed by time-resolved fluorometry. A number of control serum samples covering different MBL levels were included in all assays.

4. MBL Real-Time RT-PCR

For determination of MBL mRNA expression, RNA was isolated from adipose tissue obtained from a subset of the participants using TRIzol reagent as previously described [26]. Isolated total liver RNA was obtained from Ambion (Life Technologies Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF, UK).

Reverse transcription was performed using random hexamer primers as described by the manufacturer (GeneAmp RNA PCR Kit from Perkin Elmer Cetus, Norwalk, CT). Then, PCR-mastermix containing the specific primers and Taq DNA polymerase (HotStar Taq, Quiagen Inc., USA) was added.

The following primers were designed using the primer analysis software Oligo version 6.64: MBL2: GCAAACAGAAATGGCACGTAT and AGAGGCCTGGAACTTGACA, product length 139 bp and β-actin: ACGGGGTCACCCACACTGTGC and CTAGAAGCATTTGCGGTGGACGATG, product length 658 bp. Real-time quantitation of a target gene to β-actin mRNA was performed with a SYBR-Green real-time PCR assay using an ICycler from Bio-Rad. The target gene and β-actin mRNA were amplified in separate tubes. The increase in fluorescence was measured in real time during the extension step. The threshold cycle (Ct) was calculated and the relative gene expression was calculated essentially as described in the User Bulletin number 2, 1997, from Perkin Elmer (Perkin Elmer Cetus, Norwalk, CT). Briefly, the target gene (X0)-to-β-actin (R0) ratio in each sample before amplification was calculated as X0/R0 = kx1/((2**deltaCt)); delta Ct is the difference between Ct-target and Ct-reference, and k is a constant, set to 1. All samples were amplified in duplicate. A similar setup was used

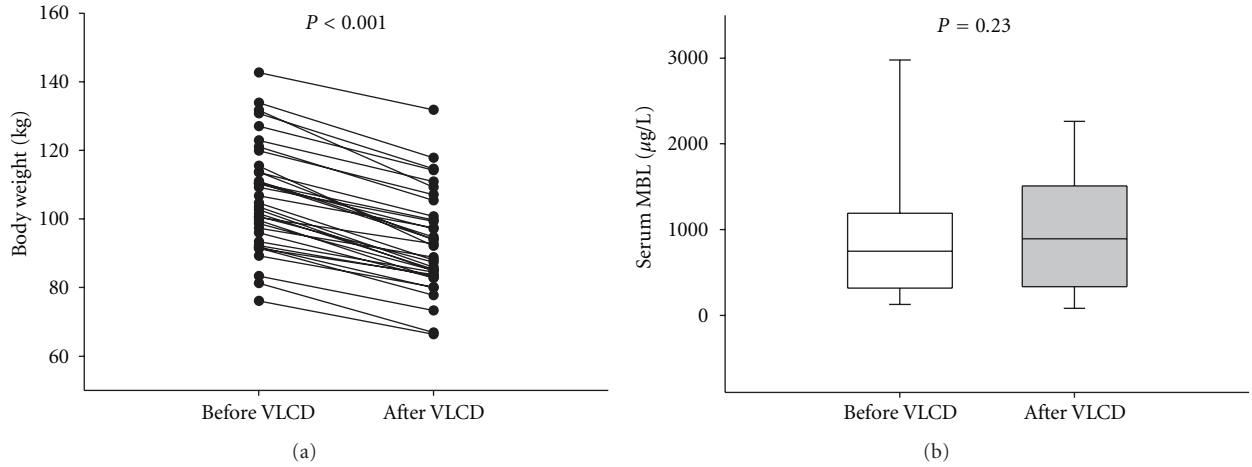

Figure 1: (a) Body weight before and after VLCD. (b) Serum MBL before and after VLCD.

for negative controls except that the reverse transcriptase was omitted and no PCR products were detected under these conditions.

5. Statistical Analysis

Values are presented as means ± SE, except for MBL, which was nonnormally distributed, and values are given as medians with interquartile ranges (IQR).

Paired or unpaired t-test was used for normally distributed variables as appropriate. For nonnormally distributed variables, comparisons between groups were performed using the Wilcoxon signed ranks test or the Mann-Whitney U-test as appropriate. Pearson's correlation was used for normally distributed variables, whereas Spearman's correlation with two-tailed probability values was used to estimate the strength of association between nonnormally distributed variables.

Statistical significance was assumed for $P < 0.05$. IBM SPSS version 20 was used for all calculations.

6. Results

The participants' mean age was 43 years (range from 24 to 62 years). Their characteristics at baseline and after 8 weeks of VLCD are presented in Table 1. They had a mean body weight of 106.3 ± 2.6 kg (range from 76.1 to 142.7 kg) and a mean body mass index (BMI) of 34.2 ± 0.5 kg/m^2 (range from 29.5 to 40.8 kg/m^2).

After 8 weeks of VLCD the mean body weight was reduced by 12.7% (106.3 ± 2.6 kg versus 92.8 ± 2.4 kg, $P < 0.001$), Figure 1(a). Mean BMI was reduced by 12.6% (34.2 ± 0.5 kg/m^2 versus 29.9 ± 0.6 kg/m^2, $P < 0.001$) and the insulin resistance (HOMA-IR) was reduced by 50.2% (2.39 ± 0.32 versus 1.19 ± 0.22, $P < 0.001$).

Measures of waist and fat mass (%) were also significantly reduced after 8 weeks.

Median serum MBL levels did not change significantly during the 8 weeks of VLCD (746 (316–1190) μg/L versus 892 (336–1511) μg/L, $P = 0.23$), Figure 1(b).

Table 1: Clinical characteristics of participants.

	Baseline	After 8 weeks of VLCD	P value
Weight (kg)	106.3 (±2.6)	92.8 (±2.4)	<0.001
BMI (kg/m^2)	34.2 (±0.5)	29.9 (±0.6)	<0.001
Waist (cm)	112.8 (±1.8)	102.5 (±1.8)	<0.001
Fat mass (%)	37.0 (±1.4)	31.6 (±1.7)	<0.001
HOMA-IR	2.39 (±0.32)	1.19 (±0.22)	<0.001
Serum MBL (μg/L) (median (IQR))	746 (316–1190)	892 (336–1511)	0.23

Median serum MBL levels did not differ significantly between men and women at baseline nor after 8 weeks and the change in MBL levels remained insignificant when looking at men and women separately (data not shown).

MBL levels did not correlate with age (data not shown).

As expected, change in HOMA-IR correlated to change in weight ($r = 0.595$, $P < 0.001$).

Correlations between serum MBL at baseline and other baseline parameters are shown in Table 2. MBL at baseline was not correlated to baseline weight, BMI, or HOMA IR. Correlations between changes in serum MBL and changes in other parameters after the 8 weeks are shown in Table 2. No correlations were found between changes in MBL and weight loss, change in BMI, or change in HOMA-IR.

MBL real-time RT-PCR showed no expression of mRNA in adipose tissue but as expected a good expression in liver tissue, Figure 2.

7. Discussion

Our results suggest that serum MBL levels do not seem to be related to weight or insulin resistance. As expected, weight loss and reduction in insulin resistance were correlated. MBL levels were not affected by weight loss or changes in insulin resistance.

Table 2: (a) Correlations (Spearman's rho) between baseline MBL and baseline weight, BMI and HOMA-IR. (b) Correlations (Spearman's rho) between change (Δ) in MBL and change (Δ) in HOMA-IR and weight and BMI.

(a)

Baseline	Baseline MBL
Weight	−0.022 ($P = 0.899$)
BMI	−0.013 ($P = 0.942$)
HOMA-IR	0.08($P = 0.964$)

(b)

Δ	ΔMBL
Weight	−0.098 ($P = 0.571$)
BMI	0.098 ($P = 0.571$)
HOMA-IR	−0.242 ($P = 0.154$)

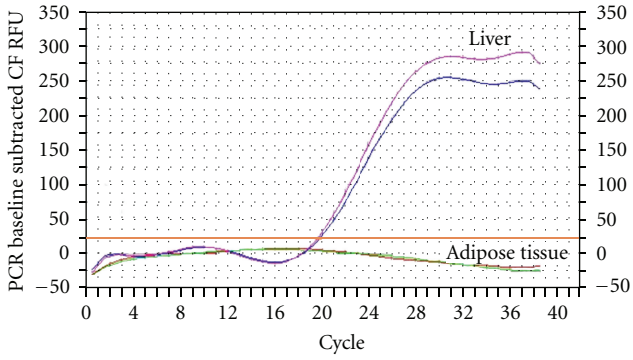

Figure 2: PCR of MBL in human liver and adipose tissue.

The effect of weight loss on MBL levels has been poorly elucidated, and the reports so far have been conflicting. A small study of 10 severely obese, nondiabetic men and women found MBL levels to increase after weight loss obtained over 2 years (after biliopancreatic diversion). Furthermore, the study concluded that the change in MBL levels were positively associated with the increase in insulin sensitivity [22]. In contrast, another study from the same group found no significant change in MBL levels after weight loss (also after biliopancreatic diversion) in 10 normal glucose-tolerant obese women [23], data in support of our findings.

We found no evidence that MBL is synthesized in the human adipose tissue. This further supports our finding of MBL not being related to weight loss.

The contradictory impacts of MBL under various conditions support a hypothesis of duality of MBL in a disease, and interventions, changing the MBL level, are currently being explored. Up to now, most of the focus has been made on antibodies to MBL or downstream complement factors, whereas the possible effect of lifestyle interventions remains sparsely explored.

8. Conclusion

We suggest that MBL levels may not be significantly modifiable by lifestyle interventions, such as weight loss, and that obesity and insulin resistance are not associated with MBL levels. MBL is not synthesized in human adipose tissue. Together, this indicates that decreased MBL levels are neither the result nor a contributing cause of obesity and insulin resistance. Rather interindividual differences in MBL depend primarily on the MBL genotype and constitute a preexisting condition, which might be an advantage or a disadvantage under different circumstances of health and disease.

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Review Article

Familial Atypical Hemolytic Uremic Syndrome: A Review of Its Genetic and Clinical Aspects

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Atypical hemolytic uremic syndrome (aHUS) is a rare renal disease (two per one million in the USA) characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure. Both sporadic (80% of cases) and familial (20% of cases) forms are recognized. The study of familial aHUS has implicated genetic variation in multiple genes in the complement system in disease pathogenesis, helping to define the mechanism whereby complement dysregulation at the cell surface level leads to both sporadic and familial disease. This understanding has culminated in the use of Eculizumab as first-line therapy in disease treatment, significantly changing the care and prognosis of affected patients. However, even with this bright outlook, major challenges remain to understand the complexity of aHUS at the genetic level. It is possible that a more detailed picture of aHUS can be translated to an improved understanding of disease penetrance, which is highly variable, and response to therapy, both in the short and long terms.

1. Introduction

Hemolytic uremic syndrome (HUS) is a rare disease characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure. It is most frequently caused by infections of Shiga-like toxin producing bacteria, such as *Escherichia coli* strain O157 : H7, O111 : H8, O103 : H2, O123, and O26 [1]. In approximately 10% of HUS cases, there is no association with Shiga-like toxin. These cases are classified as atypical HUS (aHUS) and occur with an incidence of about 2 per million in the USA [1, 2]. aHUS patients have a poorer prognosis than those with typical HUS, with acute phase aHUS mortality of about 8% [3, 4], and with 50%–80% of aHUS patients progressing to end-stage renal failure [1]. However, it is important to note that epidemiological outcomes data are relatively out of date because of the development of better diagnostic, treatment, and management strategies.

Atypical HUS can be classified as sporadic or familial. Familial aHUS is defined as the presence of aHUS in at least two members of the same family with diagnoses at least 6 months apart [1, 3, 5]. It accounts for less than 20% of aHUS cases [3]. In familial aHUS (and also sporadic aHUS), genetic (e.g., gene mutations, rare variants, and risk haplotypes) and acquired abnormalities (e.g., autoantibodies against factor H) are found in ~70% of patients [6]. Gene mutations are usually found in complement genes, such as factor H (*CFH*), factor I (*CFI*), factor B (*CFB*), complement component 3 (*C3*), and membrane cofactor protein (*MCP* or *CD46*). Evidence from familial studies indicates a high rate of incomplete penetrance, with about 50% of carriers of *CFH* or *MCP* aHUS-associated variants not developing disease [7]. The reasons underlying incomplete penetrance are unclear, although it is recognized that multiple predisposing genetic variants and risk haplotypes exist which may be relevant to disease onset in the face of environmental triggers such as pregnancy, viral infection, cancer, organ transplantation, and the use of certain drugs [8, 9].

In this paper, we focus on familial aHUS. Over the past 20 years, dozens of aHUS pedigrees have been reported, clarifying the underlying mechanisms of both familial and sporadic aHUS. In followed sections, we will discuss

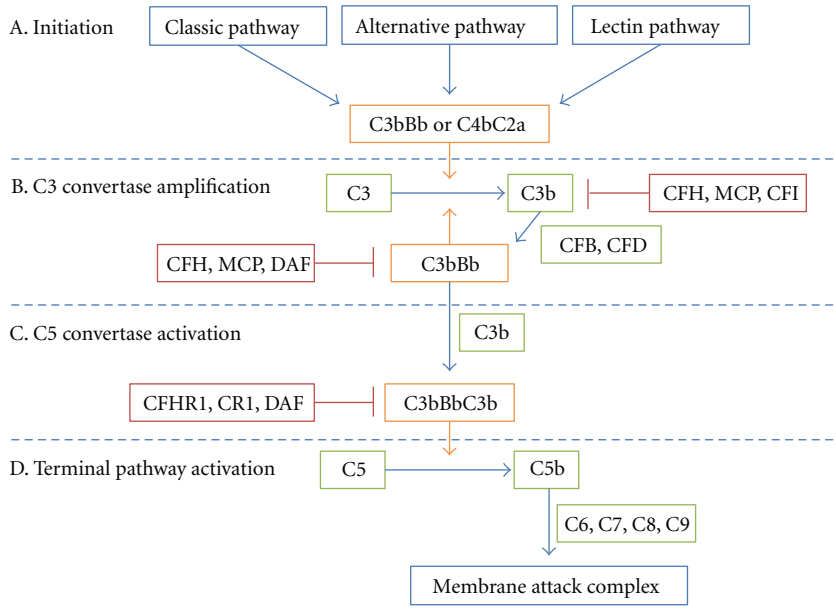

Figure 1: The complement system and its regulators. The complement system has four main steps. (A) Classical, alternative, or lectin pathway activation produces C3 convertases (C3bBb or C4bC2a) to initiate the complement cascade. (B) C3 convertase cleaves C3 into C3a and C3b. CFB binds to C3b and is cleaved by CFD into Bb, forming a new C3 convertase, C3bBb. This amplification step is tightly controlled by multiple regulators of complement (e.g., CFH, MCP, DAF, and CFI). (C) Once C3 convertase amplification is allowed to proceed, additional C3b is generated, ultimately forming C5 convertase, C3BbC3b. (D) C5 convertase cleaves C5 into C5b, which recruits C6, 7, 8, and 9 to form the membrane attack complex.

the complement system and aHUS, genetic abnormalities identified in familial studies, factors associated with incomplete penetrance, and current methods of diagnosis and treatment.

2. The Complement System

The complement system is an essential component of the innate immunity (Figure 1). Its four major steps are: (1) the initiation of the complement cascade; (2) C3 convertase activation and amplification; (3) C5 convertase activation; (4) terminal pathway activation [10]. Initiation of the complement cascade occurs through three pathways: the classical pathway [11, 12], the lectin pathway [13, 14], and the alternative pathway [15, 16]. Once activated, C3 convertases are formed (the alternative pathway forms C3bBb, and the classical pathway or the lectin pathway forms C4bC2a), which cleave C3 to C3a and C3b. C3b can indiscriminately bind to surfaces of microbes and host cells [17, 18]. On the surface of microbes or modified host cells, C3b and factor B form more C3 convertases, which produce more C3b. This amplification process exponentially increases the amount of C3b and C3 convertases.

On the surface of intact host cells, in contrast, C3b deposition and C3 convertase amplification are prevented by complement regulators. Regulators distribute in the fluid phase (CFH, CFHR1, CFP, etc.) and on cell surfaces (CR1, MCP, DAF, etc.) to control complement activity through two major mechanisms: decay acceleration activity and cofactor activity [10]. CFH, for example, acts as a cofactor with CFI to cleave C3b to an inactive form, iC3b; has the decay acceleration activity, which promotes the decay of the C3 convertase [19, 20] and competes with CFB for binding to C3b. If C3 convertase amplification is allowed to proceed unchecked, additional C3b binds to C3 convertases to generate C5 convertases (C3bBbC3b or C4bC2aC3b) [21]. C5 convertases cleave C5 to C5a and C5b to initiate the terminal pathway and form terminal complement complexes with C6, C7, C8, and C9 to lyse target cells [22, 23].

Mutations of complement genes can either change expression level or disrupt protein function. Figure 2 shows a model of dysregulation of complement regulators inducing aHUS. Mutations in complement genes impair the regulation of C3b on host cells, leading to formation of membrane attack complex and host cell damage. Most genetic abnormalities in aHUS patients are found in complement membrane regulators and C3 convertases. Multiple genetic and environmental risk factors are believed necessary to develop disease; however, relatively little is known about how environmental triggers affect homeostasis of complement system in the face of predisposing genetic variants in complement genes [8]. It is also unclear whether genetic variants in complement gene increase susceptibility to typical HUS [24, 25].

3. Genetic Abnormality in Family Cases

The term "familial aHUS" is used to describe families in which two or more persons develop aHUS at different times without exposure to common triggering infectious agents, or when disease-causing mutations are identified in one of

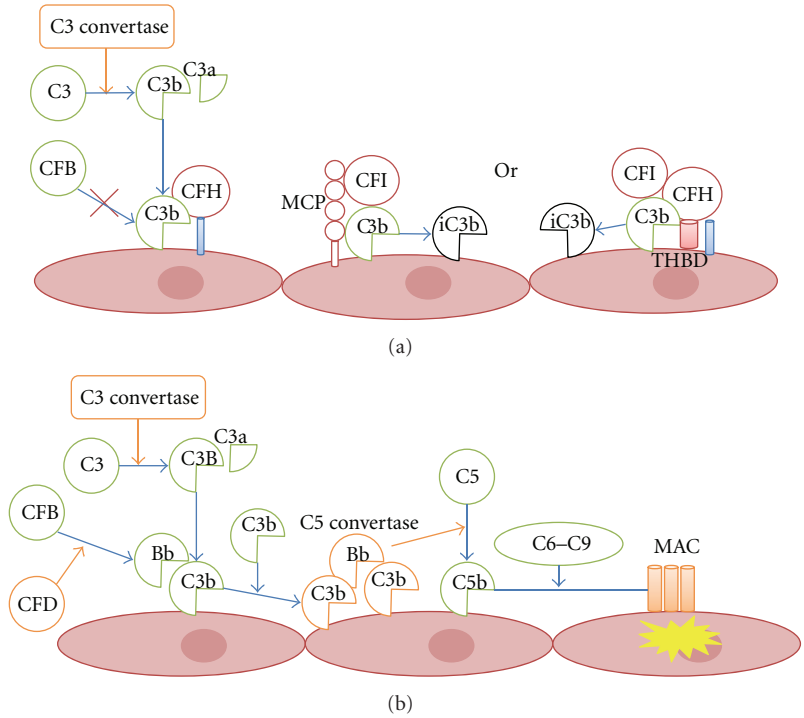

Figure 2: Regulation and dysregulation of complement activity on host cells. C3b is generated by the classical, lectin, or alternative pathways. (a) To protect normal host cells, C3b is inactivated by membrane regulators, such as factor H (CFH) and membrane cofactor protein (MCP). Factor I (CFI) cleaves C3b into iC3b and other C3 degradation products with the activity of cofactor regulators. (b) If genetic and/or environmental risk factors reduce the efficiency of membrane complement regulators, C3 convertase (C3bBb) can accumulate on cell surfaces, creating a C3b amplification loop. Formation of C5 convertase (C3bBbC3b) triggers C5 cleavage into C5b, which interacts with C6, C7, C8, and C9 to generate membrane attack complex (MAC) leading to cell damage.

the genes (discussed next) known to be associated with aHUS irrespective of familial history [1]. Thus, genetic aHUS can be multiplex (two or more affected family members) or simplex (a single occurrence in a family). Since simplex cases develop in patients who do not have a family history of disease, these cases are also referred to as sporadic [1].

The first familial aHUS case was reported in concordant monozygotic twins in 1965 [26]. Since that report, autosomal dominant and recessive familial aHUS has been reported. Familial studies have revealed important genetic factors contributing to aHUS, including mutations in *CFH*, *CFHR3*, *MCP*, *CFI*, *CFB*, and *C3* (Table 1). Most of these mutations impair protein function, causing dysregulation of the complement pathway once it has been activated. Interestingly, some genes implicated in sporadic aHUS, such as *THBD* [5], have not been associated with familial cases to date. It is reasonable to expect that comprehensive genetic screening of genes in the complement and coagulation pathways will identify variants in additional genes that impact disease penetrance, consistent with aHUS being a complex genetic disease.

3.1. CFH and CFHR Mutations. Complement factor H, encoded by *CFH* gene, is an essential inhibitor of C3 convertase and a central regulator of the complement alternative pathway. It is produced by liver as a soluble protein but can attach to and act on cell surfaces. CFH protein contains 20 repetitive units of about 60 amino acids named short consensus repeats (SCRs; also known as complement control protein (CCP) repeats or Sushi domains) [27]. N-terminal SCRs regulate binding to C3b, while C-terminal SCRs facilitate cell-surface binding and regulation. CFH regulates the complement system through three mechanisms: (1) inhibiting the assembly of C3 convertase by competitive binding to C3b, (2) accelerating the decay of C3 convertase, and (3) acting as a cofactor in the cleavage and degradation of C3b by CFI [27].

CFH is the most thoroughly studied gene in aHUS. Mutations associated with aHUS were first identified in *CFH* by a familial genetic study in 1998 when Warwicker and colleagues conducted linkage analysis in three aHUS families and mapped the aHUS risk region to a 26-cM interval on chr 1q32 with a lod score of 3.94. The linked region includes the *CFH* and *CFHR* genes, and in sequencing *CFH*, a heterozygous c.3716C>G variant changing an arginine to glycine was found to cosegregate with disease in family 2. In family 3, a *CFH* deletion, c.145_148delAGAA, was found [28].

Numerous missense transversion and transition variants in *CFH* have now been associated with aHUS through familial studies [29–34]. Typically, affected patients are heterozygous for these changes, which predominantly occur in the C-terminal SCRs 19 and 20. Since these SCRs are essential for

Table 1: Reported gene mutations and risk haplotypes in aHUS pedigrees[#].

Author	Year	Population	Size/carrier/affected	Gene: variant or haplotype	Risk genotype	Penetrance rate	SNP rs ID	MAF
Warwicker et al. [28]	1998		51/3/11*	*CFH*: c.3716C>G (p.R1197G)	C/G		rs121913051	
				CFH: c.145_148delAGAA	het			
Ying et al. [31]	1999	Bedouin-Arab	55/?/11**	*CFH*: c.3645C>T (p.S1191L)	T/T		rs460897	29.35%
Richards et al. [40]	2003	Belgian	8/0/3	*MCP*: p.237_238delDS	het	100.0%		
		German	4/1/2	*MCP*: c.822T>C (p.S206P)	T/C	66.7%	rs121909589	
		Turkish	4/2/2	*MCP*: c.822T>C (p.S206P)	C/C	50.0%	rs121909589	
Caprioli et al. [30]	2003		13/1/2	*CFH*: c.1494_1496delAAA	het	66.7%		
			5/1/2	*CFH*: c.3620T>A (p.Y1183R)	T/A	66.7%		
			10/5/2	*CFH*: c.3654G>A (p.G1194D)	G/A	28.6%		
			6/1/3	*CFH*: c.3701C>T (p.R1210C)	C/T	75.0%	rs121913059	0.02%
			37/?/10*	*CFH*: c.3579A>T	A/T			
				CFH: a 24-bp deletion in SCR20	het			
			9/2/3	*CFH*: c.3717G>A (p.R1215Q)	G/A	60.0%		
Noris et al. [41]	2003	White	4/1/2	*MCP*: c.843_844delAC	het	66.7%		
Frémeaux-Bacchi et al. [42]	2006	White	5/0/3	*MCP*: p.G162R	het	100.0%		
			4/0/2*	*MCP*: p.Y155D	het	100.0%		
				MCP: c.IVS7-2A>G	het	100.0%		
Esparza-Gordillo et al. [43]	2006	Spanish	24/11/2*	*MCP*: c.598C>T(p. P165S)	C/T	28.6%		
				MCP: MCPggaac***	het	33.3%		
				CFI: c.1610insAT(p.T538X)	het	28.6%		
Caprioli et al. [7]	2006	Sardinian	8/4/3*	*MCP*: D1S2735, D1S2796, IVS1-1G>C, ExV(SCR3), Ex XII, D1S2692	2,1,+,A,T,8	42.9%		
				MCP: c.IVS1-1G>C	G/C	42.9%		
			8/1/5*	*MCP*: c.218C>T	C/T	85.7%		
				MCP: c.147G>A	G/A	85.7%		
			5/2/2	*MCP*: c.843_844delAC	het	50%		
			21/5/2	*MCP*: c.768T>G	T/G	28.6%		
Goicoechea de Jorge et al. [53]	2007	Spanish	32/4/7*	*CFB*: c.858C>G (p.F286L)	C/G	64.0%		
				MCP: MCPggaac	het	87.5%		
Frémeaux-Bacchi et al. [50]	2008		54/?/6	*C3*: p.R570W	C/T			
Martinez-Barricarte et al. [29]	2008		6/1/3	*CFH*: p.R1210C	C/T	12.5%	rs121913059	0.02%
Lhotta et al. [51]	2009	Austrian	61/9/4	*C3*: c.1775G>A (p.R570Q)	G/A	10.0%	rs121909583	
Habibi et al. [34]	2010	Tunisian	33/10/6	*CFH*: c.3767_3771delTAGA	hom	37.5%		
Sullivan et al. [33]	2010		6/3/2	*CFH*: c.3007G>T (p.W978C)	G/T	40.0%		

Table 1: Continued.

Author	Year	Population	Size/carrier/affected	Gene: variant or haplotype	Risk genotype	Penetrance rate	SNP rs ID	MAF
			5/2/2	*CFH*: c.3619G>T (p.R1182S)	G/T	50.0%		
			4/1/2	*MCP*: c.404delG (p.G135VfsX13)	het	66.7%		
			3/?/2	*CFI*: c.491A>T (p.D164V)	A/T			
Hakobyan et al. [32]	2010		15/3/4	*CFH*: p.C853R, H402	het+H	33.3%		
Provaznikova et al. [44]	2012		3/0/2	*MCP*: c.1148C>T	C/T			
			2/0/2	*MCP*: c.404G>A	G/A			
			2/0/2	*MCP*: c.350insA	het			
			7/2/2	*MCP*: c.2T>A	T/A	50.0%		
Sartz et al. [52]	2012		20/2/4*	*C3*: c.4973T>C (p.V1636A)	T/C	100.0%		
				CFI: c.IVS12+5G>T	G/T	100.0%		
				MCP: c.1058C>T (p.A304V)	C/T	50.0%		
				CFH: c.2850G>T (p.Q950H)	G/T	50.0%		
Francis et al. [38]	2012		35/4/3	*CFH/CFHR3* hybrid	het	42.9%		

[#] Pedigrees are included if at least two family members were diagnosed with aHUS at least 6 months apart.
* More than one mutation identified within the family.
**? presents undetermined number.
***The risk haplotype *MCP* ggaac is formed by rs2796267, rs2796268, rs1962149, rs859705, and rs7144.

cell-surface attachment, this finding suggests that membrane dysregulation of the complement system is critical to the pathogenesis of aHUS.

Based on available studies, we estimate that penetrance of *CFH* mutations ranges from 12.5% to 100% (Table 1). It is remarkable that one *CFH* rare variant, rs121913059 (c.3701C>T or p.R1210C), has been reported in five families from three familial studies [29, 30, 32]. This variant decreases CFH binding to C3b, heparin, and endothelial cells, and leads to a positive sheep erythrocyte hemolytic assay [35]. The Y402H variant (rs1061170, c.1204T>C) of *CFH*, notable for its association with age-related macular degeneration [36], dense deposit disease, and C3 glomerulonephritis [37], has not been associated with aHUS. However, Hakobyan et al. have reported low expression of the CFH-H402 allele in association with other known aHUS variants in two aHUS families, suggesting that in some instances the CFH-H402 allele may contribute to the aHUS phenotype [32].

In addition to *CFH*, *CFHR3* has been linked with familial aHUS. The *CFH*-related genes (*CFHR1*, *CFHR2*, *CFHR3*, *CFHR4,* and *CFHR5*) localize next to *CFH* and share many of the functional properties of *CFH*. A recent study has reported a hybrid *CFH/CFHR3* gene caused by a microhomology-mediated deletion that is associated with familial aHUS. The transcript product of the hybrid gene contains 24 SCRs with SCRs 1–19 deriving from *CFH* and SCRs 20–24 deriving from *CFHR3*. The hybrid protein shows normal fluid-phase activity but loses complement regulation on cell surfaces [38].

3.2. MCP Mutations. MCP (*CD46*) encodes membrane cofactor protein, which acts as a cofactor for CFI to regulate complement activity by cleaving C3b and C4b deposited on the surface of host cells. MCP is a transmembrane protein with four N-terminal extracellular Sushi domains, a transmembrane domain, and a C-terminal cytoplasmic tail. Sushi domains 3 and 4 are responsible for complement regulation [39].

MCP is well studied in aHUS. In 2003, Richards et al. [40] first reported mutations of *MCP* in aHUS families. Two mutations were found in three families—a 6 bp deletion (p.237_238delDS) and c.822T>C (p.S206P, rs121909589). The c.822T>C mutation causes an amino acid change of serine to proline and leads to a significant reduction of C3b binding. Soon afterwards, Noris et al. reported another aHUS family carrying a 5 bp deletion in *MCP* gene, which causes a premature stop codon in the fourth Sushi domain [41]. Expression analysis showed around 50% reduction in MCP as compared to healthy controls. Subsequently, several more *MCP* mutations have been identified in aHUS families [7, 33, 42–44].

Studies indicate that MCP mutations account for up to 15% of aHUS patients [7, 33, 42]. Although the majority of MCP mutations are heterozygous (~75%), some homozygous or compound heterozygous mutations in MCP have been reported [45]. MCP mutations are defined as (a) type I (~75%) if they reduce expression on cell surface and (b) type II (~25%) if expression is normal but complement regulatory activity is impaired [46, 47].

The *MCP* ggaac haplotype formed by c.−652A>G (rs2796267), c.−366A>G (rs2796268), c.IVS9−78G>A (rs1962149), c.IVS12+638G>A (rs859705), and c.4070T>C (rs7144), is associated with aHUS in both sporadic and familial cases [43, 48, 49]. Further studies are needed to determine the functional or expression differences between *MCP ggaac* and normal haplotypes.

3.3. C3 Mutations. Complement component C3 is the keystone in the complement system. It undergoes spontaneous hydrolysis and is also cleaved by C3 convertase to C3b and C3a. C3b interacts with CFB to form C3bB, which is then cleaved to C3bBb by CFD forming the C3 convertase. Additional C3b leads to the formation of C5 convertase, which activates the terminal pathway. C3 products are key ligands for multiple complement regulators, including CFH and MCP. Theoretically, mutations influencing C3 binding ability or other functions could disrupt complement regulation and contribute to the development of aHUS.

Reported C3 mutations are heterozygous and localized on both the beta and alpha chains. In 2008, Frémeaux-Bacchi et al. first reported nine mutations of *C3* in 14 patients from 11 families, including a p.R570W mutation in a very large family. Five of the nine identified mutations (p.R570Q, p.R570W, p.A1072V, p.D1093N, and p.Q1139K) reduce ligand binding to MCP, making the mutant convertase resistant to cleavage by CFI thus impairing complement regulation of C3 convertase amplification on cell membranes [50]. Lhotta et al. have also reported a large Austrian family carrying the p.R570Q C3 mutation. In their study, they showed reduced or borderline C3 levels in mutation carriers [51]. Recently, another familial C3 mutation, V1636A, has been identified to cause increased affinity of CFB for C3b [52].

3.4. CFB Mutations. Complement factor B, a key component of C3 convertase (C3bBb), contains three Sushi domains, a vWFA domain, and a peptidase S1 domain. It is cleaved by CFD into Ba and Bb. Bb is a serine protease, which binds to C3b to generate the C3 convertase.

In 2007, Goicoechea de Jorge et al. reported a CFB gene mutation in an aHUS family with seven patients [53]. Sequence analysis and functional studies indicated that the missense mutation, c.858C>G (p.F286L), in the vWFA domain, caused more rapid formation and a higher level of C3 convertase. Penetrance was incomplete with seven of 11 mutation carriers developing aHUS. Interestingly, the *MCP* ggaac risk haplotype was found only in patients and one young carrier who is probably still at risk for disease, suggesting that the effects of the CFB variant are modulated by variants in other complement gene.

3.5. CFI Mutations. Complement factor I is an inhibitory regulator of complement system. It cleaves C3b or C4b with the presence of cofactors, such as CFH and MCP, to iC3b, which is cleaved to smaller C3 degradation products. Defects in *CFI* cause multiple complement-related diseases, including aHUS and CFI deficiency (OMIM: 610984), a disease characterized by recurrent infections and glomerulonephritis in some patients [54].

Most *CFI* mutations have been found in sporadic aHUS cases. These mutations either interrupt cofactor activity or impact the expression level of CFI [55]. In familial aHUS studies, CFI mutations have been reported in three pedigrees (Table 1) [33, 43, 52]. In a Spanish family, a heterozygous 2 bp insertion within the coding region of *CFI* has been identified to cause a premature stop codon, p.T538X, which reduces plasma levels of CFI by 50%. A missense mutation in *MCP* (c.598C>T) and the *MCP* ggaac risk haplotype were also identified in this family, with all patients carrying all three genetic risk factors. Nine unaffected persons carry only one or two genetic risk factors, suggesting that it is the combination of mutations and the risk haplotype that are critical to the development of aHUS [43].

3.6. Combined Mutations and Incomplete Penetrance of Familial aHUS. Multiple familial studies have reported that it is the combination of complement gene mutations that contributes to aHUS [29, 30, 32, 42, 43, 52, 53]. For instance, in a study by Sartz et al., four mutations, one each in *C3*, *MCP*, *CFI*, and *CFH*, were found in two patients from a single family [52]. The *C3* mutation, p.V1636A, increases the affinity for CFB and C3 convertase; the *MCP* mutation, p.A304V, increases the activation of the alternative pathway on cell surfaces; and although the functional significance of the *CFI* mutation (c.IVS12+5) and the *CFH* mutation (p.Q950H) is unknown, they have been reported in other aHUS cases [52]. The aggregate data suggest that accumulated dysregulation by combined mutations impairs the complement system and leads to disease [29, 30, 32, 42, 43, 52, 53]. It is unknown whether other complement factors, such as *THBD*, *CR1*, *C5-C9*, and *DAF*, contain risk variants that contribute to the mutation/variant load in aHUS.

Incomplete penetrance is widely observed, with the estimated penetrance of aHUS in mutation carriers being about 50~60% [7, 8]. Within families, affected persons may also show different symptoms and onset ages [56]. These findings strongly suggest that most aHUS-associated genetic variants predispose to rather than cause the disease. However, the genetic picture is incomplete as most studies have focused on only the common complement genes in a disease where rare genetic variants in other complement genes and genes in other pathways are likely to be contributory to the phenotype. Importantly, the effect of common variants is probably marginal as demonstrated by Ermini and colleagues who tested 501 SNPs in 47 complement genes in 220 aHUS patients and 549 controls and found disease associations for only *CFH*, *MCP*, and the *CFHRs* [49]. However, until a comprehensive rare variant screen is completed, it will remain very difficult to calculate disease risk for persons in aHUS families.

4. Diagnosis and Treatment

aHUS is clinically characterized by microangiopathic hemolytic anaemia (low hemoglobin, high lactic acid dehydrogenase, undetectable or low haptoglobin, presence of schistocytes in the peripheral blood smear, and negative Coombs test), thrombocytopenia (platelets < 150000/mm^3 or a documented rapid decrease), and acute kidney injury (AKI) (hematuria, proteinuria, and/or reduced renal function). However, as a systemic disease, aHUS can affect the endothelia of any organ, and extrarenal manifestations including involvement of the central nervous system, liver, heart, pancreas, and skin, are observed in as many as 20% of patients [3, 4]. These additional sites of involvement can blur the distinction between aHUS and other primary thrombotic microangiopathies (TMAs), such as STEC-associated HUS, thrombotic thrombocytopenic purpura (TTP), HELLP syndrome (hemolytic anemia, elevated liver enzymes, and low platelets), and transcyanocobalamin deficiency, or TMAs secondary to malignant hypertension, catastrophic antiphospholipid syndrome, or disseminated intravascular coagulation.

The treatment of aHUS is based on two main strategies: supportive treatment and cause-specific treatment. The former is focused on careful fluid, electrolyte, acid-base, and nutritional management, with the use of blood transfusion, antihypertensive medications, and/or dialysis, as needed. Cause-specific treatment includes plasma therapy provided by either plasma infusion (fresh frozen plasma, 20–40 mL/kg/day if the patient is not volume overloaded) or by high volume plasma exchange with fresh frozen plasma (150% of plasma volume daily or every other day until clinical remission). A recent impressive improvement in the management of aHUS has been reported with the use of the anti-C5 monoclonal antibody, Eculizumab, which binds to C5 thereby preventing activation of the terminal complement cascade. This relatively new (since 2009) treatment is continued until stable clinical remission. Whether life-long or recurrence-specific treatment is necessary and how genetics may or may not impact care of persons on Eculizumab have not been determined.

5. Transplantation

Only recently has renal transplantation become the treatment of choice for patients in end stage renal disease on chronic dialysis for aHUS. Until the availability of Eculizumab, transplantation was associated with a 40%–80% risk for disease recurrence [3, 57–59]. The notable exception was aHUS patients with *MCP* mutations since MCP is expressed on the renal endothelia and not in the fluid phase. Eculizumab has become a key resource for preventing recurrence following kidney transplantation and for rescue therapy in case of disease recurrence. Combined liver-kidney transplant with preemptive and perioperative plasma therapy [60], although successfully used in the very recent past, in the Eculizumab era no longer appears to be first-line treatment. Transplantation with a living-related donor is not recommended given our current incomplete understanding of the genetics of aHUS.

6. Conclusion

Over the past two decades, studies of familial aHUS have greatly increased our understanding of this disease. The identification of genetic variants in complement genes has

defined the mechanism whereby complement dysregulation at the cell surface level leads to disease. This understanding has culminated in the use of Eculizumab as first-line therapy in disease treatment, significantly changing the care and prognosis of affected patients. However, even with this bright outlook, major challenges remain to understand the complexity of aHUS at the genetic level. It is possible that a more detailed picture of aHUS can be translated into patient-specific short- and long-term therapy with Eculizumab and/or other anticomplement drugs in the developmental pipeline.

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Review Article

Initiation and Regulation of Complement during Hemolytic Transfusion Reactions

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Hemolytic transfusion reactions represent one of the most common causes of transfusion-related mortality. Although many factors influence hemolytic transfusion reactions, complement activation represents one of the most common features associated with fatality. In this paper we will focus on the role of complement in initiating and regulating hemolytic transfusion reactions and will discuss potential strategies aimed at mitigating or favorably modulating complement during incompatible red blood cell transfusions.

1. Introduction

As pathogens began to evolve elaborate antigenic structures to avoid innate immunity, vertebrates evolved an equally impressive mechanism of combating antigenic diversity among pathogens [1]. Indeed, adaptive immunity appears to possess the capacity to respond to a nearly infinite number of antigens, enabling immunological protection against a wide variety of potential pathogens [1, 2]. However, not all foreign antigens represent a pathogenic threat. Although tolerance mechanisms exist that reduce the likelihood of developing antibodies against innocuous antigens, individuals can possess significant antibodies against antigenic polymorphisms on human tissue [2]. Indeed, hemolytic transfusion reactions typically reflect the engagement of antibodies directed against antithetical antigens on donor red blood cells (RBCs).

The earliest example of human donor rejection occurred following transfusion of ABO (H) incompatible RBCs [3]. Although ABO(H) represent the first RBC polymorphic antigens described, many other carbohydrate and protein antigenic differences became apparent as transfusion practices increased [4]. Interestingly, these immune-mediated discoveries provided the first example of significant polymorphisms within the human population long before DNA was recognized as the molecular basis of inheritance [5–8]. As hemolytic transfusion reactions (HTRs) can occur following transfusion of incompatible RBCs or following transfer of antibodies present in donor units, such as platelets or plasma, significant testing occurs prior to transfusion to insure utilization of antigen compatible blood products [9, 10]. Unfortunately, these procedures occasionally fail. In addition, some patients fail to demonstrate detectable antibodies but exhibit amnestic antibody responses to previously exposed RBC antigens following transfusion [11]. Under these circumstances, cellular rejection in the form of a hemolytic transfusion reaction may occur.

Hemolytic transfusion reactions may not only cause significant morbidity and compromise the therapeutic efficacy of transfusion, but ultimately these reactions can prove fatal. Indeed, hemolytic transfusion reactions represent one of the most common causes of transfusion-related mortality. Furthermore, in highly immunized patients, securing antigen compatible blood can be difficult, if not impossible, preventing appropriate and timely life-saving intervention

[12]. As a result, a greater understanding of the factors that may influence hemolytic transfusion reactions is needed. Although many factors influence hemolytic transfusion reactions, in this paper we will focus on the potential role of complement in initiating and regulating hemolytic transfusion reactions, with a particular focus on potential strategies aimed at mitigating or favorably modulating complement during incompatible RBC transfusions.

2. Early Transfusion Reactions

While many diseases reflect complement dysregulation [13], perhaps the earliest and most potent example of complement-mediated mortality predates the discovery of microbes and immunity. In 1667, Dr. Jean-Babtiste Deny transfused several patients multiple times with either sheep or calf blood. Although the patients appeared to initially tolerate transfusion, repeated transfusions uniformly resulted in patient death [4, 14]. Subsequent attempts nearly two centuries later utilizing human donors for transfusion resulted in more favorable outcomes; however, patients receiving transfusions from human donors occasionally experience similar fatalities despite many attempts to predict favorable responses to transfusion [4].

Prompted by previous work suggesting that antigenic differences on RBCs occur between different mammalian species, Karl Landsteiner sought to determine whether similar differences may account for incompatible transfusions using human donors [3]. In 1900, Landsteiner published his seminal work demonstrating that sera isolated from patients could differentially agglutinate donor RBCs [5]. Within the next decade, the discovery of A, B, and C (O) antigens enabled accurate prediction of immunological compatibility between donor and recipient, for which Landsteiner was awarded the Nobel prize in physiology and medicine in 1930 [3].

While the factors responsible for fatal outcomes during an incompatible transfusion remained unknown for many years, naturally occurring antibodies directed against carbohydrate xenoantigens on animal RBCs or ABO(H) antigens on human RBCs likely mediated activation of complement [15]. Robust complement activation not only results in significant intravascular hemolysis, but complement products also independently induce significant physiological changes. Indeed, early transfusion reactions likely reflected significant complement-mediated hemolysis and systemic alterations that ultimately resulted in fatal outcomes [15–18].

3. Complement: A Brief History

The identification of microbes as a potential cause of human illness drove intense research by numerous investigators to understand host factors that may inhibit microbial invasion. Early studies by Pastuer and others demonstrated that inoculation of animals with microbes could induce a form of host resistance to further infection [19]. Although the specific players responsible for acquired host immunity remained unknown for many years, both cellular and serological factors appeared to possess the ability to protect animals against reinfection [20].

Early studies focused on characterizing serological factors demonstrated that blood possesses intrinsic bactericidal activity following inoculation of a specific microbe. Subsequent studies demonstrated that heating serum at 55°C effectively eliminated this bactericidal activity *in vitro*. However, infusion of heat inactivated serum isolated from previously inoculated animals protected recipients from infection. These results suggested that serum contains heat labile and heat stable components. Paul Ehrlich subsequently coined the term complement to describe the heat labile component of immunity, which he postulated worked in concert with a heat stable "amboceptor" that provided target specificity [21]. Subsequent studies demonstrate that a complex pathway of complement activation and regulation occurs following "amboceptor" (antibody) engagement of antigen.

4. Complement Initiation and Regulation: An Overview

Complement activation can be divided into three primary cascades of activation, classical (antibody), lectin, and alternative (Figure 1) [22, 23]. While the lectin and alternative pathways of complement activation play a key role in immunity [24, 25], antibodies provide the primary initiating activity of complement activation during a hemolytic transfusion [26]. Regardless of the initiating stimulus, each pathway converges on the formation of an enzyme complex capable of converting complement component C3 into active products, C3a and C3b. C3b, working in concert with additional complement components, ultimately propagates a cascade that terminates in the formation of a membrane attack complex and eventual target lysis [27].

Following transfusion of incompatible blood, antigen-antibody interactions facilitate engagement of the first component of the classical pathway, C1q. Once bound, C1q induces conformational changes in the serine protease, C1r, which allows C1r to cleave C1s, resulting in an active C1s protease [28]. This complex then cleaves C2 and C4, generating target bound C2b and C4b and the release of soluble C2a and C4a (Figure 1). Bound C2b and C4b form a complex, the C3 convertase, which cleaves C3 to form C3a and C3b [27]. C3a provides a soluble complement regulator of variety of biological pathways, including activation of mast cells, endothelial cells, and phagocytes in addition to intrinsically possessing antimicrobial activity [29, 30]. In contrast, C3b covalently attaches to the target membrane through a highly reactive thioester and thereby facilitates continuation of the complement cascade [23, 27]. Bound C3b, in complex with C2b and an additional C3b molecule, then facilitates conversion of C5 to C5a and C5b. Similar to C3a, C5a regulates a wide variety of systemic factors in immunity [31]. Bound C5b recruits additional complement factors, including C6, C7, and C8, which together facilitate the insertion and polymerization of C9 in the target membrane [32]. Ultimately, C9-mediated pore formation results in osmotic lysis of the target [32].

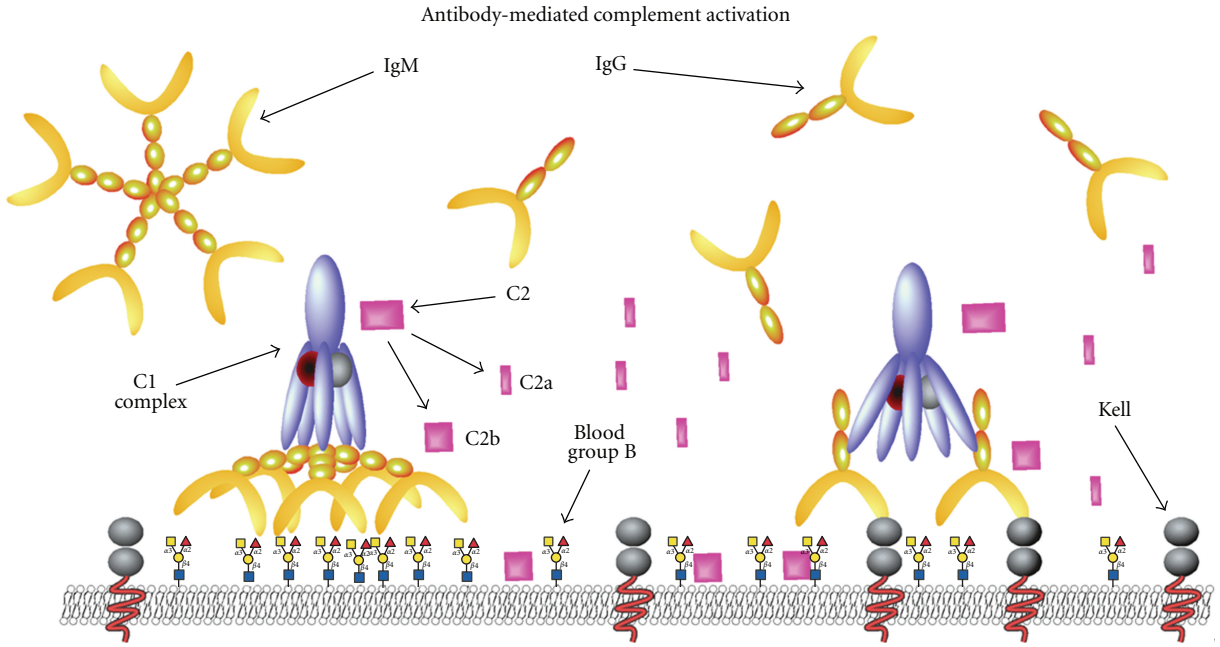

Figure 1: Antibody-mediated complement activation. IgM antibodies primarily exist as planar molecules. However, upon engagement of antigen, IgM antibodies undergo significant conformation changes that result in favorable exposure of multiple C1q binding domains. As a result, a single IgM molecule can initiate significant complement activation. In contrast, two separate IgG antibodies must engage antigen within close enough proximity to simultaneously engage the C1q fix complement.

In contrast to the antibody-mediated pathway, the lectin and alternative pathways do not require adaptive immunity to target pathogen. For example, the lectin pathway utilizes a series of innate immune lectins, such as mannan binding lectin (MBL), which target microbes by recognizing common pathogen-associated molecular motifs [25]. Upon pathogen recognition, MBL engages adapter proteins, MASP-1 and MASP-2, which cleave C4 and C2 similar to C1s in the classical pathway [25, 28]. In contrast to the classic and lectin pathways, the alternative pathway does not actively engage pathogens through a target molecule. Instead, the alternative pathway relies on spontaneous activation of C3 to form C3 (H_2O). C3 (H_2O) then binds an additional complement factor, factor B, which renders factor B sensitive to cleavage by constitutively active factor D [24]. A complex of factor C3 (H_2O) and Bb, stabilized by a factor P, then forms the C3 convertase of the alternative pathway [33, 34]. Importantly, C3b generated during antibody or lectin-induced activation can also directly facilitate D-mediated factor B activation, amplifying complement activation even when antibody alone can provide an initiating stimulus [25]. While antibody-antigen complexes primarily utilize the classic pathway, several studies demonstrate that antibody can also directly activate the alternative pathway [35]. As a result, although antibody-induced activation likely provides the primary driving force, each pathway likely contributes to the overall effect of complement-mediated transfusion reactions.

In addition to facilitating complement activation, complement factors, such as C3b, can serve as opsonins on the target membrane for a series of complement receptors [36]. Each complement receptor appears to be expressed on distinct leukocyte populations and tissue [37]. Furthermore, engagement of complement receptors on different cells can result in distinct immunological outcomes. For example, several complement receptors, including CR1 (CD35), CR3 (CD11bCD18), CR4 (CD11cCD18), and CRIg, typically facilitate phagocytosis of opsonized targets following C3b ligation [38–43]. In contrast, CR2 (CD21) engagement of complement can influence B cell activation and tolerance [44–46]. Additional complement receptors, which recognize soluble complement components, C3a and C5a, also regulate a wide variety of biological pathways following ligation [27, 31, 47]. Although not functionally important in complement activation, several complement factors actually possess unique antigenic structures used in blood group classification, such as the Cromer, Chido, and Rodgers blood groups, and therefore could also theoretically facilitate RBC removal as unique target antigens [48, 49]. Taken together, complement activation results in the production of a variety of highly active immunoregulatory components capable of influencing a broad range of biological processes.

As with all immune effector functions, unregulated activation of complement can result in significant pathological sequelae. Indeed, many disease states are defined by genetic or acquired dysregulation of complement [50]. Given the evolutionary ancient history of complement, many inhibitory pathways exist that facilitate complement regulation. For example, constitutively expressed cell surface proteins, such as CD35 (CR1), CD46 (MCP), CD55, or CD59, provide various inhibitory activities to inhibit off-target complement activation [40, 51–53]. Importantly, each of these factors displays unique regulatory activities. For

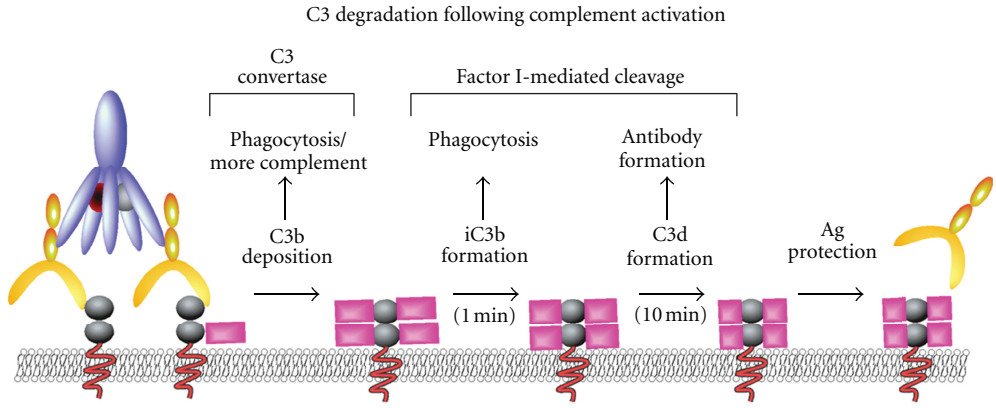

Figure 2: C3 degradation following complement activation. Conversion of C3 to C3b by C3 convertase complex results in rapid deposition of highly reactive C3b on the target surface. Degradation by inhibitory factor I, in concert with other regulatory molecules, results in conversion of C3b to iC3b. Although iC3b retains the capacity of C3b to engage complement receptors as an opsonin, it no longer participates in the production of additional complement. Additional cleavage of iC3b by factor I results in the formation of C3d, which no longer fixes complement or serves as an opsonin, although it may engage B cells and facilitate antibody formation. Decoration of antigen with C3d may result in protection of the antigen from additional antibody binding and subsequent effector function.

example, CD35 and CD46 bind C3b and C4b preventing complex formation with Bb and 2b, respectively, [40, 51, 54], thereby inhibiting active C3 convertase formation. Similarly, CD55 (decay accelerating factor or DAF) along with CD35 actively dissociates C3 convertase complexes once formed by displacing Bb and 2b [51, 53, 55]. Furthermore, CD59 (protectin) binds the C5, C6, C7, and C8 complex and inhibits C9 binding and formation of the membrane attack complex [56–58]. In concert, all of these mammalian, membrane bound receptors inhibit off-target complement deposition from actively destroying an individual's own cells.

In addition to cell surface factors, several soluble factors also aid in complement regulation. Similar to the inhibitory activities of CD35, CD46, and CD55, C4 binding protein (C4BP) can inactivate C4b by preventing binding to C2b [59], thereby also limiting C3 convertase formation [55]. In contrast, other soluble factors provide additional checkpoints in complement regulation. For example, although activated fragments of complement will readily hydrolyze in the absence of target attachment [60, 61], inhibitory factor H binds soluble C3b to further reduce off-target effects [62]. C1 inhibitor (C1INH) actually dissociates C1r and C1s from C1q, thereby directly targeting the effector arm of antibody-mediated complement activation [63]. Patients with hereditary angioneurotic edema illustrate the importance of C1INH in complement regulation.

Complement degradation also plays a key role in complement regulation. For example, factor I cleaves surface bound C3b and C4b. Although formation of the first cleavage product of C3b, iC3b retains the ability to bind complement receptors, iC3b no longer facilitates complement activation [27]. iC3b can be additionally cleaved by factor I to form C3dg and C3d which are no longer recognized by phagocytosis-inducing complement receptors (Figure 2). Indeed, CD21 serves as the primary receptor for C3dg, where CD21 ligation significantly enhances antibody production against opsonized antigen [45, 64]. Importantly, factor I requires coactivation by several other factors, including factor H, CD35, CD46, and CD55, allowing soluble and membrane inhibitory factors to work in concert to regulate complement activation [55, 65], while also directing complement inactivation toward an individual's own cells. Importantly, several studies recently described additional soluble and membrane bound regulators of complement, adding to the complexity of complement homeostasis [66, 67]. Thus, complement activation reflects a highly regulated pathway responsible for differentially targeting pathogens while protecting an individual's own cells.

5. Factors Influencing Complement Activation

While complement can result in significant pathophysiology during a hemolytic transfusion reaction, not all HTRs result in complement activation [26]. Although the precise factors responsible for dictating whether complement activation will occur remain enigmatic, several factors appears to play a role. Of these factors, the type of antibody and antigen mediating the HTR appears to play the most significant roles in determining the outcome of an incompatible transfusion.

Although different isotypes of antibody fix complement, not all antibodies possess an equivalent capacity to engage this pathway. Appropriate engagement of C1q requires accommodation of two separate CH2 domains on antibody Ig heavy chains [68]. As a result, CH2 availability can influence antibody proclivity for complement fixation. For example, while a single pentameric IgM antibody possesses five separate CH2 domains, an IgG molecule only contains a single domain capable of C1q engagement. In solution, IgM exists as a planar molecule that fails to bind significant C1q, preventing IgM-mediated complement activation in the absence of antigen. However, IgM engagement of antigen induces a conformation change that enables a single IgM to engage C1q and activate complement [69] (Figure 1). In contrast, two IgG molecules must engage distinct antigenic

epitopes within 20–30 nm of each other in order to adequately engage C1q and initiate complement [70] (Figure 1). As a result, fewer IgM antibodies must bind antigen to fix equivalent levels of complement as IgG [71]. Equally important, different IgG subclasses possess distinct hinge regions that can differentially impact the conformational flexibility of the heavy chain, thereby also potentially impacting the ability of the CH2 domain to engage complement [72, 73]. Intrinsic differences between subtypes in the CH2 domain may also directly impact C1q engagement [74]. In contrast to IgG and IgM, while IgA may induce complement activation through engagement of the lectin pathway [75], the CH2 domains of IgA, IgD, and IgE possess little affinity for C1q [73]. As a result, IgM and IgG antibodies provide the primary stimulus for classical complement activation.

In addition to intrinsic differences in the ability of Ig isotypes to induce complement activation, a variety of enzymatic and nonenzymatic posttranslational modifications can impact antibody-induced complement activation. For example, posttranslational glycosylation of the Fc domain can also impact the ability of IgG molecules to engage C1q or activate the lectin pathway of complement [76, 77]. Furthermore, nonenzymatic formation of advanced glycation end-products can also affect the ability of antibodies to induce complement activation [78]. Importantly, none of these factors in isolation predicts the ability of an antibody-antigen interaction to result in complement deposition [35]. As a result, the influence of an antibody on complement activation reflects a variety of distinct factors. As polyclonal antibodies typically mediate HTR, the unique composition, affinity, and potential modifications of antibodies within a recipient will impact the likelihood of complement activation during an incompatible transfusion.

In addition to the antibody isotype, the antigen itself appears to independently predict the likelihood of complement activation. For example, ABO(H) HTRs result in robust complement activation and rapid intravascular hemolysis [79]. Similar studies suggest that IgG antibodies against other RBC antigens, including the Kidd antigens (Jk^a and Jk^B) and Fy^a, appear to induce complement-mediated hemolysis [79, 80]. Other antigens, such as Kell, appear to result in mixed forms of clearance following antibody engagement, utilizing both complement and Fc receptors [81]. It should be noted that many of these studies employed complement deposition, clearance kinetics, or intravascular hemolysis as a surrogate for complement-mediated destruction [79]. Although intravascular hemolysis typically occurs in the presence of complement deposition, it remains to be definitively tested using mammalian animal models whether complement may be required in each of these settings. For example, while some results suggest that complement may be required for both IgM and IgG-induced hemolysis in an animal model of a glycophorin A-mediated HTR [82], a recent study using the same model system suggests that hemolysis occurs independent of Fc receptors or complement [83]. As a result, HTRs reflect complex immunological reactions that need additional investigation in genetically defined animal models to be fully elucidated.

In contrast to ABO(H) HTRs, the Rh(D) antigen, the second most common antigen implicated in HTR, appears to mediate RBC clearance independent of complement. For example, Rh(D) alloimmunized patients who receive incompatible blood display little complement activation, despite several studies demonstrating that similar IgG subclasses facilitate complement activation in other settings [84, 85]. Similarly, administration of Rh immune globulin (RhIG) results in complement-independent hemolysis, likely through Fc-receptor-dependent phagocytosis [86]. Although the Rh antigen complex provides the most compelling example of complement-independent hemolysis, several other antigens, including S and M, also appear to predominately induce HTR independent of complement [79, 87].

While the exact mechanism whereby distinct antigens dictate the mode of clearance remains unknown, the density of the antigen likely plays a significant role. Consistent with this, A and B antigens not only represent the most common complement-mediated HTR, but also display the highest antigen density, typically ranging from 700,000 to over a million antigens per cell [88, 89]. In contrast, the Rh(D) antigen typically occurs at approximately 20,000 sites per cell [90]. As a result, the lower density of Rh likely reduces the probability of successful engagement of C1q [73], especially when the antibody composition mediating an HTR primarily reflects the IgG isotype. However, additional antigen factors likely influence this process. For example, although Rh(D) and Kell both reflect low-density antigens [91, 92], Rh mediates complement-independent hemolysis while Kell can engage complement and Fc receptor effector responses [79, 81]. With low-density antigens, antibody-induced clustering likely influences the efficiency of C1q engagement and complement activation [70]. As a result, lateral mobility and location of different antigens within the RBC membrane likely play an important role. Consistent with this, recent studies demonstrate that the cytoplasmic domains of Rh antigens engage RBC cytoskeletal proteins [93], likely limiting the lateral mobility needed to effectively engage C1q. In contrast, Kell possesses a very small cytoplasmic domain with little cytoskeletal attachment [94], thereby potentially enhancing lateral mobility and antibody-induced cluster formation.

As antithetical antigens occasionally only differ at discrete binding sites, the unique orientation and presentation of antigens may also influence the ability of antibody-antigen complexes to mediate complement fixation. Indeed, several antithetical antigens responsible for HTRs only differ at a single nucleotide polymorphism [95]. As a result, effective antibody-mediated crosslinking and cluster formation may be compromised if each individual molecule only possesses a single epitope. Although some antigens may exist in higher order complexes [96], providing additional epitopes, the availability of the antigenic sites may also be sterically constrained, thus, reducing the overall ability of antibodies to engage antigen. Conversely, carbohydrate A and B antigens display high levels of expression on highly mobile glycolipids and exist as multiple copies on glycoproteins [97], directly facilitating optimal antibody engagement for complement fixation. Taken together, a variety of biochemical features

at the antigen level appear to possess the ability to impact antibody-induced complement activation.

Although the type of antibody and antigen can independently impact the probability of a complement-mediated HTR, the type of antigen itself partially dictates the type of antibody generated in response to a particular antigen. Although it remains to be formally examined, previous studies suggest that bacterial flora in the gut that expresses A- and B-like antigens stimulates anti-A and anti-B antibody formation required for an ABO(H) HTR [98, 99]. As these antigens reside on O antigen LPS molecules, they do not typically possess the T cell epitopes required for significant antibody class switching [15]. As a result, anti-A and anti-B antibodies form naturally by the fourth month of life without prior exposure secondary to transfusion and predominately reflect IgM antibodies [15, 100].

In contrast to ABO(H) antigens, most antibodies mediating HTR to protein antigens, such as Rh(D), Kell, and Duffy, do not form without prior antigen exposure. Although IgM development during a primary exposure to antigen can actually shorten the lifespan of transfused blood [101, 102], primary antibody responses do not typically result in a routinely detectable HTR. However, during this primary antigen exposure, T cell-mediated class switching results in significant production of IgG. As a result, RBCs transfused into an individual previously immunized against a protein antigen will likely encounter IgG antibodies [103]. Taken together, antibody isotype, antigen density and location, and the modes and mechanisms of antibody stimulation can significantly impact whether complement mediates a HTR.

6. Consequences of Complement Activation

Given optimal antigen and antibody conditions, incompatible transfusion can result in robust activation of complement. Complement activation may not only result in intravascular hemolysis, but complement split products, in particular C5a, can directly impact vascular permeability by activating endothelial cells and inducing significant cytokine production [27, 47]. Thus, complement can directly impact hemodynamic stability following an HTR. Free hemoglobin (Hb) released following complement-mediated hemolysis also induces significant alterations in vascular tone. In addition, Hb may also induce cytokine production, result in significant coagulopathy, and can be directly nephrotoxic [104–108]. As a result, exuberant complement activation following exposure to a large bolus of incompatible RBCs can induce significant pathophysiological changes that may ultimately result in a fatal outcome.

Although rapid complement activation can result in significant mortality, complement-mediated HTRs do not uniformly result in patient death. While several factors, including the volume of incompatible blood and titer of recipient antibody, likely influence patient prognosis following complement-mediated HTR [10, 109], RBC intrinsic factors may also play a role. For example, while ABO(H) HTR may saturate complement inhibitory factors, lower density antigens, such as Kell antigens, likely fix insufficient complement to adequately overcome regulatory pathways, causing arrest of complement activation prior to complete intravascular hemolysis [92]. Indeed, many non-ABO(H) HTRs arrest at the C3b stage of the complement cascade [92]. Although C3b-mediated phagocytic removal may increase cytokine production in these situations [110, 111], limited activation of C5a and reduced release of intravascular Hb also likely limit systemic consequences of complement-mediated HTR. These differences likely contribute to the significantly mortality rate associated with ABO(H) HTR [16].

Regulation of complement activation may also influence the rate and magnitude of RBC clearance during a complement-mediated HTR. For example, while C3 retains opsonizing activity following initial factor I-mediated cleavage [27], additional degradation to C3dg and CD3d renders RBCs relatively resistant to C3-mediated removal [112–114] (Figure 2). Consistent with this, complement-mediated HTRs characteristically result in rapid clearance during the first 10 minutes followed by an abrupt change in clearance kinetics that mirror the degradation rate of C3 to C3dg [79, 115, 116]. These results strongly suggest that alterations in clearance at least partially reflect the kinetics of factor I-mediated degradation. Interestingly, once cells accumulate C3dg, they become relatively transparent to the immune system and may circulate with C3dg virtually undetected by phagocytic cells [115–117] (Figure 2). In addition, ongoing degradation of complement may actually result in release of phagocyte-engaged cells, as several studies demonstrate that RBC can reappear hours following a complement-mediated HTR [110, 118].

A variety of additional variables can influence the extent and consequences of complement activation following an HTR. For example, although stochastic competition between activating and inhibitory stimuli likely dictates which cells will resist complement-mediated lysis, recent studies suggest that the activity of complement inhibitory proteins may be altered as RBCs age. Indeed, accumulation of advanced glycation end products on complement inhibitory proteins appears to directly diminish their function, strongly suggesting that older RBCs may be more sensitive to complement-mediated hemolysis [119]. Furthermore, the age of the patient may also impact the extent of complement-mediated hemolysis, as pediatric patients possess a less mature complement inhibitory apparatus than adults [120]. Thus a variety of cell-specific factors may also independently regulate the consequences of complement activation.

While many modifications or alterations in the expression of complement inhibitor proteins can impact RBC sensitivity to complement-mediated hemolysis, additional alterations affecting general RBC physiology may also impact complement sensitivity. In addition to hemolyzing transfused cells during HTR, occasionally patients will hemolyze their own cells, a phenomenon called bystander hemolysis [121]. Although bystander hemolysis may theoretically occur in any patient [122], individuals with sickle cell disease appear to be particularly prone [121]. Interestingly, several studies suggest that sickle cells may be intrinsically sensitive to complement-mediated lysis, suggesting that complement may in part be responsible [123, 124]. However, as several

antigens that may not activate complement, such as Duffy, can also induce bystander hemolysis [125], the actual role of complement in the pathophysiology of this process remains largely unknown. In addition, complement activation may also be involved in non-hemolytic transfusion reactions, such as transfusion-related acute lung injury (TRALI) [126], another leading cause of transfusion-related mortality [127]. Indeed, recent studies suggest that, in addition to anti-HLA antibodies, anti-neutrophil antibodies, and other factors [127], differences in complement may also contribute to the gender influence of donor plasma on the development of TRALI [128].

7. Possible Complement-Targeted Therapeutic Interventions

Once an HTR is suspected, immediate intervention takes place in the form of stopping the transfusion and initiating intravenous fluids in an effort to achieve hemodynamic stability and protect the kidneys from the nephrotoxic effects of hemoglobin. However, no intervention in routine clinical practice specifically targets the HTR itself. As some patients, particularly patients with paroxysmal nocturnal hemoglobinuria, suffer from chronic hemolysis secondary to a deficiency in CD55 and CD59, a number of complement inhibitors have been developed in an effort to treat this and other complement-related disorders. While many inhibitors exist [129–135], only a few inhibitors have been evaluated using animal models of HTRs and no inhibitors have been formally examined clinically in an HTR setting. Injection of a soluble form of CR1 significantly attenuates complement activation following transfusion and increases posttransfusion survival of incompatible blood. Similar results were obtained using small molecule inhibitors of complement or smaller fragments of CR1 [136, 137]. Specific targeting of complement inhibition to RBCs through antibody-DAF chimeras may also inhibit complement-induced hemolysis [138]. Additional experimental models also suggest that intravenous immune globulin (IVIG) can inhibit complement-mediated HTRs [139, 140]. Although many of these inhibitors demonstrate promise in animal models, other complement inhibitors, including the C5 inhibitor, eculizumab [141], which are currently available for clinical use in other settings, need to be formally examined in the setting of HTR. As a result, future studies need to explore the potential role of complement inhibition as a prophylactic agent in the event that antigen-compatible blood may not be available or as therapy following an unfortunate HTR.

Although reengagement of antibody with antigen would be expected to provide a continuous supply of active complement, RBCs represent the only cells in the human body that do not resynthesize protein following maturation. As a result, whatever antigen exists on an RBC represents the only antigen that RBC will possess for the duration of its lifetime. While this may not alter basic antibody-antigen interactions, as C3 molecules attach to target membrane through covalent association, previous studies suggest that deposition of complement on antigens can sterically or directly mask targets from additional antibody binding [115, 116, 142–144] (Figure 2). As a result, once antigen masking and complement degradation are complete, cells become relatively transparent to the effector mechanism of hemolysis. These studies suggest that deposition and inactivation of complement *in vitro* may allow complement-mediated masking of antigen prior to transfusion in an effort to generate compatible RBCs for highly immunized patients (Figure 2). While these possibilities are intriguing, more studies are needed utilizing defined animal models to determine whether complement-mediated RBC manipulation might enable transfusion across immunological barriers.

8. Conclusions

HTRs historically provided significant insight into the role of complement as an antibody effector system [79]. Fortunately for patients, extreme vigilance exercised by transfusion medicine services has drastically reduced the rate of hemolytic transfusions [9]. As a result, most detailed studies examining HTRs predate the exponential increase in genetically manipulated animal models designed to appreciate subtle, yet critical, factors responsible for complement regulation *in vivo* [145–147]. Furthermore, until recently, no mammalian models existed to study clinically relevant RBC antigens in the setting of HTR [82, 148–151]. Indeed, despite Landsteiner's discovery of ABO(H) antigens over a century ago, no animal model currently exits to formally examine the role of complement or other factors in ABO(H) HTRs. Despite the relatively decreased incidence of mistransfusion and HTRs, the risk still remains. In 2011, HTR represented the second leading cause of transfusion-associated mortality reported to the FDA [152]. Thus, additional models need to be developed with a renewed focus on the mechanisms responsible for complement-dependent and complement-independent mechanisms of RBC hemolysis. These future studies possess the potential to provide rational approaches to not only develop therapeutic interventions in the unfortunate event that an HTR occurs, but to also extend the scope of immunological compatibility by manipulating complement to render otherwise incompatible transfusions compatible. Future work will likely provide significant insight into complement activation and regulation with the potential to significantly impact patient care.

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Review Article

The Role of Complement System in Septic Shock

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Septic shock is a critical clinical condition with a high mortality rate. A better understanding of the underlying mechanisms is important to develop effective therapies. Basic and clinical studies suggest that activation of complements in the common cascade, for example, complement component 3 (C3) and C5, is involved in the development of septic shock. The involvement of three upstream complement pathways in septic shock is more complicated. Both the classical and alternative pathways appear to be activated in septic shock, but the alternative pathway may be activated earlier than the classical pathway. Activation of these two pathways is essential to clear endotoxin. Recent investigations have shed light on the role of lectin complement pathway in septic shock. Published reports suggest a protective role of mannose-binding lectin (MBL) against sepsis. Our preliminary study of MBL-associated serine protease-2 (MASP-2) in septic shock patients indicated that acute decrease of MASP-2 in the early phase of septic shock might correlate with in-hospital mortality. It is unknown whether excessive activation of these three upstream complement pathways may contribute to the detrimental effects in septic shock. This paper also discusses additional complement-related pathogenic mechanisms and intervention strategies for septic shock.

1. Introduction

Septic shock is a leading cause of morbidity and mortality among critically ill patients. Despite the use of potent antibiotics and improved intensive care, mortality rates of patients with severe sepsis and septic shock remain high (20–50%) [1–3]. A better understanding of the underlying mechanisms is important to develop future platforms of effective therapies.

Multiple mechanisms are likely involved in the development of septic shock. Host responses may initially respond to an infection but become amplified and dysregulated, resulting in hemodynamic collapse [4]. Decades of basic science and clinical research indicate that complement factors are involved in septic shock. While complement is an important defense system against bacterial infection, earlier clinical observations suggest that activation of complement factors is associated with detrimental effects in septic shock, such as multiorgan damages and poor outcome [5–8].

There are three pathways in the complement system: classical, alternative, and lectin. Different initiators activate each pathway but all converge to complement protein C3 and are followed by a common cascade (C5-9), resulting in the deposition of a membrane-attack-complex on targets and the release of chemoattractants (C3a and C5a) for inflammatory cells.

2. Pathophysiology of Complement Involvement in Septic Shock

2.1. Involvement of Complement Common Cascade in Septic Shock. A series of observations on C3 activation in septic shock patients were reported by a group of Dutch investigators led by Hack and Groeneveld. Activated C3 fragments, C3a and C3b/c, were elevated in septic shock patients and correlated with mortality [9–13]. Other clinical investigators also reported similar findings. Dofferhoff et al. found that,

in 20 sepsis patients, C3a and C3d were elevated and that C3a levels correlated with Acute Physiology and Chronic Health Evaluation II (APACHE II) scores [14]. Furebring et al. showed that, in 12 patients with severe sepsis or septic shock, C3a (as well as C5b-9) levels were increased at the time of diagnosis [15]. These clinical observations suggest that C3 fragments released during septic shock may contribute to the development of fatal complications like profound hypotension and disseminated intravascular coagulation (DIC), thereby leading to a more severe disease course and a poor outcome.

It is interesting to note that some investigations did not conclude that C3 activation was detrimental in the development of severe sepsis. For instance, Shatney and Benner reported that in traumatic patients with acute systemic sepsis, serum C3 levels decreased shortly after admission [16]. Thereafter, C3 levels gradually returned to normal, despite the onset of fulminant systemic sepsis. These investigators argued that changes in C3 levels during severe sepsis were more consistent with protective host defense functions but did not support a role for C3 in the pathogenesis of acute fulminant clinical sepsis.

Basic science researchers have used various animal models to investigate the role of complement factors (mostly C3 and C5) in the common cascade. In a study using *E. coli* to induce septic shock in anaesthetized and artificially ventilated rabbits, circulating C5a positively correlated with endotoxin and the degree of accumulation of granulocytes in the lung tissue [17]. Using a baboon model with *E. coli*-induced septic shock, high amounts of endotoxin led to uncontrolled activation of complement C3 and C5-9 [18]. In a pig model of sepsis induced by fecal peritonitis, terminal complement complexes were found to have been deposited in the kidneys [19].

The availabilities of several strains of complement factor knockout mice have greatly facilitated septic shock research, but conflicting results have been reported depending on the model that investigators chose. Yeh's group in Canada reported that like mice incapable of C3a/C3a receptor signaling, C5a receptor C5l2-deficient mice were hypersensitive to lipopolysaccharide- (LPS-) induced septic shock [20]. However, Ward's group in USA reported opposite findings using a mouse model of cecal ligation and puncture (CLP), which induces polymicrobial sepsis. In "mid-grade" sepsis (30–40% survival), blockade or absence of either C5a receptors, C5ar or C5l2, greatly improved survival and attenuated the buildup of proinflammatory mediators in plasma [21]. In "high-grade" sepsis (100% fatality), the only protective condition was the combined blockade of C5l2 and C5ar. Ward's group further showed that C5a induces apoptosis in adrenomedullary cells during experimental sepsis [22].

$C3^{-/-}$ mice had significantly reduced survival in 2 septic shock models (LPS-induced and CLP) [23, 24]. Surprisingly, $C5^{-/-}$ mice showed identical survival as wild type controls in CLP model [24]. In addition, C6-deficient rats had no significant differences in lung inflammation in an LPS-induced septic shock model [25].

These data suggest that C3 is essential to control bacteremia in 2 different models, but the functions of C5-9 seem dispensable. Depending on the type of animal model, anaphylatoxins C3a and C5a may be important factors in recruitment of inflammatory cells to combat infections but excessive release of these factors could be detrimental.

2.2. Involvement of Complement Classical Pathway in Septic Shock. Specific factors in complement classical pathway include immunoglobulins, C4 and C2. Animal studies showed that $C3^{-/-}$, $C4^{-/-}$, $Btk^{-/-}$ (immunoglobulin deficient) and RAG-$2^{-/-}$ (immunoglobulin deficient) mice were significantly more sensitive to endotoxin than wild-type controls [23, 26], suggesting an essential role for complement classical pathway to clear endotoxins.

Clinical investigators have explored the involvement of classical pathway in septic shock since the early 1980s. In a clinical study of 48 patients (19 with septic shock), those with septic shock had markedly decreased levels of C3, C4, and total complement activity as measured by the 50% hemolytic complement (CH_{50}) assay. However, after 96 h, these values returned to baseline [27]. This underlines the transitory activation of the complement system through the classic pathway in septic shock. C4 activation, manifested as decrease of C4 or increase of C4a, was also reported in other clinical studies of severe late septic shock (42 patients) [28], clinically suspected sepsis on admission (43 of 47 patients) [13], severe sepsis and septic shock (50 patients) [29, 30]. IgG has also been associated with clinical outcomes of septic shock. In a study of 50 patients with severe sepsis or septic shock, survivors showed significantly higher levels of IgG upon diagnosis than those who ultimately died [30]. Thus, complement classical pathway is activated in septic shock as an essential response to clear endotoxins.

2.3. Involvement of Complement Alternative Pathway in Septic Shock. Specific factors in complement alternative pathway include factors B and D, which are regulated by factors H, I, and P (also called properdin). The alternative pathway is a critical defense system against bacterial infection. Patients with preexisting deficiency in factors of alternative pathway, for example, factor D and properdin, could suffer fulminant meningococcal infections resulting in septic shock [31–33].

Evidence of activation of alternative pathway in septic shock has been reported in a number of clinical studies. A study of 42 patients with severe late septic shock found factor B levels were significantly lower in patients who died than in patients who survived [28]. In these patients, the alternative pathway appears to be activated early in septic shock, whereas the classical pathway is activated later. Oglesby et al. also reported that among septic shock patients (both Gram-negative and Gram-positive) with preexisting cirrhosis, factor B levels were decreased [34]. Lin et al. reported that the active fragment of factor B, Bb as well as the Bb to factor B ratio, was significantly increased in septic shock patients [29]. Brandtzaeg et al. showed that among 20 patients with systemic meningococcal disease,

ten patients with persistent septic shock had significantly higher levels of Bb than the other 10 patients without septic shock [35]. Finally, PBMCs from septic shock patients showed increased factor B mRNA expression when compared with control patients [36]. All these evidences suggest that complement alternative pathway is essential to fight against infections and is activated in clinical settings of septic shock.

2.4. Involvement of Complement Lectin Pathway in Septic Shock. Specific factors in complement lectin pathway include MBL and ficolin. Both MBL and ficolin circulate in complexes with one of three MBL-associated serine proteases (MASPs) [37, 38], with MASP-2 being the major complement-activating component among the three known MASPs. The MASP-2 is activated when MBL binds to certain carbohydrate or acetyl patterns on pathogens [39–41]. The activated MASP-2 then cleaves C4 and C2 to form the C3 convertase, C4b2a. MASP-2 activation is regulated by C1-inhibitor [42–44].

Basic research has shown that MBL and MASPs can bind to certain LPS containing a mannose homopolysaccharide. Such a binding of LPS to MBL and MASPs may cause C4 activation, resulting in the platelet response and development of rapid shock in mice [45].

However, two clinical studies from Eisen's group in Australia showed more complicated patterns of lectin complement activation in septic shock. In a study of 128 patients with sepsis and septic shock, the majority of patients did not display an MBL acute phase response on days 1, 3, 5, 7 [46]. Forty percent of these patients maintained consistent MBL levels throughout hospital stay, thirty percent of these patients had a positive acute phase response, and the remaining had a negative acute phase response [46]. In another study of 114 septic shock patients and 81 sepsis patients, MBL functional deficient patients had significantly higher sequential organ failure assessment (SOFA) scores, while higher MBL function and levels were found in patients who had SOFA scores predictive of good outcomes [47]. Thus, Eisen's group suggested that deficiency of MBL function may be associated with bloodstream infection and the development of septic shock.

Even with a likely protective role of MBL against sepsis, it is still possible that excessive activation of lectin pathway may contribute to the detrimental effects in septic shock. Sprong et al. reported two cases of MBL-deficient septic shock patients who had relatively low disease severity and mild disseminated intravascular coagulation (DIC) compared with 16 septic shock patients who had sufficient levels of MBL [48].

It is still unclear whether other molecules in the lectin pathway are involved in septic shock. We have investigated the temporal patterns of MASP-2 in 16 septic shock patients and their correlation with in-hospital mortality. Our preliminary results showed that there was no difference in the baseline levels of MASP-2 between survivors and nonsurvivors. However, there was a trend that survivors had an increase of MASP-2 over the course of 5 days, while the nonsurvivors had a decrease during the same time period (Figures 1(a) and 1(b)). The change of MASP-2 in survivors at 6 hours after diagnosis of septic shock was significantly different than that of nonsurvivors (Figure 1(c)). Kaplan-Meier survival analysis showed that patients with $\geq$10% increase of MASP-2 within 6 hours after the diagnosis of septic shock had significantly less in-hospital mortality than patients with $\geq$10% decrease of MASP-2 during the same time period (Figure 1(d)). Therefore, acute decrease of MASP-2 during the early phase of septic shock may correlate with in-hospital mortality.

It remains to be determined to what degree is lectin complement activation necessary for protective effect against infection and whether there is threshold for the activation before detrimental effects appear. Future research, especially laboratory studies, may answer these questions.

2.5. Involvement of Other Complement-Related Inflammatory Mediators in Septic Shock. The development of septic shock is multifactorial and many potential mechanisms have been reviewed extensively by others [49–52]. Thus, this paper will only briefly describe the potential links between the complement system and its related inflammatory mediators in septic shock.

Septic patients often exhibit a relative deficiency of C1-inhibitor (C1-INH) [53], which can inhibit activation of all 3 complement pathways [54–56]. C1-INH also inhibits proteases of the fibrinolytic, clotting, and kinin pathways. It is likely that during septic shock C1-INH may be depleted from the circulation by binding to factors in coagulation/fibrinolysis [57], thereby unable to control the excessive complement activation.

Cytokines and chemokines, particularly TNF-α and IL-6, are considered the first line biomarkers that drive the dynamic process of sepsis [58]. Cytokines and complement components can be activated similarly in sepsis [11, 14, 59–62] and their activation products may have overlapping biological activities [63]. Therefore, concomitant activation of cytokines and complements may amplify systemic inflammation leading to organ and system failure.

Other circulating inflammatory mediators, including plasma prostaglandin (PGI) and phospholipase A-2 (PLA-2), may be activated in parallel with complements in septic shock and have direct association with complement factors [64, 65]. Thus, like cytokines, these mediators may have synergistic effects with complements in the development of septic shock.

3. Complement-Related Therapeutic Strategies for Septic Shock

Current management of septic shock includes early identification and treatment of the causative infection [66–68], adequate and rapid hemodynamic resuscitation [52, 69, 70], treatment of organ failure, corticosteroids [71], and modulation of the immune response [72, 73]. There are many comprehensive reviews on these topics and hence we will only review those strategies related to complements.

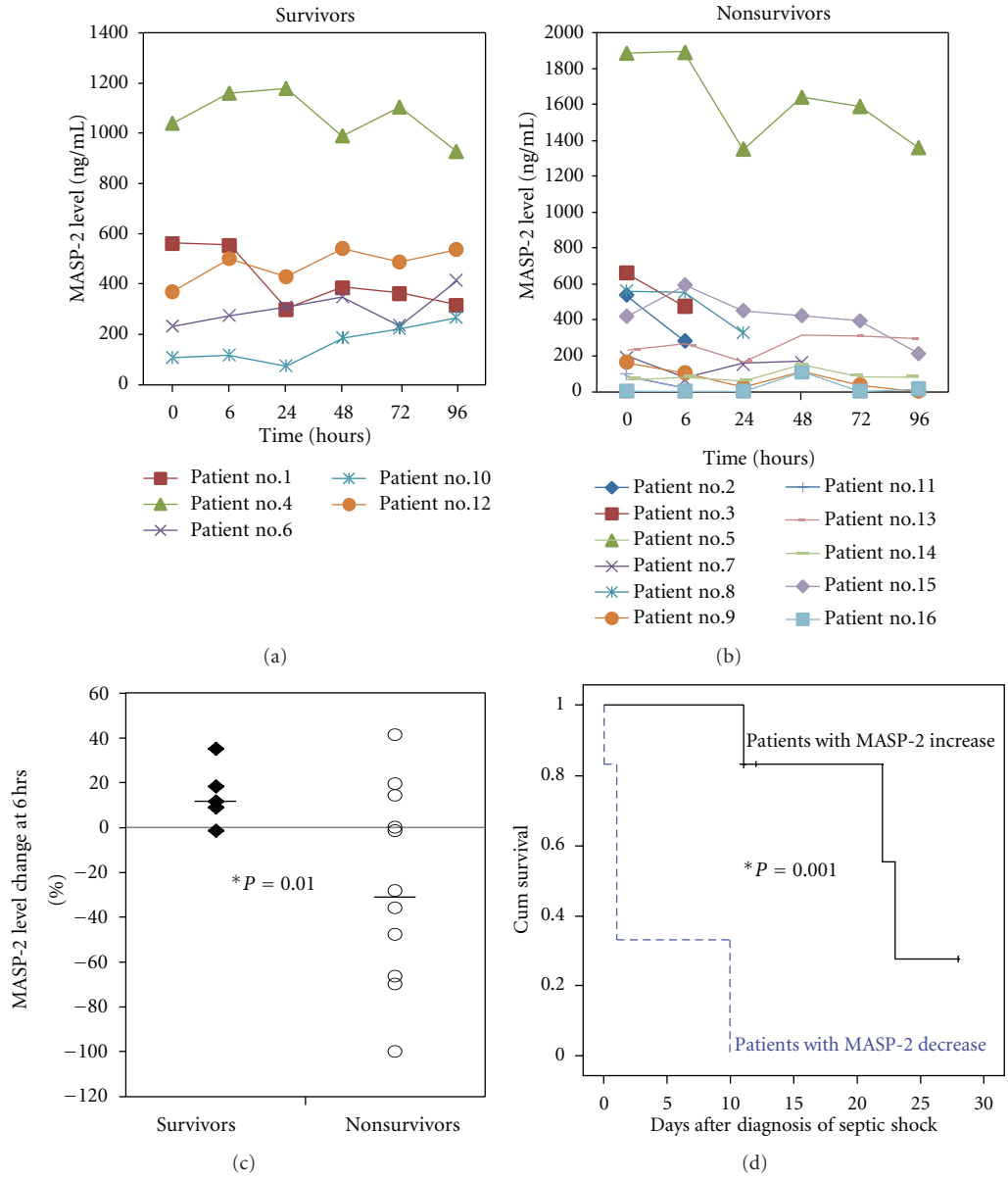

Figure 1: MASP-2 level changes in the survivors or nonsurvivors of septic shock. Plasma levels of MASP-2 were measured over the first 5 days in 16 patients after the diagnosis of septic shock. (a) MASP-2 profiles in the survivors: each line represents an individual patient; (b) MASP-2 profiles in the nonsurvivors; (c) comparison of the change of MASP-2 in survivors versus nonsurvivors at 6 hours after enrollment; (d) the association of changes in plasma levels of MASP-2 at 6 hrs after enrollment with hospital outcome. Kaplan-Meier survival analysis was performed to determine if patients with ≥10% increase of MASP-2 within 6 hours after the diagnosis of septic shock (solid line) had different in-hospital mortality rate compared with patients with ≥10% decrease of MASP-2 over the same time point (dashed line). *indicates statistical difference ($P < 0.05$).

3.1. Strategies Directly Targeting Complement System

3.1.1. Anti-C5a Treatment to Block Neutrophil Chemotaxis. Given the potential role of C5a in the development of septic shock, anti-C5a agents or C5a receptors-blockers may be reasonable therapeutic approaches. One benefit of C5a-blockade is that it still allows for terminal complement complex formation which is important to fight against infection. Anti-C5a has been tested in several septic shock models. In a primate model, anti-C5a antibody treatment significantly attenuated septic shock and pulmonary edema [74]. In a rat model of LPS-induced septic shock, pre-treatment with F(ab′)2 fragments of rabbit anti-rat C5a did not change the circulating cell counts compared with LPS alone; however, a significant improvement in the mean arterial pressure and a decrease in hematocrit were observed [75]. In a pig model, pretreatment with anti-C5a mAb resulted in a decrease of serum IL-6 activity compared to control animals [76]. In a rodent model, blockade of C5a using neutralizing antibodies dramatically improved

survival, reduced apoptosis of lymphoid cells, and attenuated the ensuing coagulopathy [77].

These anti-C5a approaches were based on the hypothesis (with supporting evidence) that C5a induced-neutrophil chemotaxis is harmful in septic shock. However, the effectiveness of anti-C5a in clinical settings remains to be tested. Even though it may have some beneficial effects, it may not be the ultimate solution for severe sepsis/shock, particularly because the role of neutrophils in sepsis may change at different stages of sepsis and inhibition of neutrophil chemotactic response could be detrimental in severe sepsis [78, 79].

3.1.2. C1-INH. Administration of C1-INH can block complement activation, and its beneficial effect, although modest, in severe sepsis or septic shock has been demonstrated in animal studies [80, 81]. C1-INH has been tested in small clinical trials. In a trial of 7 patients with streptococcal toxic shock syndrome, administration of C1-INH markedly shifted fluid from extravascular to intravascular compartments, and 6 of these 7 patients survived [82]. In another trial of 40 patients with severe sepsis or septic shock, C1-INH treatment had a beneficial, although mild, effect on organ dysfunction [83]. These modest effects of C1-INH in septic shock are likely due to nonspecific inhibition of all 3 complement pathways. In another word, it not only suppresses complement-related systemic inflammation, but also hampers complement related antimicrobial responses.

3.2. Strategies Indirectly Affecting Complement System. Anti-TNF treatment (using humanized antibody to neutralize TNF-α) has been tested in a number of clinical trials of septic shock, and this topic has been thoroughly reviewed by others recently [84]. In short, although there was a trend of beneficial effect for anti-TNF to reduce sepsis-associated risk of death, none of these trials showed significance. One interesting notion is that inhibition of TNF alone does not reduce complement activation during sepsis, as demonstrated in a baboon model [85]. Thus, it is possible that inhibiting TNF without tackling excessive complement activation only provides a partial protection against systemic inflammation in septic shock. Combination of both anti-TNF and blockage of complement activation may lead to more effective protection.

Extracorporeal immunoadsorption (ECIA) using polymyxin B-immobilized fiber column hemoperfusion has been tested in animal models [86] and clinical trials [68, 87, 88]. ECIA has been used to adsorb endotoxin, monocytes, activated neutrophils, and anandamide [88], which may indirectly decrease complement activation [89, 90].

4. Conclusion

Complement system is essential to combat infections. However, it is a double-edged sword as excessive activation may cause severe injury to the host, as seen in septic shock. Direct or indirect inhibition of complement may provide new approaches in managing septic shock. Future research directions in this area may include (1) clarifying the pattern of complement activation, for example, time course; (2) determining the degree of involvement of three complement pathways in protection and detrimental effects; (3) reconciling the observations in different animal models; (4) further testing of anti-C5a and C1-INH in randomized clinical trials; (5) developing other specific and effective targeting strategies, for example, C3a inhibition.

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Research Article

Complement Factor C7 Contributes to Lung Immunopathology Caused by *Mycobacterium tuberculosis*

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Mycobacterium tuberculosis (MTB) remains a significant global health burden despite the availability of antimicrobial chemotherapy. Increasing evidence indicates a critical role of the complement system in the development of host protection against the bacillus, but few studies have specifically explored the function of the terminal complement factors. Mice deficient in complement C7 and wild-type C57BL/6 mice were aerosol challenged with MTB Erdman and assessed for bacterial burden, histopathology, and lung cytokine responses at days 30 and 60 post-infection. Macrophages isolated from C7 −/− and wild-type mice were evaluated for MTB proliferation and cytokine production. C7 −/− mice had significantly less liver colony forming units (CFUs) at day 30; no differences were noted in lung CFUs. The C7 deficient mice had markedly reduced lung occlusion with significantly increased total lymphocytes, decreased macrophages, and increased numbers of CD4+ cells 60 days post-infection. Expression of lung IFN-γ and TNF-α was increased at day 60 compared to wild-type mice. There were no differences in MTB-proliferation in macrophages isolated from wild-type and knock-out mice. These results indicate a role for complement C7 in the development of MTB induced immunopathology which warrants further investigation.

1. Introduction

Tuberculosis (TB) remains a significant public health problem, causing nearly 2 million deaths each year [1]. Host protection against TB requires the development of a Th1 immune response [2]. Th1 responses are initiated by dendritic cell presentation of MTB antigen to naive CD4+ T-cells accompanied by synthesis of IL-12 [3]. These Th1 cells produce IFN-γ, which activates macrophages to cause phagosome acidification, phagolysosome fusion, and the generation of reactive nitrogen species that control MTB growth [2]. It is essential to understand the innate immune system functions that lead to protective T-cell responses to MTB in order to develop improved vaccines and therapies based on immune modulation.

Increasing evidence indicates that the complement system is essential for generating protective adaptive immune responses to mycobacterial infections [4–6], in addition to its clear role in both innate immunity and the initiation of adaptive immunity to a variety of other pathogens [7]. The three major pathways of complement are the classical, alternative, and lectin pathways. Each complement pathway leads to the cleavage of C3 generating C3a and C3b. C3b binding to pathogens promotes opsonization resulting in the enhanced elimination pathogens, as well as the formation of C5b-9, the membrane attack complex. Insertion of the complement component C7 into the cell membrane is the critical step in the formation of the membrane attack complex that leads to cell lysis.

The precise role of complement factors in immunity and pathology during mycobacterial infection is unclear. It was reported that mice C3 knockout mice infected with *M. avium* had no differences in granuloma formation or bacterial load in comparison to wild-type mice [8]. However, mice deficient in complement C5 have decreased survival, increased inflammation, and decreased macrophage cytokine production [4, 5, 9]. Little is known about the role of the terminal complement components in MTB infection. Thus,

complement factor C7 knock-out (C7 −/−) mice were infected with MTB and evaluated for bacterial burden, lung immunopathology, and cytokine responses.

2. Methods

2.1. Animals. Six week-old C7 deficient mice (C7 −/−) and wild-type C57BL/6 mice were a kind gift from Michael C. Braun (Baylor College of Medicine, Houston, TX). Briefly, C57BL/6J ES cells with a targeted insertion of a gene trap vector in the intronic region between exons 6 and 7 of the C7 gene were obtained from the Texas Institute of Genomic Medicine (TIGM, Houston, Texas). Single insertion of the vector was confirmed by FISH analysis, and targeting to the intronic region between exons 6 and 7 of the C7 gene was confirmed by sequence analysis. Heterozygous chimeric mice with germ-line integration were then back-crossed with C57BL/6J mice. The successful gene trapping of C7 using the targeting vector was confirmed by RT-PCR, and hemolytic assay. Six to eight mice were used per group, per time points indicated, infected within biosafety level 3 facilities. All studies were performed under the approval of the Animal Welfare Committee at UTHSC, protocol AWC-11-020.

2.2. Acute Tuberculosis Infection of Mice. MTB strain Erdman (TMC 107, American Type Cell Culture) was cultured in Middlebrook 7H9 broth, with 10% supplement, to log phase. Pelleted bacteria were resuspended in phosphate buffered saline (PBS) and diluted to 3×10^8 colony forming units (CFU) per mL using McFarland standard #1 (Thermo Fisher Scientific-Remel, Lenexa, KS). Bacteria were sonicated to disperse any clumps. C57BL/6 mice were infected using an aerosol inhalation exposure system (GLAS-COL Model #A4212 099c, Glas-Col, Terre Haute, IN) to achieve an average aerosol implantation of 100 CFUs, as described [10]. The inoculation dose was confirmed by sacrificing a subset of mice at day one after the-challenge and plating lung homogenates onto 7H11 agar plates (Remel, Lenexa, KS), which were incubated at 37°C for 3-4 weeks.

Mice were sacrificed at days 30 and 60 after the-MTB challenge. The lung, liver, and spleen were removed and sectioned. Portions used for determination of CFUs were placed into 5 mL PBS and homogenized. The organ homogenates were serial diluted and plated on Middlebrook 7H11 plates. Colonies were enumerated after a 3-4 week incubation period at 37°C.

2.3. Lung Histopathology Analysis. Lung tissue was fixed in 10% formalin and embedded in paraffin. Five μm thick sections were stained with hematoxylin and eosin by standard methods. Acid-fast staining was performed by the Ziehl-Neelsen method. Six lungs from each group at 30 and 60 days post-challenge were randomly selected for immunohistochemistry (IHC) for CD4 and CD8. Rat anti-mouse monoclonal antibodies (R&D Systems, Minneapolis, MN) were used to detect CD4+ and CD8+ cells using an anti-rat horseradish peroxidase 3,3′-diaminobenzidine cell and tissue staining kit according to the manufacturer's instructions (R&D Systems).

Histopathology images were obtained with the Nuance multispectral imaging system (CRI, Woburn, MA). This system allows the enumeration of cell types in defined areas of pathology. The entire section of the immunostained lungs was imaged using the 10x objective. Quantification of normal lung, area percentages of macrophages and lymphocytes, and total number of CD4+ and CD8+ cells was performed using the tissue and cell segmenting functions of Inform software (CRI) using the manufacturer's instructions.

2.4. Lung Cytokine Expression. Total lung RNA was isolated and used to produce cDNA as previously detailed [11, 12]. Analysis of cDNA was performed using qRT-PCR procedures, with previously detailed primers and probes [12]. The reaction mixture contained 5 μL cDNA, 1X PCR buffer (5Prime, Fisher Scientifics, Pittsburg, PA), 200 nM dNTPs (Invitrogen, Carlsbad, CA), 400 nM each for the forward and reverse primer (Integrated DNA Technologies, San Diego, CA), 100 nM probe (Biosearch Technologies, Novato, CA), 1x ROX reference dye (Invitrogen), and 1 U/50 μL Taq DNA polymerase (Fisher). The reaction was performed using the ABI Sequence Detection System (Applied Biosciences, Carlsbad, CA) by heating for 1 min at 95°C, and then 40 cycles of 95°C for 12 seconds and 60°C for 1 min. Data are expressed as fold change expression relative to naive controls after normalization to β-actin [13].

2.5. Preparation of Bone-Marrow-Derived Macrophages and Infection with MTB. Bone-marrow-derived macrophages from wild-type C57BL/6 and complement factor C7 −/− mice were generated by previously described methods [14]. Briefly, femurs were flushed with McCoy's media (Sigma, St. Louis, MO), and 2×10^6 cells were added to 24 well tissue culture plates (Corning Incorporated, Corning, NY). Cells were cultured in McCoy's media (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma), 100 μg mL^{-1} gentamycin (Sigma), 100 U mL^{-1} penicillin (Sigma), and 10 ng mL^{-1} recombinant murine granulocyte/macrophage colony stimulating factor (GM-CSF; Chemicon, Billerica, MA). The cells were cultured at 37°C in 5% CO_2 for seven days, with two media changes containing GM-CSF. Finally, cells were washed and suspended in antibiotic free Dulbecco's modified eagle's medium (DMEM; Sigma) supplemented with 10% FBS.

The cells were infected with log phage cultures of MTB Erdman at a multiplicity of infection (MOI) of 1 : 1. The supernatants were removed after 24 and 72 hours of incubation and analyzed for production of IL-6, TNF-α, IL-12p40, and TGF-β by ELISA according to the manufacturer's instructions (R&D Systems).

2.6. Statistics. The data are shown as the mean ± SD. Two-way ANOVA was used to determine the differences between groups by use of GraphPad Prism software. A P-value of less than 0.05 was defined as statistically significant.

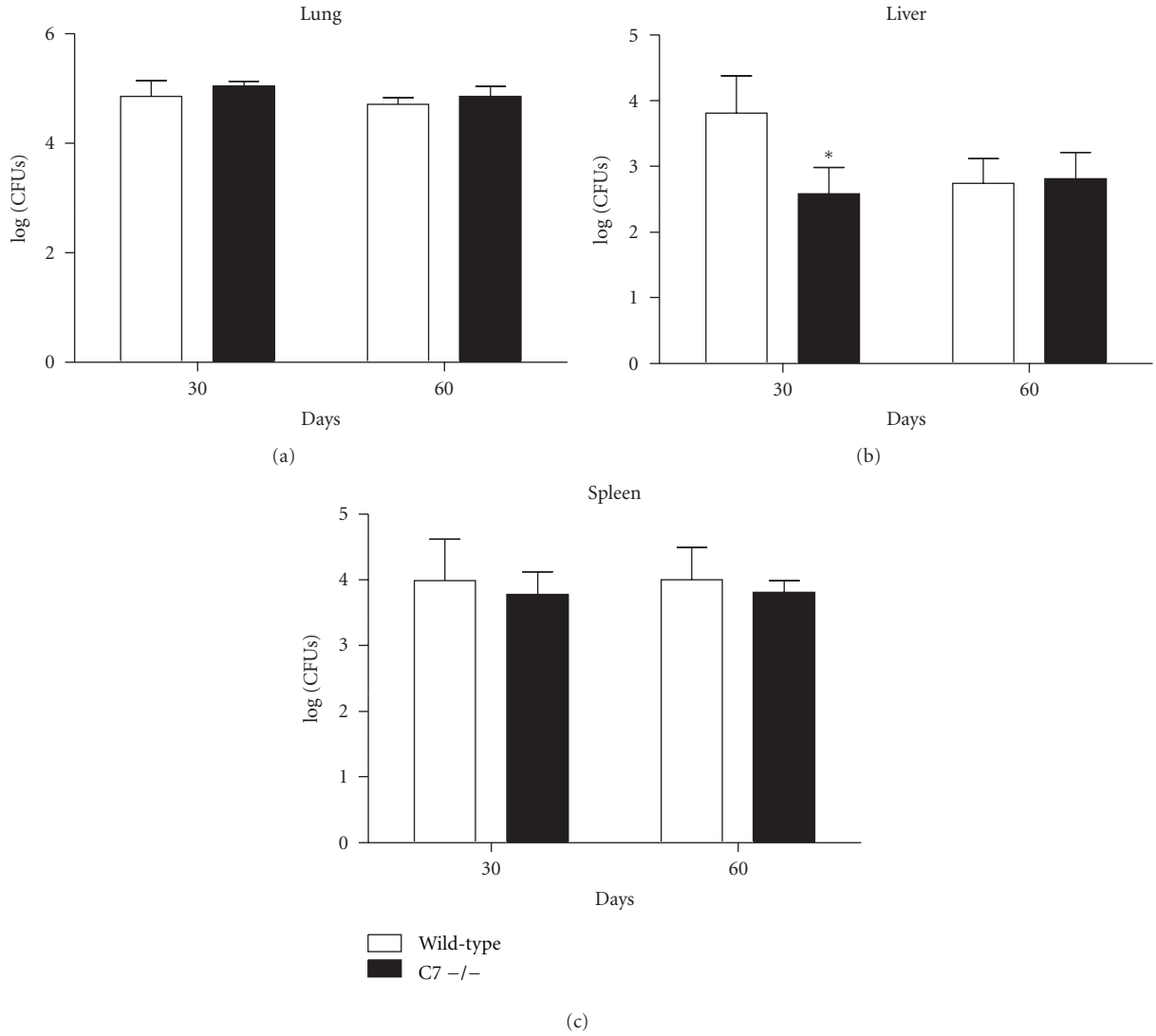

Figure 1: Decreased early bacterial dissemination to the liver in C7 −/− mice. C7 −/− mice had significantly reduced bacterial CFUs in the liver at day 30 after infection with MTB. There were no differences in bacterial burden in the lung or spleen. $^*P < 0.05$ with comparison to wild-type C57BL/6 mice.

3. Results

3.1. Decreased Bacterial Dissemination in C7 Deficient Mice. Bacterial CFUs in the lung, liver, and spleen are shown in Figure 1. C7 −/− mice had significantly reduced CFUs in the liver at day 30; specifically, liver $\log_{10}$ CFUs in wild-type mice were 3.82 ± 0.56 and 2.58 ± 0.40 at day 30 for the C7 −/− mice. No significant differences in bacterial load were observed in the lung and spleen at either time point.

3.2. C7 −/− Mice Have Decreased Lung Immunopathology. Representative images of lung histopathology for wild-type and C7 −/− mice at 60 days post-infection are shown in Figure 2. Wild-type mice demonstrate significant lung inflammatory infiltrates, which were predominantly macrophages (Figures 2(a) and 2(b)). In contrast, C7 −/− mice have reduced destructive lung pathology with smaller granulomas and decreased parenchymal inflammation (Figure 2(c) and Table 1). Abundant clusters of lymphocytes were observed in the C7 −/− mice, primarily near the vasculature and adjacent to granulomas (Figure 2(d)). Quantitative IHC demonstrated increased numbers of CD4+ lymphocytes and reduced areas of macrophages in the C7 −/− mice (Table 1). Rare multinucleated giant cells were noted in the C7 −/− mice (Figure 2(e)).

3.3. Absence of Complement Factor C7 Modulates Lung Cytokine Expression. Lung cytokine expression of TNF-α, IL-6, IL-12p40, TGF-β, IL-10, IFN-γ, and IL-17 was measured by quantitative PCR (Figure 3). Expression of all cytokines increased in challenged wild-type mice relative to naive mice. C7 −/− mice had significantly increased expression of TNF-α and IFN-γ at day 60 after the-MTB challenge, compared to wild type mice. There were no significant differences in lung

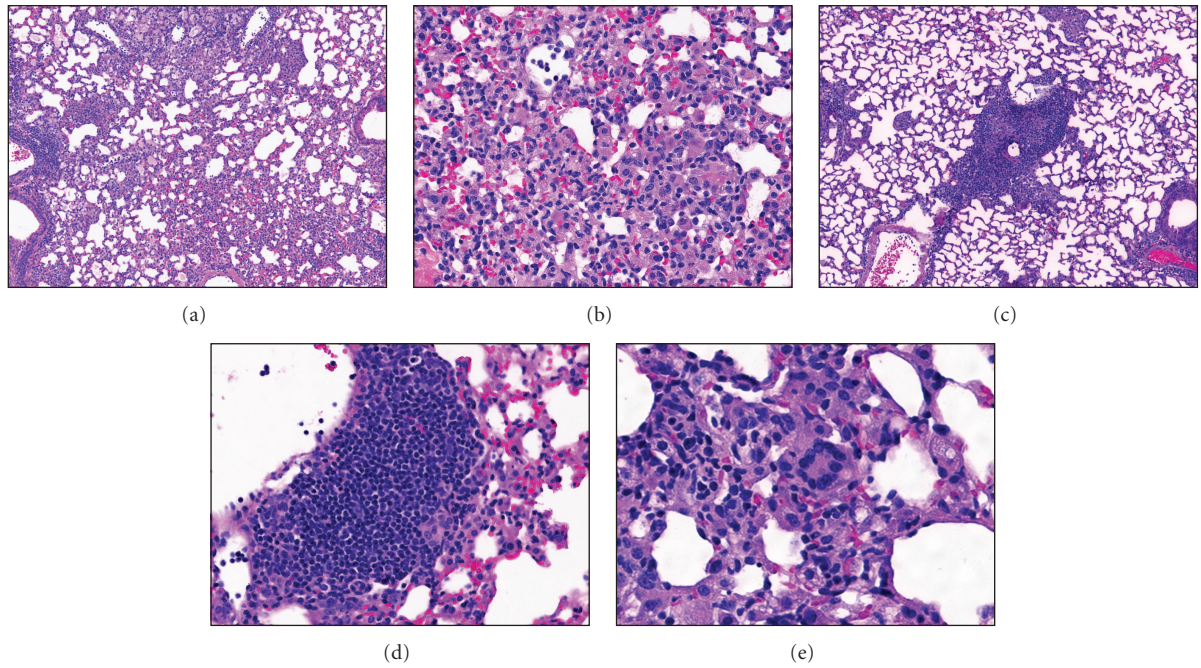

Figure 2: Lung histopathology of wild-type C57BL/6 and C7 −/− mice after the-challenge with MTB Erdman. (a) C57BL/6 mice demonstrate extensive granulomatous inflammation at 60 days post infection, 100x. (b) High power images of wild-type mice show that infiltrates are largely macrophages, 400x. (c) C7 −/− mice have reduced lung immunopathology at day 60 after the infection, 100x. (d) Abundant areas with clusters of lymphocytes are observed in C7 −/− mice, 400x. (e) Rare multinucleated giant cell in C7 −/− mice, 400x.

Table 1: Quantitative IHC of wild-type C57BL/6 mice and C7 deficient mice infected with MTB.

	Control	C7 −/−
% lung occlusion		
Day 30	50.6 ± 12.1	47.8 ± 12.3
Day 60	59.1 ± 12.8	40.8 ± 7.5*
% macrophages		
Day 30	50.8 ± 12.0	39.9 ± 8.1
Day 60	49.2 ± 13.1	30.3 ± 10.1*
% lymphocytes		
Day 30	4.3 ± 2.8	6.7 ± 3.7
Day 60	9.1 ± 3.1	17.0 ± 8.7*
CD4+ lymphocytes		
Day 30	277.2 ± 122.9	348.9 ± 239.5
Day 60	291.1 ± 155.9	973.9 ± 449.2*
CD8+ lymphocytes		
Day 30	321.3 ± 177.1	417.3 ± 169.8
Day 60	422.3 ± 284.8	440.3 ± 216.4

expression of IL-6, IL-12p40, TGF-β, or IL-17 (data not shown).

3.4. Modulation of Macrophage Cytokine Production by Complement C7. Proinflammatory cytokine production was measured from MTB-infected bone-marrow-derived macrophages isolated from wild-type and C7 −/− mice (Figure 4). The macrophages from the C7 −/− mice had slight, but significant, decreased synthesis of IL-12p40 and increased TGF-β at 72 hours post-infection. There were no differences in synthesis of TNF-α and IL-6 (not shown). Additionally, the absence of complement factor C7 did not affect MTB proliferation in macrophages (not shown).

4. Discussion

While deficiencies in the terminal complement factors such as C7 are associated with *Neisseria meningitidis* infections [15], their function in mycobacterial infections is unknown. These studies indicate a role for complement C7 in the development of lung immunopathology caused by infection with MTB. Mycobacteria are known to activate all three complement pathways [16], but the results of specific pathway activation on MTB pathogenesis are mixed. While complement activation likely promotes the uptake of mycobacteria into macrophages [8, 17], deficiency of C5 resulted in enhanced susceptibility to TB and its associated trehalose 6,6′-dimycolate glycolipid; this was evident by a reduced IL-12 production by macrophages and reduced IFN-γ synthesis and production [6, 9]. Prior investigations demonstrate that macrophages and dendritic cells produce complement C7 [18, 19]; we therefore determined the impact of complement C7 deficiency on MTB-infected macrophage cytokine secretion. A slight reduction in IL-12p40 and an increase in TGF-β were noted, possibly indicating that C7 deficiency may impact induction of T-cell responses.

The complement C7 deficient mice had increased expression of IFN-γ and TNF-α at 60 days post-challenge with MTB

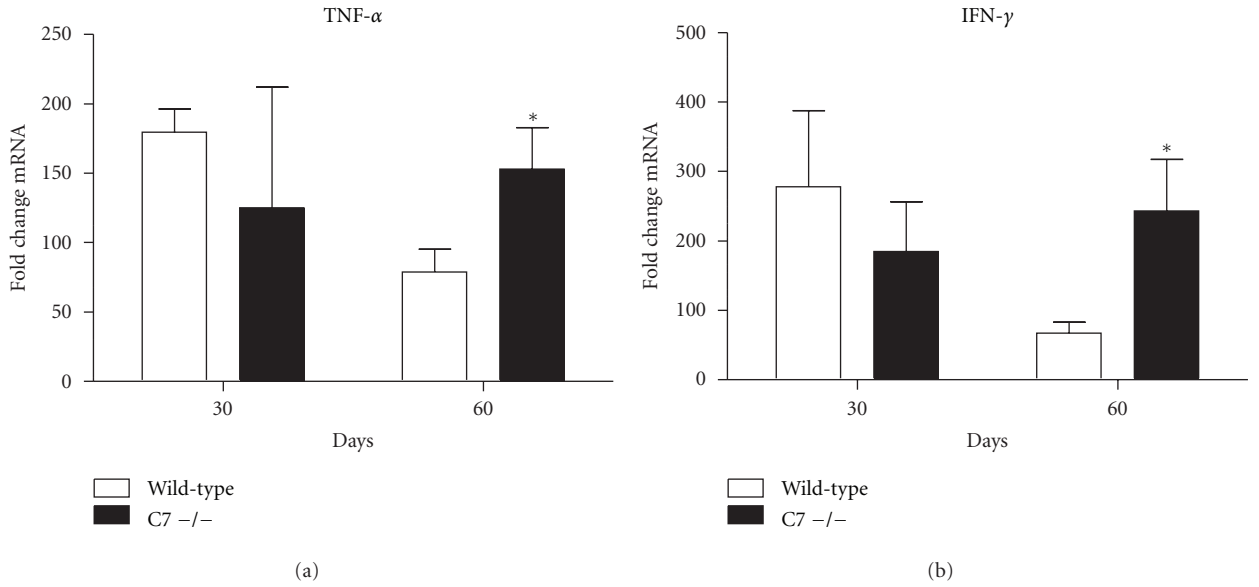

Figure 3: Lung expression of proinflammatory mediators TNF-α and IFN-γ in wild-type and C7 −/− mice. Lung cytokine expression of TNF-α, and IFN-γ as measured by quantitative PCR in wild type (solid bars) and C7 −/− (open bars) mice at 60 days post challenge with virulent MTB. Data are expressed as fold change relative to naïve mice after normalization to β-actin. Data are presented as the mean with SD, $n = 6$ mice per group, per time point. $^*P < 0.05$, comparisons are made to control mice. There were no significant differences in lung expression of IL-6, IL-12p40, TGF-β, or IL-17 (data not shown).

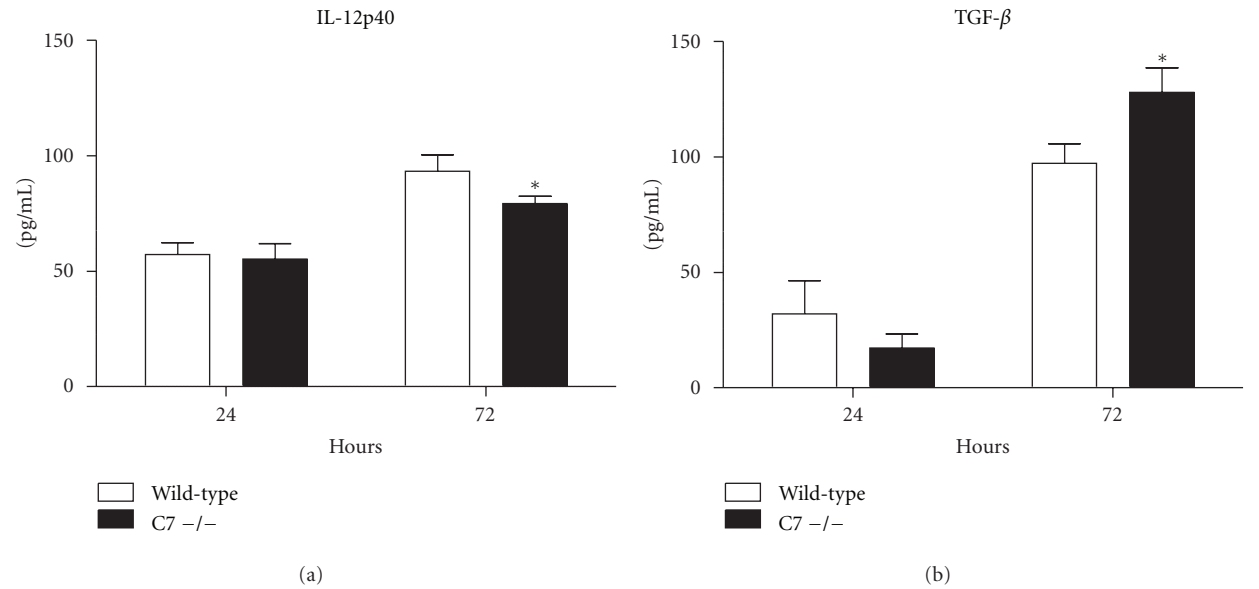

Figure 4: Cytokine production from infected bone-marrow-derived macrophages. Macrophages were isolated from wild-type and C7 −/− mice and infected with MTB at a MOI of 1 : 1. Cytokines production was measured 24 and 72 hours after infection by ELISA. $^*P < 0.05$, comparisons are made to control mice.

accompanied by increased numbers of CD4+ lymphocytes, despite a decrease in IL-12p40 by infected macrophages. Numerous investigations link the early complement cascade components to the development of Th1 responses [20]. For example, the binding of complement factors C3a and C5a to their receptors promotes the migration of antigen presenting cells to areas of infection, where they present antigen to T-cells and modulate IL-12 production that is essential for induction of IFN-γ synthesis [21–23]. Binding of immune complexes to the C1q receptor on T-cells promotes T-cell activation with production of TNF-α and IFN-γ [24]. Both CD4+ and CD8+ T-cells responses in C3 deficient mice are

decreased in viral infection models [25–27]. The precise role of complement C7 in induction of IFN-γ synthesis is unclear; however, there are reports of CD59 modulation of T-cell responses. These data suggest that the crosslinking of CD59, an inhibitor of MAC formation at the level of C9, resulted in augmented IL-2 production and cell proliferation [28]. Furthermore, CD59a deficient mice have enhanced specific CD4+ T-cell responses after challenge with recombinant vaccinia virus [29]. It should be noted that the increase in lung expression of IFN-γ in the C7 $-/-$ mice did not result in a decrease in lung bacterial burden; only an early reduction in liver CFUs was observed. Some investigators report little correlation between the levels of specific IFN-γ producing CD4+ and CD8+ cells and protection against MTB [30].

Of potential significance, the C7 deficient mice had markedly reduced lung immunopathology in this mouse model of MTB infection. The histopathology generated by the host in response to MTB possibly contributes to the persistence and dissemination of the organism [31]. For example, MTB in granulomas convert to a state of non-replicating persistence that is characterized by alterations in biochemical pathways and reduced bacterial proliferation, changes that make the bacteria resistant to antibiotics [32, 33]. The altered cytokines in the infected C7 $-/-$ mice are clearly important in granuloma induction and maintenance of structure [12]. MTB contained with granulomas are physically sequestered from cytotoxic T-cells capable of eliminating infected cells [31]. Reduction of lung immune-mediated pathology may additionally enhance the penetration of antibiotics into infected tissue, possibly enabling a more rapid response to antimicrobials [34]. Indeed, modulation of this pathology has been proposed as a novel therapeutic strategy to shorten the treatment of TB, an approach that has had some success in human clinical trials [35, 36]. Future studies are needed to explore the therapeutic potential of C7 on MTB-induced pathology.

The data presented in these studies indicate a role for complement C7 in the development of lung histopathology in MTB infection, with a potential role for dysregulation in innate interaction with lymphocytes and their subsequent production of cytokines. Future studies are needed to further investigate the role of C7, to specifically define the direct link between the regulation of response affecting MTB-induced immunopathology. In this manner, we can further understand the relationship of pathological dysregulation directly attributed to C7 compared to its contribution as related to other terminal complement components.

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