Atherosclerosis 241 (2015) 480–494

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Review

The complement system and toll-like receptors as integrated players in the pathophysiology of atherosclerosis

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ARTICLE INFO

Article history:
Received 6 January 2015
Received in revised form
8 May 2015
Accepted 29 May 2015
Available online 5 June 2015

Keywords:
The complement system
Toll-like receptors
Atherosclerosis
Inflammation

ABSTRACT

Despite recent medical advances, atherosclerosis is a global burden accounting for numerous deaths and hospital admissions. Immune-mediated inflammation is a major component of the atherosclerotic process, but earlier research focus on adaptive immunity has gradually switched towards the role of innate immunity. The complement system and toll-like receptors (TLRs), and the crosstalk between them, may be of particular interest both with respect to pathogenesis and as therapeutic targets in atherosclerosis. Animal studies indicate that inhibition of C3a and C5a reduces atherosclerosis. In humans modified LDL-cholesterol activate complement and TLRs leading to downstream inflammation, and histopathological studies indicate that the innate immune system is present in atherosclerotic lesions. Moreover, clinical studies have demonstrated that both complement and TLRs are upregulated in atherosclerotic diseases, although interventional trials have this far been disappointing. However, based on recent research showing an intimate interplay between complement and TLRs we propose a model in which combined inhibition of both complement and TLRs may represent a potent anti-inflammatory therapeutic approach to reduce atherosclerosis.

1. Atherosclerosis – an inflammatory disease

Atherosclerosis is a common disorder and a leading cause of morbidity and mortality worldwide. In many cases, individuals are asymptomatic and the disease is therefore not recognized until an acute thrombotic manifestation like myocardial infarction (MI), stroke or sudden death occurs. Moreover, the prevalence of atherosclerotic disease and its related costs are expected to increase not only in the industrialized but also in developing countries [1]. It remains a huge challenge to solve this global clinical problem.

Inflammation is a major component of atherosclerosis and considered to play a role in all developmental stages of the disease [2,3]. Illustratively, cholesterol and inflammation have been described as two partners in crime during atherogenesis [4]. Lipoproteins that are trapped and retained by matrix proteoglycans in the intimal layer of the arterial wall easily undergo oxidative modifications, and this event is followed by an immediate innate immune response [5,6]. The bidirectional interaction between inflammation and lipids will lead to an accumulation of lipid-filled macrophages in the intima and eventually form a lipid core not only including lipid-filled cells but also apoptotic and necrotic cells, cell

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http://dx.doi.org/10.1016/j.atherosclerosis.2015.05.038
0021-9150/

debris and cholesterol crystals. Provided a local cytokine profile favoring smooth muscle cell proliferation and synthesis of extracellular matrix proteins, the lesion will acquire a stable but narrowing phenotype in relation to lumen diameter characterized by the central lipid core and a thick surrounding layer of smooth muscle cells and fibrous connective tissue, a so-called fibrous cap.

However, atherosclerosis is a dynamic process and the stable lesion may be transformed into an unstable, rupture-prone lesion. In contrast to the stable plaque, a large lipid core and a thin fibrous cap characterize the unstable plaque. In addition there is consistent evidence for an imbalance between pro- and anti-inflammatory mediators towards larger infiltrates of T cells and activated macrophages, higher apoptotic rates and increased expression of pro-inflammatory cytokines, chemokines and proteolytic enzymes in unstable plaques. Despite this increasing knowledge of plaque characteristics, the complex and multifactorial mechanisms behind plaque destabilization are far from clarified.

Several types of immune cells are involved in the inflammatory arm of atherosclerosis. Overexpression of T helper 1 (Th1)-derived cytokines, including interferon (IFN)-γ and tumor necrosis factor (TNF), has been associated with advanced and unstable plaque phenotypes [7,8]. An excessive Th1 activity is thus considered to drive the development towards plaque destabilization. On the other hand, regulatory T cells seem to have atheroprotective properties by exerting anti-inflammatory and Th1 suppressive effects. Recently, B cells have also been shown to be involved in atherogenesis elicting both pro- and anti-atherogenic activities [9–11]. Thus, while B2 B cells seem to have pro-atherogenic effects, B1 B cells appear to attenuate the atherosclerotic process at least partly by secreting interleukin (IL)-10.

Macrophages, prototypical cells in the innate immune system, have for several years been known to play a key role in lipid accumulation and inflammation during atherogenesis. These cells have now been divided into inflammatory (M1) and resolving (M2) phenotypes [12,13]. Thus while LPS through TLR4 activation and in combination with IFN-γ, released from Th1 cells, promotes M1 polarization, IL-4 and IL13, released from Th2 cells promote M2 polarization of macrophages. More recently, additional subdivision of M2 macrophages has been performed, i.e. M2a, M2b, M2c and M2d macrophages [12,13]. A functional classification refers to these M2a macrophages as 'wound-healing macrophages'. M2b macrophages are induced upon combined exposure to immune complexes and TLR ligands or IL-1 receptor agonists, producing both inflammatory (e.g., IL-6 and TNF) and anti-inflammatory cytokines (IL-10), M2c macrophages are induced by IL-10 and glucocorticoids [14]. These M2c macrophages, together with M2b macrophages, are also referred to as "regulatory macrophages". Finally, M2d macrophages are induced by co-stimulation with TLR and adenosine A2A receptor agonists, characterized by high levels of IL-10 and vascular endothelial growth factor (VEGF), potentially playing a role in angiogenesis. In the atherosclerosis field, additional forms have been described including the Mhem macrophage, consistent with their presence in regions of haemorrhage [15], and M4 macrophages that are induced by CCL4 showing high expression of matrix metalloproteinases associated with plaque destabilization in carotid plaques [16]. M1 polarization is induced by TLR2 and TLR4 activation in combination with lipids. Th2 related cytokines and not TLR activation seem to be of importance for M2 macrophage polarization. Like TLRs, complement activation has been linked to M1 polarization and C3 deficient mice have been shown to have fewer M1 macrophages and more M2 macrophages [17].

Indeed several components of innate immunity including the complement system and TLRs, as mentioned above, have increasingly been targeted in atherosclerosis research [3,18]. Oxidatively modified lipoproteins in the arterial wall are potentially dangerous stressors. The innate immune system is initiating and orchestrating the elimination of these particles. In this "first line defence" a variety of pattern-recognition receptors (PRRs) are used including cellular PRRs such as scavenger receptors and TLRs, and soluble PRRs such as complement components and germline naturally occurring IgM antibodies. The innate immune response not only involves immediate pro-inflammatory actions, but also initiation of adaptive immunity and resolution of inflammation and tissue repair. The production of natural IgM antibodies to oxidation-specific epitopes by naïve B cells is one potential atheroprotective effect generated by the innate immune system [19,20].

A chronic exposure to stressors in the arterial wall may eventually lead to a loss of immune homeostasis. TLRs and complement are mediators bridging danger sensing further to adaptive immunity, thereby acting as key regulators in the maintenance of immune homeostasis. The complement system has important regulatory effects on both B cells and T cells [21,22]. Previous reviews have either addressed the interaction between TLRs and atherosclerosis [23–26] or between complement and atherosclerosis [27–29]. However, recent research indicates an extensive crosstalk between TLRs and complement, thus proposing a complex interplay between these pathways of innate immunity in atherogenesis. As discussed in the present review, this may open up for therapeutic strategies favoring the repair process and stabilization of atherosclerotic lesions.

2. The complement system

The complement system (Fig. 1) is part of our innate defence against infections, and was initially described in the late 19th century [30]. It consists of more than 40 membrane bound and soluble proteins, the latter mainly being secreted by hepatic cells, monocytes and macrophages [31,32]. The traditional view of complement as being predominantly a host defence system against microbes has expanded markedly the last decades to our current knowledge that complement is a surveillance system that quickly can be activated by sensing any danger to the host and thereby contribute to maintaining tissue homeostasis and promote tissue regeneration and repair [33]. On the other hand, undesired or uncontrolled activation of the system can induce tissue damage and organ dysfunction in the host. Forty years ago the interplay between atherosclerosis and the complement system was suggested [34], and the theory has later been maintained [29,35].

2.1. Activation pathways

Traditionally there are three known ways through which the complement system is activated (Fig. 1). The classical pathway (CP) is activated by C1q binding to antibodies when bound to their antigen, or antibody independent by other recognition molecules like the pentraxins including C-reactive protein (CRP), serum amyloid component P (SAP) and long pentraxin 3 (PTX3). The lectin pathway (LP) is activated when proteins like mannose-binding lectin (MBL), the ficolins (−1, -2 and −3) and collectin-11 recognize their ligands like sugar molecules on microbes, on dying host cells or on a subendothelial matrix [36,37]. The alternative pathway (AP) is continuously undergoing a low-grade activation due to hydrolysis of the internal C3 thiol-ester bond, and further activated when there is an imbalance between activation and inhibition e.g. on foreign surfaces or structures lacking complement regulatory proteins.

The different activation pathways lead to the common pathway with activation of C3 and C5 (Fig. 1). From this point the cascade continues to the terminal pathway with release of the biologically

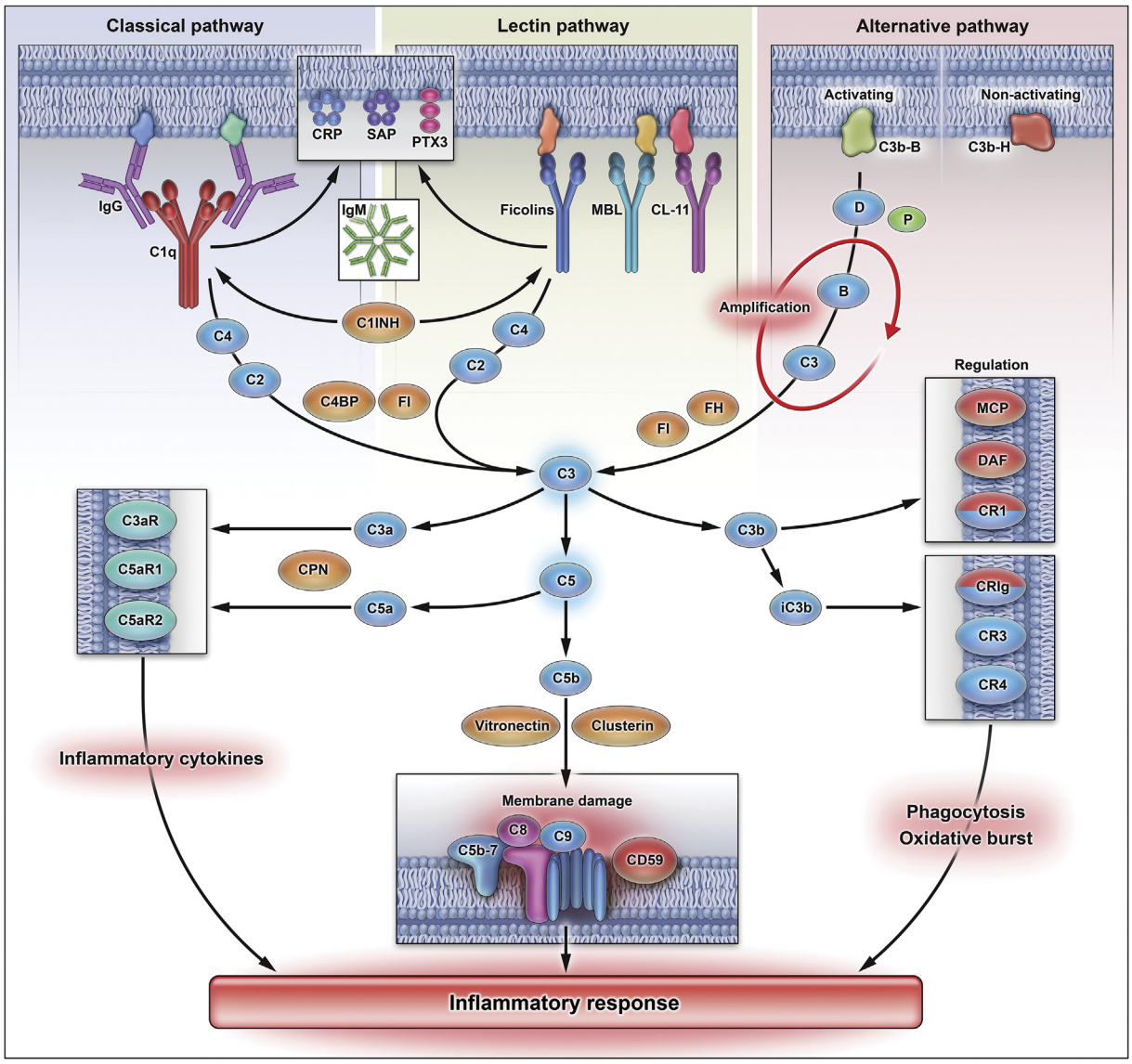

Fig. 1. The complement system. The complement system can be activated through three pathways, all converging to the cleavage of C3 to generate C3a and C3b. In the classical pathway (CP) C1q can bind to antibodies, but also pentraxins including C-reactive Protein (CRP), serum amyloid P component (SAP) and pentraxin 3 (PTX3). The Lectin pathway (LP) is activated through recognition of carbohydrates by mannose binding lectin (MBL), ficolins and collectin-11 (CL-11). Furthermore LP activation may be mediated through IgM antibodies, e.g. directed against damaged self antigens. The alternative pathway (AP) is activated by foreign or damaged own cells, facilitated by the continuous spontaneous hydrolysis of C3. AP also has an important function in the complement system providing an amplification loop enhancing C3 activation independent of which pathway that is initially activated. This effect is mainly due to properdin (P), the only positive regulator in the complement system, which stabilizes the C3 convertase. Activation of C3 leads to formation of a C5 convertase, cleaving C5 into C5a and C5b. The anaphylatoxins C3a and C5a bind to the receptors C3aR, C5aR1 (CD88) and C5L2 (C5aR2), leading to downstream production of inflammatory mediators. C5b initiates the formation of the terminal C5b-9 complement complex (TCC), which either forms the membrane attack complex if inserted into a membrane. This may lead to lysis of bacteria and cells, or in sublytic doses to activation of cells. The cleavage and inactivation of C3b generates iC3b, binds to complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18), facilitating phagocytosis, oxidative burst and downstream inflammation. The complement system is tightly regulated by soluble inhibitors, including C1-inhibitor (C1–INH), factor H (FH), factor I (FI), C4-binding protein (C4BP), carboxy-peptidase N (CPN), vitronectin (VN) and clusterin (Clust.), keeping the continuous low-grade activation in the fluid phase in check. Host cell membranes are equipped with a number of inhibitors to protect them against attack by complement, including membrane cofactor protein (MCP; CD46), complement receptor 1 (CR1) (CD35), decay accelerating factor (DAF; CD55), controlling C4 and C3 activation, and CD59 protecting against final assembly of the C5b-9 complex.

highly potent anaphylatoxin C5a and formation of the terminal C5b-9 complement complex (TCC). The terminal complement complex can appear as a soluble complex in the fluid phase (sC5b-9) if there is no adjacent surface to be attacked. sC5b-9 is formed by assembly of C5b-9 together with the regulators vitronectin and clusterin, keeping the complex soluble, sC5b-9 is a useful marker for complement activation in body fluids. If C5b-9 is formed on a membrane, the membrane attack complex (MAC) is formed. The latter may either lead to lysis of bacteria and cells by penetrating the membrane after binding C8 and additional C9 molecules, or, if formed in sublytic amounts, to stimulation of the cell to release inflammatory mediators [38]. With relevance to atherosclerosis, coagulation factors like plasmin, thrombin and other proteases have in recent years emerged as direct activators of C3 or C5, circumventing the initial pathways [39–42].

2.2. Complement regulators

As the complement system is both rapid and potent and components like C1q and C3 undergo a low-grade spontaneous

activation, there is a need for strict inhibitory regulation of the system. These regulators, of which there are both soluble and membrane-bound types, act in different steps of the complement cascade (Fig. 1). The C1-inhibitor (C1–INH) and C4b-binding protein (C4BP) control both CP and LP, the AP is inhibited by factor H (FH), while factor I (fI) acts in all three pathways. Several membrane bound receptors including complement receptor 1 (CR1; CD35), membrane co-factor protein (MCP; CD46) and decay-accelerating factor (DAF; CD55) inhibit at the level of C3 and thus contribute to keeping all the initial pathways under control when they converge at C3. The anaphylatoxins C3a and C5a are inhibited by carboxypeptidase-N, whereas protectin (CD59) inhibits the formation of TCC. The regulators are of crucial importance in order to maintain complement homeostasis. Lack of or dysfunctional regulation is often associated with a clinical disease; e.g. atypical haemolytic uremic syndrome (aHUS) or membranoproliferative glomerulonephritis type II (dense deposits disease) when FH is missing or dysfunctional [43], and paroxysmal nocturnal hemoglobinuria (PNH) when CD55 and CD59 are missing [44]. This illustrates an important principle of complement activation, namely that the system can be activated without any specific activator, the loss of an inhibitor is enough to trigger the system, leading to tissue damage and disease.

2.3. Clinical use of complement inhibitors

The complement regulator C1–INH has been in clinical use for a long time as substitution therapy in hereditary angioedema, a condition with life-threatening recurrent swellings due to low C1–INH concentrations [45]. The pathophysiology is due to bradykinin formation through the kallikrein-kinin system, in which C1–INH also plays a crucial role. Thus, C1–INH is not a specific complement regulator, but participates in several cascade systems.

A novel approach for complement therapy in the clinic is to use a specific complement inhibitor with the aim of reducing the adverse effects induced by pathological complement activation. PNH is a condition where red cells are lysed since they lack complement regulators on their surface. Blocking C5 by a monoclonal antibody, eculizumab, was first FDA-approved for treatment of PNH patients [46]. By blocking cleavage of C5, the anaphylatoxin C5a and the lytic C5b-9 complex are not formed (Fig. 1). Recently, eculizumab was also approved for the treatment of aHUS, and it is likely that more indications will emerge in the areas of kidney disease and transplantation medicine. Side effects of continuous C5 inhibition include increased susceptibility to Neisseria infections, in the same manner as patients with C5 deficiency, who otherwise are healthy [47,48]. The therapeutic potential of complement manipulation in various diseases has recently been reviewed [49].

Specific complement inhibition has also been used in cardiovascular diseases, with promising results in animal models [50], but, as will be presented below, without convincing clinical results so far [51,52]. However, studies with inhibition of several complement factors simultaneously, or the combination of complement and TLR-system inhibition is yet to be performed.

3. Toll-like receptors

The human innate immune system serves as a first line of defence, and includes a magnitude of proteins and receptors in addition to the complement system. Humans have membrane-bound receptors including TLRs and cytoplasmic sensors including NOD-like receptors, pyrin and HIN domaine-containing family members and Rig-I-like receptors [53]. TLRs recognize exogenous and endogenous stimuli [54]. The exogenous, “non-self” inducers of these receptors are labeled pathogen associated molecular patterns (PAMPs), and the endogenous “self” counterpart are called damage associated molecular patterns (DAMPs) or alarmins. The current view is that the main task of the innate immune system is to detect danger, and not simply to discriminate between “self” and “non-self” [55]. TLRs are PRRs localized on a variety of different cell types including neutrophils, monocytes/macrophages, mast cells, T- and B-cells, but also endothelial and smooth muscle cells [56]. TLRs are phylogenetically old, and in humans there are at least 10 different TLR proteins, while the number and types vary between different mammals [57]. They are all classified as type 1 transmembrane proteins, and except for TLR3 they all use the adaptor molecule Myeloid Differentiation Factor 88 (MyD88) for intracellular signaling and activating transcription of pro-inflammatory genes. TLR4 signaling can be initiated both through MyD88 dependent and independent pathways (Fig. 2). The MyD88-dependent pathway rapidly activates NF-κB and mainly takes place at the plasma membrane [58,59], whereas the MyD88-independent pathway activates interferon regulatory factor-3 (IRF3) and occurs at early endosomes [60,61]. TLR2 is activated through a MyD88 dependent signaling mechanisms (Fig. 2), however, recent studies have revealed a novel role for TRAM and TRIF also for some TLR2 responses [62]. Importantly, there are several endogenous ligands that can activate TLRs in atherosclerosis. Different types of heat shock proteins have been reported to stimulate both TLR2 and TLR4 [63–67]. Another DAMP that can be released from necrotic cells is high-mobility group box 1 protein (HMGB1), which initiates signaling both through TLR2 and TLR4 [68–70]. Lipids can also act as TLR ligands and oxidized LDL has been reported to signal both through TLR2 [71] and through CD36/TLR4/TLR6 [72]. Comprehensive reviews on endogenous TLR ligands of relevance for atherosclerosis can be found in ([26,73]). It should be kept in mind, when evaluating results from experiments regarding ligands for TLRs, that contamination with LPS is a ubiquitous source of misinterpretation [74].

TLR1, TLR2, TLR4, TLR5 and TLR6 are located on the plasma membrane and can be activated by a whole array of ligands including bacterial cell wall components (e.g. lipopolysaccharide [LPS] and lipoproteins) [75]. TLR3, TLR7, TLR8, and TLR9 are sequestered in the endoplasmic reticulum and are delivered to the endosomes, where they encounter and respond to endogenous and exogenous DNA and RNA. Once inside the endosomes, the N-terminal region of the TLRs is processed by multiple lysosomal proteases, including cathepsins and asparagine endopeptidase, to generate functional receptors that elicit signaling [75]. TLRs have a number of co-receptors, of which CD14 is of particular interest since it interacts with several of the TLRs, including TLR2, TLR3, TLR4, TLR6, TLR7, and TLR9 [76]. Recently it was demonstrated that modified LDL induces cytokine release, mediated by TLR4 and CD14, indicating possible therapeutic potential [77].

The prototypical inflammatory cytokine IL-1β as well as IL-18 are processed from their pro-forms via caspase-1 activation to their active form through assembly of inflammasomes, of which the NOD-like receptor with a PYD-domain (NLRP)3 inflammasome is the best characterized [53]. Fully activation of NLRP3 resulting in mature IL-1β and IL-18 requires two signals [78]. The first signal is transcription of pro-IL-1β and pro-IL-18 that is induced by NF-κB activation often downstream of a TLR-ligand interaction. The second signal is activation of the inflammasome that results in caspase-1 activation and maturation and release of IL-1β and IL-18. Numerous DAMPs and PAMPs activate the inflammasome complex including various types of crystals, and recently cholesterol crystals were found to be potent activators of NLRP3 inflammasomes [79]. Cholesterol crystals are frequently found in atherosclerotic lesions [80], and this phenomenon has until recently been thought to develop late in the disease. However, minute cholesterol crystals

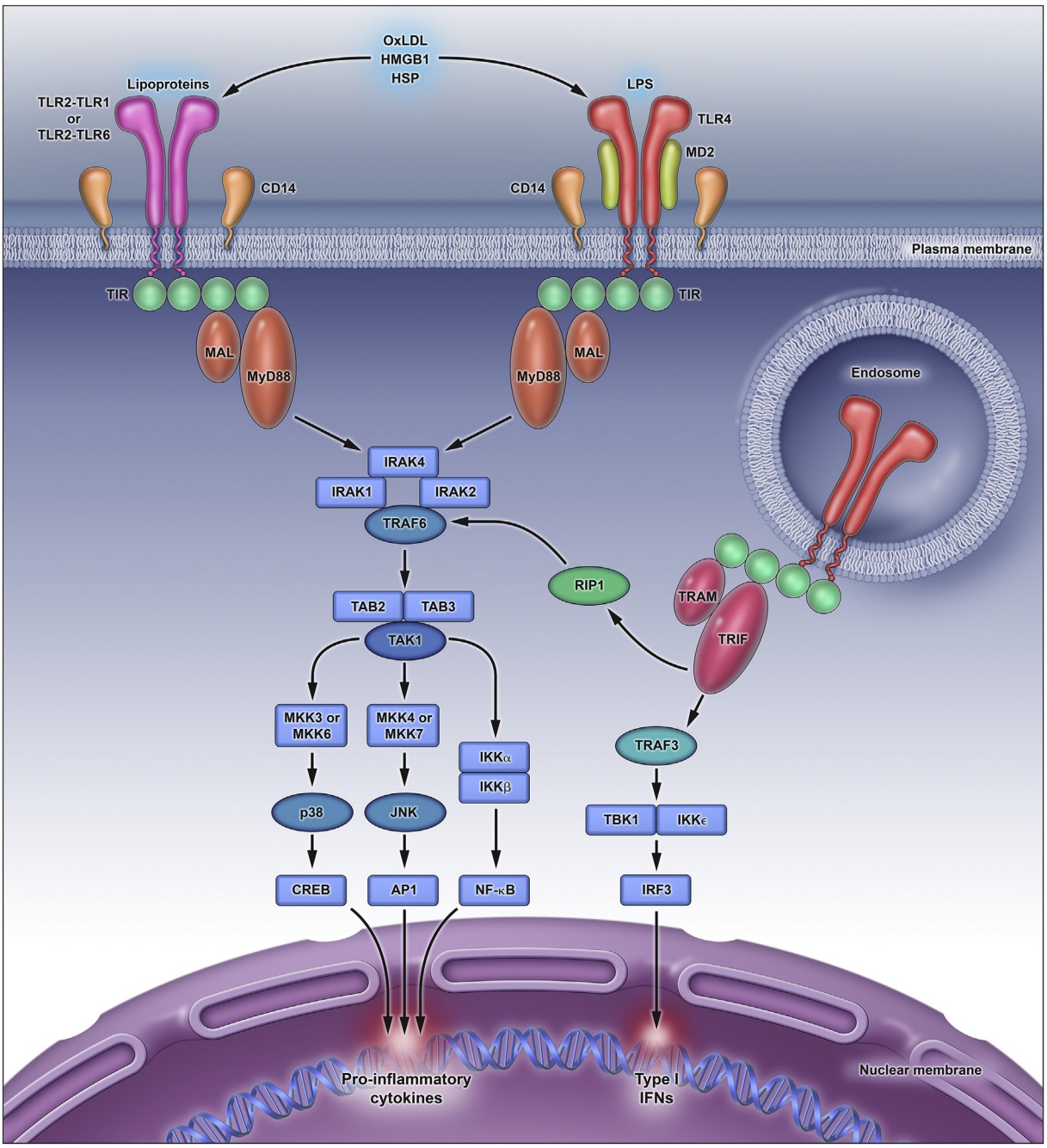

Fig. 2. Toll-like receptor (TLR) signaling illustrated by TLR2 and TLR4. The membrane bound TLR2 (associated either with TLR1 or TLR6) and TLR4 react with ligands including pathogen-associated molecular patterns (PAMPs), like lipoproteins for TLR2 and lipopolysaccharides (LPS) for TLR4, and damage-associated molecular patterns (DAMPs), like oxidized LDL (OxLDL), high-mobility group box 1 protein (HMGB1) and heat shock proteins (HSP) for both receptors. These two TLRs interact with their co-factor CD14 and TLR4-signaling is also dependent on myeloid differentiation factor 2 (MD-2). Then the adaptor proteins myeloid differentiation primary-response protein 88 (MyD88) and MyD88 adaptor-like (MAL) are engaged. TLR4 signaling may also occur from endosomes where TLR4 interacts with TIR-domain-containing adapter-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) activating interferon regulatory factor 3 (IRF3) via TNF receptor-associated factor (TRAF) 3 leading to the production of pro-inflammatory cytokines downstream. The MyD88 dependent pathway is dependent on IL-1R-associated kinases (IRAKs), TRAFs, several regulatory proteins and transcription factors. Endosomal TLR4 (MyD88 independent) may also interact with TRAF6 via TRIF and receptor-interacting protein 1 (RIP1). AP1: activator protein 1, CREB: cyclic AMP-responsive element-binding protein, IKK: inhibitor of NF-κB kinase, JNK:c-jun N-terminal kinase, MKK: MAP kinase kinase, NF-κB: nuclear factor-κB, TAB: TAK1-binding protein, TBK1: TANK-binding kinase 1.

are present in early high fat-diet induced atherosclerotic lesions in ApoE deficient mice and their appearance coincides with infiltration of inflammatory cells [79].

It has been shown that CD36 mediates uptake of oxLDL leading to crystal formation [81]. Moreover, CD14 is important in uptake of minimally modified LDL that also leads to crystal formation, albeit to a lesser extent than oxLDL [81]. These studies suggest new molecular targets, such as the NLRP3 receptor complex and IL-1β, for therapy against atherosclerosis. Recently a phase II trial confirmed anti-inflammatory effect of IL-1β inhibition in patients with type 2 diabetes and high cardiovascular risk as both CRP and IL-6 levels were significantly reduced by this treatment [82]. Challenging this concept, a recent study in mice found that IL-1α, in contrast to IL-1β, is the central mediator of atheromatous

inflammation [83]. There are, however, no data supporting that this is the case in humans.

4. Crosstalk between complement, the TLRs and other systems relevant to atherosclerosis

The complement system and the TLRs are both parts of the innate immune system and hence mediate the hosts "rapid response" to danger. Crosstalk, in this instance indicating interaction between different arms of innate immunity, the complement system and TLRs, has been described in several reviews [84–87]. By using nature's own human knock out, a genetic C5 deficient individual, we have previously determined the relative role of C5 and CD14 in the inflammatory response to Gram-negative bacteria [47]. Although interactions between these systems are barely studied in atherosclerosis, it is reasonable to suggest that such crosstalk is of importance in the pathogenesis of this condition as well.

The crosstalk between complement and TLRs involve both positive and negative feedback mechanisms. Additive and synergistic effects between complement and TLRs occur at several levels, and such potentiated inflammatory responses may be beneficial for the host in certain circumstances, including local protection against infections. This response may, however, be detrimental for the host if inappropriate and overwhelming, or if occurring systemically; thus be an attractive target for therapy [86]. A close interaction between TLR- and C5a receptor-activation has been described [88–90], and recently we showed that there was not only an additive but even synergistic effect on a number of inflammatory mediators when both complement and the TLR co-receptor CD14 were inhibited in combination as compared to separate inhibition [91]. Moreover, mice lacking DAF, an inhibitor of the C3 convertases leading to less C3a and C5a generation, are hypersensitive to TLR stimulation (TLR4, TLR2/6 and TLR9), suggesting a central role for complement in the outcome of TLR activation [92] (Fig. 3). The crosstalk is also bidirectional, and TLR activation has in both *in vitro* and *in vivo* studies been shown to potentiate the effects of C5a. Raby et al. have demonstrated that TLR activation enhanced C5a-induced pro-inflammation responses [90]. This was paralleled by a TLR mediated down-regulation of C5aR2, which serves as a regulator of C5aR1, thus sensitizing C5aR1 for stimulation with C5a, further increasing the inflammatory response (Fig. 3) [90].

Complement factor B, a component in the AP, has also emerged as an important effector of the responses to TLR activation. In a model of polymicrobial sepsis, factor B was markedly increased in serum and upregulated in several organs including the heart. This effect was dependent on the TLR and IL-1 receptor signaling adaptor MyD88. Importantly, deletion of factor B had marked protective effects in this model [93].

Complement receptor 3 (CR3) consists of the integrin CD11b and CD18 and is centrally involved in phagocytosis. CD11b takes part in negative regulation of TLR-signaling through crosstalk with MyD88, rendering mice more susceptible to septic shock [94,95], further underlining the close link between complement and TLRs (Fig. 3). Thus, the crosstalk between complement and TLRs may be a potent trigger of further inflammatory loops (Fig. 3) [96]. A dysregulated interaction between the complement system and TLRs could therefore not only lead to inappropriate inflammatory responses during the acute phase, but could also contribute to maintaining a state of non-resolving inflammation as in atherosclerosis.

Complement factors have recently been reported to promote NLRP3 activation [97]. Asgari et al. showed that C3a potentiates LPS-induced NLRP3 inflammasome activation in monocytes by regulating the efflux of ATP into the extracellular space [98]. Recently, we showed in *ex vivo* human model systems that cholesterol crystals induce complement activation through CP, which leads to cytokine release, production of reactive oxygen species and activation of the inflammasome [99]. These effects were highly complement-dependent, underscoring complement as an upstream mediator of cholesterol-induced inflammation.

The concept of inflammation in atherosclerosis is firmly established, though still not fully clarified. Increased understanding of the interplay between complement system and TLRs may add important knowledge. Based on the possibility to modulate this interaction at several levels, it is tempting to hypothesize that these systems and their bidirectional interaction could be promising targets for therapy in atherosclerotic disorders (Fig. 3).

There is also an extensive crosstalk between the complement system and the coagulation cascade [100], and among others factor Xa, thrombin and plasmin may activate the complement cascade producing C3a and C5a and inducing an inflammatory response [39,42,101]. Several links for crosstalk between complement and platelets have also been shown [102]. Furthermore oxidized LDL-cholesterol may trigger generation of tissue factor through TLR4-6 heterodimer dependent on CD36 [72,103]. The linking together of several systems including the haemostatic systems, lipids and innate immunity, including crosstalk within, could be attractive targets for therapy in atherosclerosis. Recent evidence that C3 plays an important role in lipid metabolism, obesity and diabetes type 2 emphasizes this view [104].

Microbial pathogens have acquired highly complex ways to manipulate the host's innate immunity [105,106]. More precisely the pathogens may interact with innate immunity receptors, modulating downstream inflammatory signaling [105]. Hence, it should be possible to exploit this evolutionary trait in therapy addressing inflammation. Our group has demonstrated that combined inhibition of the complement system and TLRs, more specifically combined inhibition of the TLR co-receptor CD14 and complement inhibitors of C3 and C5, has a remarkable inhibitory effect of the inflammatory reaction induced by both a number of danger signals both in animal models and in human *ex vivo* models [47,87,99,107–110]. A future goal would be to test the hypothesis of double-blockade in a human model of atherosclerosis.

Ischemia reperfusion injury is known from coronary artery disease, one of the common end stages of atherosclerosis, in which reperfusion of an occluded artery leads to damage to the tissues downstream [111]. Similarly, the ischemia reperfusion injury occurring in renal transplantation is a serious problem with respect to graft survival [112]. Crosstalk between the complement system and TLRs is seen in renal ischemia reperfusion injury, and furthermore the two systems seem both to be upregulated after brain death of the donor [113], supporting an important link between the neuro-endocrine systems and innate immunity. The role of ischemia in the development of atherosclerosis should be considered and models should be developed to study the crosstalk of complement and TLR inhibition.

MicroRNAs (miRNAs) are small non-coding RNA particles important in regulating protein synthesis. Distinct types of miRNAs may affect innate immunity, and specifically miR-146a is shown to be a negative regulator of TLR signaling, which could be of importance in neurodegenerative diseases [114]. Another miRNA, miR-155, may affect the complement system by attenuating FH in a human cell model of Japanese encephalitis [115]. Lastly, the complement system may affect miRNAs. In a mouse model of brain endothelial cells in systemic lupus erythematosus, C5a seems to regulate miRNA expression [116]. MiRNAs are attractive targets for therapeutic intervention, and currently several research projects address miRNAs, also in lipoproteins and atherosclerosis [117]. Since there seems to be a connection between innate immunity and miRNAs, this connection should also be further explored in the field of atherosclerosis.

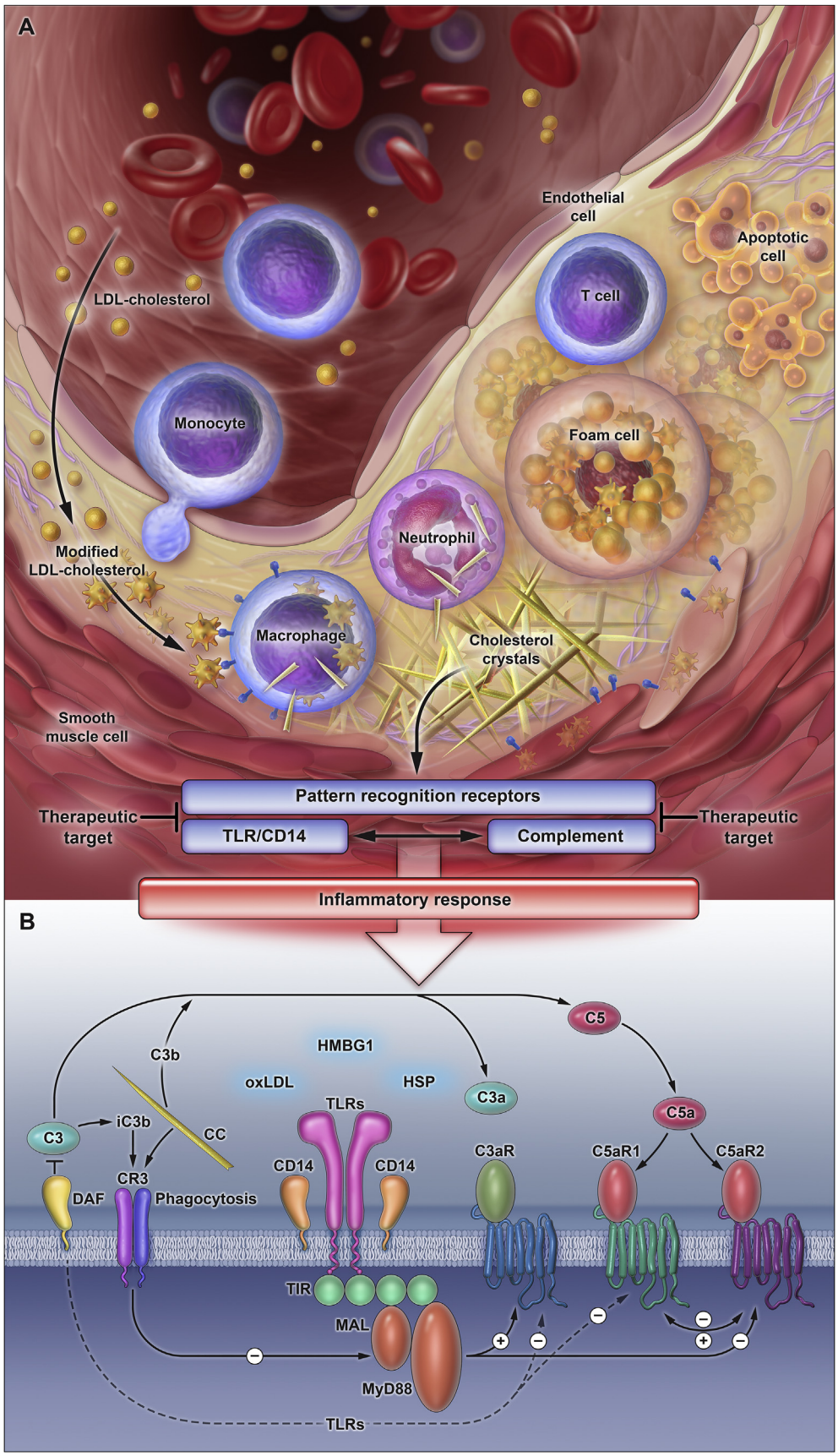

Fig. 3. Potential role for inhibition of innate immunity in atherosclerosis. The atherosclerotic plaque (panel A) is characterized by immune cells including monocytes, macrophages, granulocytes, T-cells and foam cells. LDL-cholesterol is retained in the intima where it is oxidized or otherwise enzymatically modified. There is also formation of cholesterol crystals known to activate the innate immune system. The changes in the vessel wall induce innate immune activation through pattern recognition receptors including toll-like receptors

5. Complement and TLR in experimental atherosclerosis

Much of the current knowledge in atherosclerosis has been obtained through animal experiments. Murine models have obvious advantages including reproducibility, knockout options, availability and cost. Even if mice are not men, and murine atherosclerosis is not human atherosclerosis (e.g. mice do not develop unstable coronary lesions), murine models can integrate research in lipidology and atherosclerosis as well as the immune system and provide us with important basic knowledge [118].

The complement system is important in murine models of atherosclerosis, and examples are shown in Table 1 (References are given in the table). Activation through CP may be protective consistent with the role of C1q and the classical pathway being important for tissue homeostasis by clearance of cell debris and immune complexes. AP activation, on the other hand, may be pro-atherogenic due to activation of C3 and subsequent terminal pathway activation, whereas the role of LP is less elucidated. Irrespective of the direct effect of the initial pathways, it seems that activation of C3 and beyond is detrimental to the vessel wall. Thus, both C3a and C5a have inflammatory effects in atherosclerosis, and inhibiting their respective receptors seems promising in murine models. Several of the studies referred to in Table 1 underscore the importance of CD59 in protection against atherosclerosis, supporting the hypothesis that C5b-9 formation is important in the pathogenesis, either through cell lysis or through sublytic inflammatory effects. Furthermore, complement inhibitors like CD55 and CD59 have anti-atherogenic effects in murine models through altered lipid handling and foam cell formation [119]. In rabbits, complement C6 deficiency protects against atherosclerosis in rabbits [120]. However, the demonstration that C6-deficient rabbits fed a high-cholesterol diet are protected against atherosclerosis, whereas atherosclerosis-prone ApoE−/−mice crossbred with C5-deficient mice are not protected [121], underlines the limitations of animal studies and the need for robust human studies.

Cholesterol crystals have been known for decades to activate the complement system [122]. In a human whole blood model we recently showed that the cholesterol crystal-induced cytokine response was totally dependent on complement activation [99]. A limitation of this model with respect to atherosclerosis is the lack of endothelial cells. We therefore developed a novel human whole blood model where endothelial cell activation could be studied [123]. When cholesterol crystals were incubated in whole blood on a monolayer of endothelial cells, we found that complement activation was critically important as initial event for the endothelial cell activation, and that the activation was mediated by secondary release of TNF. Despite that this is an *ex vivo* model with its limitation as such, the data support the notion that complement may play an important role in human atherosclerosis.

Several TLRs, especially TLR4 and other CD14-dependent TLRs, are central in the development of murine atherosclerosis. Some TLR-signaling pathways display anti-atherosclerotic properties, consistent with a complex inflammatory network with partially counteracting functions, and examples of studies on TLR-signaling are shown in Table 2. An example of this complexity, which also involves interaction with adaptive immunity, was the study by Subramanian et al. which showed that the suppressive effect of regulatory T cells on murine atherosclerosis was dependent on MyD88 signaling in dendritic cells [124]. Yu et al. broadened the understanding of MyD88 dependent signaling, demonstrating that the interplay between myeloid and endothelial cells in obesity associated inflammatory diseases including atherosclerosis, is MyD88 dependent [125].

Although these are animal data, and cannot be immediately extrapolated to humans, since “mice are not men” [126], they can, however, still be used to generate hypotheses for further testing in human models.

6. Complement and TLR in human atherosclerosis

6.1. Modified LDL-cholesterol and innate immune response

LDL-cholesterol is an established risk factor in atherosclerosis, and a major player in the development of the atherosclerotic plaque [127]. In the intima, the trapped LDL particles are oxidized (oxLDL) and enzymatically modified (E-LDL) facilitating uptake by the macrophages. Bhakdi et al. have demonstrated that E-LDL binds to CRP triggering complement activation in human atherosclerotic lesions [128,129]. In a human macrophage model, it was demonstrated that C1q and MBL bind to modified lipoproteins including oxLDL and enhanced macrophage uptake of these lipoproteins [130]. Furthermore, in the presence of C1q and MBL, an increased efflux of cholesterol to ATP-binding cassette transporters and HDL-cholesterol was demonstrated, indicating possible protection against early atherosclerosis.

In a human leukocyte model, modified LDL led to an increase in TLR4, as well as TLR2 and CD14, inducing an inflammatory response including tumor necrosis factor (TNF) formation [131]. Su et al., who discovered that oxLDL triggered TLR4 and TLR2, extended this finding [132]. Exploring the pathways of inflammation in cell cultures, it was found that pro-inflammatory cytokine production was induced through TLR4 signaling, also underlining the importance of the Src family kinases [133]. In a combined model including cell cultures and animal research, it was demonstrated that oxLDL-cholesterol leads to tissue factor (TF) expression via TLR4 and TLR6 signaling, hence suggesting a TLR mediated link between lipids and thrombus formation [103]. A recent study documented crosstalk between complement and TLRs in this interaction between lipids and innate immunity as oxLDL increased C3 production in human macrophages via activation of TLR4 [134].

Thus, the evidence points to a close interaction between oxLDL, E-LDL and innate immunity as demonstrated by studies in human cell cultures, possibly indicating therapeutic targets in early atherosclerosis formation.

6.2. Innate immune activation in human atherosclerotic tissues

Coronary arteries with atherosclerotic lesions differ from normal coronary arteries in that they express the anaphylatoxin receptors C3aR and C5aR1 [135]. There are two C5a receptors, C5aR1 (CD88) and C5aR2 (C5L2). Vijayan et al. noted prominent expression of C5aR2 in advanced human atherosclerotic plaques, and C5aR2 correlated with high levels of pro-inflammatory cytokines [136]. C3a and C5a have also been demonstrated in stenotic aortic valves as part of an inflammatory response [137]. Complement inhibitors have been found in stenotic aortic valves, but not in amounts sufficient to inhibit complement activation and deposition [138].

and the complement system. These systems cross-talk extensively (panel B, bottom) including both positive and negative feedback mechanisms. E.g. decay accelerating factor (DAF), a membrane inhibitor of C3 activation, cross-talks with several TLRs including TLR4, TLR2/6 and TLR9, leading to reduced responses from C3aR and C5aR1 [92]. Complement receptor 3 (CR3, CD11b/18), a main receptor for phagocytosis, inhibits TLR signaling by interfering with MyD88 [94,95]. Furthermore, TLR activation (TLR2, TLR4, TLR6 and TLR9) leads to enhanced C3aR response and to a reduced C5aR2 response [22]. The latter implies an enhanced effect of C5aR1, due to the counterbalance of C5aR1 and C5aR2 in the response to C5a.

Table 1
Selected experimental studies documenting the role of the complement system in atherosclerosis.

Author, year, ref	Animal model	Intervention	Major Endpoint	Major finding
Bhatia, 2007 [185]	Ldlr.C1qa$^{-/-}$ mice	Normal or high-fat diet	Aortic atheroma size and apoptotic cells	Increased aortic atheroma and reduced apoptotic cell clearance in Ldlr.C1qa$^{-/-}$ mice.
Lewis, 2009 [186]	C1qa.sIgM.Ldlr$^{-/-}$ mice	Low- and high-fat diet	Aortic atheroma size	Increased aortic atheroma in sIgM.Ldlr$^{-/-}$ mice, indicating IgM protection, independent of classical pathway
Matthijsen, 2009 [187]	Ldlr$^{-/-}$ mice	High-fat diet	Presence of MBL	MBL is present in early but not late atherosclerotic lesions
Orsini, 2012 [188]	Mice/rats	Cerebral artery occlusion and MBL inhibition	Presence of MBL cerebral infarct	MBL is present in ischemic areas and MBL$^{-/-}$ and MBL inhibited animals are protected
Malik, 2010 [189]	Bf.Ldlr$^{-/-}$ mice	Low-fat diet LPS	Aortic atheroma size	Aortic root atheromas were larger in Ldlr$^{-/-}$ compared to Bf.Ldlr$^{-/-}$
Shagdarsuren, 2010 [176]	ApoE$^{-/-}$ mice	Femoral artery injury and C5aR inhibition	Neo-intima formation and inflammatory cells.	C5aR blocking inhibited neo-intima formation and reduced inflammation
Manthey, 2011 [190]	ApoE$^{-/-}$ mice	CD88 antagonist	CD88 and C5L2 expression and aortic atheroma size	CD88 antagonism reduced atheroma size
Lu, 2012 [175]	Ldlr.Apob58$^{-/-}$	Immunization with peptides located at C5aR	Aortic atheroma size	Immunization reduced atheroma size
Sakuma, 2010 [191]	Daf1$^{-/-}$ mice	Femoral artery injury	Leukocyte accumulation and neo-intima thickening	Enhanced Leukocyte accumulation and neo-intima thickening in Daf1$^{-/-}$ mice
Lewis, 2011 [192]	ApoE.CD55$^{-/-}$ mice	High-fat diet	Brachiocephalic atheroma and lipid profile	ApoE.CD55$^{-/-}$ mice were protected from atherosclerosis due to better lipid profile
Wu, 2009 [119]	ApoE.mCd59 ab$^{-/-}$ mice	High-fat diet Anti-mouse C5 antibody	Aortic and coronary atherosclerosis	ApoE.mCd59 ab $^{-/-}$ had advanced atherosclerosis compared to ApoE$^{-/-}$ mice, and this response was attenuated by anti-mouse C5 antibodies.
Liu, 2014 [193]	mCd59 ab$^{+/+}$/Apoe$^{-/-}$ and mCd59 ab$^{-/-}$/Apoe$^{-/-}$ mice	CR2-Crry	Aortic atherosclerosis	Complement inhibition with CR2-Crry protected mice against atherosclerosis

Abbreviations: Ldlr: LDL-receptor. C1qa: complement factor C1qa (classical activation). sIgM: serum-immunoglobulin-M. MBL: mannose binding lectin. Bf: factor B (alternative activation). LPS: lipopolysaccharide (bacterial). ApoE: apolipoprotein E. C5aR: complement factor 5a receptor (C5aR1). CD88: C5a-receptor (C5aR1). C5L2: C5a-receptor 2 (C5aR2). Apob: apolipoprotein B. DAF1: Decay accelerating factor 1 or CD55 (blocks alternative pathway). mCd59 ab: inhibitor of membrane attack complex assembly. CR2:complement receptor 2. Crry: complement receptor 1 related gene/protein Y.

Table 2
Selected experimental studies documenting the role of TLRs in atherosclerosis.

Author, year, ref	Animal model	Intervention	Major Endpoint	Major finding
Yvan-Charvet 2008 [194]	Macrophages from Ldlr$^{-/-}$ mice transplanted with ABCG1$^{-/-}$ bone marrow	LPS challenge	Inflammatory gene expression and plaque composition	HDL induces TLR4 attenuation. ABCG1$^{-/-}$ macrophages had more inflammatory cells in the adventitia.
Liu 2010 [195]	Rat VSMC	Human recombinant CRP and TLR4 small-interfering RNA	Inflammatory response	CRP mediates pro-inflammatory actions via TLR4 signaling.
Ding 2012 [196]	Ldlr.TLR4$^{-/-}$	Diabetogenic diet	Aortic atheroma and adipose tissue inflammation	TLR4 deficiency reduced atherosclerosis without change in adipose tissue inflammation
Hayashi 2012 [197]	ApoE.TLR4$^{-/-}$mice	Infected with an oral pathogen	Aortic atheroma	TLR4 is atheroprotective in oral pathogen induced atherosclerosis
Curtiss 2012 [198]	Ldlr.TLR1$^{-/-}$ and Ldlr.TLR6$^{-/-}$ mice	High fat diet and challenged with TLR2/1 and TLR2/6 ligands	Aortic atheroma	TLR1 and-6 deficiency were neutral. TLR2/1 and 6 ligands increased atherosclerosis.
Karper 2012 [199]	ApoE3 Leiden mice	Femoral artery cuff and TLR7/9 antagonists.	TLR7/9 expression. Vascular remodeling and foam cell formation.	TLR7/9 inhibition reduced postinterventional remodeling and foam cell formation
Polykratis 2012 [200]	ApoE$^{-/-}$ mice with endothelial and myeloid cell TRAF6 deficiency	High fat diet	Aortic atheroma	TRAF6 induce pro-atherogenous changes in endothelial but not in myeloid cells in which TRAF6 signaling is anti-inflammatory and anti-atherogenous.
Koulis 2014 [201]	ApoE$^{-/-}$·TLR9$^{-/-}$	High fat diet	Aortic atheroma	TLR9 protects against atherosclerosis

Abbreviations: Ldlr: LDL-receptor. LPS: lipopolysaccharide. ABCG1 ATP-binding cassette transporter G1. HDL: High-density cholesterol. TLR: Toll-like receptor. VCMC: Vascular smooth muscle cell. CRP: C-reactive protein. RNA: Ribonucleic acid. ApoE: apolipoprotein E. TRAF6: tumor necrosis factor receptor–associated factor 6.

TLRs are also expressed in human atherosclerotic plaques, including TLR1, TLR2 and TLR4 [139–141]. The role of TLRs in the development of atherosclerosis appears to be complex. For example, expression of TLR2 on endothelial cells seems to promote atherosclerosis, whereas it has protective effects when expressed on myeloid cells [142]. Furthermore TLR3, TLR7, and TLR9 may protect against atherosclerosis in mice [143]. Thus, anaphylatoxin receptors and different types of TLRs seem to be upregulated in atherosclerotic tissues indicating a plausible connection to the inflammatory component of atherosclerosis. This should be further explored as possible therapeutical targets in atherosclerosis.

6.3. Markers of innate immunity in cardiovascular disease

Previous epidemiological studies have indicated that the complement system is associated with development of atherosclerosis and serum C3 and C4 levels have been linked to an increased risk for cardiovascular diseases [144–148]. It has also been demonstrated that an increased ratio of C3/C4 is predictive for new coronary events in a group of patients with former coronary events [149].

Hertle et al. studied persons with increased risk of atherosclerosis and found that plasma C3a was associated with an increase in carotid intima media thickness in the population as a whole, and in heavy smokers C3a was associated with overt cardiovascular disease [150]. Angiographic lumen loss detected by coronary angiography 6–8 months after percutaneous coronary intervention (PCI) with drug eluting stents (DES) was associated with higher levels of C3a and C5a at baseline [28]. Very recently a positive correlation between the anaphylatoxin receptors C3aR and C5aR and platelet activation in coronary artery disease was detected [151].

In a study of 50 patients with MI, it was shown that monocytes from MI patients compared to cells from healthy controls showed increased expression of TLR2, in particular in patients with accompanying cardiogenic shocks [152]. The monocyte expression of TLR2 was associated with circulating levels of systemic inflammation. Other TLRs were, however, not investigated [152].

Studying coronary thrombi in acute coronary syndromes, it was found that myeloid related proteins were ligands for TLR4, leading to a downstream pro-inflammatory response [153]. Consequently, TLR4 expression on monocytes was increased in patients with MI compared to controls, and in patients with MI and heart failure, TLR4 expression and corresponding pro-inflammatory cytokines were even more increased [154]. Another group found an increase in TLR4 expression on monocytes in patients with AMI, and it was demonstrated that TLR4 to a larger degree was expressed on CD14 + CD16 + cells [155]. Furthermore, in a study of 70 patients with stable angina, a significant correlation between the severity of coronary stenosis and the TLR2 and TLR4 response in monocytes was demonstrated, and two hours after PCI there was a significant decrease in these responses [156]. Thus, similar results have been obtained in both stable and unstable coronary syndromes. Finally, stimulating blood from patients with stable angina with lipopolysaccharide (a TLR4 ligand), resulted in increases in TNFα and IL-6 compared to normal controls, however the response did not reflect disease severity [157].

So, in patients with clinical coronary artery disease the complement system is activated, especially through the proinflammatory anaphylatoxins, mirroring the atherosclerotic process. The TLRs are also activated in overt coronary artery disease, mainly through TLR4, hence it would be prudent to explore this further in clinical trials.

7. Genetic studies of innate immunity in human atherosclerosis

C4A and C4B are two genes encoding complement factor 4, a protein participating in the initial activation of CP and LP. Both genes are present in most individuals, but the number of copies varies. A genetic linkage study found that a low number of C4B copies is a risk factor for short term mortality in MI patients who are smokers [158].

Patients with systemic inflammatory diseases, including rheumatoid arthritis and systemic lupus erythematosus (SLE), have increased risk for atherosclerotic diseases [159]. Interestingly, it was found that both elevated and lowered levels of MBL were associated with increased carotid intima media thickness in rheumatoid arthritis as compared to those with medium MBL levels [160], illustrating the double-edged sword of complement activity. However, MBL (MBL2) genotypes have not been predictive for coronary artery disease in statin treated patients [161]. Low MBL2 genotype, but not total serum MBL concentration, was associated with cardiovascular events in type 2 diabetics in South Asia [162]. In a large Caucasian population, MBL2 polymorphisms related to MBL deficiency were associated with increased risk for MI [163]. A similar association between MBL deficiency and arterial thrombosis has been demonstrated in SLE [164]. In patients with type 2 diabetes and MI, high levels of soluble TCC predicted future cardiovascular events, and low levels of MASP-2 at admittance predicted poorer prognosis [165]. In contrast, a recent study showing different levels of MASP-2 and other MASP molecules in cardiovascular disease compared to controls, could not document a correlation between the concentration and disease outcome [166]. Lastly, in a genetic linkage study, the C5 rs17611 GG genotype correlated with levels of circulating C5a, indicating increased risk for outcome in patients with known carotid atherosclerosis [167]. It has been reported that CH50 and small high-density lipoprotein (HDL) particles were associated with subclinical atherosclerosis in patients with SLE [168]. However, whereas CH50 can measure functional complement capacity, it is not an accurate method of measuring complement activation, making interpretation of this study difficult.

Age-related macular degeneration (AMD) is a common disease that clearly is linked to complement dysregulation [169]. AMD and atherosclerosis may share partially overlapping pathogenesis. A non-synonymous SNP (rs1061170/Y402H) in the FH gene encoding FH is robustly associated with increased risk of AMD, however, no association to cardiovascular events has been demonstrated [169]. This at least suggests, that it is not genetically determined dysregulation of the AP that is the most important factor in complement-dependent atherogenesis. In a large multicentre study with patients with familiar hypercholesterolemia, presence of the Y402H polymorphism in the FH gene was associated with a two-fold reduction in risk of cardiovascular disease [170].

No association was found between genetic variations in TLR4 or TLR2 and carotid intima media thickness in a large community population [171]. However a very recent study has documented that TLR4 is upregulated in stroke patients, and furthermore indicating that polymorphisms in the TLR4 gene promoter region influences TLR4 gene expression [172]. A genetic variation in the TLR4 gene was found to be associated with reduced risk for MI [173], and it has been reported that SNP1350 T/C in the TLR2 gene was less frequent in patients with MI and hypertension, suggesting a possible protective effect of this SNP [174].

8. Human interventional studies

As C5-inhibitors have been shown to reduce atherosclerosis in murine models indicating C5 as a possible therapeutic target [175,176], several groups have studied inhibition of cleavage of C5 into C5a and C5b, in humans. Two early trials tested pexelizumab, a precursor of the recombinant anti-C5 antibody eculizumab, in coronary artery bypass grafting (CABG) with results indicating reduced mortality [177,178]. However, a recent study, combining results from the two trials including more than 7000 CABG patients found only a non-significant 6.7% reduction in 30-days mortality [52]. Nevertheless, there was a mortality benefit for high-risk surgical patients in an explanatory analysis of the combined data [179]. In a systematic overview from 2006, Mahaffey et al. found that pexelizumab reduced 30-day mortality in patients with acute MI [180]. In 2008, Testa et al. published a larger meta-analysis including more than 15,000 patients with STEMI or undergoing

CABG [51]. There was no survival benefit for the group as a whole, but a significant reduction in mortality in the CABG group. Thus, C5 inhibitors have so far yielded disappointing results when used in patients with acute coronary events. However, it should be emphasized that pexelizumab was administered after some delay in most of the trials, and a recent study documented that the effect of C5 inhibition was not satisfactory since an increase in soluble TCC was found prior to infusion of the drug, possibly explaining the lack of effect [181].

Notably, no long-time effects of complement inhibition on development of atherosclerosis have been reported. Genetic complement deficient patients, except for MBL, are so scarce that clinical intervention studies in these patients have been impossible. Furthermore, no clinical interventional studies targeting TLRs have been performed. However, in a study on murine and human monocytes, inhibition of TLR2 and CD14 (co-receptor of TLR4) by a small oxidized phospholipid leads to reduction of downstream inflammatory cytokines [182]. The same small molecule fed to rabbits reduced atherosclerosis [182]. Thus, it remains to be shown whether TLR-targeted strategies – either alone or combined with complement inhibitors - will prevent human atherosclerosis.

9. Concluding remarks and future perspectives

There is abundant evidence that the innate immune system, including the complement system, the TLRs and the inflammasome, is an essential player in inflammation in general, whether this is induced by exogenous PAMPs like in infection, or by endogenous DAMPs like in sterile inflammation [183], the latter of particular importance for atherosclerosis. We have put forward a hypothesis that combined inhibition of key “bottle necks” molecules in the complement system (e.g. C3 or C5) and in the TLR-family (co-receptor CD14), acting early in the recognition phase, might be a novel general therapeutic regimen in various inflammatory disease conditions whether induced by PAMPS or DAMPS [184]. Animal studies using the combined inhibitory approach in experimental atherosclerosis are still missing and highly warranted, as are studies with refined human models. Thus, future studies are needed to test the hypothesis that upstream modulation of complement and TLRs might be a rationale for treatment of atherosclerosis in humans (Fig. 3).

Acknowledgments

We thank Scott Leighton, CMI, Medicus Media for creating the illustrations.

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