Inflammation and coagulation in atherosclerosis

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Summary
Cardiovascular diseases remain to be the leading cause of death in Western societies. Despite major findings in vascular biology that lead to a better understanding of the pathomechanisms involved in atherosclerosis, treatment of the disease has only changed slightly within the last years. A big body of evidence suggests that atherosclerosis is a chronic inflammatory disease of the vessel wall. Accumulation and peroxidation of LDL-particles within the vessel wall trigger a strong inflammatory response, causing macrophage and T-cell accumulation within the vessel wall. Additionally, B-cells and specific antibodies against LDL-particles, as well as the complement system are implicated in atherogenesis. Besides data from clinical trials and autopsy studies it was the implementation of mouse models of atherosclerosis and the emerging field of direct gene modification allowed for thorough introduction of suitable animal models of atherosclerosis in mice and men complicated the translation of experimental data into clinical practice. Despite these limitations, new anti-inflammatory medical therapies in cardiovascular disease are currently being tested in clinical trials.

Keywords
Inflammation, atherosclerosis, CRP

Entzündung und Gerinnung in der Atherosklerose
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Zusammenfassung

The most dangerous complication of coronary atherosclerosis is the rupture of such a plaque, exposing the highly pro-coagulatory plaque content to the blood stream that may cause thrombotic occlusion of the coronary vessel, leading to acute myocardial infarction or sudden cardiac death.

Our understanding of the underlying pathophysiological mechanisms of atherosclerosis evolved substantially within the last decades. For much of the 20th century, atherosclerosis was considered to be a mere cholesterol storage disease, caused by elevated levels of circulating lipids passively accumulating within the vessel wall (3). Later, the emerging field of vascular biology shifted the focus on smooth muscle cell migration and proliferation, a process triggered by endothelial damage (4, 5). In the early nineteenth, first evidence for a participation of the immune system was generated by detecting immune cells and immune mediators within the vessel wall. The introduction of suitable animal models of atherosclerosis and the ability of selective gene modification allowed for thorough investigation of the pathophysiological mechanisms involved in the disease and created overwhelming evidence for a participation of the immune system. The con-
cept of atherosclerosis as a chronic inflammatory disease of the vessel wall was born (6–10). Today, it is believed that the immune system constitutes the key regulator between risk factors and vessel wall alterations.

Despite strong evidence arising from preclinical work, we currently have no tailored atherosclerosis therapy exhibiting anti-inflammatory effects.

Serious limitations of the established mouse models have to be considered when trying to extrapolate experimental data to humans. Despite major disparities in atherosclerotic disease of mice and men, such as differences in general immune mechanisms, location of the disease and lack of important clinical manifestations in the mouse model, first immunomodulating experimental attempts are being carried out today.

Nearly more than two decades after first evidence accumulated for participation of the immune system in atherosclerosis, two large randomized controlled clinical trials investigating anti-inflammatory agents for its cardiovascular effects, are in the recruiting phase (11, 12). Therefore, the aim of this review is to summarize the role of the immune system in initiation, progression and complication of atherosclerosis and to give an outlook on possible future therapeutic options.

Overview

The association of elevated cholesterol levels with cardiovascular disease was established decades ago (3). Low-density lipoprotein (LDL), a lipoprotein containing an apolipoprotein B100 molecule is a transporter of cholesterol in the blood. LDL particles may migrate into the intima, the innermost layer of the artery, where they are bound to proteoglycans and „trapped” within the vessel wall making them prone to oxidative modification (13). Peroxidation of fatty acid residues generates oxidatively modified LDL, so-called oxLDL, triggering a strong inflammatory activation of the surrounding tissue (14). Activation of the endothelium induces the upregulation of adhesion molecules, such as selectins, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (15, 16). Together with the production of chemokines, such as monocyte chemoattractant protein-1 (MCP-1), these mechanisms induce attraction, adhesion and migration of monocytes, T-lymphocytes and dendritic cells (DC) into the intima.

Within the intima, under the influence of macrophage colony stimulating factor (M-CSF), monocytes become macrophages, express scavenger receptors (SR) and start taking up oxLDL. The lipid accumulation within the macrophages give them a „foamy” appearance under the microscope, thus they were termed „foam cells” (9, 17). The continued migration of fat and immune cells into the vessel wall leads to the formation of the first atherosclerotic plaques, the so-called „fatty streaks”. Due to migration of smooth muscle cells (SMC) into the intima and the production of collagen, a fibrous cap that protects the pro-thrombotic plaque content from the blood is formed. The growing plaque stands at the crossroads between increased inflammation, leading to further destabilization with the possibility of plaque rupture or increased fibrosis and stabilization. Variable mechanisms such as reduced synthesis and increased degradation of collagen mediated by T-cells and macrophages cause cap thinning. Subsequently the cap may rupture triggering thrombosis and vessel occlusion (18, 19). On the other hand, stable, but growing plaques, might induce intermittent ischaemic pain caused by blood flow limitation (20).

Innate immunity

Lipid accumulation and oxidative modification within the vessel wall poses a strong inflammatory trigger for innate immune reactions. Other potential activators include angiotensin II, circulating inflammatory cytokines, hyperglycaemia and smoking. Once activated, endothelial cells express adhesion molecules and further produce cytokines, causing leukocyte adhesion, migration and accumulation within the intima, particularly at branching points in the coronary vascular tree that are characterized by turbulent blood flow (16, 21, 22).

Activation of the innate immune system is dependent on ligation of so-called pattern recognition receptors (PRR), a primitive and highly conserved part of the immune system necessary for pathogen recognition. Leukocytes expressing PRRs recognize conserved characteristic patterns on the surface of pathogens and „dangerous” molecules generated after tissue damage. The two major classes of PRRs are endocytosolic scavenger receptors (SR), necessary for pathogen uptake and antigen presentation, and the family of toll-like receptors (TLR) that, once activated, induce a strong inflammatory response. Within the growing plaque, besides cell debris, oxidized lipids are recognized and absorbed via scavenger receptors on activated macrophages. Scavenger receptors that recognize oxLDL include SRA-1, SRA-2, MARCO, CD36, SR-B1, LOX-1 and PSOX (23). Despite a clear role for subintimal cholesterol accumulation and foam cell formation, gene deletion experiments in mouse models generated conflicting results. A possible atheroprotective mechanism of SR might be the orchestration of cholesterol efflux from the lesion (24). Knock-out studies identified TLR-1, 2, 4 and 6 as potential pro-atherogenic mediators, while TLR-3 and TLR-7 might exhibit atheroprotective effects (25–27). Deletion of MyD88, an important adaptor protein for most TLRs resulted in the largest reduction of atherosclerotic plaques (28).

Potential antigens activating TLRs within the plaque include the endogenous stress molecule heat shock protein 60 (HSP60) and oxLDL. TLR-ligation activates the nuclear factor KB (NFkB) and the mitogen-activated protein kinase (MAPK) pathways, triggering the production of pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α) and IL-1β as well as matrix metalloproteinases, a group of peptidases critically involved in plaque destabilization (29, 30).

Very recently, the inflammasome, a macromolecular aggregation of intracellular PRRs that induces IL-1β production in macrophages via caspase-1, was implicated
Monocyte subsets can be distinguished via their surface expression of CD14 and CD16 into three main subtypes, namely "classical monocytes" (CD14++CD16–), "intermediate monocytes" (CD14++CD16+) and "non-classical monocytes" (CD14+CD16++), each exhibiting a distinct array of surface molecules used for extravasation (53). Current evidence suggests a developmental relationship between monocyte subsets. However, knowledge on the developmental fate of distinct monocyte subtypes once they become resident within the tissue is missing. Classical monocytes act as anti-microbial cells due to their strong phagocytosis capacity, while intermediate and non-classical monocytes represent pro-inflammatory cells, evidenced by their enhanced production of inflammatory cytokines. Furthermore, intermediate subsets were implicated in angiogenesis, while non-classical monocytes show a "patrolling" behaviour at the vessel wall. Within the vessel wall, monocytes may differentiate into dendritic cells, professional antigen presenting cells, or distinct macrophage phenotypes, such as M1 or M2 macrophages, as well as foam cells, lipid-laden macrophages representing the cellular hallmark of early atherosclerotic lesions. Similar to monocytes, macrophages exhibit strong diversity and plasticity, dependent on their environment. M1 macrophages exhibit a more pro-inflammatory phenotype and thus might be associated with plaque destabilization, while M2 macrophages express anti-inflammatory mediators, probably stabilizing the growing lesion. However, M2 macrophages further promote angiogenesis that is usually implicated in plaque destabilization.

CD: cluster of differentiation; IL: interleukin; MCP-1: monocyte chemoattractant protein-1; TLR: toll like receptor; MHC II: major histocompatibility complex II; IL-1RA: IL-1 receptor antagonist; TGF-β: transforming growth factor β; MMP: matrix metalloproteinase; TNF-α: tumor necrosis factor α; ROS: reactive oxygen species
in atherogenesis. Accumulating cholesterol often crystallizes within the plaque as the plaque advances to a more mature phenotype. Two papers published recently demonstrated that cholesterol crystals activate the inflammasome NLRP3 thereby triggering IL-1β production (31, 32). Additionally, bone-marrow reconstitution in LDL-R⁻/⁻ mice on western diet from mice deficient in NLRP3 significantly attenuated atherosclerosis extent. As IL-1β is a major proatherosclerotic factor, targeting the interplay of cholesterol crystals and the inflammasome may pose an elegant new therapeutic approach for potential immune-modulating cardiovascular therapies (33).

The complement system

The complement system constitutes an important part of the innate immune system, playing an important role in many inflammatory diseases. It is therefore not surprising that it has been implicated in atherogenesis (34, 35). Besides its well-known pro-inflammatory effects, the complement system is also involved in removal of debris and apoptotic cells. Thus, complement activation via the alternative pathway triggering the formation of the anaphylatoxins C3a and C5a and the terminal complement complex (TCC) exhibits pro-atherogenic effects, while activation via the classical and the lectin pathway has anti-atherosclerotic effects by facilitating debris removal.

Complement components are detectable in the atherosclerotic vessel wall, whereas the healthy vessel is free of complement factors. In rabbits on a high-fat diet, the formation of early fatty streaks with lipid accumulation was accompanied by complement activation in the vessel wall even before leukocytes started to migrate into the newly formed lesions (36). The activated complement component C5a was hardly detectable in stable plaques, while in unstable lesions it co-localized with MMP-1 and MMP-9 situated around cholesterol clefts (37). Furthermore, C5a was able to induce proinflammatory cytokines as well as MMP-1 and MMP-9 production in plaque macrophages (38). An autopsy study revealed a much higher C3b concentration in ruptured plaques when compared to stable plaques (39). These results, together with evidence for increased complement activation in plasma obtained from the site of thrombus formation in MI-patients (40), suggest an important role of C3a and C5a in matrix degradation leading to plaque rupture.

Gene-modification in atherosclerosis mouse models further established the dual role of complement activation in atherogenesis, as reviewed elsewhere (34). Additionally, both treatment with anti-C5a-antibodies and direct blocking of the C5a-receptor attenuated atherosclerosis extent and induced a more stable plaque phenotype in animal models, thus laying the foundation for a possible treatment involving the complement system (41, 42).

In patients, changes in circulating levels of complement components were implicated in all stages of atherogenesis. Patients with detectable atherosclerotic lesions exhibited elevated levels of C3a and C4a, while plasma levels of C3a, C5a and the TCC were predictive of cardiovascular events in different patient cohorts (43–46). Furthermore, serum levels of C5a predicted restenosis after femoral balloon angioplasty and C3a and C5a were predictive of late lumen loss after treatment with drug eluting stents in stable CAD patients (47, 48).

Monocyte and macrophage heterogeneity

Monocytes and monocyte-derived macrophages play a crucial role in every phase of atherogenesis, from initiation and progression to destabilization of the lesion. In the late 1980s it was shown that monocytes comprise a heterogeneous cell population that can be distinguished according to their CD14 and CD16 surface expression into a CD14⁺CD16⁻ and a CD14⁻CD16⁺ phenotype (49). Soon after the first evidence of monocyte heterogeneity, CD16-positive monocytes were considered to be a pro-inflammatory subset of monocytes, as they exhibited an increased production of inflammatory cytokines such as TNF-α upon stimulation and were shown to be elevated in numerous inflammatory diseases (50–52). However, strong evidence accumulating over the past 20 years suggested the further division of CD16-positive monocytes into two distinct populations. This was realized in a consensus document on leukocyte nomenclature published in 2010 suggesting to classify monocytes (53) as

- "classical monocytes" (CM; CD14++CD16⁻);
- "intermediate monocytes" (IM; CD14++CD16⁺) and
- "non-classical monocytes" (NCM; CD14⁺CD16⁻⁺).

The concept of a subset-specific role in atherogenesis is mainly derived from animal models. In mice, surface expression of Ly6C, an epitope of Gr-1, is used to distinguish monocyte subsets (54–56). In steady state conditions, an equal distribution of Ly6C⁺ high and Ly6C⁺ low monocytes can be seen. However, hypercholesterolemia in mouse models of atherosclerosis induces monocytosis that is mainly driven by Ly6C⁺ high monocytes (57). This led to the assumption of Ly6C⁺ high monocytes to exhibit a rather "pro-inflammatory" phenotype and were further implicated to play a crucial role in atherogenesis (58). However, differences in subset distribution, surface markers and functional aspects complicate translation from animal data to human disease. Therefore and to keep a clinical focus in this review we will concentrate on monocyte and macrophage heterogeneity in humans from this point forward.

In adipose patients, the percentage of NCM was strongly increased and correlated with fat mass and fasting glucose (59). Additionally, the NCM-proportion decreased together with patient’s weight and intima-media thickness. The correlation between BMI and NCM was further confirmed in a larger study including 500 individuals (60). Several papers demonstrated a negative correlation between NCM and HDL-cholesterol (61), while total cholesterol, LDL-cholesterol and triglycerides showed a positive correlation with non-classical monocytes (62). However, when corrected for BMI-levels, the described correlations lost their statistical significance.
Several cross-sectional studies suggested elevated levels of CD16+ monocytes in patients suffering from atherosclerosis. In a cohort of 500 apparently healthy individuals, the proportion of CD16+ monocytes correlated with intima-media thickness (60). In a very heterogeneous CAD-population including both stable CAD patients and patients after AMI, the CD16+ monocyte proportion was increased when compared to healthy controls (63). Additionally, CD16+ cells were correlated with plaque instability and were inversely correlated with plaque stabilization (64, 65).

Further studies evaluated monocyte subset distribution as a possible prognostic marker in cardiovascular disease. In several smaller studies, intermediate monocytes were shown to be predictive for adverse outcome in patients with chronic kidney disease (CKD) (66, 67). The largest study so far evaluating monocyte subset distribution for its prognostic abilities demonstrated that IM are predictive for cardiovascular events in more than 900 patients undergoing elective coronary angiography (68).

Similar to monocytes, also macrophages exhibit heterogeneity and plasticity. In vitro treatment with IFN-γ and LPS skew macrophages to the M1 phenotype characterized by increased secretion of pro-inflammatory cytokines, ROS, tissue factor and metalloproteinases and is implicated with plaque destabilization and rupture. On the other hand, M2 macrophages defined by a high expression of CD206, IL-10 and arginase-1 arise via in vitro stimulation with IL-4, IL-10 or IL-13 and exhibit anti-inflammatory and tissue regenerating effects, thus implicated in plaque stabilization. Therefore, modulation of macrophage phenotype could be a promising approach for plaque stabilization in future (69, 70).

Adaptive immunity

Strong evidence from both clinical and preclinical research suggests a crucial role for adaptive immunity in atherogenesis. The detection of circulating antibodies against oxidation-specific epitopes (OSE) on oxLDL and the detection of T-cells within atherosclerotic plaques were first hints on the involvement of adaptive immune responses in atherosclerosis (30).

CD4-positive T-helper (Th)-cells show a vast heterogeneity and plasticity depending on their microenvironment. Th-1 cells act as pro-atherogenic cells, as they produce increased levels of IFN-γ, a cytokine implicated in reducing collagen synthesis while increasing expression of proteases, chemokines and cytokines, thus resulting in further plaque destabilization. Knockout of IFN-γ reduced plaque extent in mice, while treatment with IFN-γ augmented the magnitude of the disease (74, 75). Two other typical Th-1 cytokines, IL-12 and IL-18, accentuate atherosclerosis in rodents as well (76, 77). However, conflicting data exists for the role of Th2-cells, as some of their signature cytokines such as IL-4 were shown to exhibit both pro- and anti-atherosclerotic effects (78, 79), while IL-13 was shown to attenuate atherosclerosis (80). Additionally, also data for the role of IL-33 in atherosclerosis, a cytokine that is implicated in Th-2 polarization, is conflicting (81, 82). Likewise, the role of IL-17-producing Th-cells, the Th17-cells, is unclear, as both pro-atherosclerotic, neutral and anti-atherosclerotic effects were shown (83, 84). On the other hand, regulatory T-cells (Tregs) were shown to exhibit atheroprotective effects. They produce the anti-inflammatory cytokines IL-10 and transforming growth factor-γ (TGF-γ), a counterpart to the pro-inflammatory IFN-γ deriving from Th-1 cells, therefore causing plaque stabilization (85).

B-cells are primarily found in lymphoid tissue of the adventitia of atherosclerotic vessels. Initially they were considered atheroprotective as they represent the source of oxLDL specific antibodies. Additionally, splenectomy in ApoE−/− was shown to aggravate atherosclerosis, while B-cell transfer exhibited atheroprotective effects in these mice.69 However, the fact that treatment with a B-cell depleting anti-CD20-antibody was shown to be atheroprotective revealed a more complex picture of B-cell involvement in atherogenesis and induced the investigation of B-cell heterogeneity (87).

B1 cells are the main source of so-called „natural antibodies”, IgM antibodies produced without previous infection, vaccination or other form of exposure, based on natural selection. Remarkably, more than one third of all natural antibodies recognize and react with OSE on oxLDL (88, 89). Many of these antibodies resemble antibodies directed against common pathogens. Indeed, immunization against S. pneumoniae induced oxLDL-reactive IgM antibodies thus attenuating atherosclerosis in a mouse model (90). Mice that lost their ability to produce IgM due to genetic deletion showed a strongly aggravated disease (91). Recently, an equivalent to the murine B1-cells was found in men, the CD20+CD27+CD43+ B-cells that have the ability to produce natural IgMs that were shown to inversely correlate with cardiovascular events (92, 93). It should be noted...
that several monoclonal antibodies targeting CD20 are currently approved and being used in a variety of diseases.

Conversely, B2-cells, represent the “conventional” B-cells responsible for IgG-production after contact with T-cells. IgG against OSE can be detected both within plaques and in the circulation of patients with atherosclerotic disease (30). Genetic deletion of the B-cell activating factor-receptor (BAFF-R) selectively reduces the amount of B2-cells without affecting B1-cells, thus attenuating atherosclerosis in mice (94).

Clonal T-cell expansion within atherosclerotic lesions and co-localization of T-cells with macrophages and dendritic cells suggest a local immune activation within the plaque (95). Besides bacterial and viral antigens, possible antigens include autoantigens such as the stress molecule HSP60 and native or oxidatively modified LDL. HSP60 is highly conserved and abundantly expressed under stress triggering a strong innate and adaptive immune response (29). Interestingly, parenteral immunization with HSP-60 aggravated atherosclerotic disease while oral tolerization was shown to be atheroprotective (96, 97).

LDL and especially oxLDL may represent another potential antigen, as IgM and IgG titers against oxLDL are elevated in patients with atherosclerosis. Furthermore, T-cells isolated from human atheromas were reactive against oxLDL and transfer of LDL-reactive T-cells aggravated atherosclerosis in mice. As expected, immunization against oxLDL showed atheroprotective effects, while, surprisingly, also immunization against native LDL and native ApoB100 attenuated atherosclerosis (98–101). As native LDL comprises an important plasma component, immunologic tolerance against it is essential for survival. It was therefore thought that by eliminating LDL-reactive T-cell clones, central tolerance against LDL is established and that the later oxidation of LDL would create neoantigens recognized by cells that were not removed during development of the thymus. The fact that central tolerance against LDL is by far not perfect brings peripheral tolerance into play. Recent evidence points to regulatory T-cells as an important player in achieving peripheral tolerance against LDL (102, 103).

### Fig. 2: Heterogeneity in innate immune mechanisms implicated in atherogenesis

Within the growing lesion, dendritic cells take up antigens such as LDL, HSP-60 and oxidatively modified LDL and apoB100 particles and subsequently migrate from the intima into secondary or tertiary lymphoid tissue where they present peptides of these antigens to naive T-cells via MHC-receptors, inducing a clonal expansion and differentiation into effector T-cells. These cells leave the tissue into the circulation where they extravasate into atherosclerotic lesions and get reactivated by macrophages and DCs expressing the same antigens, inducing a strong pro-inflammatory answer by both the effector T-cells and resident macrophages. Recently, B-cell heterogeneity was implicated in atherogenesis. Activated effector T-cells such as Th1-cells trigger production and release of IgG by classical B-cells, the B2-subtype, that was implicated in atherosclerosis promotion. On the contrary, B1-cells produce and release natural antibodies of the IgM type that were shown to be atheroprotective, a mechanism dependent on IL-5 secreted by Th2-cells. HSP-60: heat shock protein-60; Treg: regulatory T-cells; Th: T-helper cell; TCR: T-cell receptor; LDL: low density lipoprotein; IL: interleukin; MHC II: major histocompatibility complex II.
The beneficial effects seen in immunization experiments were shown to be dependent not only on antibody generation but also on the generation of oxLDL or ApoB100-specific Tregs. Recently, a T-cell receptor reactive to ApoB100 was identified and cloned, demonstrating for the first time that naturally occurring autoimmunity against LDL does exist (104). The existence of LDL-specific T-cell receptors furthermore implies the existence of LDL-specific Tregs, probably responsible for peripheral tolerance against LDL. Immuno-modulation with ApoB100 immunization might represent an interesting and promising therapeutic approach.

Currently, many study groups throughout the world try to translate these experimental findings into clinical practice. One interesting target might be malondialdehyde-(MDA)-modified ApoB100 p45 peptide, as immunization with this peptide triggered the generation of reactive antibodies of the IgG class. Isolation, recombinant production and application attenuated atherosclerosis in a mouse model, inducing oxLDL removal and interfering with pro-inflammatory effects against oxLDL (105–107). These antibodies are currently being tested in the GLACIER-trial, a phase II proof-of-activity study that has already been completed (according to www.clinicaltrials.gov, NCT01258907, as of June 1st, 2013) (101).

**Impact of the coagulation system**

Various mechanisms, including inflammatory activation of macrophages, increased apoptosis within the plaque and thinning of the cap, may destabilize the lesion rendering it prone to rupture. Plaque rupture exposes collagen and tissue factor to the blood triggering platelet and coagulation activation resulting in athrothrombosis. The important role of platelets in atherothrombotic disease has become evident from many studies investigating platelet inhibition. However, the impact of coagulation components and the crosstalk of coagulation and inflammation in this disease is less well established (108).

The coagulation system and platelets promote each other during thrombus formation, as platelets supply the coagulation system with cofactors, while thrombin further promotes platelet activation and aggregation via activation of proteinase-activated receptor 1 (PAR-1) and PAR-4 on platelets (109). The immune system, in particular leukocytes such as monocytes and neutrophils, actively participates in thrombus formation at many stages. During arterial thrombosis, activated platelets of the growing clot release chemokines and express adhesion molecules such as P-selectin, causing subsequent recruitment of monocytes and neutrophils into the growing thrombus. Activated platelets form aggregates with monocytes that express the ligand for P-selectin, namely P-selectin glycoprotein ligand-1 (PSGL-1). These monocyte-platelet aggregates (MPA) were shown to be elevated in patients with unstable angina, acute myocardial infarction and after coronary intervention in stable patients. Interestingly, abrogation of platelet-leukocyte adhesion blocked leukocyte accumulation within the thrombus and inhibited the deposition of fibrin within the thrombus (110–113).

The classical notion of TF as a mere subendothelial protein has been challenged by the detection of TF-expressing monocytes within the blood stream. This cell-surface bound TF is silenced under physiologic conditions but may be activated during inflammation and coagulation. Intravascular TF and TF within atherosclerotic plaques may represent the main trigger of atherothrombosis following plaque rupture. Monocytes may further enhance coagulation by the release of TF-expressing microparticles. Microparticles are small, cell-derived membrane vesicles, that are shed from the plasma membrane of activated and apoptotic cells. Monocyte-derived microparticles were shown to express TF, phosphatidyserine that may act as an important co-factor for coagulation and PSGL-1, suggesting a potent, pro-coagulatory phenotype of these microparticles (114).

Important hints for a participation of the coagulation system also in stable atherogenesis were first derived from plaque analysis showing the presence of almost all coagulation proteins and factors within lesions. Data from animal models investigating the effects of hypercoagulability on atherosclerotic disease revealed aggravated atherogenesis in different hypercoagulable genotypes such as factor V Leiden mutation and protein C deficiency (111). However, in men the association of inherited hypercoagulopathy and atherosclerosis is rather moderate and true only for rather young patients. A large meta-analysis including approximately 150 000 factor V Leiden mutation carriers, revealed a relative risk for coronary disease of 1.17 and 1.31 for two different mutations, respectively (115). Furthermore, data for the association of hypocoagulability and atherogenesis is less well established. Hae-mophilia was traditionally thought to exhibit atheroprotective effects, however, a recently published meta-analysis could not confirm this assumption (116).

Thrombin is a serine protease converting soluble fibrinogen into fibrin, a protein necessary for thrombus formation. Besides its key role in clotting, thrombin exhibits pro-inflammatory effects by activating PAR-receptors that are expressed by cells throughout the atherosclerotic lesion. Thrombin generation may pose an important contributing factor to early atherogenesis, as it was shown to exhibit pro-atherosclerotic effects such as inflammation, endothelial dysfunction, monocyte activation and increased apoptosis (112, 117, 118). In an autopsy study it was demonstrated that early atherosclerotic plaques exhibit an enhanced pro-coagulatory phenotype compared to advanced atherosclerotic lesions (119). Direct thrombin inhibition with me-lagatran and dabigatran reduced plaque formation, promoted plaque stability and improved endothelial function in ApoE−/− mice (120, 121). Despite major advances in the treatment of acute coronary syndromes (ACS) including timely reperfusion and dual-antiplatelet therapy, patients remain at increased risk for a recurrent cardiovascular event after ACS (122). Increased circulating levels of thrombin after an event were related to this increased risk (123). Preclinical evidence for beneficial effects of PAR-inhibition (124) led to the conduction of the TRACER-study, evaluating the thrombin-receptor antagonist vora-
paxar in patients after ACS without ST-elevation. However, the study was terminated early based on the recommendation of the data safety monitoring board, as vorapaxar treatment increased bleeding risk (125). In another secondary prevention trial including more than 25,000 patients with a history of cardiovascular disease, vorapaxar reduced the risk of cardiovascular events, but significantly increased the risk of bleeding (126).

In recent years, anticoagulation therapy in CAD is enjoying a renaissance. A meta-analysis evaluating the effects of warfarin treatment on top of aspirin treatment in approximately 6000 patients after ACS revealed beneficial effects for warfarin treatment (127). The ESTEEM trial randomizing patients after MI to placebo or ximelagatran, an oral direct thrombin-inhibitor, showed a reduced event rate for patients in the ximelagatran arms (128). However, ximelagatran was pulled off the market due to hepatotoxic effects. The results of the recently published ATLAS-ACS2-TIMI51 study showed a decreased event rate in patients after ACS treated with rivaroxaban, an oral factor Xa-inhibitor, on top of standard of care (129). The subsequent approval of rivaroxaban for secondary prevention after ACS in combination with standard antiplatelet therapy in Europe may represent a new treatment strategy in patients after ACS.

**Limitations of preclinical models**

Our knowledge on the role of immune processes in every step of atherogenesis is overwhelming. Still, after twenty years of investigating its role in the development of atherosclerosis, there is still no cardiovascular therapy available that directly and effectively targets the immune system in this setting. It has to be noted, that our knowledge on inflammation in atherosclerosis is largely derived from animal models. As described above, the introduction of animal models and the ability of selectively modifying genes made a thorough investigation of every step of atherosclerosis possible. However, serious limitations of animal models have to be considered when trying to translate findings from animal models to patients (8). In recent years, the predominantly used species in atherosclerosis research is the mouse. All mouse models including the most frequently used ApoE−/− and the LDL-R−/− mouse require severe hyperlipidemia to generate lesions, in this case achieved by a combination of genetic manipulation and diet, resulting in severely elevated cholesterol levels usually not seen in humans with atherosclerosis (130–132). The mouse model is probably best suitable to study initiation and progression of atherosclerosis. However, mice practically never experience plaque rupture, a condition that often subsequently triggers thrombus formation and myocardial infarction in patients. The endpoint in mice experiments is often defined as plaque extent in large proximal arteries, such as the proximal aorta. However, life-threatening complications in patients usually occur in small, muscular arteries such as coronary, carotid or cerebral arteries. These vessels differ significantly from elastic arteries in structure, haemodynamics and even embryonic origin of smooth muscle cells. Additionally, the murine immune system differs in important aspects from the human immune system (133). However, if one is aware of the limitations of atherosclerosis mouse models, they represent important means to elucidate the mechanisms in the pathophysiology of the disease.

**The translation from bench to bedside**

After establishing the involvement of inflammatory processes in atherogenesis in preclinical research, we now witness the challenge to translate these findings into daily clinical work. The implementation of inflammatory biomarkers for risk prediction and as a tool in clinical decision making might be a first and important step. Besides practicability, low costs and reproducibility, a potential novel biomarker needs to be predictive of cardiovascular events independent of other risk markers and secondly, a positive test result should result in therapy that otherwise would not have been initiated. Amongst many, high sensitive C-reactive protein (hsCRP) stands out and might fulfill these criteria. CRP has a long half-life and a high stability without diurnal variation. Plasma levels of CRP were shown to be predictive of future events in apparently healthy patients (134), in patients with stable CAD (135) and after a coronary event had occurred (136). When added to traditional risk factors, the measurement of CRP significantly improved cardiovascular risk prediction (137). Furthermore, patients with elevated CRP levels exhibit an impaired fibrinolytic capacity, possibly an important contributor to increased event rate (138).

Important work from the Emerging Risk Factors Collaboration suggests that hsCRP and total cholesterol (TC) are equal in predicting cardiovascular risk (139). In this meta-analysis, data from 166 596 disease-free patients from 38 prospective studies was included. In a model for cardiovascular risk prediction including age, smoking, systolic blood pressure and diabetes, the addition of TC changed the C-statistic by 0.0043, further addition of HDL-cholesterol (HDL-C) improved the C-statistic by additional 0.0050. Interestingly, the magnitude of change for addition of hsCRP levels on top of TC and HDL-C was 0.0039, thus fully comparable to TC and HDL-C. Furthermore, the multivariable adjusted hazard ratio for increase in 1 standard deviation was 1.20 for hsCRP and 1.17 for TC (140).

This strong epidemiological data led to the investigation of hsCRP as a potential marker of therapy monitoring and clinical decision making. A post-hoc analysis of the AFCAPS/TexCAPS study, a primary prevention study, demonstrated that patients with low levels of LDL-C but elevated levels of hsCRP benefited from therapy with lovastatin, whereas patients with both low hsCRP and LDL-C did not experience a benefit (141). In a pre-specified subgroup of the PROVE-IT TIMI 22 study, a secondary prevention trial randomizing patients to placebo or statin therapy, patients reaching LDL-levels below 70 mg/dl and hsCRP levels below 2 mg/l experienced the largest benefit (142). These findings were confirmed in a post-hoc analysis of the A to Z-study (143). The arising hypothesis of statins exhibiting beneficial effects in patients with below-threshold cholesterol lev-
els but slightly elevated levels of hsCRP was than prospectively tested in the JUPITER study. More than 17,000 patients free from cardiovascular disease with LDL levels below 130 mg/dl but hsCRP levels of above 2 mg/dl were randomly assigned to 20 mg rosuvastatin or placebo (144). The primary endpoint was defined as a composite of myocardial infarction, stroke, arterial revascularization, hospitalization for unstable angina and cardiovascular death. Upon request of the data safety monitoring board the study was stopped early due to an overwhelming relative risk reduction of 44% considering the primary endpoint. Upon further analysis, rosuvastatin treatment reduced the occurrence of myocardial infarction by 54%, stroke incidence was reduced by 48%, revascularizations by 46% and total mortality by 20%.

It has to be noted, that despite the impressive results of the above described JUPITER study, we cannot draw a formal conclusion on the mechanism of action. To directly test the inflammatory hypothesis in a clinical setting, we need to test substances that exhibit mere anti-inflammatory effects without impacting other risk factors and components involved in atherothrombosis, such as cholesterol levels or platelet aggregation. Currently, two large, randomized, placebo-controlled clinical trials trying to answer this question are in the recruiting phase, the "Cardiovascular Inflammation Reduction Trial (CIRT)" and the "Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS)" (11, 12). In the CIRT-study, 7,000 stable CAD-patients after myocardial infarction with persistent elevations of hsCRP despite optimal medical therapy including high-dose statin therapy will be included. Patients will be randomized in a 1:1 manner to placebo or low-dose methotrexate (10 mg/week) therapy and followed for three to four years. Primary endpoint was defined as a composite of non-fatal MI, non-fatal stroke and cardiovascular death. Low-dose methotrexate exhibits an acceptable risk profile and is currently used in rheumatoid arthritis, a disease bearing resemblance to CAD. It was shown to be associated with reduced cardiovascular mortality and event rate in these patients in observational studies. The study design of CANTOS strongly resembles the CIRT design. Approximately 17,000 stable CAD patients post MI with persistent hsCRP elevation despite optimal medical therapy including a high dose statin should be included and randomized to therapy with placebo or canakinumab in one of three dosages once every three months. Canakinumab is a monoclonal antibody directed against IL-1β. The composite endpoint includes non-fatal MI or stroke and cardiovascular death. In a recently published phase IIb study in diabetic patients with elevated cardiovascular risk, canakinumab dose-dependently reduced circulating levels of IL-6 and fibrinogen without influencing HbA1c, glucose, HDL or LDL (145). These findings confirm canakinumab as a mere anti-inflammatory substance ideal for testing a potential anti-inflammatory cardiovascular therapy.

Conclusion

Two decades ago, the concept that immune mechanisms play a major role in atherogenesis was generated. Clinical and histopathological studies and particularly data from genetically modified animal models established a role for innate and adaptive immune mechanisms in initiation, progression and complication of atherothrombotic disease. Recently, the cross-talk between the immune system and the coagulation system became a focus of attention. However, strong limitations of preclinical models used for atherosclerosis studies complicated translation of preclinical findings into the clinical setting. Currently, new potential anti-inflammatory therapies for atherothrombotic disease are being tested in large randomized clinical trials. The upcoming years should provide further evidence for immune mechanisms in atherogenesis and might introduce first anti-inflammatory therapy options in atherothrombotic disease.

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Conflict of interest

The authors declare that they have no conflict of interest.

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