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REVIEW

Novel therapeutic concepts

Disordered haematopoiesis and athero-thrombosis

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Atherosclerosis, the major underlying cause of cardiovascular disease, is characterized by a lipid-driven infiltration of inflammatory cells in large and medium arteries. Increased production and activation of monocytes, neutrophils, and platelets, driven by hypercholesterolaemia and defective high-density lipoproteins-mediated cholesterol efflux, tissue necrosis and cytokine production after myocardial infarction, or metabolic abnormalities associated with diabetes, contribute to atherogenesis and athero-thrombosis. This suggests that in addition to traditional approaches of low-density lipoproteins lowering and anti-platelet drugs, therapies directed at abnormal haematopoiesis, including anti-inflammatory agents, drugs that suppress myelopoiesis, and excessive platelet production, rHDL infusions and anti-obesity and anti-diabetic agents, may help to prevent athero-thrombosis.

Keywords Athero-thrombosis • Haematopoiesis • Atherosclerosis • Monocytes • Neutrophils • Platelets

In Western societies, the consumption of diets high in calories, saturated fat, and cholesterol combined with a sedentary lifestyle lead to a high prevalence of atherosclerotic cardiovascular disease (CVD). High-circulating blood lipids, including elevated low-density lipoproteins (LDL) and triglyceride-rich lipoproteins, result in increased entry and retention of these particles in the arterial wall, leading to a macrophage-dominated chronic inflammatory process and eventuating in atherosclerotic plaque rupture or erosion, myocardial infarction, or thrombotic stroke.[1] While elevated plasma cholesterol levels have an essential role in atherogenesis, co-morbidities such as smoking, hypertension, and diabetes accelerate atherosclerotic CVD. In addition, chronic kidney disease,[2,3] recurrent infections,[4,5] myeloproliferative neoplasms (MPNs),[6–10] and autoimmune disease such as rheumatoid arthritis[11,12] and systemic lupus erythematous[13] also greatly increase the risk of athero-thrombosis. A common theme linking these diseases to athero-thrombosis is an overactive immune system, mediated in part by increased production and activation of innate immune cells.

Leukocytosis as a risk factor for cardiovascular disease

There is strong epidemiological evidence detailing the link between elevated white blood cells (WBCs) and CVD.[14–16] Numerous studies of people with and without pre-existing CVD at baseline measurements show that WBC counts predict the incidence of cardiac events,[17–27] largely irrespective of race or gender[15,28–30] and after adjusting for confounding factors such as smoking, age, BMI, and lipids.[17,28] It appears that the myeloid compartment of the WBCs, namely monocytes[31–33] and neutrophils[14,34–37] are the strongest predictors of cardiac events, while lymphocytes generally have no correlation or an inverse relationship.[20,32] Preclinical animal models of atherosclerosis have identified monocytes, neutrophils, and platelets as important players in the disease process, where the levels of these cells in the blood influence disease initiation and progression[38–45] (*Figure 1*). Cross-talk between these cells can also promote thrombotic disorders.[46–49] In humans, the abundance of leucocytes can affect heart failure following a myocardial infarction,[50,51] while also contributing to secondary cardiovascular events.[52]

Neutrophils and atherosclerotic cardiovascular disease

Neutrophils are the major WBC in humans accounting for ~60% of the circulating WBCs. Neutrophils are relatively understudied in the setting of atherosclerosis as they are not always present within stable atherosclerotic lesions. However, it is appreciated that neutrophil activation can influence coronary artery disease (CAD).

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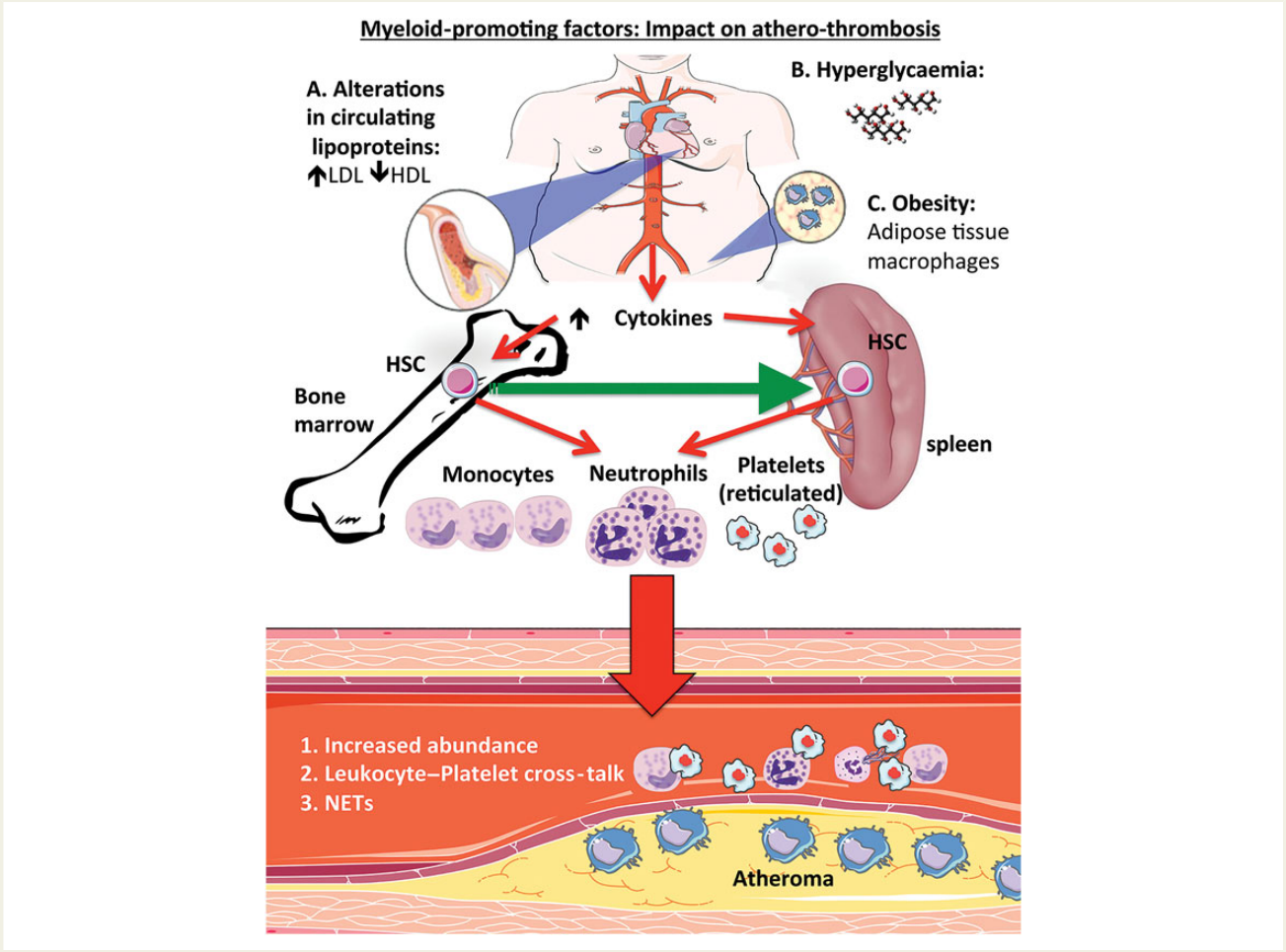

Figure 1 Cardiovascular risk factors promote myelopoiesis and contribute to athero-thrombosis. (*A*) Increased plasma low-density lipoproteins and decreased high-density lipoproteins levels, (*B*) hyperglycaemia, and (*C*) obesity are major cardiovascular risk factors. Through various mechanisms, these risk factors directly or indirectly stimulate the production of myeloid cells (monocytes, neutrophils, and reticulated platelets) increasing the abundance in circulation. Hypercholesterolaemia also promotes the mobilization of haematopoietic stem cells (HSCs) to the spleen resulting in extramedullary haematopoiesis further contributing to the circulating pool of myeloid cells. (1) The increased abundance in circulating myeloid cells enhances the progression and impairs the regression of the atheroma. (2) There is also increased platelet–leucocyte interactions that enhance the recruitment of the leucocytes to the atherosclerotic lesion. (3) Neutrophils activation can also result in the formation of neutrophil extracellular traps (NETs), which contribute to enhanced atherogenesis and athero-thrombosis by binding platelets.

People with unstable angina have higher levels of neutrophil activation as measured by lower myeloperoxidase (MPO) levels (i.e. degranulation) compared to people with stable angina or healthy controls.[53] Further, MPO levels were found to be inversely associated with the inflammatory molecule C-reactive protein (CRP).[53] C-reactive protein could be playing a causative role in neutrophil activation particularly when CPR dissociates into a monomeric form.[54] This monomeric form of CRP is formed by and activates platelets,[55] and in turn platelets via P-selectin can activate neutrophils in acute coronary syndromes.[56] Importantly, neutrophils have been identified in human carotid atherosclerotic plaques where their abundance correlates positively with features of vulnerability, i.e. lipid core area, macrophage abundance, vessel density, and negatively with collagen and smooth muscle cells.[57] In murine models, neutrophils were shown to play a direct role in initiating atherosclerotic lesion formation.[38] Neutrophil levels have also been shown to predict ischaemic heart disease over a 10-year period in the Caerphilly and Speedwell studies[14] and also predict the risk of recurrent ischaemic events.[58] Conversely, Yemenite Jews, who frequently present with benign hereditary neutropenia,[59] rarely suffer from myocardial infarction,[60] and those who do are generally not neutropenic.[61] Neutropenic preclinical models also support the observations that lower neutrophil levels afford some protection against vascular disease, particularly in the early stages of disease development.[38] There may be an important role for co-morbidities in driving the production of neutrophils and how they influence CVD, for example, in Type 1 diabetics (T1D) who have CVD compared with T1D without CVD.[43] There are a number of theories as to how neutrophils may contribute to CVD, including releasing proteases that contribute to the rupturing of atherosclerotic lesions[62,63] and MPO, which modifies proteins, particularly lipoproteins,[64,65] and is raised in people with CAD.[66,67]

Neutrophil extracellular traps and athero-thrombosis

Recent findings suggest that neutrophils may also play an important role in athero-thrombotic events, by releasing DNA NETs (neutrophil extracellular traps) that may have a primary role in protecting against bacterial infections but also promote thrombus formation.[68–71] These NETs, while primarily comprised DNA, also hold proteins of specific neutrophil granules including elastase, MPO, and gelatinase, all of which have atherogenic and plaque-destabilizing properties. NETosis with or without death of the neutrophil leads to release cytoplasmic proteins including damage-associated molecular pattern molecules (DAMPs) such as S100A8/A9 (myeloid-related protein 8/14; MRP8/14) and high mobility box 1 (HMGB1), which can further potentiate inflammation and coagulation. These DAMPS can also be presented by activated platelets to promote NETosis.[72] The nucleic and chromatin contents of NETs can be found systemically and are positively associated with CAD, pro-thrombotic state, and adverse vascular events.[73] Recent studies have suggested that neutrophil nets are present in atherosclerotic plaques in WTD-fed *Apoe*$^{-/-}$ mice and that reducing net formation by DNAse injection or by crossing mice with neutrophil elastase/proteinase 3-deficient mice reduced formation of atherosclerotic lesions. However, the major impact was on advanced rather than early lesions, in contrast to earlier studies that had implicated neutrophils in early lesion development.[38] Moreover, the critical genetic test, involving atherosclerosis susceptible mice with genetic knockouts of peptidylarginine deiminase 4 (PAD4) (which has an essential role in chromatin decondensation and net formation),[74] has not yet been reported. Neutrophils can be stimulated to release NETs *in vitro* following incubation with cholesterol crystals;[71] however, it is uncertain whether neutrophils have significant contact with cholesterol crystals within atherosclerotic lesions. In another study of human atherosclerotic lesions with eroded plaques, nets appeared to be formed at the surface of plaques, where they were proposed to contribute to endothelial cell apoptosis and pro-thrombotic effects.[70] Platelet–leucocyte interactions have also been described to trigger neutrophil activation and NET formation in sepsis, and while this was primarily in the liver sinusoids,[75] this could be a potential mechanism in athero-thrombotic disease (*Figure 1*). Thus, additional studies on the role of nets in atherosclerosis may prove informative, especially in settings where neutrophils and platelet/neutrophil aggregates are increased. Along with the strong evidence for a role in thrombosis,[69] these studies suggest that NETs may have an important role in promoting athero-thrombosis.

Monocyte subsets and atherosclerotic cardiovascular disease

Monocytes account for around 10% of the WBCs and are also a heterogeneous population of cells. They can be divided into three sub-populations based on the expression of the cell surface markers CD14 (co-LPS receptor) and CD16 (FcγIII receptor). Classical monocytes are defined as CD14^{+}CD16^{-}, intermediate are CD14^{+}CD16^{+}, while non-classical are CD14^{dim}CD16^{+}.[76] These subsets differ in many respects, including in their expression of adhesion molecules, chemokine receptors, and functionality.[76] CD14^{+} monocytes are more phagocytic, produce larger amounts of ROS and cytokines in response to bacterial cues, while CD16^{+} monocytes appear to be akin to murine Ly6-C^{lo} monocytes in that they can patrol the endothelium and appear to be adapted for viral rather than bacterial immunity. CD16^{+} monocytes selectively produce TNF-α, IL-β, and CCL3 in response to viruses and immune complexes containing nucleic acids via TLR7 and TLR8.[76] The role of monocyte subsets in CVD is not well established, but there are some reports associating levels of specific subsets with disease. Patients with CAD[77–79] or unstable atherosclerotic plaques[80] have higher numbers of CD16^{+} monocytes. On the other hand, a decrease in these CD16^{+} monocytes is associated with plaque stabilization.[81] Elevated levels of CD16^{+} monocytes have been shown to independently predict cardiovascular events[82] and correlate with markers of atherosclerosis including carotid intima media thickness (cIMT) and risk algorithms (Framingham and SCORE).[83] These associations with the CD16^{+} monocytes are observed in people with diseases associated with CVD including obesity[83] and chronic kidney disease,[84] and abundance in CD16^{+} monocytes after stroke[85] and myocardial infarction[86] can predict the clinical course and inform on the prognosis. A recent study has also shown that Ly6-C^{lo} monocytes adhere more readily to endothelium and extravasate into tissues resulting in macrophage accumulation in the setting of hypertriglyceridaemia.[87] Given that the CD14^{+}CD16^{-} monocytes are the most abundant monocytes in humans and these cells are similar to the CCR2^{+}Ly6C^{hi} monocytes in mice, which are consistently been reported to more readily enter atherosclerotic lesions,[44,45] it seems likely that both CD16^{-} and CD16^{+} monocytes contribute to atherogenesis in humans and the distinct roles of different subsets require further investigation.

Platelets and platelet–leucocyte aggregates in athero-thrombosis

Platelets also have a major role in the initial and advanced stages of athero-thrombotic disease.[39,40,42,88,89] Meta-analyses with combined data from over 140 randomized trials show that anti-platelet therapy reduces the risk of vascular events.[90] However, platelet counts as predictors of disease have been far less studied. Thaulow *et al.*[91] found that platelet counts and platelet reactivity independently correlate with CVD mortality in a cohort of healthy males. Infusion of activated platelets into *Apoe*$^{-/-}$ mice resulted in an increase in atherosclerosis, reflecting binding of platelets and platelet/leucocyte aggregates to arterial endothelium over atherosclerotic plaques with release and binding of chemokines to arterial endothelium. Sreeramkumar *et al.*[92] have recently shown that activated platelets may bind to neutrophils after neutrophils have adhered to activated endothelium (*Figure 1*). The interaction of platelets with leucocytes is initiated by the binding of platelet P-selectin to P-selectin glycoprotein ligand-1 (PSGL-1), which localizes to the uropod (tail) of neutrophils as they bind to endothelium. Platelets and monocytes may be involved in a similar interaction on arterial endothelium. Platelet–leucocyte interactions trigger a series of events that contribute to the

inflammatory reaction of the vessel wall and promotion of atherogenesis. Platelet–leucocyte interactions are also important as this can activate adhesion molecules (i.e. CD11b/c) and trigger cytokine expression (i.e. IL-1β) in leucocytes (i.e. CD11b/c) causing adhesion to arterial endothelium and promotion of atherosclerotic lesion formations.[93] This process also activates the platelets and triggers the release of an array of atherogenic chemokines including CCL5, CCL2, and CXCL4, which promote entry of monocytes and neutrophils into lesions[93] (*Figure 1*). Platelet–leucocyte transcellular metabolism of arachidonic acid leads to the synthesis of inflammatory, vasoconstrictive leukotrienes, and thromboxane A2, while also generating pro-resolving mediators such as lipoxins.[94,95] The factors determining the balance of these opposing factors are poorly understood but could be important in determining the resolution of inflammatory processes such as atherosclerosis.

Monocyte–platelet interactions are observed in the setting of MI and are increased for at least 1 month after the acute event, suggesting that this mechanism could contribute to a secondary MI.[86] There is also a clearly defined relationship between platelet size as determined by mean platelet volume (MPV), a marker of platelet production, and CVD.[96–98] Mean platelet volume is increased in high-risk groups including those with diabetes, obesity, metabolic syndrome, after acute MI, and in restenosis of coronary angioplasty.[99] In a study of over 200 000 people with a median follow-up of 4.6 years, MPV was found to predict mortality due to ischaemic heart disease.[100] Mean platelet volume is also elevated in people with low levels of HDL.[101] Increased MPV suggests an enriched population of larger immature or reticulated (RNA rich) platelets that are more reactive than mature platelets[102] and do not respond as well as mature platelets to anti-platelet therapies such as aspirin and clopidogrel[102] (*Figure 1*). This is possibly because reticulated platelets carry RNA allowing *de novo* synthesis of cyclooxygenase (COX)-1 and COX-2, overcoming effects of aspirin on these targets.[103] This is particularly relevant in people with diabetes who typically have elevated reticulated platelets,[102–106] increased MPV,[106–108] and respond poorly to anti-platelet therapies.[109,110] A high MPV was also shown to markedly increase CVD in people with diabetes.[111] The increase in these platelet parameters appears to be driven by diabetes (hyperglycaemia) as the increase independently correlates with the severity of diabetes.[112] Obese individuals also have an elevated MPV,[113] which is reduced upon weight loss.[114] Significantly elevated platelet counts also correlate with CVD in diabetic subjects.[115] These observations suggest the importance of anti-platelet therapy in diabetics. Moreover, a deeper understanding of the mechanisms of platelet overproduction in diabetes could lead to the development of new approaches to preventing CHD in diabetics.

Athero-thrombosis in myeloproliferative disorders

Myeloproliferative neoplasms are blood disorders where increased production of myeloid cells is strongly associated with venous and arterial thrombosis (MI and stroke). These MPNs include essential thrombocytosis (ET), polycythemia vera (PV), and myelofibrosis. Cardiovascular disease is a major cause of morbidity and death in patients with MPNs.[6–10] Essential thrombocythemia is also associated with thrombosis including CAD.[116–119] Randomized clinical trials have shown that aspirin on top of other anti-thrombotic strategies (thromboprophylaxis) reduced the risk of vascular events;[120] however, patients with MPNs still remain at significantly higher risk of thrombotic and athero-thrombotic events. It is becoming apparent that thrombosis in ET is linked to leukocytosis,[121] potentially providing a major link to thrombotic events in MPNs as leukocytosis remains the strongest risk factor for thrombosis in people with PV.[122–124] When people with PV are intensively treated with phlebotomy and/or hydroxyurea to achieve a haematocrit target of $<45\%$, there was significantly fewer cardiovascular events compared with the less aggressively treated group. Interestingly leucocyte, but not platelet levels, reflected the abundance of haematocrit in these people,[125] providing further evidence for the importance of WBC levels in people with MPNs and cardiovascular events. While the mechanisms are not completely understood, it is likely that enhanced leucocyte–platelet aggregates resulting in leucocyte and platelet activation and chemokine/cytokine release (as discussed above) are intimately involved.[126–129] In addition, increased circulating leucocytes, possibly via the formation of platelet–leucocyte aggregates in MPN patients may give rise to NETS that likely contribute to both arterial and venous thrombosis as seen in other thrombotic disorders.[48] Patients with MPNs often have increased monocytes, which may lead to increased entry into atherosclerotic plaques and contribute to increased macrophage foam cell formation. Interestingly, some genetic changes that promote MPNs, such as in *LNK/SH2B3*, may also be associated with platelet and leucocyte counts and with increased CVD in the general population,[130,131] suggesting common mechanisms promoting athero-thrombosis in MPN and in the general population.

Disordered cholesterol metabolism links haematopoiesis to athero-thrombosis

Monocytosis is prominent in animal models of atherosclerosis and is increased in response to diets high in saturated fat and cholesterol.[41,44,45,132] Moreover, monocytosis is associated with increased monocyte entry into plaques, and limitation of monocytosis or entry of monocytes into plaques reduces atherosclerosis, suggesting a causal relationship.[41,43–45,133] While monocytosis may be related in part to increased inflammatory cytokines such as IL-3, GM-CSF, M-CSF, IL-1β, etc.,[134–138] recent studies have uncovered a role of both hypercholesterolaemia and defective cholesterol efflux pathways in haematopoietic progenitors, both in the bone marrow (BM) and in the spleen, in promoting myelopoiesis in mouse atherosclerosis models.

Active cellular cholesterol efflux is mediated by ATP-binding cassette transporters, including ABCA1 that mediates cholesterol efflux to lipid-poor apoA-1 and minimally lipidated apoA-I particles, and ABCG1 and ABCG4 that mediate cholesterol efflux to HDL particles, especially larger HDL species.[139] Unexpectedly, *Abca-1/Abcg1* double knockout mice were found to develop marked monocytosis and neutrophilia that were associated with a dramatic expansion of the haematopoietic stem and multipotential progenitor cells (HSPCs) in the BM, uncovering an important role of cholesterol efflux pathways in the regulation of myelopoiesis[140] (*Figure 2*). Another important

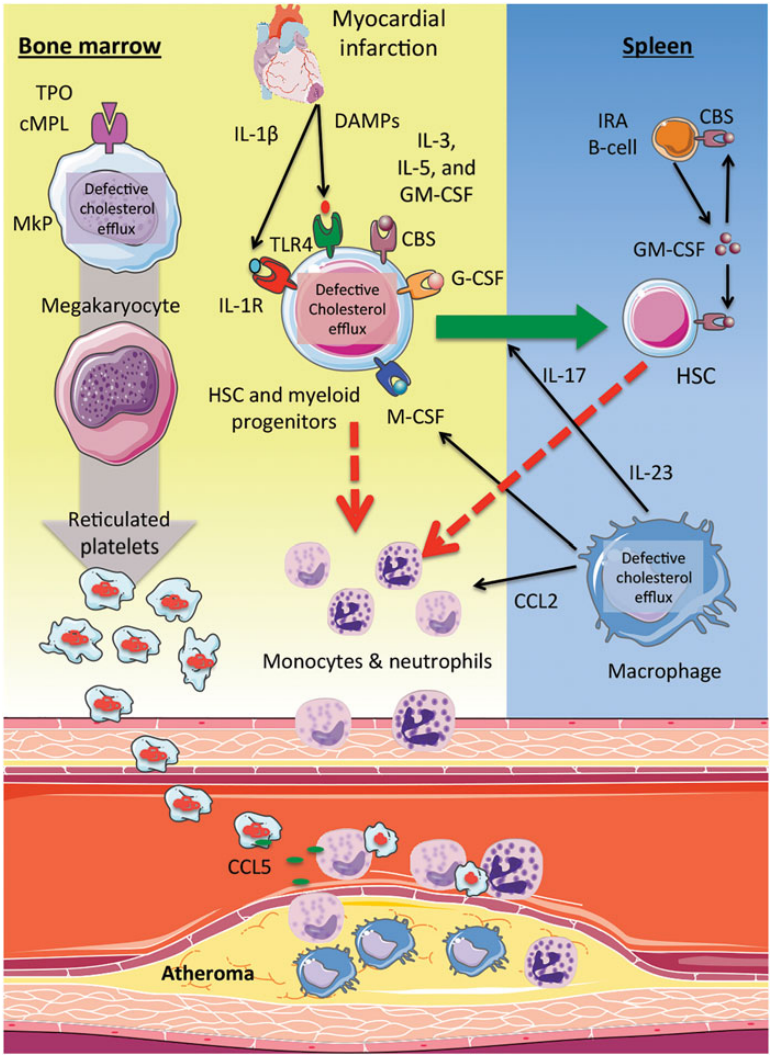

Figure 2 Defects in cellular cholesterol efflux pathways trigger myelopoiesis, extramedullary haematopoiesis, and enhanced atherosclerosis. In the bone marrow, defects in intrinsic cellular efflux pathways in haematopoietic stem (HSC) and myeloid progenitor cells result in increased membrane cholesterol levels and increased sensitivity to growth factor and cytokines. Deletion of ABCG4 in megakaryocyte progenitors (MkPs) results in increased c-MPL expression and enhanced thrombopoietin (TPO) signalling. This stimulates the production of immature reticulated platelets that can enhance atherogenesis via a number of mechanisms including deposition of cytokines (CCL5) and binding and activating leucocytes. Defective cholesterol efflux in haematopoietic stem cell and myeloid progenitors increased the cell surface abundance of the common β-subunit (CBS) of the IL-3, IL-5, and GM-CSF receptors resulting in enhanced proliferation. Inflammatory stimuli from a myocardial infarction including damage-associated molecular pattern molecules and IL-1β can influence haematopoietic stem cell proliferation and lineage fate. HSCs can also mobilize and migrate to the spleen when efferocytosis fails in macrophages with defective cholesterol efflux as there is a failure to shut down the expression of IL-23; thus, IL-17 and in turn G-CSF levels remain increased. In the spleen, there is an increased abundance of the innate response activator B cells (IRA B-cells) in the setting of hypercholesterolaemia, which produce GM-CSF driving the haematopoietic stem cells to produce monocytes and neutrophils. Defective cholesterol efflux in splenic macrophages also promotes M-CSF production to enhance myelopoiesis and CCL2 to promote monocyte migration. Together, the increased abundance of platelets, monocytes, and neutrophils all contribute to promoting the accumulation of macrophages in the atherosclerotic lesion.

cholesterol efflux pathway suppressing monocytosis and HSPC proliferation is mediated by apolipoprotein E (apoE), which may interact with ABCA1/G1 in haematopoietic stem cells and in multipotential progenitor cells, i.e. the $Lin^{-}Sca1^{+}cKit^{+}$ population (HSPCs) to promote cholesterol efflux.[41] In both *Abca1*$^{-/-}$/*Abcg1*$^{-/-}$ and *Apoe*$^{-/-}$ mice, HSPCs showed evidence of increased cholesterol in the plasma membrane associated with increased cell surface levels of the common β-subunit of the IL-3/GM-CSF receptor (CBS), allowing these cells to more readily sense these cytokines.[41,140] ABCA1/ABCG1-deficient splenic macrophages also show increased expression of cytokines including M-CSF, which provide an additional stimulus of myelopoiesis[141] (*Figure 2*).

In line with these findings, injection of neutralizing antibodies to IL-3 and GM-CSF in WTD-fed *Apoe*$^{-/-}$ mice significantly reduced

Table 1 Mechanisms of monocyte production in CVD and potential interventions

Disease complication	Initiating cell/ligand	Target cell/receptor	Intervention	References
Hypercholesterolaemia and defective cholesterol efflux (ABCA1/G1 and apoE)	Spleen – IRA B cell/IL-3 and GM-CSF – Macrophage/M-CSF and CCL2	HSPC/CBS	rHDL	41,44,45,134,138,140,141,142
Myocardial infarction	Damaged myocardium – DAMPs – IL-1β	HSPCs – TLR4 – IL-1R	?Paquinimod ?Anakinra, ?Canakinumab	52,157,159,162,164,165,166
Diabetes/hyperglycaemia	Neutrophil/S100A8/A9 ?	CMPs/RAGE Defective efflux in HSPCs	SGLT2i ?Paquinimod Anti-miR33	43,192
Obesity	ATM/IL-1β	CMP and GMP/IL-1R	Anakinra ?Paquinimod	167

the production of splenic monocytes and in turn triggered apoptosis.[142] IL-3 and GM-CSF appear to be made in IRA B-cells, which arise from peritoneal B1a cells.[143] The IRA B cells play an important role in the spleen and are not only expanded in *Apoe*$^{-/-}$ mice but also produce more cytokines[134] (*Figure 2*). *Ldlr*$^{-/-}$ mice transplanted with *Apoe*$^{-/-}$*Cbs*$^{-/-}$ BM had fewer circulating monocytes and neutrophils, which was accompanied by a reduction in BM and splenic stem and progenitor cells compared with mice that received *Apoe*$^{-/-}$ BM.[138] Interestingly, the IRA B cells are sensitive to the hypercholesterolaemic environment (or the inflammation associated with it), which is dependent on the expression of the CBS. Deletion of the CBS lowered the numbers and proliferation of the IRA B cells. Initially, deletion of the CBS translated into smaller atherosclerotic lesions with fewer macrophages. However, when studies were extended to look at more advanced lesions, it was discovered that deletion of the CBS resulted in lesions of similar size again with fewer macrophages, but these lesions had significantly larger necrotic cores. The decrease in macrophages and larger necrotic cores was linked to increased macrophage apoptosis. This appeared to be due to a complete absence of *Abcg1* expression, which has been shown to render macrophages more susceptible to apoptosis.[144] Interestingly, GM-CSF has previously been identified to stimulate *Abcg1* expression via PPARγ, suggesting an important pro-survival role for this cytokine in atherosclerosis. The increase in necrotic core formation associated with lower GM-CSF signalling in advanced plaques suggests the need for caution when using therapies that inhibit GM-CSF, e.g. in people with autoimmune diseases that are also at high risk of CVD, e.g. rheumatoid arthritis.[145]

Increased LDL cholesterol has also been suggested to contribute to the production of WBCs (monocytes and neutrophils) in preclinical models,[38,41,44,45,146,147] and this can be dampened when cholesterol levels are restored to normal levels by diet[147] or statin[44] interventions. However, statins do not appear to effectively lower WBCs in people with CVD,[148] suggesting that targeting LDL levels alone via statins may not be sufficient to reduce excessive WBC production. Studies in genetically modified mice showed that hypercholesterolaemia (i.e. increased LDL) along with decreased HDL levels due to an *Apoa1* gene mutation were both required to influence monocyte counts.[149] Similarly, in children with heterozygous familial hypercholesterolemia who were statin naive, HDL cholesterol levels were inversely related to blood monocyte counts.[149] Together these findings support the hypothesis that HDL inversely correlates with monocyte levels, particularly in the setting of hypercholesterolaemia, and suggest that to reduce leucocyte production it may be necessary to increase cholesterol efflux in myeloid progenitors as well as lowering LDL levels, for example by rHDL infusion or LXR activator treatment[41] (*Table 1*).

Enhanced thrombopoiesis and atherosclerosis

As discussed above, platelets play an important role in CVD, and studies have linked their enhanced production and activation to cardiac events.[39,40,42,88,89] Platelets promote atherogenesis via a multitude of pathways, from priming circulating monocytes and neutrophils so they are ready to adhere to the endothelium, to depositing potent chemokines such as RANTES (CCL5) and platelet factor 4 (CXCL4) on monocytes and the endothelium lining the atherosclerotic lesion.[39] The membrane-bound platelet adhesion molecule P-selectin appears to be required for this to occur, and P-selectin-deficient platelets do not elicit the same response.[39] It should also be noted that a soluble form of P-selectin occurs *in vivo* and has been shown to activate leucocytes from people with peripheral arterial occlusive disease (PAOD).[150,151] However, injections of sP-selectin only moderately promote atherosclerosis,[152] suggesting the platelet–leucocyte interaction is key in this atherogenic pathway.

While the study by Huo *et al.* was seminal in understanding the contribution of platelets to atherogenesis, they relied on adoptive transfer of activated platelets. More recently, models of enhanced thrombopoiesis have confirmed the important contribution that platelets play to atherogenesis.[42,153] The cholesterol transporter ABCG4, which promotes cholesterol efflux to HDL, was shown to regulate platelet production.[42] ABCG4 was highly expressed in MkPs, and haematopoietic deficiency of *Abcg4* resulted in enhanced atherogenesis, which again appeared to be attributable to an increase in total platelets, reticulated platelets, enhanced activation, and increased platelet–leucocyte aggregates[42] (*Figure 2*). Deletion of *Abcg4*$^{-/-}$ resulted in enhanced expression of the thrombopoietin (TPO) receptor, c-MPL on MkPs making them more sensitive to TPO signalling. It was found that LYN kinase acts a sensor of membrane cholesterol, where LYN kinase is protected from

phosphorylation when cellular cholesterol levels increase. This precludes LYN kinase from activating the E-3 ubiquitin ligase c-CBL and in turn prevents c-MPL from degradation. Interestingly, SNPs associated with platelet counts revealed a connection to *c-CBL*, which is in tight linkage disequilibrium with *ABCG4*. This suggests that SNPs could alter the expression of either or both these genes and could be responsible for altered platelet production.

Infusion of rHDL in WT mice, but not *Abcg4*$^{-/-}$ mice, significantly lowered platelet numbers, by reducing MkPs and cell surface c-MPL levels. The therapeutic potential of this pathway was demonstrated by infusion of rHDL which suppressed platelet counts in a mouse model of myelofibrosis and ET caused by an activating mutation in c-MPL (c-MPL^{W515L}). These suggest a role for rHDL infusions or therapies that increase HDL production in the treatment of myeloproliferative disorders. While leucocyte levels were not measured in this study, rHDL does reduce monocytes and neutrophils,[41] decreases the activation of these cells,[154,155] and reduces platelet activation,[156] suggesting that HDL therapies could reduce thrombotic risk in people with MPNs.

HSC mobilization, extramedullary haematopoiesis, and monocytosis with myocardial infarction

The high rate of a secondary athero-thrombotic events after an initial myocardial infarction may in part reflect an acceleration of the underlying atherosclerotic process caused by enhanced myelopoiesis.[52] Swirski *et al.*[157] initially showed the spleen was home to a population of monocytes that were ready to be mobilized after an inflammatory insult, such as an MI. While splenic reserves dropped during the first 24 h following an infarct, by Day 6 post-MI there was a significant expansion of splenic monocytes fuelling monocytosis and entry into the infarct area.[158] While this had a beneficial effect on myocardial function, Dutta *et al.*[52] discovered that after an MI, myeloid cells, particularly Ly6-C^{hi} monocytes, also infiltrated atherosclerotic lesion causing a significant increase in plaque size and a shift towards an unstable phenotype. Interestingly, the continual supply of monocytes reflected an increased number of HSCs in the spleen. In this setting, there was an expansion of a myeloid-biased subset of activated HSCs that express CCR2,[159] previously identified to be responsive to inflammatory cues *in vivo*.[160] Genes that are regulated by myeloid translocation gene on chromosome 16 (*Mtg16*) were found to be enriched in the CCR2^{+} HSCs, and deletion of *Mtg16* resulted in depletion of CCR2^{+} HSCs and monocytes after an MI. The CCR2^{+} HSCs have significantly higher TLR2 and 4 expressions in the steady state suggesting that they are ready to respond to inflammatory signals. HSCs express functional TLRs and may directly sense their ligands.[161,162] While the endogenous TLR ligand(s) post-MI remains unknown, these could be DAMPs that have been released from dying or activated myocardial cells (*Figure 2*). Dutta *et al.* found that injection of HMBG1, a DAMP found to be increased in patients after an MI,[163] significantly increased CCR2^{+} HSC proliferation. Other DAMPs that are increased after MI include S100A8/A9,[164] which are correlated with leucocyte counts.[165] Additionally, Sager *et al.*[166] has also discovered that IL-1β is increased and can accelerate haematopoiesis, not to dissimilar has to what was discovered with IL-1β in the setting of obesity[167] (*Table 1*). Extramedullary haematopoiesis is not only initiated after an MI, but also in inflammatory diseases that are associated with increased risk of CVD including autoimmune diseases and MPNs, suggesting a pro-atherogenic role in these settings as well.

Promoting cholesterol efflux inhibits haematopoietic stem cell mobilization and extramedullary haematopoiesis

In murine models of defective cholesterol efflux (i.e. *Abca1*$^{-/-}$/*Abcg1*$^{-/-}$ and *Apoe*$^{-/-}$ mice), there is chronic HSC mobilization into the circulation and spleen[168] (*Figure 2*). Interestingly, the mechanisms contributing to HSC mobilization were not intrinsic to the HSC and instead reflected changes in the BM niche in response to exogenous signals. This process was initiated by splenic macrophages and dendritic cells where defective cholesterol efflux resulted in enhanced production of IL-23. This stimulated a signalling axis involving IL-23, IL-17, and G-CSF,[169,170] a well-known HSC-mobilizing cytokine. These processes were reversed by transgenic overexpression of human *APOA-I* in *Abca1*$^{-/-}$/*Abcg1*$^{-/-}$ or infusion of rHDL in *Apoe*$^{-/-}$ mice, suggesting a novel therapeutic approach to suppression of extramedullary haematopoiesis in CVD, MPNs, and leukaemia.

Metabolic diseases, myelopoiesis, and atherosclerosis

Metabolic diseases including obesity, insulin resistance, and diabetes (Types 1 and 2) greatly increase the risk of CVD[171–174] and are associated with elevations in WBCs.[83,175–183] Even when LDL is lowered by statins in diabetics, there is increased residual CVD risk. Using an experimental model of atherosclerotic lesion regression caused by LDL lowering, Parathath *et al.*[184] showed that the induction of diabetes with streptozotocin prevented regression of atherosclerotic lesions. In this model, hyperglycaemia promoted BM myelopoiesis,[43] independent of changes in plasma cholesterol or insulin levels. The mechanism involved neutrophils sensing increased levels of blood glucose and responding with production and release of S100A8/A9. S100A8/A9 induced myelopoiesis, via binding to the pattern recognition receptor RAGE on common myeloid progenitor cells (CMPs) in the BM and initiation of an autocrine/paracrine loop via the induction of key myeloid-promoting cytokines M-CSF and GM-CSF in CMPs (*Figure 3*). As CMPs are upstream of MkPs, and diabetics have more reticulated platelets,[102–106] this or a related mechanism could also contribute to the enhanced production of platelets in diabetes (*Figure 3*). Together, the increased production of myeloid cells and platelets may contribute to atherothrombotic complications in diabetes. The importance of glycaemic control in promoting atherosclerotic lesion regression was shown by treatment with the SGLT2i dapagliflozin (Farxiga). SGLT2i treatment lowered blood glucose, decreased monocytosis, recruitment of monocytes into lesions, and promoted lesion regression[167]

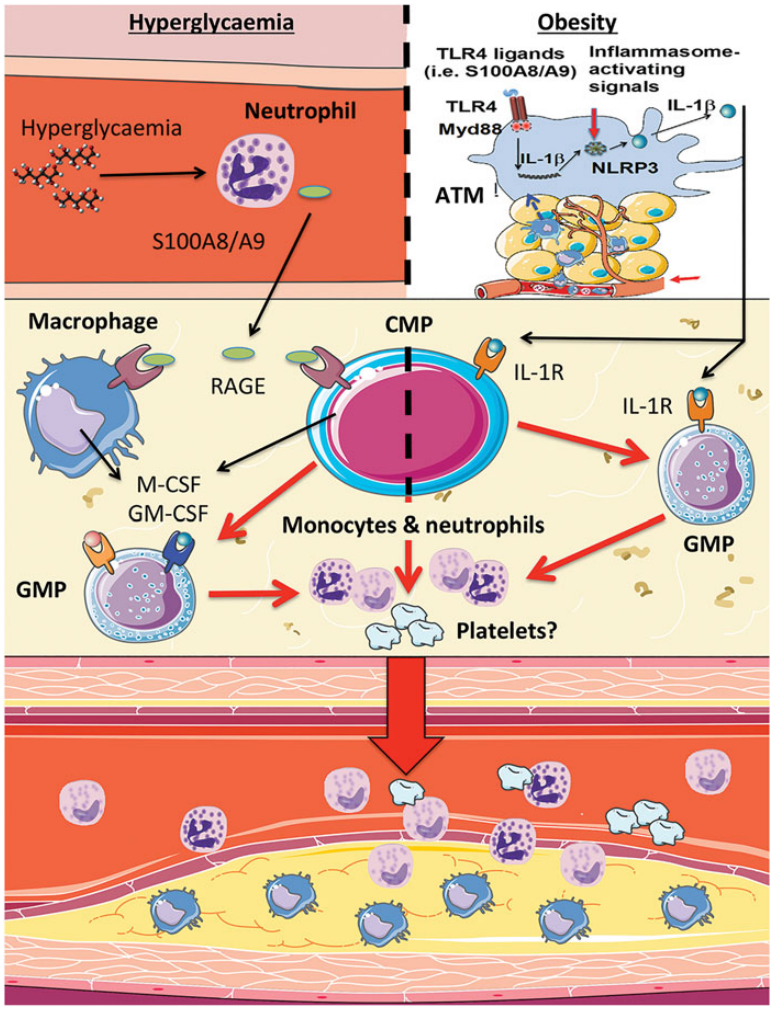

Figure 3 Mechanisms contributing to myeloid production in metabolic disorders. Hyperglycaemia: In the setting of elevated blood glucose, neutrophils are stimulated to produce S100A8/A9, which travels to the bone marrow to interact with RAGE on the surface of macrophages and common myeloid progenitors (CMPs) triggering the production of M-CSF and GM-CSF. These cytokines increase the abundance of common myeloid progenitors and granulocyte–macrophage progenitors (GMPs) promoting the production of monocytes and neutrophils. Obesity: In the context of obesity, local inflammation in the adipose tissue occurs which appears to be initiated by S100A8/A9 interacting with TLR4 on adipose tissue macrophages (ATMs). This induces IL-1β, which is processed by the NLRP3 inflammasome to its mature form. IL-1β then travels to the bone marrow and binds the IL-1 receptor, which is up-regulated on common myeloid progenitors and granulocyte–macrophage progenitors in the obese state. This interaction drives myelopoiesis. As people with diabetes and obesity have increased diabetes and common myeloid progenitor cells are precursors of megakaryocytes, this may be a mechanism contributing to increased platelets. The enhanced production of myeloid cells in diabetes impairs the regression of atherosclerotic lesions due to persistent entry of monocytes.

(*Table 1*). These studies imply that tight blood glucose control (without transient hyperglycaemic spikes)[185–187] by use of glycosuric and other agents may dampen monocyte production and entry of inflammatory monocytes into lesions, adding to the benefit of lipid-lowering strategies in diabetics. In this regard, a recent CVD outcomes trial (EMPA-REG OUTCOME)[188] in over 7000 participants has been reported to show benefit of the SGLT2i empagliflozin (Jardiance).[189] However, it should be noted that this was largely attributed to a reduction in heart failure, and more studies are required to explore this anti-atherogenic mechanism in people with diabetes.

Decreased levels of ABCA1[190] and ABCG1[191] in macrophages and myeloid progenitors have been reported in diabetic animal models and could also contribute to enhanced myelopoiesis.[192] Conversely, increased *Abca1* and *Abcg1* expression in mice achieved by administering anti-miR-33 was able to restore the defect in CMP/GMP *Abca1* and *Abcg1* gene expression and to reduce the abundance of CMPs and GMPs in the BM and monocytes in the blood of diabetic mice. While miR-33 antagonism appears to have pleiotropic effects,[193–195] more specific approaches to increasing HDL levels, such as rHDL infusions, might also be effective at reducing monocytosis in diabetic mice.

Obese Ob/Ob leptin-deficient and diet-induced obese mice that are also insulin resistant and models of early T2D also develop profound monocytosis and neutrophilia.[167] These animals were only mildly hyperglycaemic, and lowering blood glucose had no effect on monocyte counts indicating an underlying mechanism distinct from the STZ diabetes model (*Figure 3*). Using a fat transplantation model, visceral adipose tissue was shown to directly contribute to enhanced myelopoiesis. Moreover, the same ligand as in the T1D models, S100A8/A9 was involved, albeit through a different mechanism. Local, but not systemic increases in S100A8/A9, were shown to signal via $CD11c^+$ adipose tissue macrophage (ATM) TLR4/MyD88 to trigger IL-1β expression,[167] which promoted proliferation of BM CMPs and GMPs, leading to increased myelopoiesis, which presumably further promoted ATM accumulation and atherogenesis. A second study also corroborated a role for $CD11c^+$ ATMs in promoting leucocyte production in obesity.[196] While a variety of cytokines are likely involved in this inflammatory signalling, the effect could be blocked using the IL-1R antagonist, Anakinra, indicating the key role of IL-1β (*Table 1*). Clinical trials are underway targeting the IL-1 pathway in CVD,[197] and these results suggest that there could be a particular benefit in patients with obesity and increased ATMs.[198,199] A common molecule identified in diabetes and obesity appears to be S100A8/A9, which may provide a therapeutic target particularly as paquinimod (ABR-215757) that blocks the interaction and function of these DAMPs is an orphan drug for systemic sclerosis and appears to be well tolerated, suggesting that it could be used as a chronic therapy (*Table 1*).

Conclusion

In conclusion, elevated levels and activation of leucocytes and platelets promote atherosclerosis, arterial and venous thrombosis. Formation of platelet/leucocyte aggregates in the bloodstream and on arterial endothelium promotes the entry of monocytes and neutrophils into atherosclerotic lesions. Recent studies have implicated a role for neutrophil-derived DNA nets in venous and arterial thrombosis and possibly in atherogenesis. While elevated levels of atherogenic lipoproteins and reduced HDL-mediated cholesterol efflux promote macrophage foam cell formation and inflammation at the level of the arterial wall, they also act in the BM and spleen driving myelopoiesis and platelet production and worsening atherogenesis. Cholesterol accumulation in haematopoietic stem and progenitor cells promotes myelopoiesis, while macrophage foam cell formation in the spleen leads to cytokine release, further myelopoiesis and mobilization of haematopoietic stem cells to the spleen, further driving extramedullary haematopoiesis. The ability of the spleen to act as a reservoir for monocytes and neutrophils may be particularly important in the setting of myocardial infarction, and while having a beneficial effect for the healing process, may also promote further entry of leucocytes into atherosclerotic lesions. In addition, Type 1 and 2 diabetes also promote excessive production of inflammatory cells, involving distinct mechanisms invoked by hyperglycaemia or adipose tissue inflammatory macrophages, production of S100A8/A9, and expansion and activation of BM common myeloid progenitors. These mechanistic insights suggest a variety of novel approaches to the prevention of athero-thrombosis. Even with dramatic improvements in LDL lowering, there will be a large burden of residual athero-thrombotic disease, and a need for further novel therapies aimed at limiting the production and activation of inflammatory cells, especially in patients with myeloproliferative disorders, obesity, and diabetes.

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