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Myeloid cell contributions to cardiovascular health and disease

Matthias Nahrendorf

Center for Systems Biology, Massachusetts General Hospital and Harvard Medical School, Simches Research Building, 185 Cambridge St., Boston, MA 02114, USA.

Cardiovascular Research Center, Massachusetts General Hospital and Harvard Medical School, 185 Cambridge Street, Boston, MA 02114, USA.

Abstract

Recent advances in cell tracing and sequencing technologies have expanded our knowledge on leukocyte behavior. As a consequence, inflammatory cells such as monocyte-derived macrophages, their actions and products are increasingly considered as potential drug targets to treat atherosclerosis, myocardial infarction and heart failure. Particularly promising developments are the identification of harmful arterial and cardiac macrophage subsets, the cells' altered, sometimes even clonal production in hematopoietic organs, and epigenetically entrained memories of myeloid progenitors and macrophages in the setting of cardiovascular disease. Given monocytes' and macrophages' roles in host defense, intricately understanding the involved cellular subsets, sources and functions is essential to design precision therapeutics that preserve protective innate immunity. Here I review how new clinical and preclinical data, often linking the cardiovascular, immune and other organ systems, propel conceptual advances to a point where cardiovascular immunotherapy appears within reach.

INTRODUCTION

Recent conceptual and technological breakthroughs increase the resolution and depth of our knowledge on myeloid cells and their functions in cardiovascular disease (CVD). Such breakthroughs span the entire biomedical spectrum. On the clinical side, the CANTOS trial provided the first convincing proof that modulating inflammation improves patients' cardiovascular health[1]. This large-scale trial (Box 1), which examined the effects of an antibody that neutralizes the cytokine IL-1b in atherosclerosis, launches the era of immunotherapy in CVD. The association of CVD with clonal hematopoiesis[2,3], i.e. the massive expansion of leukocytes that derive from just one hematopoietic stem cell, is another clinical discovery that creates excitement since it is conceptually linked to translational studies on interactions among cardiovascular risk factors, hematopoiesis and inflammation in cardiovascular organs[4–11]. Furthermore, rapid diagnostic technology development (Box 2) has spurred multidimensional, unbiased data collection in macrophages triggering a deeper understanding of their functional diversity[12–18]. On the fundamental science end of the spectrum, preclinical studies revealed the dichotomy of

Correspondence: Matthias Nahrendorf, Center for Systems Biology, 185 Cambridge Street, Boston, MA 02114. Tel: (617) 643-0500. Fax: (617) 643-6133. mnahrendorf@mgh.harvard.edu.

cardiovascular tissues' innate immune cells according to their source (local proliferation versus recruitment from blood)[12,13,19–21]. These discoveries bridge today's chasms dividing clinical cardiology, immunology and hematology, indicating a need for vigorous interdisciplinary exchange. Here I aim to integrate new cardiovascular, innate immunity and hematopoiesis data with a focus on monocyte and macrophage biology, which I review for a broad readership.

Uncovering the mechanisms leading to atherosclerosis and MI, i.e. coronary risk factors, lowered individual cardiovascular mortality markedly[22]. Understanding and then treating cardiovascular risk factors, listed in Table 1, provided the foundation for this success story[22]. However, longer survival and changes in life style keep CVD at the top of world-wide mortality statistics, and managing risk factors such as high cholesterol, hypertension and diabetes prolongs life but does not necessarily provide a cure. Some of the residual cardiovascular risk is attributed to inflammation[23], which damages the arterial wall and myocardium. Deciphering how cardiovascular risk factors propel inflammation may provide new, orthogonal therapeutic targets that could repeat what statins have achieved in the past: breaking the vicious cycle leading to often catastrophic organ ischemia. Cholesterol and macrophage deposits accumulate below the vascular endothelium. Other immune cells, including lymphocytes, dendritic cells and neutrophils, participate in arterial wall remodeling[24]. Plaques may either erode or rupture, causing downstream ischemia. Heart failure may ensue as a consequence of extensive necrotic muscle loss. During all disease stages, monocyte-derived macrophages replace tissue-resident macrophages and modulate cardiovascular tissue health.

INNATE IMMUNE CELLS IN CARDIOVASCULAR HEALTH

The steady state arterial wall

In the steady state, arterial resident macrophages populate the adventitia and renew through local proliferation; however, they are mostly derived from circulating monocytes[12]. Interestingly, mouse arterial resident macrophages derive from a brief post-birth monocyte migration period[12], perhaps triggered by events that occur at that time, such as exposure to bacteria in the environment, closing of fetal heart shunts and blood oxygenation in the lung. Data obtained in mice in which CD11c expressing cells are labeled[25] suggest that dendritic cells likewise populate arterial and valvular tissue. There is an ongoing debate on the degree to which these cells are distinct from macrophages. It is generally assumed that arterial resident macrophages support tissue homeostasis and pursue surveillance, while organ specific functions have not been reported. How old age, which modifies tissue resident macrophages' phenotypes[26], and other cardiovascular risk factors affect arterial resident macrophage numbers and functions is currently unknown.

The steady state myocardium

We now understand that macrophages constitute 7–8% of non-cardiomyocytes in the normal adult mouse[20,27], an insight that has been recently enabled by cell-specific fluorescent reporters, optical clearing, fluorescence myocardial slab imaging and quantitative multidimensional flow cytometry of myocardial specimen. Imaging of myocardium

confirms that macrophages are also present in healthy humans[14]; FACS protocols to enumerate, isolate and phenotype human heart macrophages and their subsets are currently in development[28]. Cardiac macrophage numbers, distribution, subsets and phenotypes are heterogeneous and change as a function of age and disease in mice and, although less well studied at this time, likely also in humans.

During heart development, macrophages are involved in shaping the vasculature[29]. Macrophages appear in the organ on embryonic day 12.5–14.5 in mice and populate different locations depending on subset; $CCR2^{+}$ cells, arising from fetal monocytes, are close to the endocardium while $CCR2^{-}$ cells, which derive from the yolk sac, reside throughout the compact myocardium and in the vicinity of developing coronary arteries. In $Csf1^{op/op}$ mice that lack resident macrophages, erratic cardiac vascular development leads to increased vessel branching[29]. That cardiac macrophages participate in vascular development is consistent with their support of regeneration, via influencing angiogenesis, after ischemic injury to the newborn murine heart[16,30].

Whether cardiac macrophage numbers and phenotypes change in the aging human heart is currently unclear, though emerging preclinical data support this idea[26,31]. Age-dependent phenotypic alteration of macrophages[26], including increased secretion of IL-10[32], promote myocardial fibrosis and diastolic dysfunction in older mice. In parallel to the increased myeloid cell production in aging, the number of circulating neutrophils and monocytes rises in senescent mice, as does these cells' recruitment to the heart[31,32].

Leukocytes and cardiac conduction

Given their sensitivity to inflammatory stimuli[33], macrophages' response to systemic or local danger may divert steady state macrophage functions. Such functions, which promote organ tasks rather than host defense, may be compromised by inflammation when tissue-resident macrophages die and are replaced by monocytes, for instance after ischemic injury[7]. Of particular relevance to CVD, because macrophages participate in electrical conduction[14], their depletion leads to atrioventricular block and milder conduction abnormalities in the atria and ventricle in mice. Macrophages influence the membrane potential of conducting cardiomyocytes by electrotonic communication through Cx43-containing gap junctions, which are small membrane channels that connect the plasma of two cells. This crosstalk shortens the duration of cardiomyocytes' action potential and may enable "bridging" between two cardiomyocytes that are otherwise not electrically coupled[14]. The long macrophage dendrites observed in the heart support this "bridging" hypothesis. Overall, macrophages' participation in conduction leads to more reliable atrioventricular node function[14]. Patch clamp of co-cultured murine cardiac macrophages and ventricular cardiomyocytes documented altered membrane potentials in communicating cells[14]. It is therefore likely that the gap junction-enabled charge exchange between macrophages and conducting cells occurs not only in the conduction system but everywhere in the heart, raising the possibility that atrial and ventricular fibrillation involve altered macrophage activities. Atrial fibrillation, which leads to embolic stroke and worsens cardiac output in patients with heart failure, associates with atrial[34,35] and systemic inflammatory activity[36,37]

in patients. If monocyte-derived macrophages participate in normal or aberrant conduction remains unclear.

INFLAMMATION IN CORONARY HEART DISEASE

The inflamed arterial wall

Most deaths from cardiovascular disease are preceded by both systemic and local arterial inflammation. Innate immune cells live in the healthy arterial wall and become more numerous and more inflammatory as atherosclerotic plaques, which are subendothelial deposits of cholesterol, cells, matrix and debris, evolve. The largest leukocyte population in the mouse aorta, and by extension other arteries, are macrophages[12,17,18,21].

Cellular fate mapping studies in mice confirm that macrophages residing in atherosclerotic plaque derive from recently recruited monocytes, although these macrophages also proliferate within the plaque, especially if the atherosclerotic lesion is advanced[21]. Some foam cells, which are large lipid-filled phagocytes, may also arise from local smooth muscle cells[38]. Because of the technical challenges involved, in vivo cellular imaging of the mouse arterial wall has only recently been established at a resolution that allows meaningful in vivo data collection[39,40]. This breakthrough will provide information about cell-cell interactions and plaque cell motility. Questions that can only now be addressed include whether or not plaque macrophages depart into the circulation or alternatively die locally in the plaque, what exit routes they take and with which cells they interact.

It is hypothesized that plaques arise because of failed efferocytosis, i.e. plaque macrophages' impaired capability to remove apoptotic cells. The process of efferocytosis, which would counteract the growth of inflammatory lesions, depends on mitochondrial fission in macrophages and Ca-enabled phagocytosis of several apoptotic cells[41]. It is conceivable that macrophages exit atherosclerotic plaques after they consume a meal; however, a macrophage departing an atherosclerotic plaque has never been directly visualized. In an elegant ex vivo experiment, the aortic arches of healthy mice were perfused[25], followed by flow cytometry of the aorta's perfusate. These data suggest that dendritic cells exit the aorta after infectious stimuli. In principle, departure of macrophages, perhaps laden with cholesterol, could reduce the vulnerability and size of atherosclerotic plaques. Important unresolved questions include whether plaque macrophages depart in a similar fashion to dendritic cells via reverse transmigration and whether their exit leads to atherosclerosis regression. Intravital cell tracking, perhaps in conjunction with photoconverting fluorescent proteins[42] expressed by macrophages in plaque, is poised to provide a definitive answer. Such imaging already showed that monocyte patrolling increases on plaque-lining endothelium[43] and revealed that macrophages in atherosclerotic lesions are "dancing on the spot" i.e. extending and retracting their dendrites[39] while sampling their environs. Understanding and modulating the supply and phenotype of arterial wall macrophages, or inhibiting their inflammatory products that destabilize tissue, may provide the key to preventing plaque rupture and myocardial infarction. As will be discussed below, neutralizing the macrophage-derived cytokine IL-1β in patients[1] confirmed this concept clinically (Box 1).

Acute MI

Acute ischemia triggers rapid accumulation of millions of leukocytes in the under-perfused myocardium. These immune cells' defense abilities, which evolved as a response to nonsterile injury, harm cardiovascular organs in acute MI[15]. A subset of macrophages, identified by single-cell RNA-sequencing of infarct leukocytes on day 4 after acute MI in mice, detects danger via the interferon regulatory factor 3 (IRF3)/ type 1 interferon pathway, leading to macrophage transcription of inflammatory genes usually associated with viral infection. Interferon-inducible macrophages were termed "IFNICs"[15]. Global deletion of either the IRF3 or the type 1 interferon receptor gene improved post-MI recovery and survival compared to wild type mice[15], thereby indicating that mitigating this danger-sensing pathway rebalances the overall innate immune response to myocardial ischemia from one optimized for killing pathogens towards repair support. Although this is currently unknown, IFNICs may not only be harmful but also pursue additional, beneficial roles in wound healing such as removal of debris, regulating angiogenesis or matrix deposition. Targeting specific cell functions that are harmful may be an option to preserve those that are needed for recovery from MI.

As in generic wound healing, ischemia of the heart triggers a temporally defined inflammatory response that is dominated by divergent leukocyte phenotypes[44–46]. First, cardiac resident macrophages die alongside ischemic myocytes[7]. Massive cell death recruits neutrophils and inflammatory monocytes (Ly6C^{high} monocytes in the mouse[44], CD16^{low} monocytes in humans[47]). Surviving cardiac resident macrophages and monocytes recruited very early may participate in neutrophil attraction[48]. Since monocytes patrol the endothelium in steady state[49], they are likely present during the initiation of an inflammatory response to acute MI. Ly6C^{high} monocyte-derived macrophages dominate the leukocyte population even while inflammation resolves[46]. During the first three days after injury, macrophages assume inflammatory phenotypes, which later transition to promoting repair[50]. Flow cytometric analysis currently relies on the cell surface markers Ly6C, MHCII and CCR2 to identify macrophage subsets; however, this nomenclature is in flux. Unbiased single-cell RNA-sequencing suggests that, at least on a transcriptional level, there are up to seven infarct macrophage subsets, which can be identified using unsupervised clustering of gene expression in sorted CD45$^+$ leukocytes[15]. Among these subsets are the aforementioned IFNICs, which sense DNA from dying cardiomyocytes[15]. IFNICs are MHCII$^+$ CCR2$^+$ and currently the only subset of the seven mentioned above that has been examined using gene deletion[15]. Whether the other six subsets diverge functionally, and how stable macrophage subset characteristics are over time is currently unclear.

Such diversity of macrophage function, viewed together with other in vivo data[17,18,30,45,50,51], indicates that the frequently used M1/M2 subset framework does not fully describe cardiac or arterial macrophages. Further, cardiac macrophages do not always express the genes ascribed to each polarization state[30,45,50,51]. Even though recruited infarct macrophages proliferate locally[46], the vast majority of infarct macrophages are monocyte derived; thus, subdividing infarct macrophages by ontogeny is not always helpful. Unbiased, high-dimensional studies using CyTOF or single-cell RNA-sequencing are identifying novel myeloid subsets in blood[52] and cardiovascular tissues[17,18]. It is now essential to follow up

such studies by examining whether detected subsets differ functionally. Defining subsets is only constructive if they are functionally distinct. For instance, subdividing infarct macrophages should depend on their functional properties, e.g. interferon-inducible IFNICs[15], inflammatory or reparatory macrophages.

Failing myocardium

Cardiac inflammation is not limited to ischemic myocardium. Despite the wealth of studies describing innate immune pathways in the failing myocardium, cellular resolution of such data is only now emerging. Using flow cytometry, it is possible to isolate pure cardiac cell populations, and cell-specific promoters enable gene deletion in all major cell types, including macrophages. Sensitive flow cytometric leukocyte quantification reveals that even after acute MI, the non-ischemic remote myocardium recruits inflammatory cells, peaking around day 10 after ischemia in mice[53]. This process continues progressively as the left ventricle remodels. The local macrophage population expands due to both local macrophage proliferation and monocyte recruitment from the blood and hematopoietic organs[51]. Recruited macrophages' net effect on remodeling non-ischemic myocardium post-MI is likely detrimental, as using RNA silencing to inhibit monocyte recruitment, starting 1 week after MI, reduces left ventricular fibrosis and dilation[51].

Heart failure with preserved ejection fraction (HFpEF) is a condition with rising incidence and lack of treatment options. HFpEF does not compromise the contraction of the heart muscle but rather its diastolic filling with blood. In mice in which HFpEF is triggered by hypertension, aldosterone infusion and renal failure, cardiac macrophage numbers rise due to local macrophage proliferation and monocyte recruitment[32]. In addition, cardiac macrophage phenotypes shift, as these cells produce significantly more IL-10, an interleukin that promotes fibrosis. Macrophage-specific deletion of IL-10 improves diastolic dysfunction in mice, indicating that macrophages are causally involved in deleterious matrix buildup. However, macrophages likely continue to pursue salutary functions that promote myocyte health. For instance, macrophages provide angiogenic factors that regulate capillary density adaption to hypertrophy. It will therefore be important to therapeutically target a subset of detrimental macrophages, or, if such a subset remains elusive, to target only injurious macrophage functions. When choosing such targets, potential side effects are relevant. For instance, neutralizing IL-10 may lead to enhanced production of inflammatory cytokines.

While myeloid cells are the most numerous leukocyte population in the healthy, ischemic and failing myocardium, others, including mast cells[54] and lymphocytes, may either directly influence fibrosis, angiogenesis and hypertrophy or regulate macrophage phenotypes[55,56] and supply. Systemically expanding B lymphocytes trigger monocyte bone marrow release and recruitment via CCL7 in mice with MI[57]. This discovery led to a clinical trial (NCT03072199) that explores repurposing the CD20 B cell-depleting antibody rituximab, a drug approved for autoimmune disease and lymphoma, as a therapy for acute MI. In CVD, it is well known that non-leukocytes such as cardiomyocytes, fibroblasts and endothelial cells participate decisively in inflammatory processes either as targets of inflammatory activity or by enabling it. For example, activated endothelial cells recruit circulating leukocytes via

adhesion molecule expression[58]. Stromal cells may produce long-range signals that reach leukocyte production sites, as recently described for GM-CSF-producing fibroblasts[59].

LEUKOCYTE SUPPLY

The number of innate immune cells increases systemically in mice[24] and patients with CVD[60]. These leukocytes are recruited to tissues via chemokines and adhesion molecules expressed on activated endothelial cells[61]. Such endothelial cell activation may arise from increased sympathetic nervous system activity[58]. Once they take up residence in cardiovascular tissues, monocyte-derived macrophages proliferate[21,46,51] and assume inflammatory phenotypes that trigger atherothrombotic coronary occlusion and, consequently, MI. Likewise, inflammatory cell activity contributes to cardiac fibrosis and heart failure[19]. That circulating myeloid cells have life spans of a day or less[7,62] has motivated investigation of leukocyte supply, i.e. hematopoiesis, in CVD. In humans and mice with atherosclerosis or MI, upstream hematopoietic stem and progenitor cells (HSPC), instructed by changing bone marrow environments in the direct vicinity of HSPC, increase proliferation, acquire a myeloid lineage bias and accelerate the output of leukocytes that promote disease (Fig. 2)[5,8,9,11,21,51,59,63]. Hence, understanding how CVD changes steady state hematopoiesis (Box 3) may become the next frontier in conquering residual cardiovascular risk.

Hematopoiesis in atherosclerosis and after MI

Driven by circulating leukocytes' fast turnover[7,62], CVD's clinical association with leukocytosis[60] and a plethora of evidence that leukocytes propel atherosclerosis[24,61], immune cell production in mice with hyperlipidemia began to be explored a decade ago[10,64]. This work revealed a causal relationship between increased blood lipid levels and compromised reverse cholesterol transport out of HSPC[11] with accelerated myeloid cell production[8,10,64], which also occurs in the spleens of mice with atherosclerosis[9]. Studies in mice with coronary ligation identified lineage$^-$ Sca-1$^+$ Ckit$^+$ CD150$^+$ CD48$^+$ CCR2$^+$ HSC as the most upstream hematopoiesis activation point after acute MI[63].

One trigger for increased myeloid cell production after ischemia is sympathetic nervous signaling to bone marrow niche cells, which decreases HSPC quiescence and dampens retention signals in the hematopoietic niche[4,5]. Increased sympathetic activity also instigates higher leukocyte production in mice with heart failure[51]. Signals that accelerate myeloid cell output include Toll-like receptor ligands[63], GM-CSF[59] and IL-1β[65], the cytokine that was targeted in the CANTOS trial (Box 1)[1]. Parabiosis experiments, in which all blood-borne factors are shared between two surgically attached mice, indicate that factors circulating in blood alert the marrow after MI[59,63,65]. These circulating cytokines and growth factors act directly on HSPC and bone marrow niche cells that instruct HSPC proliferation, lineage bias and migration to the spleen (Fig. 2). In addition, dying heart cells release alarmins, which are recognized by HSPC and their progeny, into circulation. In mice[5,8,9], and perhaps in humans with CVD, myelopoiesis expands beyond the marrow into the spleen. Clinical data on splenic hematopoiesis in CVD currently rely mostly on ^{18}FDG positron emission tomography imaging[66,67]. ^{18}FDG is a radioisotope-labeled glucose which enriches in

metabolically active cells in the entire body. While this technique is not cell specific, it detects bone marrow and spleen activation in humans with acute MI[66,67].

Cardiovascular risk factors, comorbidities and leukocyte supply

In addition to hyperlipidemia, other cardiovascular risk factors may influence hematopoiesis (Table 1 and Fig. 1). Diabetes associates with increased circulating leukocytes in patients[68]. In mice, hyperglycemia enhances myeloid cell production[69,70] via neutrophil release of S100A8/S100A9, a ligand that binds the receptor for advanced glycation endproducts (RAGE) on HSPC. Diabetic mice lacking RAGE fail to develop leukocytosis[70]. Since neutrophil depletion reduced monocyte counts in diabetic mice, a self-enhancing vicious cycle that expands inflammatory myeloid cells has been proposed[70] in which increased myeloid cell levels further enhance myelopoiesis.

Diabetes also affects bone marrow niche cells[69]. Specifically, it reduces mesenchymal stem cells' responsiveness to β-adrenergic signaling and as a consequence the growth factor G-CSF. By impairing niche cells' ability to reduce the retention factor CXCL12 after G-CSF injection, diabetes inhibits HSPC mobilization from the marrow with consequences for the clinical transplantation setting[69]. While this explains why many diabetic patients are "poor mobilizers", the implications of diabetes for the hematopoietic niche, and hence on leukocyte production in CVD, are still mostly unexplored. In mice, long-term exposure to diabetes causes microangiopathy and may thus exhaust the HSPC pool[71]. Further strengthening the argument that metabolic syndrome may affect CVD via leukocyte production, mice with diet-induced obesity have increased myelopoiesis[72]. In obese mice, visceral adipose tissue macrophages release IL-1β into the systemic circulation. This cytokine then stimulates monocyte and neutrophil production by binding HSPC receptors[72].

Psychosocial stress is yet another CVD risk factor[73] contributing to inflammation. In mice, adrenergic signaling, which regulates hematopoietic circadian rhythms[74], shapes the hematopoietic niche environment after exposure to psychosocial stress. Acting via β3-adrenergic receptors on bone marrow niche cells, stress-induced noradrenaline lowers the quiescence-promoting cytokine CXCL12 and thus accelerates HSPC proliferation and leukocyte supply[6,74]. HSPC and mature leukocytes also express β-adrenoreceptors; thus, catecholamines may directly act on hematopoietic cells. Human data indicate that, as in mice, chronic stress activates hematopoiesis[67] and increases leukocyte blood counts[6].

Despite these promising beginnings, how CVD and its risk factors modulate hematopoiesis and blood cell composition and to what degree this determines outcomes in humans remain mostly unclear. For instance, aging, smoking and hypertension associate with increased myeloid cell counts and with CVD; however, we lack mechanistic studies into causal relations. Rheumatoid arthritis and other autoimmune diseases that associate with CVD may also partly exert their adverse influence on cardiovascular health via reshaping immune cell supply and leukocyte phenotypes. It is unclear if and how health-promoting behavior — or its absence — alters hematopoiesis; i.e. the influence of regular exercise, sleep, nutrition and microbiota on the hematopoietic system are mostly unexplored in the setting of CVD.

Clonal hematopoiesis

The odds ratio for developing CVD doubles in people with clonal hematopoiesis and even quadruples for early-onset MI[2,3]. In clonal hematopoiesis, HSPC deriving from one ancestor cell give rise to a variably large share of the blood leukocytes (Fig. 3). The phenomenon is a premalignant condition, and its association with CVD was discovered by studying the exome sequence in blood cells for genes that are mutated in leukemia[2,3]. For a person with clonal hematopoiesis, the risk of developing hematologic cancer is ten times higher, at about 1% per year, if the clone size is >20%[75]. If cancer develops, malignant cells carry the mutation previously observed in the clone, thereby indicating that the clone indeed gives rise to cancer. Loss of function mutations in DNMT3A, TET2, ASXL1, TP53 and JAK2, among other genes, are frequently found in the expanded cell clones. These driver mutations affect genes for epigenetic regulators of cell proliferation; the TET2 gene encodes an enzyme that catalyzes DNA hydroxymethylation[75], an intermediate stage towards demethylation. TET2 deletion increases both cell proliferation and the odds ratio for premature MI[3]. Some cases of clonal hematopoiesis are detected because of passenger mutations, i.e. mutations that may not confer a competitive advantage over other cells.

Clonal hematopoiesis strongly associates with increasing age. Hypothetically, this relationship may contribute to increasing cardiovascular risk in the elderly. According to exome sequencing of candidate driver genes, 20% of people older than 90 have clonal hematopoiesis, many of whom never develop cancer or CVD. Clonal hematopoiesis barely occurs in people younger than 40[75]. More sensitive whole genome sequencing puts the incidence even higher – at >50% – for the elderly[76]. Clonal hematopoiesis is further associated with male sex, ethnicity, smoking and type 2 diabetes. If the clone size affects 20% of blood cells, CT-derived coronary calcium scores, an imaging surrogate biomarker for coronary artery plaque burden, are higher while smaller clone sizes carry a lower risk for developing CVD[3].

The highest hazard ratio (12.0) for the risk of coronary heart disease was reported for mutation of JAK2[3]. Only for TET2, which is the second most frequently altered gene and also carries a particularly high odds ratio for premature MI, preclinical data establish causality: deleting TET2 increases atherosclerosis in mice[3,77]. One study examined chimeric mice in which a portion of the marrow was TET2-deficient[77], and a second investigated atherosclerotic mice with complete $TET2^{-/-}$ marrow replacement[3]. Both studies report increased aortic lesion size while circulating leukocyte counts remained unchanged. $LysM^{Cre}$-driven deletion of TET2 from myeloid cells and their hematopoietic progenitors likewise caused larger aortic plaques and inflammatory macrophage phenotypes, including increased production of, yet again, IL-1β[77].

Given that CVD's association with clonal hematopoiesis is a recent discovery, interesting questions remain open. For instance, some clinical and preclinical studies state that clonal hematopoiesis, despite increasing macrophage numbers in the arterial wall and other organs, may not lead to blood monocytosis[2,77]. The number of available data points in the clinical study[2] may be too small to exclude monocytosis for some mutations, and monocytosis could evolve at later time points. A whole exome sequencing study in Icelanders[76] does report an association of clonal hematopoiesis with blood monocytosis but does not mention an

association with CVD. Prior data document an association between leukocytosis and CVD in patients[60]. If there is a hematopoietic clone with an advantage, why does this not increase blood monocyte levels? Does this observation imply that unknown counter-regulatory mechanisms, which are disabled in individuals with leukocytosis, tightly control blood monocyte numbers? Interestingly, TET2 deficiency acts on two different processes: increasing HSPC proliferation and nudging plaque macrophages towards more inflammatory phenotypes[77]. The current working model is that HSPC lacking TET2 expand and give rise to monocyte clones and plaque macrophages that have a higher inflammasome activation and are thus more atherogenic. TET2 deficiency also impairs mouse recovery from acute MI[78]. We do not yet know what is causing the disease acceleration: clonal hematopoiesis, the inflammatory phenotype of the progeny or a combination of both. TET2 is currently the only mutation that has been tested for causally increasing atherosclerosis in mice. Future experimentation will determine if other driver mutations are also causal for CVD, and why. We must still decipher whether clonal hematopoiesis without driver mutations increases CVD risk, and what mechanisms lead to clonal hematopoiesis in the absence of a driver mutation. Finally, how clonal hematopoiesis affects other blood cells that promote CVD, especially neutrophils and platelets, should be explored.

TRAINED IMMUNITY

Whether the arterial wall and myocardium are inflamed depends not only on the number of locally present leukocytes but also on their phenotypes. Since most cells in an individual contain the same DNA sequence, the vastly different cellular phenotypes, for instance between an endothelial cell and a myocyte or an inflammatory monocyte-derived macrophage and a tissue-resident macrophage, are caused by epigenetic regulation of gene expression. Epigenetic DNA modification also regulates cell production rates, fate decisions during myelopoiesis[79] and phenotypes of mature macrophages residing in different tissues[80]. DNA methylation and histone tail modifications, such as methylation and acetylation, are the most well-studied epigenetic alterations that regulate DNA accessibility and, consequently, gene expression. Such epigenetic marks, which are passed on during cell division, are modulated by environmental stimuli.

Some cardiovascular risk factors represent environmental triggers that entrain memory to the innate immune system via epigenetic DNA modification. The trained immunity concept, which describes how monocytes' differential reactions to a recall stimulus depend on their prior exposure to (or "training" with) either LPS or β-glucan[81], components of the bacterial and fungal cell walls, respectively, has been extended to CVD-relevant signals. Oxidized LDL, long known to propel atherosclerosis by damaging the arterial wall, leads to epigenetic modification, specifically lower histone 3 lysine 4 trimethylation (H3K4me3), which renders monocytes more inflammatory upon a secondary stimulus[82]. Such epigenetic monocyte modification may enhance plaque inflammation. Investigating monocytes' long-lived hematopoietic progenitors and downstream tissue macrophages may reveal more durable effects. Indeed, $LDLR^{-/-}$ mice that switched from western type to normal diet retained epigenetic modifications in granulocyte-macrophage progenitor cells for weeks, resulting in more inflammatory innate immune cell phenotypes despite normalized blood cholesterol levels[83,84]. IL1β and GM-CSF mediate trained immunity on the HSPC level[84]. These

studies[83,84] considerably broaden the trained immunity concept, extending it to triggers such as cholesterol, cytokines and growth factors acting on hematopoietic progenitor cells rather than mature myeloid cells. The resulting epigenetic marks may persist in circulating monocytes and macrophages recruited to sites of inflammation, including atherosclerotic plaques.

Tissue-resident macrophages in cardiovascular organs could also be modulated by trained immunity, although this has not been tested experimentally. In the steady state, macrophages' tissue context-dependent phenotypes are a product of epigenetic modifications[80]. Likely, lifestyle related risk factors and inflammatory comorbidities affect macrophages in parallel, and these epigenetic modifications could have consequences for macrophage activity, tissue integrity and the individuals' propensity to develop CVD. Thus, even if exposure to cardiovascular risk factors ends, epigenetic modifications that may persist in HSPC and macrophages could serve as therapeutic targets to reduce inflammatory cells in the arterial wall and myocardium. Experiments using genetic disruption of the epigenetic mechanisms involved in trained immunity are needed to document the causality and specificity of connections between this compelling concept and the role of innate immune cells in CVD.

THERAPEUTIC STRATEGIES

Immunotherapy

The CANTOS trial is the first successful immunotherapy trial in CVD (Box 1). A neutralizing antibody (canakinumab) against IL1β, an inflammatory cytokine made by myeloid cells, reduced CVD events by 15%[1]. All cause and cardiovascular mortality declined by 31% in responders identified by a pre-specified decline in C-reactive protein (CRP), a circulating inflammation biomarker, after the first canakinumab dose[85]. This trial changes concepts of clinical cardiovascular care, which currently does not target inflammation. As the targeted cytokine is involved in epigenetic regulation of hematopoiesis, trained immunity[84] and clonal hematopoiesis[3,77], canakinumab's effects may extend to the phenotypes and numbers of systemic leukocytes.

CANTOS demonstrates that inflammation is a worthwhile drug target in CVD and provides a blueprint for future trials. The higher treatment efficacy in patients with drug-induced decline of CRP suggests that screening for patient subsets with high inflammatory activity is effective, as such screening achieves larger drug effects that can be detected in precisely defined, more economically sized patient populations. The concept of concise patient selection may be extended to other criteria, for instance presence of clonal hematopoiesis or of somatic mutations in circulating leukocytes. Embracing surrogate endpoints such as CRP or imaging of inflammation in cardiovascular organs, for instance by immuno-PET with radioisotope labeled antibodies or nanoparticles, provides another cost-saving strategy, which may afford more pilot studies, scaled 1–2 orders of magnitude smaller, and thus de-risk the following outcome trial. Such measures are commonplace in oncology[86], where imaging shortly after first drug application identifies non-responders. Likely, CANTOS will motivate a search for other safe, efficacious targets and other applications for canakinumab, for instance in the setting of acute MI, wherein the drug may not only reduce re-infarction,

which is particularly common during the inflammatory surge post-MI, but also improve myocardial healing. Preclinical studies document that IL-1β increases myelopoiesis after MI and that neutralizing IL-1β reduces post-MI heart failure[65]. Since CANTOS monitored hundreds of MI while patients were treated with canakinumab, safety concerns specific to infarct rupture are now addressed. CANTOS reported a higher rate of infections with canakinumab treatment. The experience with similar therapeutics in patients with autoimmune and malignant disease indicates that such risk can be managed, but it will likely require careful risk-benefit analyses and patient selection.

Supporting infarct healing

Timed myeloid cell depletion experiments in mice indicate that the phenotype transition between inflammatory and reparatory myeloid cells is functionally important for infarct healing[44]. Since delayed transition compromises tissue repair and recovery from MI, there is an ongoing effort to identify factors that usher in resolution of inflammation. These factors may include macrophage-intrinsic programs, macrophage phenotype alteration as a result of efferocytosis[87–90], autonomic tissue innervation[58,91], resolvins[92], macrophage interaction with lymphocytes[55] and/or continued provision of inflammatory Ly6C^{high} monocytes from hematopoietic organs[44]. Because reparatory macrophages support tissue rebuilding and regeneration (via VEGF, IL-10 and others), it is desirable to augment them. One way of doing so is to curb over-recruitment of inflammatory leukocytes, which improves outcomes in mice[93–95]. Alternatively, macrophages could be nudged towards reparative phenotypes by manipulating their gene expression[96]. In patients, heightened and prolonged inflammation associates with increased post-MI heart failure[97]. Theoretically, many options exist to dampen inflammatory activity or cell supply, including modulating macrophage phenotypes, inhibiting overproduction and decreasing cell recruitment.

Regenerating myocardium

Newborn mice regenerate injured myocardium[30], and anecdotal evidence indicates this may occur in newborn humans[98]. This observation suggests that if we can recreate the yet-to-be-discovered newborn conditions that give rise to myocardial regeneration in adult and elderly myocardium, we may be able to heal failing hearts. Because the immune system changes after birth, and macrophages are involved in many organs' homeostasis and repair, including salamander limb regeneration[99] and mouse hematopoiesis[100], investigating the cells' role in newborn heart repair may provide clues regarding how to therapeutically shape post-MI innate immunity. In mouse neonates, infarct macrophage numbers are higher, and their phenotypes differ from adults[30]. Macrophage depletion abolishes scar-free regeneration in newborn mice, which associates with a lower angiogenic response to ischemia[30]. Macrophages may also influence myocyte proliferation[16]. After myocyte necrosis, the mouse neonatal heart does not recruit monocytes but rather expands the MHCII^{low} CCR2^{-} resident macrophage subset by local proliferation[16]. Which unique properties of neonate macrophages support heart regeneration are not known, and their relative contribution to regeneration remains unclear. The data obtained in neonates support the hypothesis that post-MI progenitor cell therapy may modulate macrophages[101], since it is now commonly accepted that intra-myocardially injected progenitor cells do not survive. Phagocytosis of apoptotic cells, for instance neutrophils, dampens inflammatory macrophage activity[102].

Whether progenitor cells injected into the heart elicit a specific regenerative macrophage phenotype that is distinct from the one triggered by dying myocytes or neutrophils remains to be tested formally.

Technologies on the horizon

Emerging therapeutic technologies include gene editing and RNA interference to modulate immune cell production and phenotypes. Both methods were recently applied in promising preclinical studies[103] and early studies on human cells, including CRISPR gene editing in HSPC[104]. As HSPC are routinely collected and re-transplanted in humans, it is conceivable to edit HSPC to correct genetic diseases such as β-thalassaemia[104], to repair driver mutations causing malignancies or clonal hematopoiesis and to alter inflammatory genes in HSPC-derived leukocytes. Using non-viral delivery strategies[105], any gene in immune[95,106] or endothelial[58] cells, including multiple or previously difficult to reach targets such as transcription factors, could be edited, deleted or silenced in vivo.

At this time, the intersection of CVD and immunology is highly dynamic, with successful inroads being made from several directions. Clinically, CANTOS' proof of concept for CDV immunotherapy will inspire subsequent trials targeting inflammation. Fundamentally, the field of CVD research has identified and advanced on forward-looking problems including cell identity, source, fate, communication and subversion of steady state tasks by inflammatory responses that compromise cardiovascular health. Embracing these conceptual and technological advances will generate precise drug targets on a large scale. Historically disconnected research areas are closing ranks, as immunology and hematology increasingly focus on residual cardiovascular risk, and the rigorous standards of these fields, for instance how to identify leukocytes and their progenitors, are embraced across the board. Harnessing the improved understanding of immunity's role in oncology already saves lives. Immunotherapy is now poised to accomplish similar feats for CVD, as the required interdisciplinary effort emerges to tackle this task.

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Text box 1:

CANTOS (Canakinumab Antiinflammatory Thrombosis Outcome Study)

This trial[1] is the first convincing evidence that the inflammation hypothesis translates to improving outcomes in patients. It enrolled 10,061 patients with stable atherosclerosis and history of prior MI and a C-reactive protein (CRP) > 2mg/L. Patients were randomized to treatment with placebo, 50, 150 or 300mg canakinumab, an IL-1β neutralizing antibody, which was injected subcutaneously every 3 months. The treatment led to a 15% reduction of “hard” cardiovascular events and a 31% reduction of all-cause and cardiovascular mortality if the CRP declined below the median in response to the first treatment.

Text box 2:

What's new in the cellular tool box?

Mass cytometry (CyTOF) and single-cell RNA-sequencing of atherosclerotic plaque[17,18] and diseased myocardium[15] provide unbiased and much improved definition of cell subset repertoires in cardiovascular organs. These methods may identify cell subsets that are primarily harmful and thus targets for precision therapeutics.

Rapidly expanding optical imaging technology pushes the boundaries in image resolution, comprehensive sampling of large areas and the number of biomarkers sampled in parallel[107]. In particular, tissue clearing enabled whole organ microscopy in the human and mouse heart, artery and hematopoietic organs. As a consequence, spatial relationships between cells, for instance macrophages and conducting cardiomyocytes[14], or hematopoietic progenitors with stromal niche cells[108] that alter leukocyte supply in CVD, are revealed in large, representative tissue volumes. In vivo microscopy of cell-cell interaction in plaque[39], myocardium[109] and marrow[110] will provide dynamic information on proliferation, clonal cell expansion, leukocyte recruitment, departure, antigen presentation and phagocytosis, all processes that hold promise for therapeutic intervention. Completely noninvasive optical, magnetic resonance and positron emission tomography imaging of cardiovascular inflammation in mice and humans[107] are poised to disrupt drug development, as these modalities will report on drug targets days to weeks after therapy initiation, much earlier that traditional end points which accrue over years.

Gene editing[103,104,106] in immune and hematopoietic progenitor cells which eventually give rise to macrophages accelerates our ability to test the functional relevance of proteins in animals, and first human trials indicate potential translatability to human patients.

Text box 3:

Hematopoiesis

The bone marrow harbors hematopoietic tissues, which produce billions of blood cells every day. Some of these cells, especially if supplied in excess, promote CVD. Hematopoietic organs are also exceptionally well vascularized; however, very little is known about how the hematopoietic system adapts to and drives cardiovascular pathology. The marrow contains two major cell types: i) hematopoietic stem and progenitor cells (HSPC) that give rise to circulating blood cells and ii) niche cells that provide the environment in which HSPC thrive, proliferate and differentiate into blood cells. HSPC and their progeny resemble a pyramidal hierarchy with about 10,000 bona fide hematopoietic stem cells (HSC) on top[111]. HSPC are equipped with Toll-like, cytokine, growth factor and adrenergic receptors, which can sense the circulating signals that rise while CVD develops. HSPC also rely on niche cell-derived information passed on by bone marrow endothelial cells, macrophages, mesenchymal cells, osteoblasts and perivascular cells[112,113]. Upon integrating such signals, HSPC adapt their activity to either remain quiescent or produce mature blood cells. Which cells they give rise to depends on their lineage commitment, which they assume as a result of incoming signals and epigenetic modification[79].

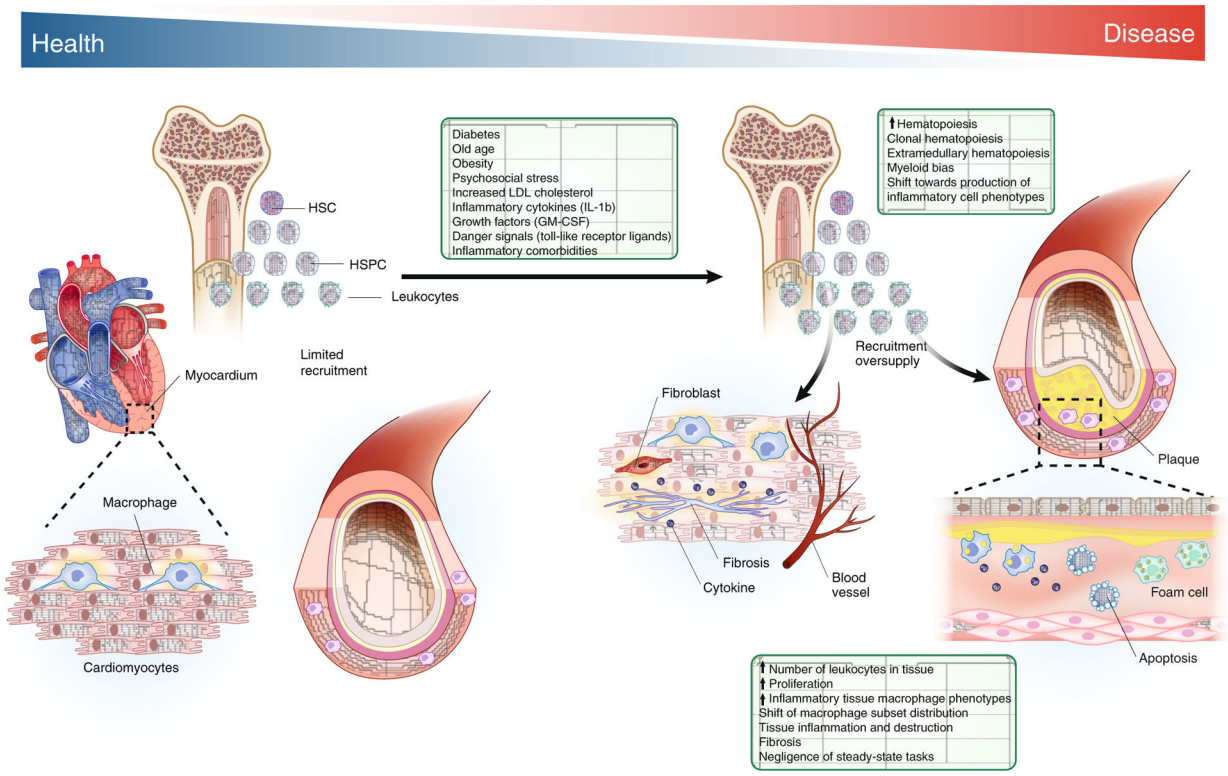

Figure 1: Hematopoiesis and CVD.

The cartoon summarizes interaction of risk factors for CVD and changes in innate immune supply that promote inflammation in the cardiovascular system.

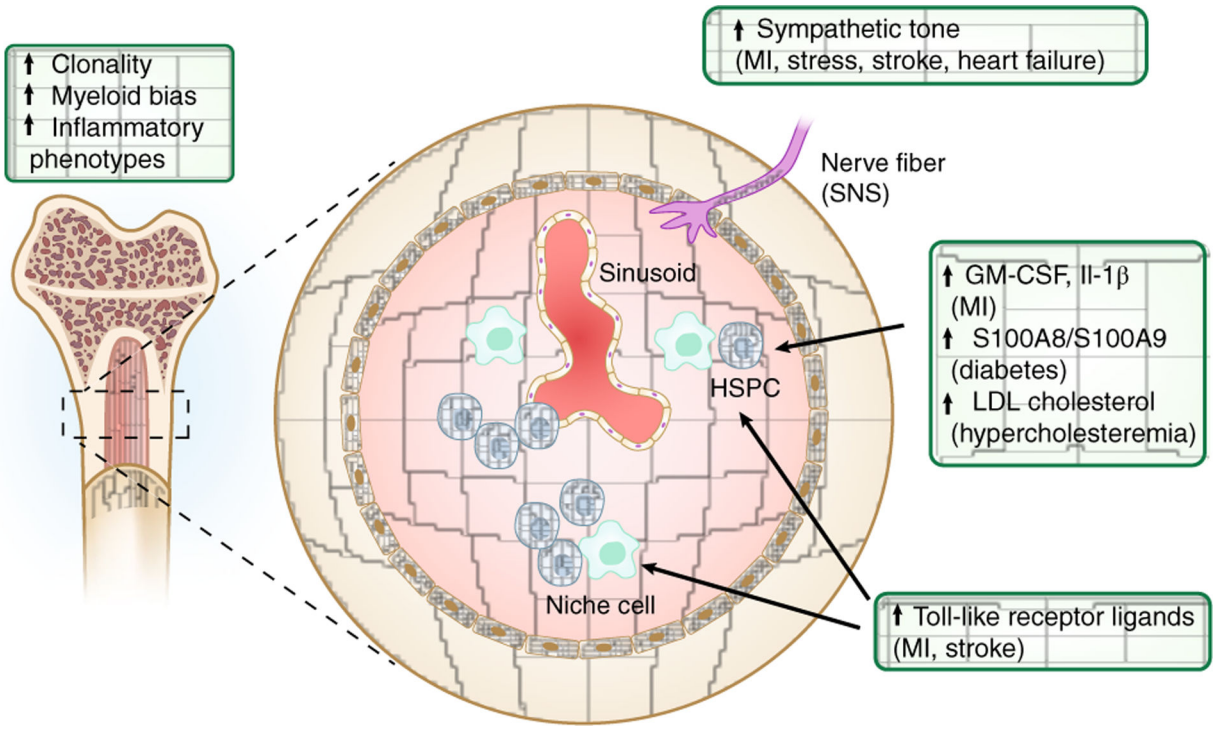

Figure 2: The hematopoietic niche and CVD.

The cartoon summarizes interaction of risk factors with the hematopoietic stem cell niche that senses systemic danger and regulates production of CVD-promoting leukocytes.

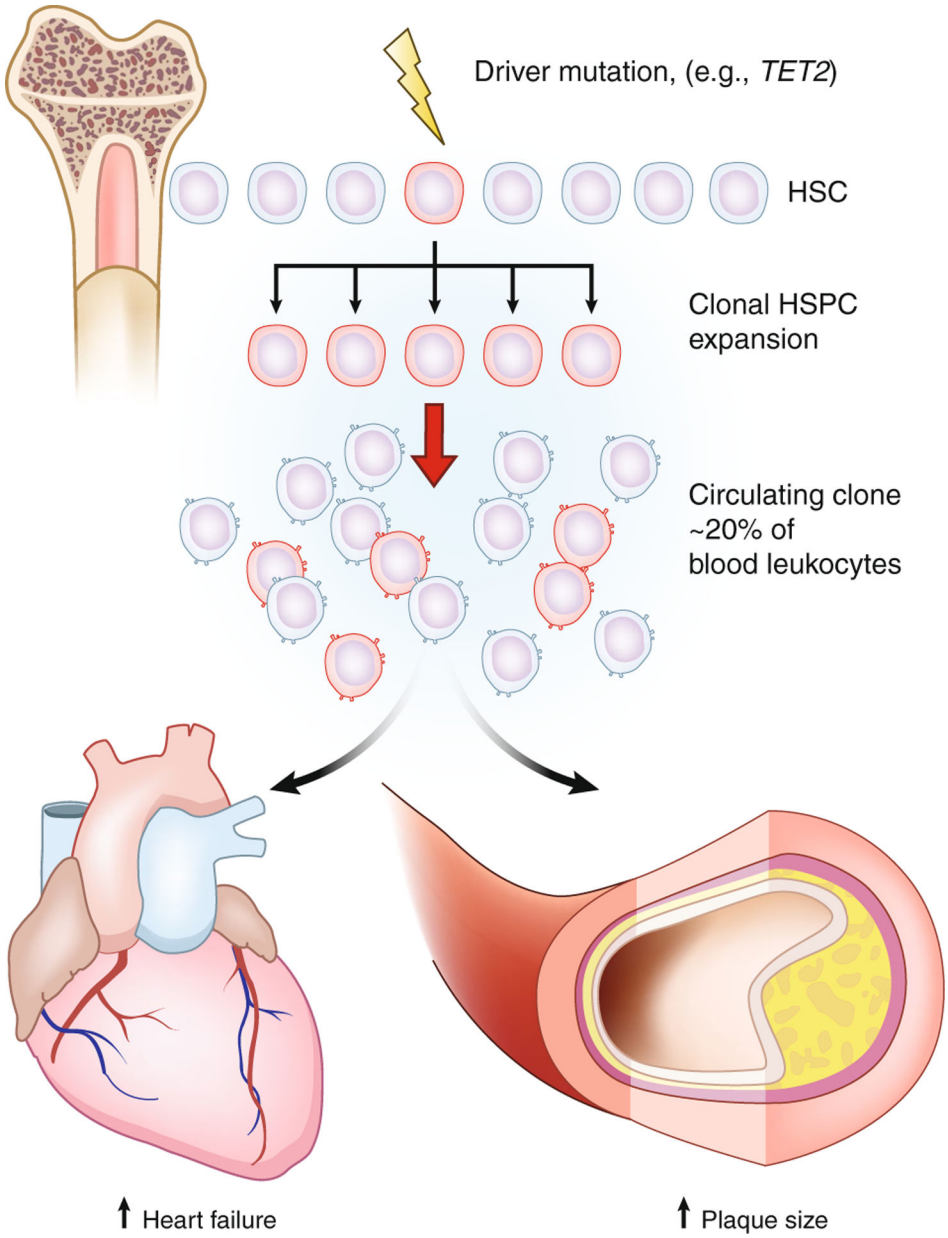

Figure 3: Clonal hematopoiesis.

A driver mutation, for instance in the gene Tet2, leads to expansion of a hematopoietic stem cell (HSC) clone, which gives rise to a variably sized leukocyte clone in circulation. Clonal hematopoiesis associates with higher incidence of MI in patients[2,3]. In mice, Tet2 deletion leads to larger, more inflamed plaques[3,77] and impaired recovery from acute MI[78].

Table 1.

Cardiovascular risk factors, current standard therapy and potential action through innate immune cells and their production (hematopoiesis).

	Modifiable?	Therapy	Link to innate immune cells
Hyperlipidemia	yes	Life style modification, statins, fibrates, PCSK9 inhibitors	Hematopoiesis ↑ (ref 10, 64), modulation of macrophage phenotype, trained immunity (ref 82)
Obesity	yes	Life style modification	Adipose tissue harbors inflammatory macrophages, hematopoiesis ↑ (ref 72)
Diet	yes	Life style modification	Via microbiome?
Hypertension	yes	Life style modification, antihypertensive drugs	Leukocytes ↑ in vascular wall and in the hypertrophic myocardium (ref 32)
Physical inactivity	yes	Life style modification	Via modulation of leukocyte phenotypes and hematopoiesis?
Psychosocial stress	yes	Life style modification	Hematopoiesis ↑ via sympathetic signaling, altered leukocyte number and phenotype (ref 6)
Smoking	yes	Life style modification	Leukocytosis and altered leukocyte phenotype?
Diabetes	yes	Diet, oral antihyperglycemic agents, insulin	Hematopoiesis ↑ via RAGE ligands etc. (ref 70)
Age	no	Currently none, possibly targeting aging hematopoietic immune system in future	Via clonal hematopoiesis (refs 2,3) and myeloid bias? Via altered tissue resident macrophage repertoire in heart (ref 26, 32) and vasculature?
Sex	no	n/a	Via sex hormone signaling to HSPC and leukocytes?
Family history/ genetics	no	Currently none, possibly gene editing in future	Via hyperlipidemia and possibly hematopoiesis etc.