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REVIEW

Cardiac resident macrophages: Spatiotemporal distribution, development, physiological functions, and their translational potential on cardiac diseases

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Abstract Cardiac resident macrophages (CRMs) are the main population of cardiac immune cells. The role of these cells in regeneration, functional remodeling, and repair after cardiac injury is always the focus of research. However, in recent years, their dynamic changes and contributions in physiological states have a significant attention. CRMs have specific phenotypes and functions in different cardiac chambers or locations of the heart and at different stages. They further show specific differentiation and development processes. The present review will summarize the new progress about the spatiotemporal distribution, potential developmental regulation, and their roles in cardiac development and aging as well as the translational potential of CRMs on cardiac diseases. Of course, the research tools for CRMs, their respective advantages and disadvantages, and key issues on CRMs will further be discussed.

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1. Introduction

Cardioimmunology is gradually receiving much-needed attention from cardiologists and biological scientists, and making breakthroughs to become an independent discipline[1]. Cardiac resident macrophages (CRMs) originate from the embryo and migrate to the heart before delivery[2]. The scRNA-seq analysis results show that they dominate the cardiac immune microenvironment in mice, accounting for approximately 7%−8% of non-cardiomyocytes[3,4] and 58.7% of $CD45^+$ cells, followed by B cells (18.2%), NK cells (8.6%), T cells (4.3%), monocytes (3.7%), and neutrophils (3.1%)[5]. During the past decades, CRMs research mainly focused on their roles in the repair and regeneration following cardiac injury[6,7], and their origin, maintenance, and renewal[8−10]. These topics have been extensively reviewed[11−14]. Recently, with the development and application of single-cell multi-omics technology and spatial transcriptomics technology, it has been found that CRMs can be educated by the specific microenvironment of different anatomical niches in the heart with unique phenotypes and transcriptional characteristics, and ultimately acquire specific functions. Our previous findings also showed that the composition of CRMs changes with age. Furthermore, more and more data also clarify the physiological function of CRMs[15,16]. Therefore, the present review will mainly focus on the spatiotemporal distribution of CRMs, explore their potential developmental regulation and their roles in cardiac development and aging, and their translational potential. Of course, the research tools for CRMs and their respective advantages and disadvantages will also be discussed. Furthermore, the review will also highlight the important issues that need to be addressed in future research on CRMs.

2. The highly heterogeneous spatiotemporal distribution of CRMs

With increasing age, the structure and microenvironment of the heart undergo continuous changes, and the phenotype of CRMs will also be reprogrammed. Our previously published research data showed that there are four consecutive subsets of CRMs with different phenotypes in the mouse heart during steady-state, namely $CX3CR1^+CCR2^-Ly6C^-MHCII^-$ (MP1), $CX3CR1^{low}CCR2^{low}Ly6C^-MHCII^-$ (MP2), $CX3CR1^-CCR2^+Ly6C^+MHCII^-$ (MP3) and $CX3CR1^+CCR2^-Ly6C^-MHCII^+$ (MP4). MP1 cluster is mainly enriched in the embryonic and neonatal stages, which is conducive to the proliferation of cardiomyocytes. MP2/3 cluster exhibits a relatively stable status throughout the life cycle. MP4 cluster is highly expressed in the hearts of adult mice[17]. Another perspective is that multiple subsets of CRMs are established at different stages of cardiac development. During the embryonic stage, a group of CRMs co-express the phosphatidylserine receptor T cell immunoglobulin and mucin domain containing 4, lymphatic vessel endothelial hyaluronic acid receptor 1 and folate receptor 2, but lack the expression of CCR2 and MHCII (TLF^+ CRMs). TLF^+ CRMs are mostly located near blood vessels, which may be conducive to their development[18]. MHCII is highly expressed in embryonic monocytes/macrophages ($MHCII^+$ CRMs), which are located close to nerves. Hematopoietic monocytes/macrophages appear in the heart after birth, and their CCR2 expression is increased ($CCR2^+$ CRMs). Nevertheless, the above two classification methods have a certain degree of overlap. MP1/2, MP3, and MP4 may correspond to the TLF^+, $CCR2^+$, and $MHCII^+$ CRMs subsets, respectively.

Generally, there are several cell subsets with different phenotypes in the microenvironmental niches of tissues and organs. Because of the tissue specificity in development and adulthood, and the specific function of microenvironmental anatomical niche, we believe that the strong heterogeneity of resident macrophages is inevitable[19]. Studies in multiple tissues and organs have confirmed this notion. For example, the resident macrophages in the lungs can be divided into alveolar macrophages and pulmonary interstitial macrophages, and pulmonary interstitial macrophages can further be divided into subsets according to more precise tissue localization. Alveolar macrophages, mainly located on one side of the lumen of the alveoli, are responsible for the turnover of pulmonary surfactant and the removal of dead cells in the alveoli. Pulmonary interstitial macrophages mainly exist in the interstitial space around the bronchial vascular bundle. Pulmonary interstitial macrophages around nerves are mainly involved in inflammation and antigen presentation, while pulmonary interstitial macrophages around blood vessels are mainly involved in wound healing and tissue repair[20−22]. Similar findings have been shown in tissues such as the spleen, brain, skin, and intestine[19,23,24]. The heterogeneity within these tissues is caused by the specificity of tissue structure and microanatomical niche. As the heart is a hollow muscular organ with a pumping function, divided into four compartments with different shapes and functions along with the atrioventricular septum, and pericardial cavity, each anatomical region is subjected to various pressure gradients and physical stimuli resulting in structural changes, which subtly shapes and coordinates heterogeneous macrophage populations[25]. Conceptually, four independent heart chambers may each have a resident macrophage subset with a specific phenotype and function. Nathan and colleagues have demonstrated significant chamber specificity in human CRMs. Among a total of 320 differentially expressed macrophage-related genes, the most significant differences are noted between the atrium and ventricle. Moreover, the macrophages in the right atrium are the most distinct, as they contain a subset of CRMs that is almost nonexistent in other chambers (94% of this subset is in the right atrium). By comparison, the difference between the CRMs contained in the left and right hearts is significantly smaller[26]. Considering the differences between cardiomyocytes in the atrium and ventricle, resulting in their unique physiological functions, electrical signal transmission system, and contractile characteristics, the local microenvironment shapes a highly heterogeneous CRMs subset. If the specific phenotype and function of CRMs in each chamber can be defined and analyzed, it will be a new opportunity and target for the treatment of cardiovascular diseases related to individual chambers. Besides, studies have shown that the mouse pericardial cavity is also a unique macrophage ecological niche, including $Gata6^+$ macrophages. The transcription profile of $Gata6^+$ pericardial CRMs is similar to the macrophages in the peritoneal cavity and pleural cavity, but it varies greatly between the CRMs in various cardiac chambers[27]. Another group of CRMs with distinct profiles are in the valve. $CD206^+$ CRMs are mainly located in the subendothelial co-fusion area, and $MHCII^+$ CRMs are located at the distal end of the aortic valve and mitral leaflet. Such distribution is maintained until adulthood in mice. Nevertheless, the roles that these CRMs can play in physiological states are not yet fully determined, and they may contribute to maintaining valve homeostasis, extracellular matrix remodeling, and damage repair[28,29]. Recent research by Hulsmans et al. shows that high-density CRMs are enriched in the atrioventricular node (AN) of the human and mouse hearts, more

densely than the left ventricle. Working through Cx43, they regulate the electrophysiological activity of cardiomyocytes, to control the electrical conduction between the atria and ventricles and ensure the coordinated contraction of these chambers (Fig. 1)[30]. Interestingly, according to the trajectory analysis, CRMs that settle in the ecological niche of the heart in a steady state are also likely to migrate into the infarcted area after the heart injury to respond to emergencies[27]. There is every indication that multiple subsets of CRMs with spatial heterogeneity exist in the heart, which are shaped into subsets with different phenotypes and functions. However, the specific details of the distribution and whether their sources and maintenance are consistent remain unclear. In general, the spatial heterogeneity of CRMs exists, which may provide clues for events in different physical ecological niches in the heart in the future.

3. The origin, maintenance, and transcriptional characteristics of CRMs

3.1. *The origin of CRMs*

Based on novel technologies such as genetic fate mapping and lineage tracing, we have gained a clear understanding of the origin of CRMs. CRMs generally have two independent origins: one is the self-sustaining and renewable macrophages established during the embryonic stage[31,32]. Specifically, during embryonic development, the precursors of CRMs can be divided into yolk sac progenitor cells and fetal liver monocyte progenitor cells, which come from various progenitor cells in different regions outside or inside the embryo, and they fill the heart in the embryonic stage[33]. From embryonic (E) day E7.0 to E9.0, macrophage precursors were seeded into the heart. From E12.5 to E17.5, the heart mainly receives monocyte progenitor cells from the fetal liver. The second type of origin is macrophages derived from monocytes that migrate to tissue. From E17.5 to adulthood, mature monocytes can replenish multiple macrophage lineages in different tissues. Compared with the first origin, the contribution of resident macrophages from the second one is relatively small, with a limited lifespan, and is mainly associated with pathological conditions[23,31,33].

3.2. *Renewal and maintenance of CRMs*

With more and more understanding of the macrophage lineage, under stable conditions, CRMs populations are expanded locally in the heart through *in situ* proliferation, and the contribution of blood monocytes to the CRMs in healthy hearts is relatively small[33]. It has also been suggested that there are specialized macrophage progenitor cells residing in the tissue[8]. Recent studies suggest that CRMs can be roughly divided into the following three categories according to the way of macrophage maintenance and renewal. Firstly, TLF$^+$ CRMs can almost completely replenish through *in situ* proliferation[34]. Despite some insights, the specific

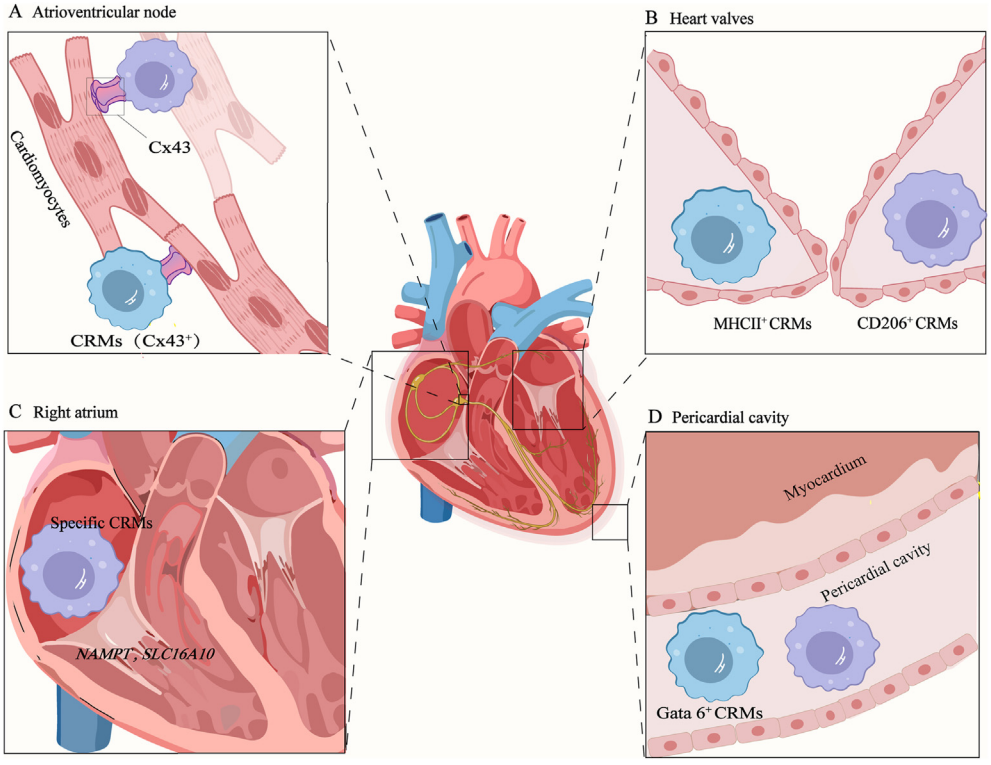

Figure 1 CRMs subsets residing in different anatomical niches. (A) Macrophages in the AN. High-density CRMs in the AN control the electrical conduction of cardiomyocytes *via* Cx43, thus controlling the contractile function of the heart. (B) CRMs in the heart valve. CRMs within heart valves can also continue to be subdivided, with CD206$^+$ CRMs located primarily under the endothelium at the region of coaptation, and MHCII$^+$ CRMs located at the distal end of the aortic valve and mitral leaflets, which may be associated with valve homeostasis and repair. (C) CRMs in the right atrium. Most notably, CRMs in the right atrium show strong genetic differences from those in other chambers, but the specific physiological functions remain unclear. (D) Macrophages in the pericardial cavity. The Gata6$^+$ CRMs in the pericardial cavity have a similar transcriptional profile with peritoneal and pleural macrophages. AN means atrioventricular node.

mechanism of CRMs self-proliferation remains incompletely understood. M-CSF has been proven to have a significant impact on macrophage proliferation. It not only strictly regulates the number of macrophages, but also is a requirement for maintaining the survival and function of macrophages[35,36]. Moreover, mice with CSF1R$^{-/-}$ exhibit a loss of resident macrophages in many tissues[37]. Studies have shown that, in the non-cardiac tissues, IL-4 can promote rapid proliferation of resident macrophages[38], which seems to be mediated by PI3K/AKT signaling[39]. Although IL-4R can directly promote macrophage proliferation, the regulation of macrophages by IL-4 has high environmental requirements[37,38], whether the same phenomenon exists in the heart remains to be confirmed. An interesting mechanism that has been identified to promote the proliferation of CRMs is the activation of the A-type scavenger receptor by binding to various ligands, including oxidized low-density lipoprotein, and bacterial components, and then induces intracellular activation of the cellular-myelocytomatosis viral oncogene (c-Myc: a proliferative gene) by coupling the downstream TAK1/p38/c-Myc/SIRT1 signaling pathway[10] (Fig. 2a).

Secondly, CCR2^{+} CRMs are likely to be replaced by the circulating monocyte[16]. Conversely, MHCII^{+} CRMs renewal is a compromise between the first two. Part of this group will be replaced by monocytes[16]. Although CRMs have highly proliferative potential in the heart of newborns, the proliferation rate decreases significantly with age[17]. TLF^{+} CRMs decrease, conversely, CCR2^{+} CRMs gradually increase, throughout their life MHCII^{+} CRMs almost unchanged, and only some are replaced by monocytes (Fig. 2b). We speculate that the composition of every subset of CRMs has a clear continuous evolution pattern that changes over time, and CRMs at different stages also have specific functional impacts on the heart. However, the spatiotemporal distribution pattern of CRMs is also worth discussing. Additionally, although CRMs exhibit high spatiotemporal heterogeneity, it remains unclear whether there is a similar change tendency of CRMs in different chambers of the heart. Of course, during different stages of cardiac development, even if the phenotype of CRMs is consistent, their sources may be different. Because during cardiac stress, recruited Ly6C^{+} blood monocytes settle within the heart, differentiate into all CRMs subsets, and subsequently expand and restore homeostasis[40]; even without inflammation or damage, in the postnatal heart development, embryonic-derived CRMs are also partially replaced by monocyte-derived macrophages[41]; which is one of the key reasons that CRMs have the same phenotype with different function. Additionally, tissue requirements also assign CRMs specific functions.

Furthermore, it remains controversial about the difference between embryonic-derived macrophages and peripheral monocyte-derived macrophages. Epelman et al.[40] and Cohen et al.[42] illustrated the differences by genetic fate mapping and found that the striking differences were in the expression of genes associated with inflammation and antigen presentation. MHCII^{+} CRMs can efficiently present antigens, whereas CCR2^{+} CRMs more highly express NLRP3-inflammasome-associated genes and

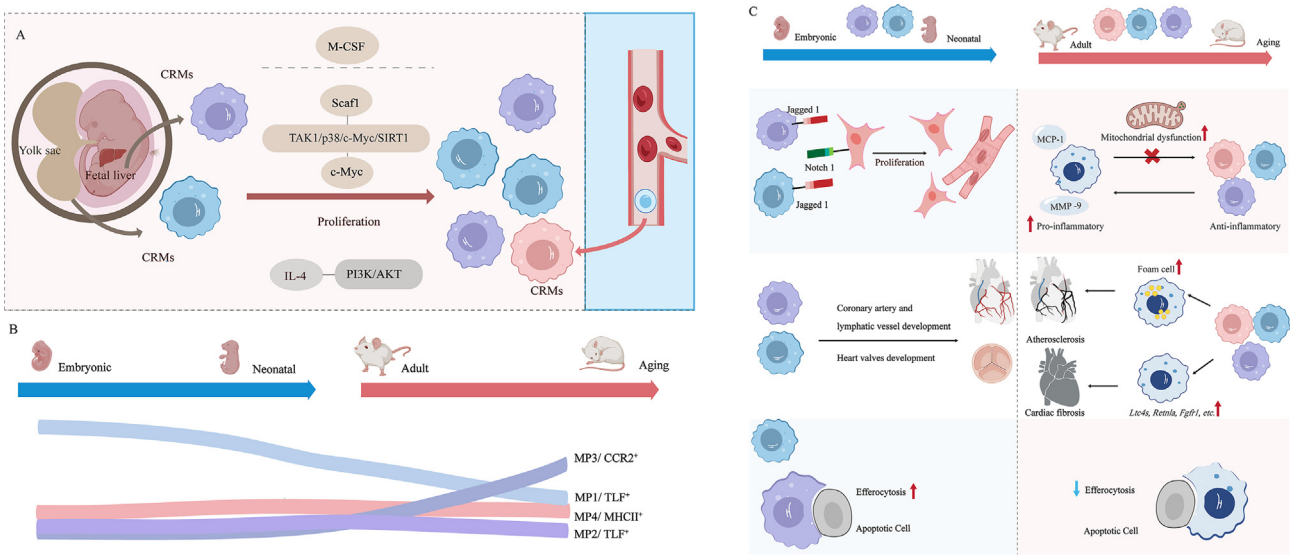

Figure 2 The changes of CRMs in life cycle, and their influences on cardiac development and aging. (A) The origin and maintenance of CRMs. Yolk sac and fetal liver-derived CRMs settle into the heart prenatally and can maintain renewal through self-proliferation. M-CSF and IL-4 can promote the proliferation of macrophages, additionally, SR-A1 binds to ligands, thereby activating c-Myc to promote CRMs proliferation. Macrophages derived from monocytes contribute relatively small to the heart at homeostasis. (B) The changes of CRMs in the life cycle. MP1 subset is the dominant group in the embryo and neonatal stage, MP4 dominates in the adult stage, MP2/3 subsets are throughout the life cycle. The tendency of MP1/2, MP3, and MP4 may be consistent with those of TLF^{+}, CCR2^{+}, and MHCII^{+} CRMs, respectively. (C) CRM's influences on cardiac development and aging. Yolk sac and fetal liver-derived CRMs promote the development of heart valves, coronary artery and lymphatic vessels, cardiomyocyte proliferation, and removal of apoptotic cells. Cardiac aging causes mitochondrial dysfunction, which eventually inhibits the reprogramming of pro-inflammatory macrophages to anti-inflammatory phenotypes, resulting in the release of pro-inflammatory mediators such as MMP-9 and MCP-1. Aging macrophages can also become a "foam cell" by phagocytosis of deposited lipoproteins. The expression of fibrosis-related genes in CRMs during aging was up-regulated, and the efferocytosis effect was significantly down-regulated. MP1-4 means CX3CR1^{+}CCR2^{-}Ly6C^{-}MHCII^{-}, CX3CR1^{low}CCR2^{low}Ly6C^{-}MHCII^{-}, CX3CR1^{-}CCR2^{+}Ly6C^{+}MHCII^{-} and CX3CR1^{+}CCR2^{-}Ly6C^{-}MHCII^{+}; Scaf1 means A-type scavenger receptor.

produce IL-1β following cardiac stress. TLF$^+$ CRMs can effectively phagocytose particles and metabolic waste and are involved in the scarless repair of damaged heart[40,42]. Ginhoux et al.[43] and Lichanska et al.[44] also demonstrated that yolk sac-derived macrophages have an evolutionally conserved characteristic among mammals, birds, and fish, settle and differentiate in tissues before the onset of blood circulation, and play important roles for embryonic development.

3.3. *Transcriptional characteristics of CRMs*

Anatomical location and cell origin are the two main factors determining the characteristics of macrophages[45]. The tissue microenvironment will shape a heterogeneous macrophage population, which achieves organ-specific functions by inducing the expression of a series of specific transcription factors[23]. For instance, after embryonic macrophages entering the lungs, embryonic macrophages destined to become alveolar macrophages upregulate transcription factors such as *Pparg*, *Klf4*, *Atf5*, and *Cebpb*[32]. Interestingly, the resident alveolar macrophages are partially replaced by monocytes in a damaged state and obtain the phenotype, but the transcriptional characteristics remain different, indicating that environment is not the only condition for shaping resident macrophages[21,46]. Therefore, we speculate that the demand of organ function may be another important reason. For the liver, the mRNA abundance of *Cd163*, *Marco*, *Ric3*, *Colec12*, and *Timd4* in Kupffer cells derived from the yolk sac was higher than that derived from bone marrow[47]. The expression of specific transcription factors by resident macrophages in a tissue niche is not a case in point. In the spleen, the transcription factor Spi-C is selectively expressed by red pulp macrophages, which help to engulf senescent red blood cells that have passed through the red pulp. Correspondingly, Spi-C$^{-/-}$ mice exhibit highly specific loss of red pulp macrophages[19,48]. Similarly, whether CRMs also selectively express transcription factors and perform specific functions remains to be explored. For example, pericardial CRMs express a larger number of genes related to protein and nucleic acid metabolism along with genes of all cavity macrophage populations, such as *Gata6* and *Icam2*[27]. On the other hand, CRMs express more genes related to structural tissue and inflammatory mediators. Intriguingly, CRMs in the AN and left ventricle exhibit similar expression profiles. They express both ion channel and exchanger genes (such as *Scan1b*, *Atp1a1*, *Cacna1c*, etc.). Microarray data also demonstrate that all of them express genes involved in electrical conduction[30]. Furthermore, differential gene expression analyses show that CRMs in the right atrium express specific genes such as *Nampt* and *Slc16A10*, which are different from all other CRMs[26]. However, their specific functions remain to be explored. Overall, the different ecological niches of the heart not only program CRMs with different phenotypes but also exhibit specific transcriptional features for exercising different biological functions. At present, it seems that there is still a lack of comprehensive and specific research to define and validate the CRMs contained in various cardiac niches, as well as to analyze their specific transcriptional characteristics and biological functions.

4. CRMs and heart development

4.1. *Embryonic heart development*

Cardiac development is a dynamic process that is strictly regulated through continuous differentiation and proliferation events[49]. Different lineages of embryonic macrophages exist in the developing heart and play important roles[50]. Our research shows that CRMs in embryonic and neonatal mouse hearts can directly promote cardiomyocyte proliferation through the Jagged-1−Notch1 axis, and they can significantly improve heart damage after myocardial infarction[17]. The development of the cardiovascular system, including the coronary circulation system and lymphatic network, is inseparable from the contribution of CRMs, which generally begin to develop around mid-pregnancy. Furthermore, the distribution of CRMs in the developing cardiac subepicardial space is consistent with the emergence of new lymphatic vessels, and macrophages are also closely related to the new lymphatic capillaries[51]. Other researchers also show that CCR2$^-$ macrophages derived from the yolk sac are necessary for coronary artery development. They use a lineage tracking system to demonstrate that embryonic LYVE1$^+$ CRMs are associated with neovascularization and regulate coronary artery development through insulin growth factor 1 signaling[50]. What's more, CRMs also have a significant effect on the development of heart valves. They exist in the endocardial cushion at the early stage of embryonic formation, helping to remove apoptotic cells. After gene knockout of CRMs, the heart valves of adult mice are thickened[52]. In addition, CRMs remove apoptotic cells by efferocytosis during embryonic heart development, to prevent fetal congenital heart block (Fig. 2c, left)[53,54].

4.2. *Cardiac development after birth*

Cardiomyocyte maturation undergoes a series of changes, including increased volume, cell cycle withdrawal, polyploidization, and a shift in metabolic mode from glycolysis to oxidative phosphorylation[55−57]. However, it remains unclear whether CRMs are involved in cardiomyocyte maturation. Sporadic reports show that depletion of CRMs or CRM-derived insulin-like growth factor-1 leads to the termination of adaptive growth of cardiomyocytes, which eventually progresses to cardiac dysfunction and adverse ventricular chamber dilation during hypertensive stress[58]. Few studies have suggested that the white blood cells present in mouse heart valves after birth are mainly macrophages and dendritic cells, and the number of macrophages significantly increases in the first few weeks of life: a critical period of valve elongation and physiological extracellular matrix remodeling[29]. All these data suggested that CRMs may play a crucial role in the maturation of the heart after birth (Fig. 2c).

4.3. *The cardiac aging*

Cardiac aging usually manifests by cardiac hypertrophy, high vascular permeability, chronic inflammation, and mild cardiac physiological impairment, which are the main risk factors leading to the increase of incidence rate and mortality of heart disease in the elderly[59]. It has been speculated that heart aging is an indirect effect of cardiac immune system changes[41]. Compared to young mice, CRMs in the elderly have a reduced reactivity to various pro-inflammatory or anti-inflammatory effects, and their efferocytosis is also significantly downregulated[60]. Although CRMs proliferate to varying degrees from embryonic to elderly stages, changes in their composition, gene expression, and function precede the damage caused by chronic inflammation after heart aging[41]. The enhanced chronic inflammatory state is a hallmark of heart aging, and a shift in macrophage phenotype to a pro-inflammatory type directly leads to an enhanced inflammatory response[60,61]. As to the roles of CRM in this process, Chiao et al.[62] studied the plasma markers of cardiac aging phenotype.

The results showed that most of the substances increased in the heart of aging mice were inflammatory markers related to macrophage function, including MMP-9 and MCP-1. The increase of the inflammatory markers was related to the increase of end-diastolic size during aging. In addition, an increase of macrophage density was found in the left ventricle of the aging heart, which serves as the main source of MMP-9. Fibrosis is also a sign of cardiac aging and analyses have shown that the expression of fibrosis-related transcripts in CRMs is upregulated in aging. The list of the transcripts includes *Ltc4s, Retnla*, *Fgfr1*, *Mmp9*, and *Ccl24*[41]. Aging macrophages may also show a "foam cell" phenotype by phagocytosis of deposited lipoproteins, as seen in the atherosclerotic plaque, triggering a range of heart diseases in the elderly[63]. They further found that immune system aging leads to organs aging throughout the body and transplanting young immune cells can ameliorate aging[64].

On the other hand, it has also been speculated that the alteration of macrophages occurs due to the aging of tissues and organs. Research has shown that metabolic changes brought about by aging can affect the phenotype and function of macrophages. The mouse macrophages with pro-inflammatory phenotype mainly rely on glycolysis metabolism, while the anti-inflammatory macrophages are more inclined to oxidative metabolism. Multiple evidence indicates that the acquisition of the anti-inflammatory phenotype in macrophages is associated with mitochondrial respiration, but aging causes mitochondrial dysfunction, thereby inhibiting the reprogramming of pro-inflammatory macrophages towards the anti-inflammatory phenotype, which represents a weakening of the anti-inflammatory phenotype. This ultimately progresses to chronic inflammation and aging. All of such processes seem to have a causal relationship with a positive feedback loop. Therefore, the metabolic changes caused by aging affect and limit the phenotype and function of macrophages[65–67]. Taken together, the aging of the heart and macrophages seems to be mutually influenced. The upregulation of pro-inflammatory phenotype and fibrosis genes in macrophages can cause cardiac aging, which in turn affects the phenotype and function of macrophages. Of course, it is also possible that these occur simultaneously (Fig. 2c).

5. The physiological functions of CRMs

5.1. CRMs and electrical conduction

The main function of the heart is to use the electrical conduction of cardiomyocytes to produce contraction and relaxation to pump blood and provide oxygen for other tissues and organs. Myocardial contraction is strictly regulated by excitation-contraction coupling, which converts electrical stimulation into muscle contraction in synchronous mode through different cardiac chambers[16]. CRMs form a network with cardiomyocytes and are at the key position of cardiomyocytes to exchange signals. The electrical conduction may form by the direct connection between CRMs and cardiomyocytes[68]. Connexin is mainly located in the "linker", and each linker connects to the complementary linker from the next cell, thus forming intercellular channel-mediated conductive conduction. Abnormal number, function, or location of connexin will damage electrical transmission[69]. There are three major connexins expressed in the heart: connexin 43 (α1 connexin), connexin 45 (α6 connexin), and connexin 40 (α5 connexin), of which Cx43 is the only connexin known to be expressed in adult myocardium[70]. Hulsmans et al.[30] demonstrate for the first time that CRMs are abundant in the AN. It is located at the bottom of the right atrium, to maintain the unique electrical conduction between the atrium and ventricle. Moreover, these atrioventricular lymph node CRMs will interfere with cardiomyocytes through Cx43 to accelerate cardiomyocyte repolarization and electrical conduction. Depletion of CRMs or Cx43 on CRMs delays and blocks the AN conduction in mice[12,30,33,62].

Furthermore, CRMs may be related to the occurrence of atrial fibrillation (AF). AF is a very common arrhythmia in the West. Pathogenesis of AF may be considered from both molecular and electrical perspectives. Clinical research indicates that electrophysiological disruption is the fundamental cause driving AF. Electropathology refers to electrical conduction disorders, also known as systolic dysfunction, caused by molecular changes in atrial tissue that drive structural changes, leading to AF. The imbalance of Cx40 and Cx43 is a decisive molecular mechanism of AF[71]. Systemic inflammation increases the risk of AF. The infiltrations of inflammatory cells such as macrophages and mast cells to the heart may be related to the changes in intercellular communication of the heart, triggering the accumulation of fibrous tissue in the atrial myocardium and the electrophysiological rearrangement of atrial cardiomyocytes, thus facilitating the occurrence and progression of AF[72]. Some studies have shown an *in vitro* crosstalk between macrophages and atrial myocytes. Additionally, rapid stimulation of atrial myocytes will lead to polarization of proinflammatory macrophage. Conversely, activation of macrophages with lipopolysaccharide will reduce the current and refractory period of Ca^{2+}, leading to an increase in the probability of arrhythmia of atrial myocytes (Fig. 3)[73].

5.2. CRMs and cardiac homeostasis maintenance

Recent studies have shown that CRMs make a unique contribution to cardiac homeostasis by efferocytosis[74]. Efferocytosis is a characteristic function that defines macrophages and can be mediated by multiple receptors alone or in combination. To this extent, myeloid epithelial generative tyrosine kinase (MerTK) and the milk fat globule epidermal growth factor are highly expressed on the surface of macrophages. MerTK is the potent phagocytic receptor, and can recognize PtdSer on dead/dying cells by binding to "bridging" molecules Gas6 and Protein S, resulting in dimerization of receptors with MerTK itself or other tyrosine kinase receptors (including Tyro3 and Axl). Ultimately, it triggers the activation of Rac-1 and the phagocytosis of target cells[75]. As well known, cardiomyocytes are long-lived, rarely renewed cells, subject to intense mechanical stress and metabolic demands, and eject large amounts of dysfunctional mitochondria and other cargo. CRMs will actively eat up the dysfunctional mitochondria and other metabolic waste, depletion of CRMs or deficiency in MerTK results in defective elimination of mitochondria from the heart, activation of the inflammasome, accumulation of anomalous mitochondria in cardiomyocytes, metabolic alterations, and ventricular dysfunction[76]. Some data also demonstrate that CRMs-derived legumain can benefit cardiac repair by promoting clearance and degradation of apoptotic cardiomyocytes by efferocytosis after myocardial infarction, legumain deficiency on CRMs leads to cardiac function exacerbation, accompanied by the accumulation of apoptotic cardiomyocytes[6]. Interestingly, *in vitro* co-culture system, the phagocytosis of primary macrophages on adult apoptotic cardiomyocytes was inefficient, which may be related to the cardiomyocytes-induced MerTK inactivation[77].

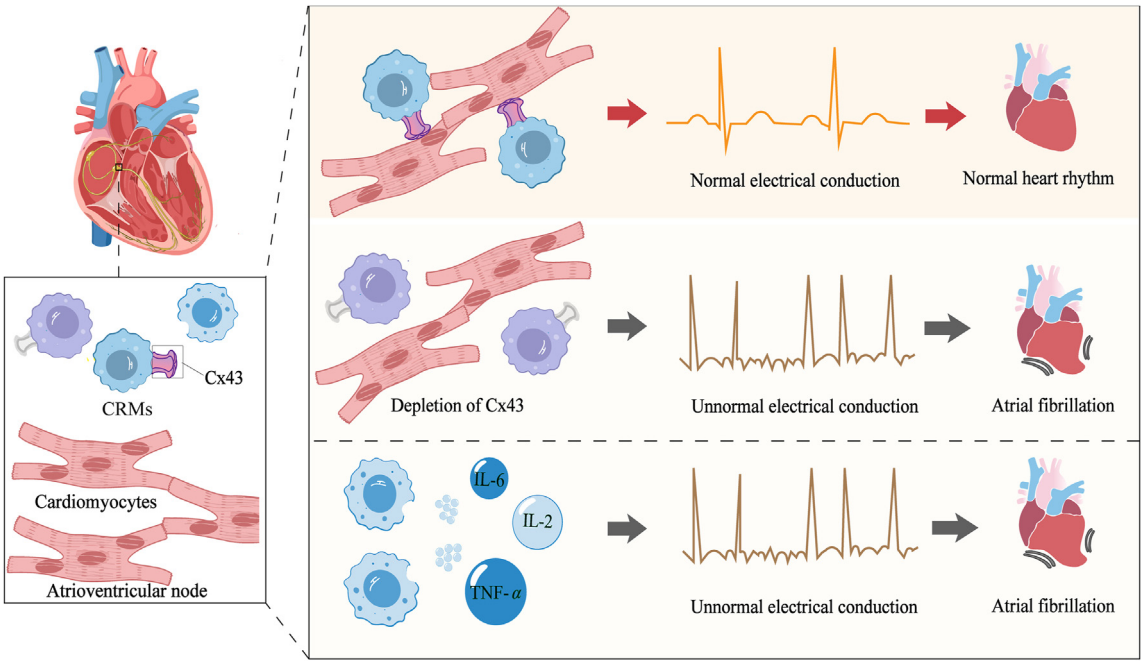

Figure 3 CRMs involve cardiac electrical conduction and their associated AF. CRMs interspersed between cardiomyocytes form a network, they are densely enriched in the AN and are at a key position for cardiomyocytes to exchange signals. They connect to cardiomyocytes *via* Cx43 to generate electrical conduction, which eventually converts electrical signals between heart chambers into muscle contractions. Depletion of Cx43 delays and blocks electrical conduction of the AN in mice, leading to AF. In addition, infiltrated macrophages produce inflammatory factors and result in an increased risk of AF. AF and AN mean atrial fibrillation and atrioventricular node, respectively.

6. The advantages and disadvantages of CRMs research tools and methods

Several exogenous or genetic methods are used to study the macrophages[78]. From the perspective of *in vivo* studies, to verify specific macrophage function, clodronate liposomes are used to knock out or deplete macrophages[79,80]. The efficiency of this method is limited to the steady-state myocardium. In contrast, transgenic mice that use macrophages to express diphtheria toxin receptors (DTR), such as CD169-DTR and CD11b-DTR mice, have some specificity in targeting depleted macrophages[81]. Although genetic methods provide more experimental options, the lack of organ specificity and the fact that most of the knockout mice are born with chronic immune system defects may have a negative impact on the experimental results[30,82]. Exploring the origin of macrophages can be achieved through fate mapping, which utilizes genetic recombination principles to permanently label cells based on the recombination-induced reporter genes. Genetic technology has now developed for induction systems regarding time-controlled recombination, which helps to accurately label embryonic populations and track them until adulthood. This approach, when combined with symbiotic and adoptive transplantation research, enables us to distinguish the relationship between macrophages and peripheral circulating monocytes. The limitation of fate mapping, also known as Cre-line, is that incomplete markers of monocyte and macrophage populations are usually obtained during embryonic development. Due to the limited duration and level of Cre expression, this approach may result in the inability of rapidly dividing progenitor cells to recombine[83]. The other limitation may be due to the leakage in some cells, which cannot ensure high specificity of targeted gene expression[81]. For example, $LysM^{Cre}$ mice are generally used to knock out target genes in monocytes/macrophages and neutrophils, but they also mistakenly knock out target genes in developing septal cardiomyocytes[84]. Fate mapping has preliminarily resolved the origin of CRMs. However, it cannot continue to provide answers about the spatiotemporal heterogeneity of macrophages. The single-cell multi-omics analysis[25,85] and spatial sequencing technology are being increasingly used[5]. Single-cell multi-omics techniques can elucidate gene regulation mechanisms at various omics levels. However, the information about spatial distribution is insufficient. Spatial sequencing techniques, including individual transcriptomics, epigenomics, and proteomics, can provide some information about the spatial distribution of resident macrophages. However, they may pose a challenge to integrate and link various mechanisms at the omics level with difficulty[86]. Although fate mapping and sequencing analysis may be used to primarily analyze the source and distribution heterogeneity of tissue-resident macrophages, they cannot ultimately determine the transcriptional characteristics and functions of macrophage subsets in specific tissue[26].

Recently, some relatively novel research models such as *in vitro* models of live myocardial slices and engineered heart (EHT) have been applied[87,88]. The cardiac tissue slice is considered an innovative multicellular *in vitro* model for advanced cardiac research. This model is cut off from the vascular circulation network and is not affected by migrating inflammatory cells. Therefore, it is used to study the response of CRMs to immune regulation and mechanical stimulation[87,89]. In addition, it can also be used to study the transmural electrophysiological differences between cardiac fibroblasts and myocardium[90,91]. EHT is constructed by 3D culture of cardiomyocytes through engineering technology, and the system can realize real-time analysis of heart function. At present, research has found that incorporating CRMs

into the EHT model can improve the contractility and electrical conductivity of the heart. Furthermore, the EHT model can help us understand the *in vitro* behavior of macrophages in complex multicellular humanized environments, providing convenience for *in vitro* experiments[12].

7. The potential clinical translation of CRMs in cardiac diseases

Cardiac diseases mainly include three aspects: inflammatory damage, cardiac fibrosis, and heart regeneration. Therefore, the potential translation of CRMs can be carried out from two aspects. Firstly, based on the high plasticity of CRMs, drug intervention, epigenetic modifications or delivery of specific regulatory genes can control the reprogramming of CRMs, and even develop therapeutic macrophage lines with specific functions *in vitro*. For example, epigenetic editing tools, such as CRISPR/Cas9, have been employed to precisely manipulate gene expression in macrophages in cancer diseases[92]. Furthermore, recent advancements in nanomedicine have led to the development of nanoparticle-based delivery systems designed to target immune cells selectively. These nanoparticles can encapsulate therapeutic agents, such as small molecules or RNA-based therapies, and deliver them to CRMs, thereby modulating their function[93–95]. In addition, the immune cell membrane can also be bound to nanoparticles to enhance their orientation and avoid being cleared[96,97]. This innovative approach holds significant potential for cardiac applications. While these methods have shown promise in preclinical studies and other disease contexts, their translation to cardiac-related diseases necessitates careful consideration. The unique microenvironment of the heart, coupled with the precise timing of interventions, may influence the efficacy of these approaches. Additionally, potential off-target effects and the need for long-term safety assessments underscore the complexity of implementing these strategies in clinical settings.

Secondly, targeting proinflammatory CRMs for selective elimination has emerged as a promising therapeutic avenue. Proinflammatory CRMs suicide can be induced through various strategies, but the challenge lies in achieving precise targeting while minimizing potential side effects. Clodronate liposome is a well-documented tool for depleting macrophages in various disease contexts[98]. In the context of heart disease, studies have explored its potential to selectively target proinflammatory macrophages. Research conducted by Haider et al.[99] demonstrates that liposomal clodronate administration in a murine model of cardiac injury leads to a reduction in proinflammatory macrophages within the myocardium. This reduction correlated with improved cardiac function and reduced fibrosis, highlighting the therapeutic potential of macrophage suicide in heart disease. Another approach could be blocking the growth factors of cardiac macrophages like CSF1/2 with specific antibodies or antagonists to induce faster apoptosis of pro-inflammatory macrophages or inhibit their proliferation is another direction worth exploring. While these approaches show promise, several challenges must be addressed for their clinical translation. Precise targeting of proinflammatory macrophages while sparing resident or anti-inflammatory macrophage populations remains a major hurdle. Additionally, the timing of drug administration is crucial, as interfering with macrophage function at inappropriate stages of cardiac repair may hinder healing and potentially lead to new inflammation or adverse effects.

The role of CRMs in heart diseases is increasingly recognized, and strategies targeting these cells hold great promise for therapeutic intervention. However, careful consideration of timing, potential side effects, and the complex interplay of macrophages in cardiac repair and inflammation is essential[100]. Lessons learned from other fields, such as oncology, provide valuable insights, and ongoing research is poised to unlock new avenues for treating cardiac diseases by modulating CRMs.

8. Key issues and highlights of CRMs future research

Over the past decades, significant progress has been made in the understanding of the heterogeneous distribution, ontogenesis, maintenance, and physiological functions of macrophages. However, there are still many key issues that have not been resolved. For example, the development process of highly heterogeneous CRMs with the spatiotemporal distributions of the heart remains unclear and requires a systematic and comprehensive detection and analysis, which is closely related to their physiological functions in the heart. Although it has been established that CRMs are involved in cardiac development, further research is needed to decipher their roles and mechanisms. The relationship between heart aging and the immune system aging represented by macrophages is also a question worth further exploration. Although there is a large body of research focused on acute cardiac injury[6,7,101], little is known about the role of CRMs in chronic cardiac damage. In addition, researchers have clearly shown that CRMs are associated with the repair of cardiac injury, and clinically relevant treatment strategies targeting them need to be developed.

The development of resident immune cell protocols has always been a key focus, and precise and cutting-edge research technologies and methods can bring breakthrough progress to the current research bottleneck. Recently, the important role of CRMs in cardiac homeostasis and diseases has attracted attention. However, due to the small number of CRMs in the heart and their susceptibility to polarization during *in vitro* culture[4,102], it is difficult to achieve the idea of targeting specific CRMs subset. *In vitro*, it is difficult to obtain enough primary cardiac CRMs directly, so it is often more dependent on bone marrow-induced or peritoneal-derived macrophages[7,85], which are prone to transcriptomic and epigenetic variations and have initial origin differences between them. None of the other sources of macrophages used for *in vitro* studies can truly represent CRMs. To avoid these limitations, CRMs can originate from induced pluripotent stem cells or human embryonic stem cells *in vitro* by providing tissue-specific clues or activating specific transcription factors[12,103].

In conclusion, in different regions of the heart, CRMs exhibit diverse phenotypic profiles and functions. These distinctions arise from the unique microenvironments and cues to which they respond. This inherent heterogeneity among CRMs presents a formidable challenge when contemplating strategies to target them for therapeutic benefit in the context of heart-related diseases. Precisely defining and characterizing these CRMs subsets, as well as deciphering their dynamic responses to pathological cues, are essential steps toward harnessing their potential for cardiac disease. Such understanding will enable the design of interventions that selectively modulate specific CRMs to promote cardiac repair while mitigating detrimental inflammation.

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Author contributions

Jing Jin, Yurou Wang, and Yueqin Liu collected the material and wrote the draft, Subrata Chakrabarti provided the ideas and edited the manuscript, Zhaoliang Su provided the ideas, funds and prepared the manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

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