

# Novel functions of macrophages in the heart: insights into electrical conduction, stress, and diastolic dysfunction

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Received 13 December 2018; revised 12 February 2019; editorial decision 5 March 2019; accepted 25 March 2019; online publish-ahead-of-print 3 April 2019

Over a century ago, Élie Metchnikoff described the macrophages' ability to phagocytose. Propelled by advances in technology enabling phenotypic and functional analyses at unprecedented resolution, a recent renaissance in macrophage research has shed new light on these 'big eaters'. We here give an overview of cardiac macrophages' provenance in the contexts of cardiac homeostasis and stress. We highlight the recently identified mechanism by which these cells regulate electrical conduction in the atrioventricular node and discuss why we need a deeper understanding of monocytes and macrophages in systolic and diastolic dysfunctions.

## Keywords

Macrophages • Monocytes • Myocardial infarction • Conduction • Diastolic dysfunction

## Macrophages and their precursors

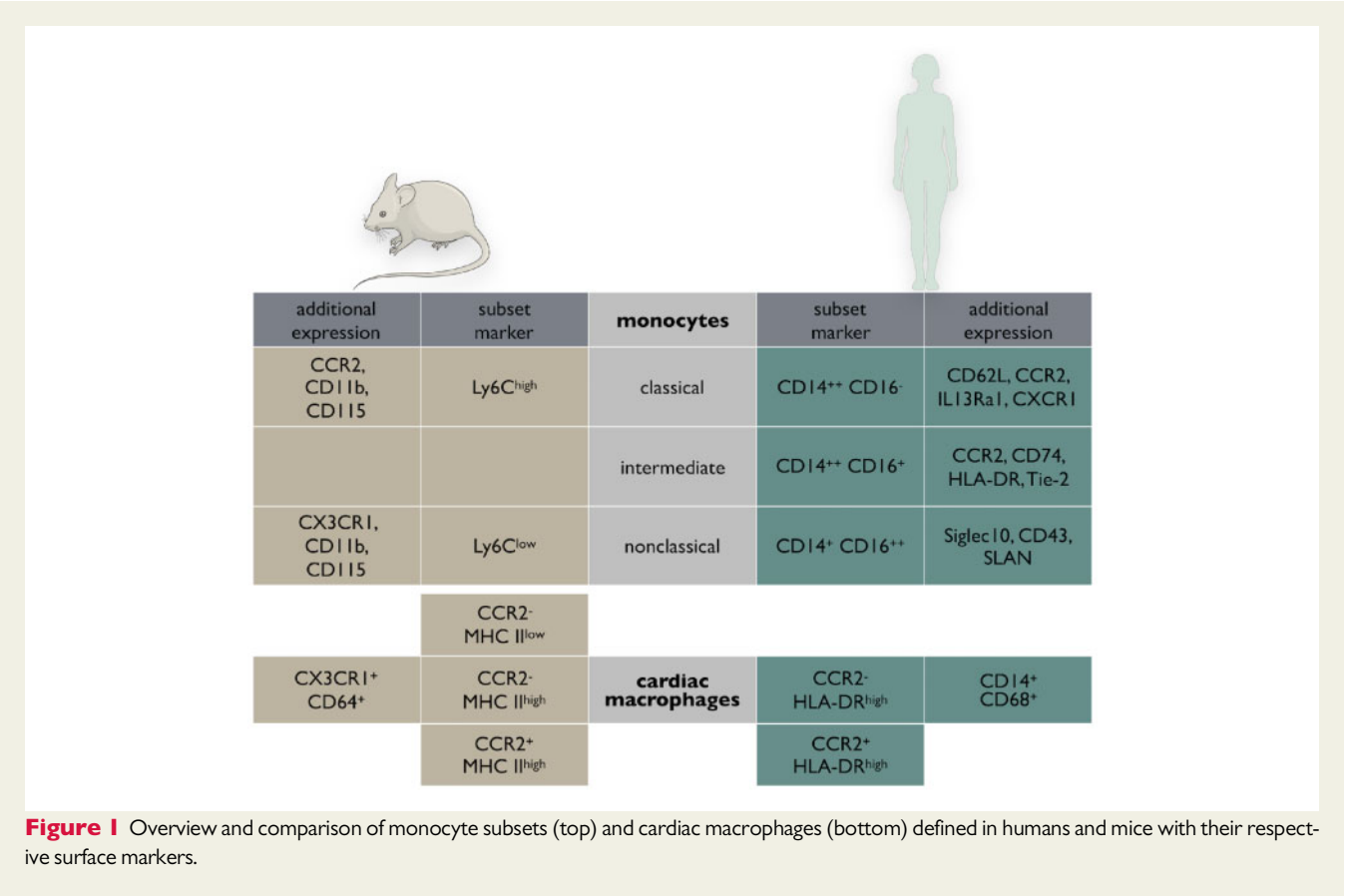
Macrophages are versatile immune cells strategically positioned throughout the body. They are crucially involved during organ development as well as tissue homeostasis and repair, and they exert multiple functions in inflammation.<sup>1</sup> In the 1960s, van Furth and Cohn<sup>2</sup> proposed that macrophages originate from circulating blood monocytes. An integral part of the vertebrate innate immune system, monocytes comprise 5–20% of peripheral blood mononuclear cells in humans and about 2–4% of blood leucocytes in mice. During embryonic development, monocytes are produced in the Foetal liver, and in adult haematopoiesis they arise from haematopoietic stem cells (HSCs) in the bone marrow. During this process, HSCs differentiate into common myeloid progenitors (CMPs), at which point they no longer express the surface markers CD117 (c-kit), Sca-1, and CD34. By increasing CD16/32, CMPs differentiate to granulocyte-macrophage progenitors.<sup>3</sup> Further differentiation steps can include generating a monocyte-macrophage dendritic cell precursor<sup>4</sup> and a common monocyte progenitor.<sup>5</sup> This linear development may vary depending on heterogeneity in precursor populations and earlier lineage commitment.<sup>6</sup> In addition, certain inflammatory triggers, such as

atherosclerosis or myocardial infarction (MI), trigger extramedullary monocyte production in the spleen.<sup>7,8</sup>

In humans, three monocyte subsets can be classified based on expression of CD14 and CD16: classical (CD14<sup>++</sup>CD16<sup>-</sup>), non-classical (CD14<sup>+</sup>CD16<sup>++</sup>), and intermediate (CD14<sup>++</sup>CD16<sup>+</sup>) monocytes.<sup>9</sup> Recent cytometer time-of-flight mass cytometry and transcriptional profiling by single-cell RNA sequencing (RNA-seq) suggest the intermediate population in particular appears to be substantially heterogeneous.<sup>10,11</sup> In mice, mature monocytes can be identified by their expression of CD11b and CD115 and distinguished by the surface marker Ly-6C (Figure 1). Ly-6C<sup>high</sup> chemokine (C-C motif) receptor-2 (CCR2)<sup>high</sup> chemokine (C-X3-C motif) receptor-1 (CX3CR1)<sup>low</sup> monocytes, which are considered the murine equivalent to human classical monocytes, preferentially accumulate at sites of inflammation. Ly-6C<sup>low</sup> CCR2<sup>low</sup> CX3CR1<sup>high</sup> monocytes, on the other hand, patrol the vasculature via LFA-1 integrin, remove damaged endothelial cells and maintain vascular integrity and homeostasis. "Non-classical" Ly6C<sup>low</sup> monocytes differentiate from Ly6C<sup>high</sup> monocytes, in a process that depends on Nr4a1,<sup>12</sup> and have previously been described as 'vascular macrophages'. Transcriptionally, however, they cluster with monocytes lacking core macrophage transcripts such as Mer tyrosine kinase (MertK).<sup>13</sup>

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**Figure 1** Overview and comparison of monocyte subsets (top) and cardiac macrophages (bottom) defined in humans and mice with their respective surface markers.

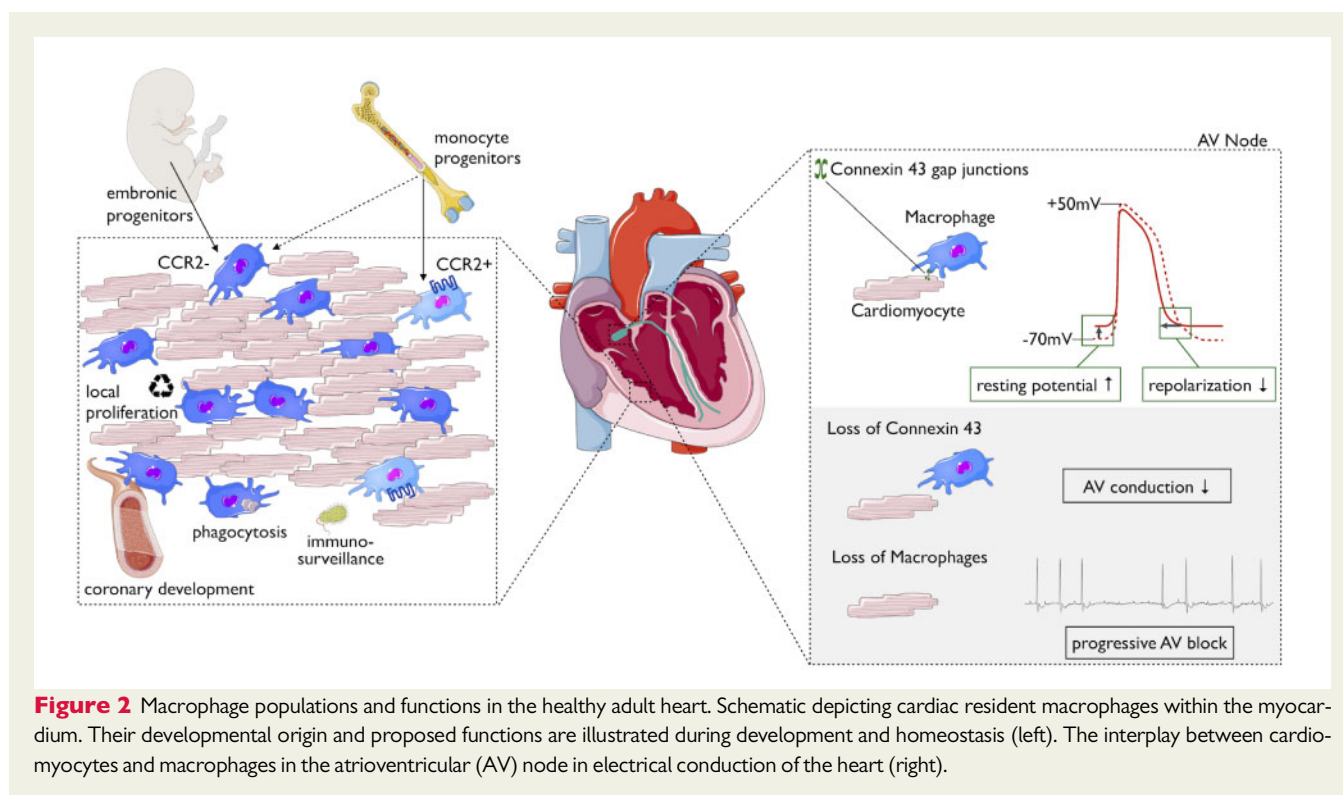
Genetic lineage tracing in mice revealed that macrophages can also arise independently from monocytes: FMS-like tyrosine kinase 3 (FLT-3), also known as CD135, is a cytokine receptor expressed on definitive but not primitive macrophages. While the latter are progeny of primitive haematopoietic progenitor cells that are present in the embryonic yolk sac, definitive haematopoietic progenitor cells can be found in both embryos (yolk sac, foetal liver) and adults (bone marrow and spleen).<sup>14</sup> Experiments involving Flt3-Cre mice demonstrated that the heart (and a variety of other organs) contains macrophages originating from both primitive and definitive haematopoietic lineages.<sup>15</sup> In addition, Mass *et al.* recently demonstrated that during pre-natal development, erythro-myeloid progenitors generate ‘pre-macrophages’ (pMacs), macrophage precursors that simultaneously colonize different organs of the whole mouse embryo and then derive their tissue-specific identity locally.<sup>16</sup>

Macrophages are best defined by their function (e.g. phagocytosis, immunity); surface (e.g. CD68, F4/80) or transcriptional markers (e.g. MertK); morphology (e.g. phagosome inclusion) or location in the investigated organ.<sup>17</sup> As they are highly plastic, the widely used M1/M2 subset classification, which is certainly applicable for the *in vitro* conditions it was defined by, holds a number of shortcomings when it comes to *in vivo* macrophage subsets and phenotypes. In order to develop a classification that better captures *in vivo* phenotypes, a common macrophage-activation nomenclature was recently proposed that suggests the description of macrophages based on origin, activation, and a collection of molecular markers.<sup>18</sup> Such markers may include transcription factors, SOCS proteins, chemokines, cytokines,

scavenger receptors, or amino acid metabolism.<sup>18</sup> While this novel macrophage activation terminology makes cell descriptions more granular, its inherently necessary simplification does not fully capture macrophage phenotypes *in vivo* either. In tissue, macrophage differentiation may be based on whether macrophages are resident or monocyte derived. Resident macrophages from different tissues may exhibit fundamental functional differences, in part driven by local input emanating from the specific organ of residence.<sup>13,19</sup> Further, a distinction depending on pro-inflammatory vs. reparatory macrophage functions appears quite useful, in particular in the context of myocardial healing.

### Cardiac resident macrophages

In the murine heart, macrophages constitute 7–8% of the cells that are noncardiomyocytes.<sup>20,21</sup> Macrophages intersperse the entire heart, where they closely associate with vessels and are enriched in the conduction system.<sup>22</sup> Spindle-shaped cardiac resident macrophages can be distinguished by their expression of major histocompatibility complex (MHC) II and CCR2. During murine embryonic development, the first macrophages seed in the epicardium (around E11.5).<sup>23</sup> They are derived from primitive yolk sac progenitors, express low levels of MHC II and CCR2 and may impact later stages of coronary vasculature development.<sup>24</sup> From E14.5 on, MHC II<sup>low</sup> CCR2<sup>high</sup> macrophages infiltrate the endocardial surface.<sup>24</sup> This macrophage subset’s function in the heart is currently unidentified.



**Figure 2** Macrophage populations and functions in the healthy adult heart. Schematic depicting cardiac resident macrophages within the myocardium. Their developmental origin and proposed functions are illustrated during development and homeostasis (left). The interplay between cardiomyocytes and macrophages in the atrioventricular (AV) node in electrical conduction of the heart (right).

Both described cardiac macrophage populations postnatally up-regulate their MHC II expression, and with age, the number of macrophages that express high levels of MHC II further increases. Shortly after birth, an additional CCR2<sup>-</sup> population accumulates in the heart. This Flt3<sup>Cre</sup><sup>+</sup> population definitively originates haematopoietically and presumably descends from foetal monocytes.<sup>15</sup> The healthy adult mouse heart contains at least three distinct macrophage populations: (i) CCR2<sup>-</sup> MHC II<sup>low</sup>, (ii) CCR2<sup>-</sup> MHC II<sup>high</sup>, and (iii) CCR2<sup>high</sup> MHC II<sup>high</sup>. Monocytes, meanwhile, are identified as CCR2<sup>high</sup> MHC II<sup>low</sup>.<sup>25</sup> The two CCR2-negative macrophage subpopulations stem from embryonic origins and are maintained throughout life by local proliferation, independent from blood monocytes.<sup>15,20</sup> In contrast, the CCR2-positive subset detectable in the adult heart is derived from and maintained by the influx of blood monocytes (Figure 2). The CCR2-positive subpopulation is small (5–15%) in healthy hearts. Elegant work has recently confirmed that analogous CCR2-positive and CCR2-negative macrophage populations are also present in human hearts.<sup>26</sup>

## Cardiac macrophages facilitate electrical conduction

Our knowledge of cardiac tissue macrophages' remains limited. In the steady state, cardiac resident macrophages are thought to serve as sentinels for injury and circulating infectious agents, such as bacteria.<sup>27</sup> MHC II<sup>high</sup> macrophages are considered particularly crucial for cardiac immune surveillance and adaptive immune responses,

while MHC II<sup>low</sup> macrophages support tissue homeostasis by removing material shed by surrounding cardiomyocytes and fibroblasts and adapting to altered tissue strain.

Gene expression analysis comparing cardiac macrophages to their counterparts from the spleen and brain revealed enriched genes involved in angiogenesis and immune quiescence.<sup>21</sup> However, we still lack functional *in vivo* data that clearly define cardiac macrophages' role in these processes for the steady state heart. It is increasingly clear that macrophages can endorse tissue-specific functions in other organs, such as modulating norepinephrine levels in adipose tissue<sup>28</sup> or alveolar macrophages removing surfactant in the lungs.<sup>29</sup> Brain macrophages (microglia) actively contact synaptic clefts and other neural components.<sup>30</sup> Direct cell–cell interaction was found in both human and murine hearts where macrophages connect with cardiomyocytes by forming connexin 43 (CX43; also known as GJA1)-containing gap junctions.<sup>22</sup> This enables electrical coupling and impacts both cell types: patch clamp analysis revealed that macrophages rhythmically depolarize when they are coupled to cardiomyocytes and cardiomyocytes show a more positive depolarized resting membrane potential in co-culture with macrophages. Further, a coupled cardiomyocyte's action potential has lower upstroke and overshoot, which leads to earlier repolarization and a shorter action potential and refractory period. Histological analyses show that one cardiomyocyte may couple with up to four macrophages, and mathematical modelling suggests that macrophages' influence on conducting cells increases with their ratio to cardiomyocytes.<sup>22</sup> Optical clearing of hearts showed more macrophages in the atrioventricular (AV) node and around other conduction system structures. Macrophage-specific genetic ablation of connexin 43 delayed

conduction through the AV node, and depletion of CD11-positive cells in CD11b DTR mice resulted in progressive AV block. These data demonstrate that macrophages are required for normal conduction in the AV node. Thus far, we do not know if a specific cardiac macrophage subpopulation is enriched for connexin 43, nor whether stress, inflammatory conditions or aging may impact macrophage function in conduction.

Another intriguing question is if resident macrophages are causally involved in the pathophysiology of atrial fibrillation (AF). Several studies in humans have described an association between inflammation and AF. Inflammatory conditions, such as rheumatoid arthritis or sepsis, may trigger AF.<sup>31,32</sup> In small autopsy series, histological analyses of atria from patients with AF showed increased numbers of monocytes and macrophages compared with control samples. In addition, levels of interleukin (IL)-6, IL-8, and tumour necrosis factor (TNF) were increased in tissue of AF patients.<sup>33,34</sup> In a prospective, placebo-controlled clinical study with 104 patients, administration of glucocorticoids reduced the recurrence of AF.<sup>35</sup> Other clinical trials have reported conflicting results and mechanistic insights are still scarce. Therefore, further research is warranted. In a murine model of lipopolysaccharide-induced AF, depletion of macrophages with clodronate liposomes reduced the inducibility of AF.<sup>36</sup> The description of spontaneous AF in certain mouse strains and in particular the increased AF inducibility in certain transgenic mice may fuel further investigation in this area.<sup>37</sup>

## Events that perturb the heart's leucocyte niche

The composition of resident cells changes as the heart ages. Over time, monocyte-derived cardiac macrophages' contribution increases, and it has been suggested that CCR2<sup>high</sup> monocytes may differentiate into CCR2<sup>low</sup> macrophages.<sup>38</sup> General immune system changes that come with age—also termed 'inflammaging'—may result in myeloid bias, impaired tolerance, or cytokine dysregulation, which all can impact cardiac macrophage homeostasis. In addition, drastic alterations in cardiac cell balance follows events such as injury, infection, and haemodynamic stress (Figure 3).

## Myocardial infarction

Ischaemic injury induces forceful changes in cardiac cell composition. Upon coronary occlusion and subsequent cell death, cardiac resident macrophages and cardiomyocytes produce and secrete pro-inflammatory cytokines and chemokines that trigger myeloid cell production and recruitment of these cells to the infarcted heart.<sup>39</sup> CCR2<sup>+</sup> macrophages mediate neutrophil extravasation in mice by producing the chemoattractants CXCL (C-X-C motif chemokine ligand) 2 and CXCL5.<sup>40</sup> Intravital 2-photon microscopy of beating hearts revealed that monocyte recruitment occurs as early as 30 min after the onset of ischaemia.<sup>41</sup> The primary monocyte subset recruited to the mouse heart are Ly-6C<sup>high</sup> cells that infiltrate in response to up-regulation of monocyte-chemoattractant protein-1 (MCP-1, CCL2).<sup>42–45</sup> Macrophages already present in the heart greatly impact monocyte accumulation: while tissue-resident CCR2<sup>+</sup> macrophages promote recruitment via a MYD88 dependent mechanism, resident CCR2<sup>+</sup> macrophages can hinder monocyte invasion.<sup>46</sup>

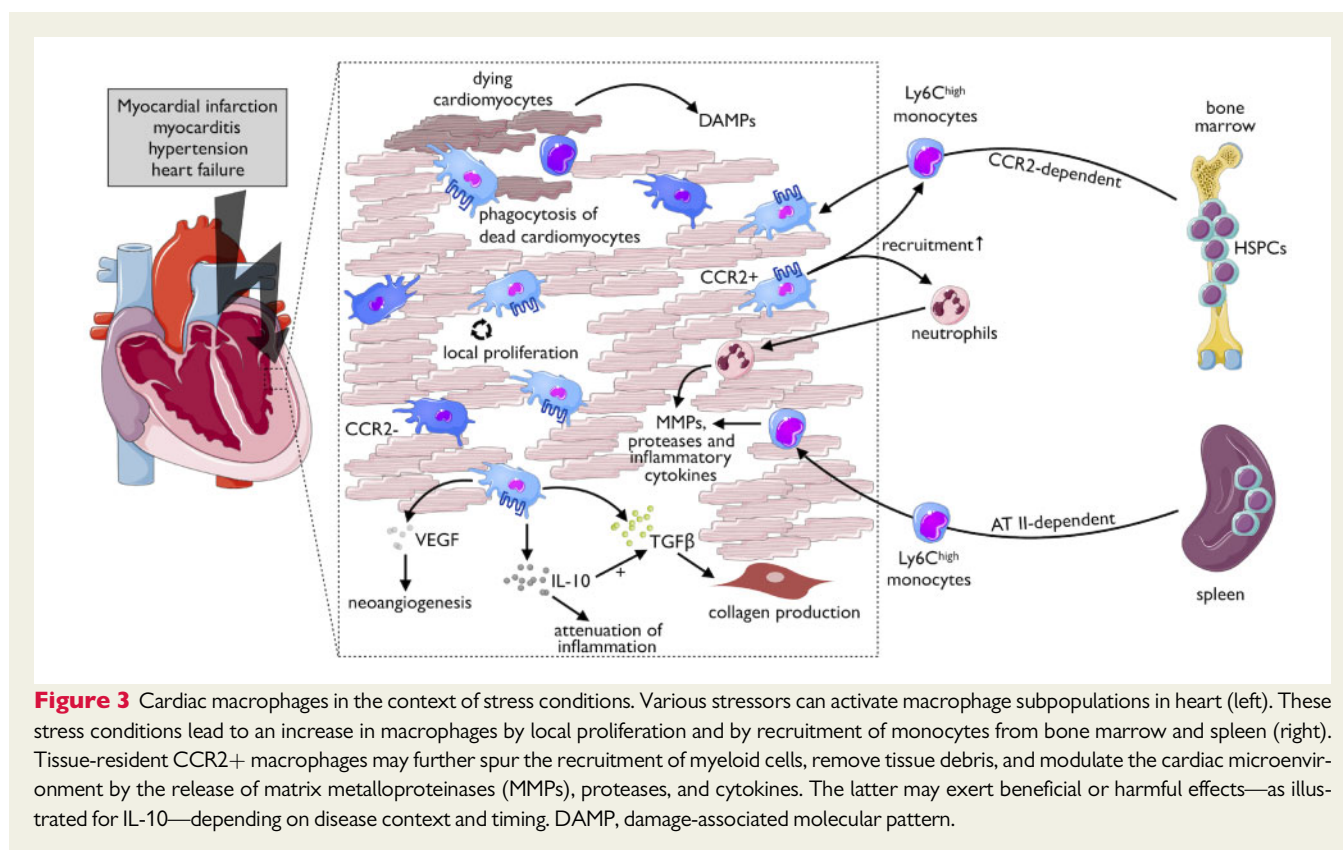
Shortly after ischaemia, cardiac fibroblasts locally produce granulocyte/macrophage colony-stimulating factor (M-CSF) that activates neighbouring myeloid cells, which further enhance the neutrophil and monocyte recruitment.<sup>47</sup> During the first days, the accumulating leucocytes' main function is removing necrotic tissue in an active process that depends on MertK, other scavenger receptors and the production of proteolytic enzymes.<sup>48,49</sup> Exposed DNA in the infarcted heart is sensed by interferon regulatory factor 3 (IRF3)-positive macrophages<sup>50</sup> that enhance the inflammatory process by locally releasing pro-inflammatory cytokines, such as IL-1 and IL-6, among others. Ly6C<sup>high</sup> monocytes rapidly turn over during ongoing inflammation and can differentiate locally into Ly6C<sup>low</sup> macrophages.<sup>7,51</sup> Those macrophages are considered to be reparative, may reside in the injured heart for several days and can undergo local proliferation.<sup>51</sup> At later stages, lower numbers of Ly6C<sup>low</sup> monocytes are also recruited via chemokine (C-X3-C motif) receptor-1 (CX3CR1) with currently unclear functional consequences. During the reparative phase, macrophages reduce IL-6, TNF, and matrix metalloproteinase 9 (MMP9) expression levels while tissue levels of IL-10 increase.<sup>51,52</sup> Macrophages secrete both transforming growth factor (TGF)- $\alpha$  and - $\beta$ , thereby inducing neighbouring fibroblasts to convert into myofibroblasts, and macrophage-derived vascular endothelial growth factor impacts endothelial cells and stimulates angiogenesis.<sup>43</sup>

Taken together, monocytes and macrophages play vital roles in infarct healing and may represent a promising therapeutic target in patients with acute MI and exaggerated systemic inflammatory activity, as discussed in detail elsewhere.<sup>53</sup>

## Myocarditis

Myocarditis in patients is diagnostically heterogeneous and treatment options are limited.<sup>54</sup> Cardiac inflammation is among the most common causes of non-congenital, non-ischaemic sudden death in otherwise normal, healthy young adults.<sup>55</sup> The best studied and characterized models of myocarditis are viral infection with the enterovirus Coxsackie B3 (CB3) and autoimmune mediated inflammation in mice.<sup>56</sup> In both of these models, cells of the monocyte and macrophage lineages comprise the majority of infiltrating inflammatory cells.<sup>57</sup> CB3 infiltrates cardiomyocytes via the coxsackievirus and adenovirus receptor and triggers the release of damage-associated molecular patterns (DAMPs) from infected cells. Such release results in the recruitment and activation of monocytes and dendritic cells.<sup>58</sup> The latter impact cardiac inflammation and subsequent heart failure (HF) by generating antigen-specific lymphocytes.<sup>59</sup> Measuring viral titres from patient endomyocardial biopsies to optimize the treatment regime is a matter of ongoing debate. A retrospective analysis from a large single-centre study indicates that the number of detectable leucocytes—rather than the virus load—in the patient's biopsy sample predicts the outcome.<sup>60</sup> Selectively immunomodulating myeloid-derived M-CSF in CB3-induced myocarditis reduced cardiac monocyte and macrophage numbers without impacting the virus titre.<sup>61</sup> In certain susceptible strains of mice, cardiac inflammation lingers beyond the clearance of infectious virus and leads to dilated cardiomyopathy. This is caused by autoreactive adaptive responses, for example, due to congruent epitopes of CB3 and cardiac myosin or production of autoantibodies against Troponin I.<sup>62,63</sup> During the acute phase of autoimmune cardiac inflammation, monocytes can differentiate into TNF- $\alpha$ - and nitric oxide synthase 2-producing





dendritic cells (TipDCs) that may limit antigen-specific T-cell expansion.<sup>64</sup> Blocking monocyte accumulation with nanoparticle-encapsulated siRNA against CCR2 reduced cardiac fibrosis and resulted in improved left ventricular function.<sup>65</sup> A thorough understanding of the involved pathways and timing must precede any translational therapeutic approach. This is intriguingly illustrated by work using an IL-17A-deficient mouse autoimmune myocarditis model. While immunization with a myocardiogenic peptide showed similar disease severity compared with control mice during acute myocarditis, the absence of IL-17A led to reduced monocyte infiltration and prevented post-myocarditis remodelling, thus demonstrating IL-17A's role in further disease progression.<sup>66</sup>

## Hypertension and heart failure

While MI and myocarditis both induce fundamental changes in the heart's macrophage populations, other common conditions cause more subtle changes. For example, the inflammatory response is crucial to the progression of both pressure overload and hypertensive cardiac stress, but the intensity of that response is orders of magnitude lower than after acute ischaemic injury.<sup>67</sup> The exact mechanisms that initiate the inflammatory response in cardiac pressure overload are still poorly understood but may involve angiotensin-mediated pro-inflammatory effects, reactive oxygen species production or cardiomyocyte death.<sup>68</sup> Commonly used models of cardiac pressure overload include transverse aortic constriction (resembling cardiac stress in aortic stenosis) and administering angiotensin II or aldosterone. If the stimulus is continued long enough (or the constriction

remains in place), fibrotic tissue develops, and adverse remodelling leads to impaired cardiac function.

Similarly, angiotensin II-induced hypertension raises resident macrophage proliferation in the heart.<sup>69</sup> Prolonged exposure to angiotensin II also increases the HSC proliferation in the spleen and augments monocyte recruitment to the heart.<sup>24,69</sup> During hypertensive stress, monocyte-derived macrophages show up-regulation of genes associated with the NLRP3 inflammasome. This pathway is also activated in diabetic mice's cardiac macrophages. This results in the production of IL-1 $\beta$ ,<sup>70</sup> which increases the risk for arrhythmias, systolic dysfunction, and HF.<sup>15</sup> Heart failure affects around 26 million people worldwide and can be considered a global pandemic.<sup>71</sup> Failing hearts host elevated numbers of macrophages that multiply by either local proliferation or differentiation from accumulating monocytes,<sup>20,69</sup> generated by the bone marrow but also extramedullary haematopoiesis in the spleen. Splenectomy resulted in ameliorated cardiac function in a model of ischaemic cardiomyopathy.<sup>72</sup>

Macrophages not only impact disease progression in cardiac pathologies with impaired systolic function but also play an important role in HF with preserved ejection fraction (HFpEF). Nearly half of all HF patients suffer from HFpEF, in which impaired cardiac performance is thought to be a consequence of increased left ventricular filling pressure caused by diastolic dysfunction.<sup>73</sup> While there is a paucity of specific treatment options, known risk factors for HFpEF include hypertension and aging.<sup>74</sup> In blood from patients with HFpEF, inflammatory markers<sup>75</sup> and circulating monocytes are elevated.<sup>76</sup> To mimic HFpEF in mice, a combination of salty drinking water, unilateral nephrectomy, and aldosterone infusion with osmotic

minipumps (SAUNA) is administered.<sup>77</sup> This regimen resulted in increased haematopoiesis in bone marrow and spleen and elevated numbers of monocyte-derived macrophages in the heart. Analysis of hearts from ageing mice corroborated these findings. Cardiac macrophages from SAUNA-treated mice were predominantly MHC II<sup>high</sup> and produced more IL-10 than macrophages from healthy hearts. Monocyte- and macrophage-specific deletion of IL-10 using CX3CR1-Cre and IL-10-floxed mice resulted in improved diastolic function after SAUNA treatment. Macrophage-derived IL-10 does not act directly on cardiac fibroblasts but rather indirectly, in collaboration with TGF, induces collagen deposition by myofibroblasts.<sup>77</sup> Interleukin-10 is known for its potent anti-inflammatory activity and its administration prevents adverse cardiac remodelling after MI and pressure overload,<sup>52,78</sup> which illustrates that timing and context is crucial for any therapeutic translation. The same holds true for modulating macrophage biology to prevent atherosclerotic plaque progression and rupture, as reviewed in detail elsewhere.<sup>79,80</sup>

## Conclusions

In recent years, we have gathered fundamental new insight into the ontogeny, dynamics, and function of cardiac macrophages. Given these observations we must be selective and precise in any manipulative approaches targeting cardiac macrophages in the context of HF or conduction abnormalities. These discoveries, however, also show the tremendous potential and force of this heterogeneous cell population and the potential therapeutic possibilities arising from better understanding of their roles. Future studies will need to decipher harmful and beneficial functions of monocyte/macrophage subsets and their involvement in cardiac (patho-)physiology.

## Acknowledgements

We gratefully acknowledge Kaley Joyes for editing the manuscript.

## Funding

This work was funded in part by federal funds from the National Institutes of Health Grants HL139598, HL128264, HL125428, and HL131495; the Massachusetts General Hospital Research Scholar Program; the German Research Foundation (DFG, Heisenberg Programme); the DZHK (German Centre for Cardiovascular Research), and the BMBF (German Ministry of Education and Research; Projects DeCaRe and coNfirm).

**Conflict of interest:** M.N. has been a paid consultant or received research support from Novartis, GSK, Medtronic, Verseaux, Sigilon, Alnylam, IFM therapeutics, and Molecular Imaging, Inc. The authors declare no conflicts of interest, financial or otherwise.

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