

## STATE-OF-THE-ART REVIEW

# Heart development and regeneration—a multi-organ effort

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development; gut; heart; immune system; inter-organ interactions; microbiota; nervous system; regeneration

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Development of the heart, from early morphogenesis to functional maturation, as well as maintenance of its homeostasis are tasks requiring collaborative efforts of cardiac tissue and different extra-cardiac organ systems. The brain, lymphoid organs, and gut are among the interaction partners that can communicate with the heart through a wide array of paracrine signals acting at local or systemic level. Disturbance of cardiac homeostasis following ischemic injury also needs immediate response from these distant organs. Our hearts replace dead muscles with non-contractile fibrotic scars. We have learned from animal models capable of scarless repair that regenerative capability of the heart does not depend only on competency of the myocardium and cardiac-intrinsic factors but also on long-range molecular signals originating in other parts of the body. Here, we provide an overview of inter-organ signals that take part in development and regeneration of the heart. We highlight recent findings and remaining questions. Finally, we discuss the potential of inter-organ modulatory approaches for possible therapeutic use.

## Introduction

No organ is an island. Organs in the body do not exist in isolation, and maintenance of their homeostasis requires the organs' ability to timely react to a dynamically changing environment through interactions with other parts of the body. Given its life-supporting function, it is not surprising that a growing body of evidence continues to reveal yet new

ways by which the heart receives and relays information from and to adjacent and distant neighbors. Disturbance of cardiac homeostatic state by injury or stressful conditions that can jeopardize its ability to match the body's circulatory demands, thus, triggers systemic responses that go far beyond the heart [1–5]. *Vice versa*, a spatially and temporally regulated

**Abbreviations**

ACTH, adrenocorticotrophic hormone; AREG, amphiregulin; B2AR,  $\beta$ two adrenergic receptor; CB2-R, cannabinoid-2 receptor; CCR2, C-C chemokine receptor type 2; CRH, corticotropin-releasing hormone; CSF2, colony-stimulating factor 2; CVD, cardiovascular disease; CX3CR1, C-X3-C Motif Chemokine Receptor 1; FFAR, free fatty acid receptor; FGF21, fibroblast growth factor 21; FOXP3, forkhead box P3; GATA4, GATA-binding protein 4; GC, glucocorticoid; GPR, G-protein-coupled receptor; GR, glucocorticoid receptor; HDAC, histone deacetylase; HPA, hypothalamic-pituitary-adrenal; IGF, insulin-like growth factor; IL13, interleukin 13; IL22, interleukin 22; IL4, interleukin 4; IL4R, interleukin 4 receptor; IL6, interleukin 6; iPSC, inducible pluripotent stem cell; MI, myocardial infarction; NF- $\kappa$ B, nuclear factor kappa B; NGF, nerve growth factor; NLRP3, NLR family pyrin domain containing 3; Nppb, natriuretic peptide B; Nrg1, neuregulin1; Olfr78, olfactory receptor 78; OSM, oncostatin M; PIGF, placental growth factor; RAS, renin-angiotensin system; S100A8, S100 calcium-binding protein A8; S100A9, S100 calcium-binding protein A9; SCFA, short-chain fatty acids; STAT3, signal transducer and activator of transcription 3; STAT6, signal transducer and activator of transcription 6; TGF- $\beta$ , transforming growth factor- $\beta$ ; TMAO, trimethylamine N-oxide; Tnfa, tumor necrosis factor alpha;  $\alpha$ 7nAChR, alpha7 nicotinic acetylcholine receptor.

network of intricate crosstalk between diverse organ systems orchestrates timely recovery of oxygen supply and thereby dictates the outcomes of acute and chronic responses to cardiac damage in myocardial infarction (MI) and heart failure [2,6,7]. Interestingly, emerging evidence has revealed a less appreciated role of inter-organ communications as important contributors to heart development.

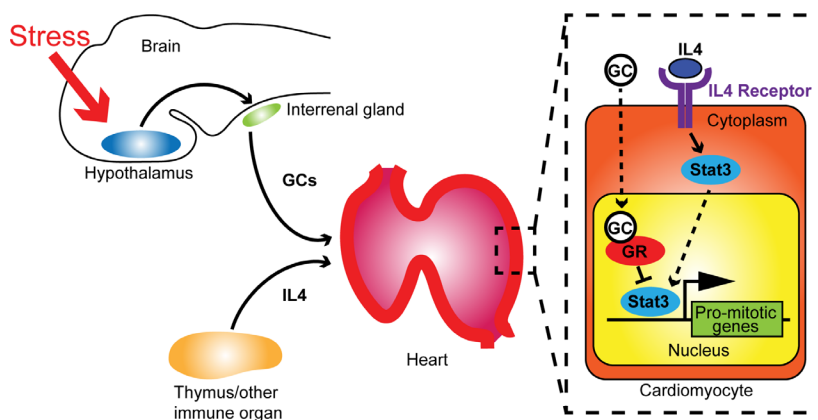
In this review, we summarize the current state of knowledge focusing on the interplay between the heart and immune, nervous, endocrine, lymphatic, and gastrointestinal systems during cardiac development and repair. We highlight the influences of these extra-cardiac organ systems on the capacity of the heart to overcome fibrotic scarring, and instead replenish dead muscles with new functional ones. Finally, we discuss how future studies elucidating further mechanistic insights into the crosstalk hold important therapeutic promise.

## Multi-systemic crosstalk in heart development

### Cardiovascular-immune interactions

While the role of the immune system as a first line of defense and critical player in the restoration of tissue homeostasis is well established, little is known about the involvement of immune cells and signaling components in physiological growth of the heart in absence of any insult. Only very recently, the developmental role of the immune system has started to be uncovered.

Yolk sac-derived CCR2 macrophages are instrumental in remodeling of the primitive coronary plexus by promoting endothelial proliferation in perfused vessels, a mechanism that might involve macrophage secretion of insulin-like growth factor (IGF) [8]. A subpopulation of circulating B cells, primarily showing a follicular naive cell expression profile, has been documented to contact microvascular endothelium of the heart from which a small fraction transits into the myocardium [9]. Unexpectedly, B cells deficiency lowers myocardial mass, a consequence of smaller cardiomyocytes, and increases left ventricular ejection fraction, unveiling a previous unappreciated role of B lymphocytes in cardiac structural and functional development [9]. In addition to coronary remodeling and myocardial cell enlargement, mitotic activity of cardiomyocytes is also under control of an immune signaling pathway. In zebrafish, interleukin 4 (IL4), through the atypical mediator signal transducer and activator of transcription 3 (STAT3), mediates cardiomyocyte cell cycle entry in the developing myocardium [10]. IL4 receptor (IL4R)-STAT3 pathway in cardiomyocytes drives expression of cell cycle progression genes, including *c-myc*, *cyclind1*, and *gata3* (Fig. 1). Notably, the pro-proliferative role of IL4 is evolutionarily conserved, since neonatal mice lacking the IL4 receptor alpha (IL4Ra) display reduced cardiomyocyte proliferation [10], and application of exogenous IL4 stimulates proliferation of rat neonatal cardiomyocytes in culture [11]. Consistent with the pro-mitotic role of IL4 in cardiomyocytes through IL4Ra activation, the cytokine IL13, which can signal through the same receptor, was reported to be a positive



**Fig. 1.** Scheme summarizing the proposed regulation of cardiomyocyte proliferation during development by IL4 signaling and stress in zebrafish. Activation of the GR in cardiomyocytes by GCs, produced following activation of the HPA axis (in zebrafish, the inter-renal gland is the homolog of the mammalian adrenal gland), counteracts IL4 receptor signaling initiated upon binding of IL4, possibly released by immune cells originating from the thymus. GCs inhibit and IL4 signaling enhances transcription of pro-mitotic genes downstream of STAT3 in cardiomyocytes. The GC-IL4 signaling crosstalk was shown to be important also for development of the mouse heart. Figure reproduced from [10].

regulator of cardiomyocyte cell division in the developing myocardium. The heart of IL3-deficient mice displays decreased cardiomyocyte cell cycle activity and increased expression of natriuretic peptide B (Nppb), indicating hypertrophic growth during early postnatal life [12].

Collectively, these results have likely uncovered only the tip of the iceberg. The collection of diverse functions of elements of the immune system in cardiac morphogenesis and functional maturation will continue to grow. We anticipate that further discoveries in the next few years will contribute to paint a complete and more complex picture of heart-immune interactions in cardiac development.

### **Autonomic and neuroendocrine communications with the heart**

The uninnervated primitive human heart starts beating by 21–22 days post-fertilization. Some degree of neurogenic regulation of cardiac conduction occurs later on by 8 weeks of gestation [13]. In mice, cardiac innervation starts at embryonic days 10–11 (E10–11) [14]. Therefore, although neurons play important roles in functional maturation of the heart through direct connections of the autonomic nerves, cardiac morphogenesis is commonly thought not to be under influence of the developing nervous system [15]. During the early postnatal period when cardiac sympathetic innervation is established, neurogenic inputs become fundamental for the organization of myocardial architecture. Evidence from both rodent and human hearts demonstrated uneven distribution of sympathetic nerve density, with a strong preference in the epicardial layer, which correlates with cardiomyocyte size [16]. Genetic and pharmacological interference with sympathetic signaling revealed that the cardiomyocyte trophic input might depend on  $\beta$ 2 adrenergic receptor (B2AR)-mediated suppression of cardiomyocyte proteolysis by the ubiquitin ligase MuRF1 [16].

Besides through direct autonomic connections with the heart, the nervous system can shape myocardial development via neuroendocrine pathways. For example, exposure of the fetus during late gestation to the key stress-related hormones glucocorticoids (GCs) [17,18] is critical for maturation of cardiovascular function [19–22]. Secretion of GCs is under control of the hypothalamic–pituitary–adrenal (HPA) axis, comprising of neurons in the hypothalamic paraventricular nucleus (PVN) that release the neuropeptide corticotropin-releasing hormone (CRH). CRH stimulates release of adrenocorticotrophic hormone (ACTH) from cells of the pituitary gland, which in turn triggers

release of GCs from the adrenal glands. Surprisingly, in zebrafish inhibition of glucocorticoid receptor (GR) induces hyperplastic growth of the myocardium, suggesting that a basal level of GC signaling is required to prevent hyperproliferation of cardiomyocytes [10]. On the other hand, neuronal stress interferes with physiological growth and morphogenesis of the heart, at least in part, by counteracting the IL4 pathway to suppress cardiomyocyte proliferation. Consequently, hypertrophic growth ensues, ultimately compromising contractility of the developing myocardium [10] (Fig. 1). These findings, thus, highlight a fundamental role of the crosstalk between a cytokine and stress signaling in shaping cardiac developmental program. Induction of cytokine signaling might have the potential to prevent cardiac developmental defects resulting from exposure to early life stress. Endogenous GC-facilitated cell cycle exit has been proposed to take part in the transition from a pro-regenerative state in early neonates to pro-fibrotic repair in adult mammals. Consistent with a dramatic rise in GC levels in late gestation, GR expression increases in cardiomyocyte during postnatal development. Cardiomyocyte-restricted GR deficiency increases cardiomyocyte self-renewal in the infarcted juvenile and adult myocardium, suggesting the potential of GR antagonization to promote heart regeneration [23].

In addition to GCs, other neuroendocrine signaling molecules, thyroid hormones, were revealed to serve as crucial regulators of heart development. Polyploidy and multi-nucleation are hallmarks of postnatal cardiomyocyte cell cycle exit in mammals. Other vertebrates such as zebrafish and newt, on the contrary, maintain diploid mononuclear cardiomyocytes throughout adulthood [24,25]. Phylogenetic analysis revealed inverse correlations between diploid cardiomyocyte abundance and metabolic rate, body temperature, and plasma thyroxine (T4) [26]. Cardiomyocyte-specific expression of a dominant negative form of the thyroid hormone receptor- $\alpha$  (TR- $\alpha$ ) increased total cardiomyocyte numbers and prevalence of diploid cells observed in mice at postnatal day (P) 14, demonstrating the role of intrinsic thyroid hormone signaling as a brake for mitotic growth of the myocardium once perinatal stage is reached [26]. RNA sequencing of the TR- $\alpha$  signaling deficient heart showed enrichment of components of oxidative phosphorylation and tricarboxylic acid cycle (TCA), with downregulation of many mitochondrial genes. Consistent with downregulation of mitochondrial respiratory genes, TR- $\alpha$  signaling-deficient hearts also contain less mitochondrial DNA and reactive oxygen species (ROS) [26]. These findings, together, suggest that

increased levels of thyroid hormones in the circulation may mediate a trade-off of myocardial mitotic growth for thermogenic capability and acquisition of endothermy [27] in development and evolution of mammals.

### The gut–heart axis

*In utero* colonization of the gut microbiota might also influence fetal heart growth. Increased left ventricular posterior wall thickness of the human heart detected before birth was correlated with lower density of microbial community and enrichment of inflammation-related bacteria [28]. However, fetal microbial colonization has been a contentious topic [29], and further work will be required to confirm a causal relationship between gut microbiota and cardiac developmental abnormalities. The endodermal precursor of the developing gut might influence early cardiac morphogenesis through mechanical cues and paracrine signals. Computational simulations and observations in chick embryos demonstrated that contraction of the anterior intestinal portal generates tension that contributes to the elongation of the primitive heart tube [30]. The presence of endodermal cells in inducible pluripotent stem cell (iPSC)-derived organoids was reported to improve expansion and maturation of cardiomyocytes and structural maturation of cardiac tissue [31]. Our knowledge about the influence of the gut on cardiac morphogenesis is currently very limited, and more work is required to fully understand how and to what extent this organ contributes to shape a growing heart. Collectively, the gut plays a critical role in shaping the developing heart through physical interactions and, together with its resident microflora, may act as an endocrine organ that emanates a diverse array of secretory factors and metabolites capable of modulating the immune system.

### The cardiac lymphatic system

The lymphatic system provides a unidirectional conduit essential not only for regulating interstitial fluid homeostasis [32] but also to prevent the spread of pathogens by modulating both innate and adaptive immune responses [33,34]. Starting from around E12.5 in mice, the first lymphatic endothelial cells enter the heart before expanding along the base-to-apex axis on both dorsal and ventral surfaces by E15 [35]. Lineage tracing revealed that these cells derive from both venous and non-venous sources [36–38]. Conditional deletion of *Prox1*, a master regulator of lymphatic endothelial cell fate specification and maintenance, or

a point mutation in the kinase domain of VEGF receptor 3 (VEGFR3), caused nearly complete loss of all cardiac lymphatics, and formation of smaller hearts detected at E17.5 [39]. The reduction in heart size was found to result from decreased cardiomyocyte mass, in association with lower proliferation and higher apoptosis of cardiomyocytes, but not other cardiac cell types. Embryonic hearts lacking lymphatics showed a substantial reduction in Reelin (RELN) [39], an extracellular matrix glycoprotein well known for its role in laminar formation of the cerebral cortex [40]. Notably, embryos with deletion of *Reln* specifically in lymphatic endothelial cells also exhibited impairment of cell proliferation and increased apoptosis in cardiomyocytes, indicating the indispensable requirement of lymphatic endothelial cell-derived RELN in cardiac development.

The lymphatic system in zebrafish has many similarities in common with the mammalian one, including heterogeneous origins of the lymphatic endothelial cells and the mechanisms of cardiac lymphatic growth [41–43]. Expansion of cardiac lymphatics in zebrafish takes place at about 21–34 days post-fertilization [43]. Depletion of *Vegfc* or its receptor *Flt4* (*Vegfr3*) in zebrafish severely impaired ventricular lymphatic formation, but did not alter ventricular size [43], implying that other cardiac cells might be able to provide lymphoangiocrine signals to cardiomyocytes to support their proliferation and survival. Additional to its direct roles in shaping heart development, given its requirement in immune surveillance, it is likely that the lymphatic system could also regulate cardiac growth and patterning through lympho-immune interactions that still await to be discovered.

## Inter-organ communications orchestrate heart regeneration

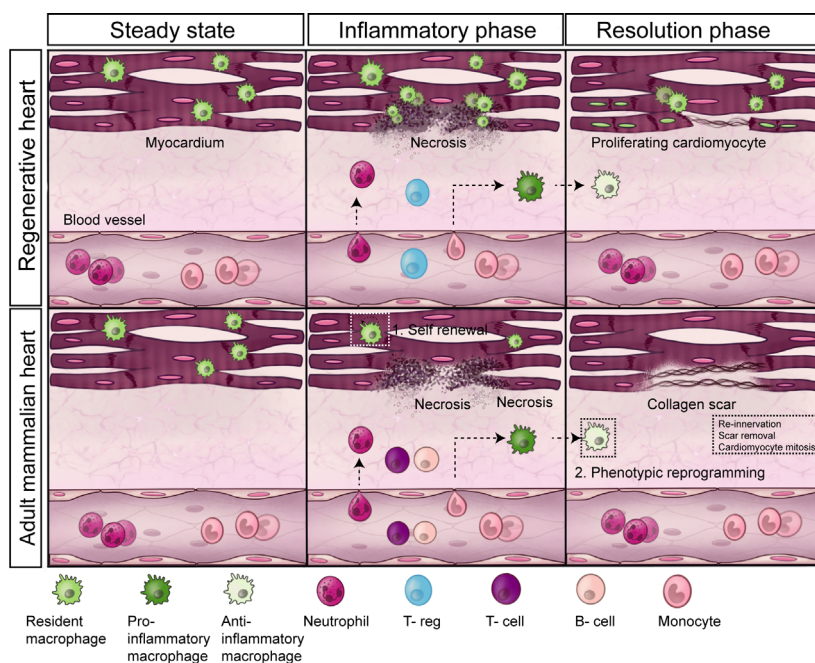
### Cardioimmunology

Ischemic heart disease is a major cause of heart failure, the long-standing leading cause of morbidity and mortality worldwide. Blockage of oxygen and nutrients supply to the myocardium leads to a drastic loss of cardiomyocytes. The regenerative ability of the adult human heart is grossly inadequate to compensate for the lost cells. Instead, dead muscle is replaced by non-contractile fibrotic scar which maintains structural integrity but compromises cardiac function [44–47]. By contrast, neonatal mouse, pig, and human hearts have been reported to retain regenerative capacity shortly after birth [48–50]. Proliferation of pre-existing cardiomyocytes and ultimately functional recovery of the injured heart in young mammals resemble the

well-known regenerative potential of adult zebrafish [48,51]. Emerging evidence demonstrates unequivocally contributions of the immune response to myocardial self-renewal. Phenotypic and functional diversities of immune cells across species and developmental stages might explain differences in the regenerative potential.

Cardiac cell death post-MI triggers an immediate response from innate immune cells to clear the tissue environment from cellular debris. The professional phagocytes, macrophages and recruited monocytes, mount a biphasic response commonly divided into an initial pro-inflammatory phase superseded by an inflammatory-resolution one [52–55] (Fig. 2). Contrary to an earlier view in which inflammation is only deleterious to cardiac repair by promoting tissue damage and adverse remodeling post-MI [56,57], we now know that it plays an essential role in cardiac regeneration. Neonatal mice depleted of macrophages were unable to regenerate the myocardium and formed fibrotic scars [58]. Similarly, delayed macrophage recruitment and blunted acute inflammation were observed in

medaka's heart, which is incapable of regeneration, whereas stimulation of inflammation promoted the process [59]. In line with these findings, a recent study demonstrated that acute inflammation, detectable from robust accumulation of monocytes in the injured heart, instead of generation of new cardiomyocytes or endothelial cells, is responsible for the benefits of stem cell therapy in cardiac repair and functional recovery [60]. A recent study in adult zebrafish demonstrated that a pro-inflammatory subset of macrophages (positive for tumor necrosis factor alpha (tnfa)), in addition to triggering acute inflammatory responses, promoted an early phase of fibrosis post-MI [61]. Macrophages, in fact, can contribute directly to scar formation via cell-autonomous collagen deposition [62]. A decreased prevalence of tnfa+ macrophages during the subsequent inflammatory resolution stage has been linked to scar removal [61] (Fig. 2). Transient collagen deposition, followed by complete removal of the scar, determines successful myocardial regeneration [63]. How monocytes/macrophages in the regenerative animal



**Fig. 2.** Biphasic immune response of regenerative and non-regenerative hearts. During the inflammatory phase of an infarcted adult mammalian heart, neutrophils, monocytes, T cells, and B cells are recruited to the damaged tissue, leading to elevated levels of pro-inflammatory cytokines. Infiltrated monocytes also differentiate into pro-inflammatory macrophages, contributing to cytokine production and matrix remodeling. In contrast, the initial immune response in neonates is marked by the presence of an embryonic-derived macrophage population, which is rapidly depleted after MI in the adult mammalian heart. T cells are prone to differentiate into Tregs at this stage. Shifting of inflammatory macrophages to a reparative phenotype, characterized by release of anti-inflammatory cytokines and angiogenic factors, marks the transition into the reparative phase. A macrophage subset required to promote cardiomyocyte differentiation and resolve collagen scar can also be recruited from the circulation as shown in adult zebrafish hearts. Strategies (dashed arrows) to replenish our heart with “regenerative macrophages” (dashed boxes) by 1) promoting their self-renewal and maintenance, 2) differentiation from the pro-inflammatory pool, or 3) recruitment from circulation might bring us closer to the goal of scarless repair.



models execute temporally restricted and intricately balanced inflammatory and fibrotic responses to coordinate scarless repair is still largely unknown (Fig. 2).

Heterogeneity of monocytes/macrophages present at different developmental stages making diverse contributions to fibrosis and regenerative mechanisms might, at least in part, underlie the loss of regenerative capability in adult mammalian hearts [62]. Different subsets of cardiac macrophages of adult monocytes and embryonic origin, distinguishable by C-C chemokine receptor type 2 (CCR2) expression, exhibit distinct functions upon injury [64,65]. The CCR2<sup>-</sup> macrophage pool orchestrates cardiomyocyte proliferation and neo-vascularization in neonatal hearts, whereas the CCR2<sup>+</sup> pool is responsible for robust inflammatory cytokine/chemokine production and induction of neutrophil influx [64]. Further fate mapping and single-cell transcriptomics identified a specific subset of embryonic CCR2<sup>-</sup> cells that were capable of self-renewal with negligible monocyte input under steady state, but were nearly abolished in infarcted adult myocardium. In the adult zebrafish heart, a macrophage pool recruited from the kidney marrow, a functional homolog of the mammalian bone marrow, might have critical regenerative functions [66]. Strategies to promote self-renewal or replenishment of these “regenerative” cells, which are possibly responsible for efficient scar removal and promoting cardiomyocyte proliferation, from blood monocytes or earlier progenitors might present a new therapeutic opportunity (Fig. 2). Additionally, plasticity of pro-inflammatory monocytes and macrophages to activate a pro-reparative phenotype in the infarcted heart after the initial inflammation phase subsides [54,67] raises the important translational consideration that the ability to regulate temporally restricted monocyte/macrophage fate specification might yield more effective infarct healing and ameliorate adverse remodeling [68] (Fig. 2). Toward this end, much work is required for better understanding of the cell-intrinsic and -extrinsic signals emanating from cardiac and extra-cardiac tissues, and regulating development, maintenance, and activation state of cardiac macrophages under injurious conditions.

In addition to macrophages, other innate immune cells, including neutrophils and mast cells, have been implicated in cardiac repair. Both detrimental and protective functions of these cells have been reported [34–36], suggesting that their role in the recovery process after MI may be similarly complex as for macrophages, with possible participation of different cell subsets.

Besides innate immune cells, participation of adaptive immune cells in cardiac regeneration has begun to be revealed. B cells are associated with aggravated

tissue injury and deteriorating myocardial function through antibody production [69] and stimulation of Ly6C<sup>high</sup> monocyte influx into the heart post-MI [70]. On the other hand, Treg cells, critical mediators of immune suppression, promote healing of the infarcted area by inducing differentiation of macrophages toward an anti-inflammatory phenotype [71]. Comparison of CD4<sup>+</sup> T cells from different developmental stages showed an intrinsic mechanism rendering CD4<sup>+</sup> T cells more prone to Treg differentiation upon T-cell receptor stimulation in neonatal mice. This property is attenuated after the first two postnatal weeks [72], consistent with a common view that maturation of an adaptive immune system equipped with matured T cells reactive to foreign antigens in adult mammals, in contrast to neonates and non-mammalian vertebrates, might be a trade off with regenerative capability [55]. In line with this view, Treg cells, potentially by enhancing monocyte/macrophage recruitment and cardiomyocyte proliferation, are indispensable for cardiac regeneration in both zebrafish [73] and neonatal mice [74]. A mechanistic understanding of Treg cells regenerative functions would be crucial to devise new T-cell-based methods to promote cardiac repair in the adult mammalian heart.

### Cytokine signaling mediating immuno-cardio interaction

A diverse array of cytokines plays central roles in regeneration of the heart not only through immune modulation but also by regulating responses of different cardiac-resident cells to injury. IL13, for example, promotes cardiac regeneration through regulation of cardiomyocyte mitotic response in neonatal mice [12,75,76]. In cultured neonatal rat cardiomyocytes, IL13 stimulated cardiomyocyte cell cycle entry through a pathway involving STAT3 and STAT6 activation and upregulation of the matricellular protein periostin [75]. In neonatal mice with cardiac apical resection, IL13 deficiency impaired regenerative capability of the myocardium, causing cardiomyocyte hypertrophy, increased fibrosis, and left ventricular dilation [12]. Interestingly, IL13 expression was shown to be down-regulated in mice lacking the transcription factor GATA-binding protein 4 (GATA4) specifically in cardiomyocytes, which displayed impaired cardiac regeneration. Systemic administration of IL13 could rescue the regenerative response in cardiomyocyte-restricted GATA4 knockout neonatal mice, suggesting that the cytokine may act directly or indirectly downstream of the GATA4 in the regenerative pathway [77]. IL13 may signal through IL4Ra in cardiomyocytes since IL4ra deletion specifically in these cells reduced their

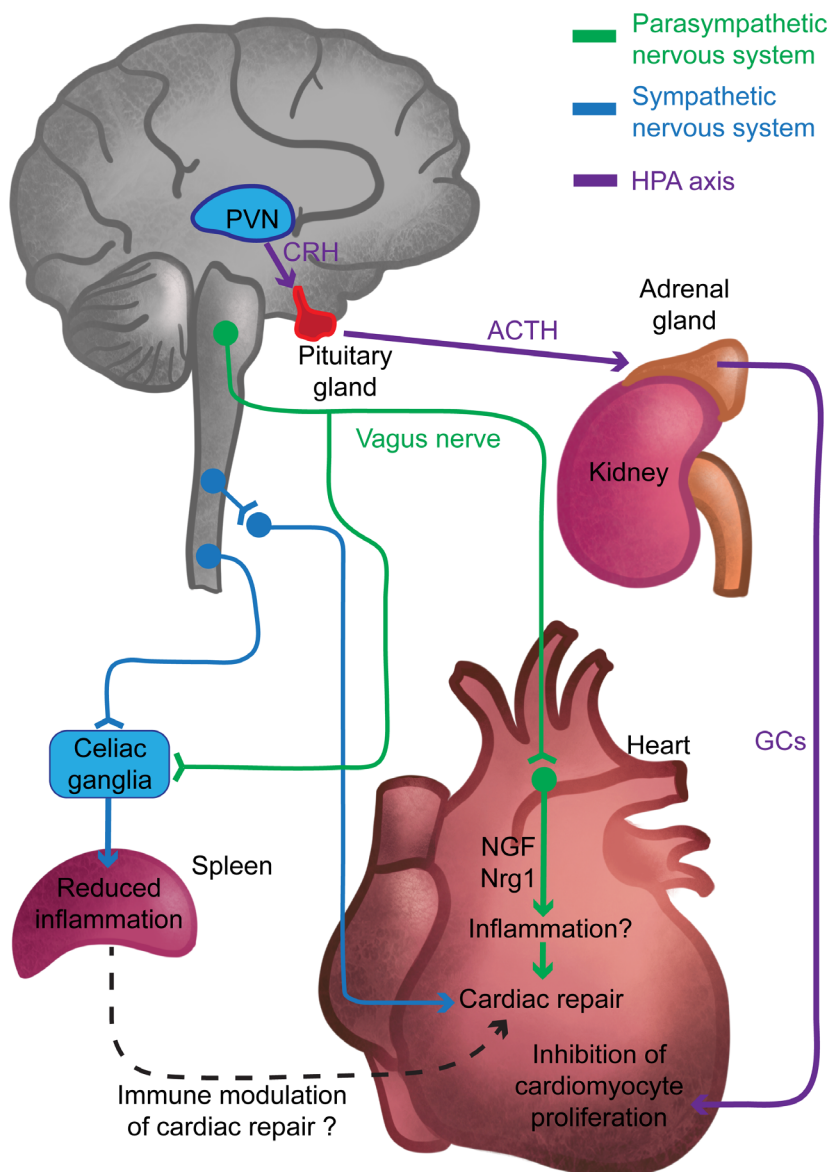
proliferation and impaired neonatal myocardial regeneration [76]. In addition to IL13, the cytokine IL6 serves as a positive driver of regenerative repair. IL6-deficient neonatal mice with cardiac apical resection exhibited reduced numbers of proliferating cells in association with downregulation of cell cycling (cyclinD), anti-apoptotic (Bcl-2), and pro-angiogenic (VEGF) proteins in the injured ventricle [78]. This pro-mitotic action of IL6 might affect mainly endothelial cells to drive neovascularization in the regenerating heart, since exposure of target cardiac cells to IL6 increased endothelial proliferation, whereas neonatal cardiomyocyte mitotic activity was largely unaffected. The same study reported that IL4, on the other hand, efficiently stimulated cardiomyocyte cell cycle entry and reduced collagen type I gene expression in myofibroblast [11]. Another cytokine of the IL6 family oncostatin M (OSM) has also been shown to play a key role as an upstream regulator of cardiomyocyte dedifferentiation and proliferation after cardiac injury [79,80]. In the injured neonatal mouse heart, OSM secretion from macrophages stimulates cardiomyocyte proliferation via its heterodimeric receptor, composed of the OSM receptor and glycoprotein 130 (gp130), present in cardiomyocytes [80]. The pro-proliferative effect of gp130 is mediated by Src-induced yes-associated protein (YAP) activation [80]. More recently, an indispensable role of IL11, another IL6 family member that also signals through a receptor complex containing gp130, to limit fibrotic scarring and promote regeneration has been demonstrated in different tissues of zebrafish including the heart, fin, and scales [81]. Mechanistically, IL11, through its cognate receptor Ill1ra and activation of STAT3 in endothelial cells, antagonizes transforming growth factor- $\beta$  (TGF- $\beta$ )-mediated endothelial-to-mesenchymal transition, thereby restricting myocardial scarring and facilitating cardiomyocyte repopulation [81].

Together, the examples of different cytokine signaling pathways participating in cardiac regenerative repair presented here clearly illustrate central roles of this broad array of proteins, once thought to act solely as immunomodulating agents, as molecular links between the immune and cardiovascular systems. Considering the pleiotropic actions of these cytokines, we anticipate that their broad influences on different cardiac cells and cellular processes coordinating cardiac regeneration are only beginning to be uncovered.

### Brain–heart crosstalk

Postnatal cardiac regeneration is under the influence of autonomic nerves, both sympathetic and parasympathetic, as demonstrated by the loss of regenerative

capability upon chemical sympathectomy [82] and vagotomy [83] (Fig. 3). Administration of neuregulin1 (Nrg1) and nerve growth factor (NGF) could partially rescue the negative effect of hypoinnervation on myocardial repair, possibly indicating that Nrg1 and NGF secreted from nerve terminals aid the regenerative response [83]. In the same study, transcriptional profiling of injured neonatal mouse and zebrafish hearts following vagotomy or pharmacological inhibition of cholinergic transmission revealed a blunted expression of inflammatory genes [83], suggesting a route by which the autonomic nervous system regulates cardiac regeneration by influencing immune function. A cardioprotective effect of vagal nerve stimulation observed in different ischemic models has also been proposed to involve a cholinergic anti-inflammatory pathway and attenuation of cardiomyocyte mitochondrial dysfunction in association with a shift of cardiomyocyte metabolism toward beta oxidation [84,85]. Cholinergic control of inflammation is a well-characterized nerve–immune interaction by which the brain modulates peripheral inflammatory responses through suppression of cytokine production from splenic immune cells, predominantly monocytes/macrophages, via activation of the  $\alpha 7$  nicotinic acetylcholine receptor ( $\alpha 7$ nAChR) [86–88] (Fig. 3). Interestingly, the cholinergic receptor is also indispensable for splenic vagal-driven sympathetic nerve discharge that primes adaptive immune response in hypertension [89]. Mechanistically, celiac vagus nerve stimulation induces, through activation of alpha-adrenergic receptors, upregulation of placental growth factor (PIGF), which in turn stimulates selective egression of CD8<sup>+</sup> T cells in the spleen [90]. Elaborated nerve–immune communications initiate a variety of immune modulatory effects, both suppressive and stimulatory [88,91,92], that can profoundly influence cardiovascular health [93]. It remains undetermined how acute injury of the heart impacts nerve–immune crosstalk in lymphoid organs and whether the efferent arc of the so-called inflammatory reflex is present in the heart. Immune cell types and molecular mechanism underlying neuronal regulation of cardiac repair remain still to be revealed (Fig. 3). Importantly, the mechanistic details of how this communication contributes to regenerative repair are mostly unknown. Together, compelling evidence highlights the importance of neuronal signaling in the regenerative response of the heart while, at the same time, underscore the need for better mechanistic understanding. Harnessing the potential of nerve-guided cardiac regeneration may present a new opportunity for development of much needed therapeutic strategies for MI.



**Fig. 3.** Diagram showing the main routes by which the nervous system can influence heart regeneration. The HPA axis inhibits cardiomyocyte proliferation in infarcted myocardium. The HPA axis is initiated by activation of neurons in the PVN secreting the peptide CRH, which in turn stimulates production of ACTH from the pituitary gland. ACTH then triggers release of GCs from the adrenal gland, which influence cardiomyocyte proliferation by binding to the GR. The parasympathetic nervous system promotes regeneration through release of the NGF and Nrg1, and possibly by regulating inflammation in the injured heart. The parasympathetic nervous system may also modulate cardiac repair by regulating inflammatory immune cells in the spleen, via the celiac ganglion. There is also evidence supporting a pro-regenerative role of the sympathetic nervous system, although the underlying mechanisms are not clear yet.

Brain–heart communications in cardiac regeneration, as in development, are also mediated by the neuroendocrine system. In addition to glucocorticoids, as mentioned earlier, several hormones have been shown to be involved in cardiac regenerative capacity. For instance, thyroid hormones and estrogen were suggested to underlie the inter-species and sex-dependent differences in regenerative ability of the heart. In accord with the developmental role of thyroid hormone signaling as a negative regulator of cardiomyocyte cell division, expression of cardiomyocyte-specific dominant negative TR- $\alpha$  increased cardiomyocyte proliferation and improved systolic function of the adult mouse heart following ischemic reperfusion injury [26]. Compared to

endothermic mammals, thyroid hormone levels and standard metabolic rates are lower in ectotherms, including fish and amphibians [26]. Thyroid hormone signaling is thought to underlie the loss of regenerative response in parallel with ectotherm-to-endotherm metabolic transition [26]. Indeed, treatment of adult zebrafish heart with exogenous triiodothyronine ( $T_3$ ) led to reduced cardiomyocyte proliferation and persistent scar tissue after ventricular apical resection [26]. In addition to inter-species differences, the regenerative capability of the myocardium may also be determined by sex [94]. In fact, sex-specific prevalence, pathophysiology, and outcomes of diverse cardiovascular diseases (CVD) have long been recognized [95]. Epidemiological studies



suggested that women during reproductive age have lower risk of CVD as compared to age-matched men [96–98]. Evidence indicates that the main female sex hormone estrogen (E2) plays a key cardioprotective role through its pleiotropic effects on the cardiovascular system [99]. In line with these studies, female zebrafish were shown to regenerate their heart more efficiently than age-matched males. Female fish showed more pronounced upregulation of two estrogen receptors, *esr1* and *esr2*, in the heart as well as elevated plasma E2 levels upon cardiac cryoinjury [94]. Treatment of female hearts with tamoxifen, an estrogen receptor antagonist, decreased cardiomyocyte proliferation at the lesion border zone and increased scar volume, indicating impairment of the regenerative response. By contrast, male hearts treated with E2 displayed increased cardiomyocyte proliferation and improved scar resolution [94]. Transcriptomic analysis showed that immune-related pathways were highly enriched in injured female hearts. For example, several components of the  $\text{Ifn}\gamma$ -Stat1 signaling pathway were highly upregulated in an estrogen-dependent manner [94]. This study, therefore, identified estrogen-mediated induction of inflammatory response as a key mechanism of sexually dimorphic regenerative response of the myocardium. Whether this pathway is involved in the pathogenic mechanism of other CVD is to be determined.

### Lymphatic drainage of the heart

In addition to cardiomyocyte death, MI also induces adverse remodeling of epicardial precollector and collector lymphatics that, despite robust capillary lymphangiogenesis, reduces fluid drainage and thereby causes myocardial edema [100]. Excess interstitial fluid eventually potentiates fibrosis, further compromising heart function [100,101]. Augmentation of injury-induced lymphatic sprouting, indeed, could improve prognosis of MI in mammalian models [100,102]. Besides reestablishing fluid balance, endogenous cardiac lymphangiogenic response also aids clearance of leukocytes, particularly innate immune cells infiltrating the ischemic heart to phagocytose dead cell debris, to regulate inflammatory resolution [100,103]. While lymphatic response of the adult mammalian heart is insufficient to serve these fluid and immune homeostasis restorations critical to facilitate optimal repair and prevent scarring, ample evidence points to a requirement of the lymphatic system and lymphoangiocrine signals in cardiac regeneration in both zebrafish and neonatal mice, as thoroughly reviewed earlier [104]. In injured *vegfc* or *flt4* mutant zebrafish hearts, largely devoid of lymphatic vessels, the scar area was

increased and remained unresolved even after 2 months post-injury [43]. In another zebrafish model with nearly complete loss of lymphatic vessels due to genetic deletion of both genes coding for Flt4 ligands, *vegfc* and *vegfd*, a subset of the mutant heart, displayed impaired capacity to clear dead cells and resolve fibrotic scar after cryoinjury [105]. Proliferative responses of cardiomyocytes, by contrast, were unaffected by the lack of cardiac lymphatics. Surprisingly, majority of the double-mutant fish could complete regeneration. Nonetheless, RNA sequencing suggested that the lack of lymphatic response in non-regenerative mutant hearts heightens TRAF6-IRF7 and interferon alpha/beta-related inflammation [105]. In line with these findings, inducible expression of soluble Flt4 (sFlt4), which competitively binds to Vegfc and thereby functions as a dominant inhibitor of Flt4 signaling, in zebrafish, caused persistence of cardiac cryoinjury-induced scar tissue without altering cardiomyocyte proliferation or epicardial activation [106]. Large amounts of neutrophils persisted in the injured heart even after 14 days post-injury, suggesting that blocking lymphatic responses impairs immune cell removal and prolong inflammation [106]. In the neonatal mouse heart, expression of the lymphoangiocrine signal regulating heart growth RELN steadily decreased from P2 to P14, coinciding with the loss of cardiac regenerative potential [39]. Myocardial infarction at P2 induced formation of new lymphatics within the infarct zone and its periphery, and reactivated RELN expression. Impairment of regenerative capacity upon RELN loss of function was evidenced from worsened cardiac function, decreased cardiomyocyte proliferation, and increased fibrosis in *Reln*<sup>-/-</sup> mice [39]. Moreover, delivery of RELN with bioengineered collagen patches improved cardiac function and reduced scarring in the adult mouse heart [39].

These studies highlight the importance of lymphangiogenesis in interstitial fluid and immune cell clearance, preventing myocardial edema and shifting the initial pro-inflammatory cardiac microenvironment to a pro-regenerative one. Better understanding of the molecular mechanisms underpinning lymphatic immune cell interactions could provide a basis for developing therapies to improve MI repair. Several important questions remain to be addressed. If and how selective clearance of specific immune cell subsets by the cardiac lymphatic system is accomplished, for instance, is currently unknown. Moreover, further studies are required to understand whether the lymphatic system contribute to recruitment of both innate and adaptive cells to the injured heart. Lastly, how the cardiac lymphatics influence maintenance of the pro-

regenerative subpopulations of primitive macrophages is yet to be determined.

## Immune signals mediating extra-cardiac organ influence on cardiac repair

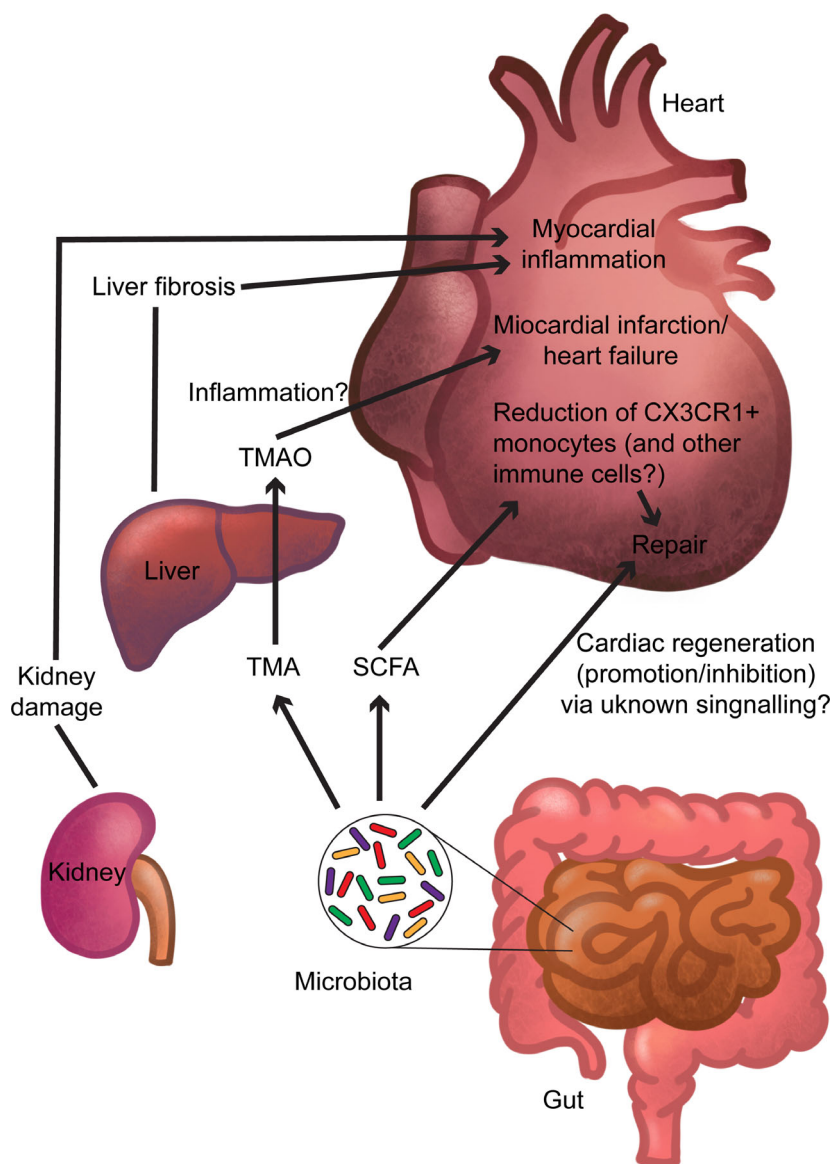
### The gut and its resident microbiota

The gut harbors a vast array of microflora that generates numerous metabolites from dietary nutrients. Dysregulation of microbial communities is associated with diverse disease states including CVD [107–111]. Alteration of intestinal microbiota by antibiotic and probiotic treatments influenced MI size in a rat model [112]. In MI patients, translocation of gut microbiota to the circulation was reported to be correlated with systemic inflammation and adverse cardiovascular events [113]. Although evidence demonstrating participation of the gut and its inhabitants in the regenerative capability of the heart is still lacking, antibiotic treatment increased the rates of ventricular rupture and mortality post-MI in a mouse model, suggesting an involvement of microbiota in myocardial repair [114]. The cardioprotective impact might be mediated in part by a class of metabolites generated mainly from microbial fermentation of indigestible carbohydrates, short-chain fatty acids (SCFAs), that is, required for recruitment of the C-X3-C motif chemokine receptor 1 (CX3CR1)<sup>+</sup> monocyte subset to the peri-infarct zone [114] (Fig. 4). In contrast to the generally salutary effects of SCFAs, trimethylamine N-oxide (TMAO) is commonly viewed as a risk factor for CVD. TMAO is generated by hepatic cells from trimethylamine (TMA), a molecule synthesized by gut bacteria as a metabolite of dietary components such as choline, betaine, and carnitine (Fig. 4). Increased plasma levels of TMAO have been associated with elevated risk of MI and heart failure in a patient cohort [115].

Commensal microbiota takes critical part in development and training of the host's innate and adaptive immune system, thereby imposing a major impact on inflammatory/immune diseases, as summarized in several excellent reviews [116–119]. Several lines of evidence highlight the immunomodulatory potency of SCFAs, in particular the most abundant ones in the mammalian gut acetate, propionate, and butyrate [120,121]. SCFA modulation of immune responses is attributed to two main pathways: 1) activation of G-protein-coupled receptors (GPR) 43 and 41 (also known as free fatty acid receptor (FFAR) 2 and 3,

respectively), GPR109a, and olfactory receptor 78 (Olfr78), expressed at different levels in diverse types of leukocytes [122]; and 2) inhibition of histone deacetylase (HDAC) activity and, as a result, enhancement of acetylation of histones, rendering the chromatin more accessible to transcription factors/regulators such as nuclear factor kappa B (NF- $\kappa$ B), forkhead box P3 (FOXP3), and STAT3 [123]. These pathways can regulate MAPK signaling and modulate expression of different cytokines and chemokines. Butyrate can drive differentiation of monocytes toward a non-inflammatory pro-antimicrobial macrophage state through HDAC3 inhibition and a shift of cellular metabolism [124]. Treatment with butyrate enhanced IL4 induction of an alternatively activated/M2 profile in bone marrow-derived macrophages by suppressing HDAC1 expression and increasing histone H3K9 acetylation [125]. In addition to influencing monocyte/macrophage polarization, SCFAs also elicit anti-inflammatory signatures in T cells. Depletion of resident microbiota reduced number of murine Treg and IL17A-producing T cells (Th17) and increased susceptibility to mucosal infection, effects which were partially controlled by SCFA treatment [126]. Propionate supplementation in multiple sclerosis patients augmented differentiation of Tregs and enhanced their immune-suppressive capacity [127]. Although the immunosuppressive role of SCFAs is well appreciated, SCFAs have also been reported to promote inflammation, for example, via stimulation of pro-inflammatory cytokine expression in intestinal epithelial cells and lung mesenchymal cells [128,129] and expansion of inflammatory T cells [130]. The contribution of TMAO in CVD is also often ascribed to immune derangement [131]. TMAO could induce splenic macrophage polarization toward a pro-inflammatory phenotype of classically activated/M1 subset [132] in a NLR family pyrin domain containing 3 (NLRP3)-dependent manner [133]. Together, given the immune modulatory roles of bioactive microbial metabolites affecting development, expansion, and functions of different immune cell types uncovered so far, an impact of the gut microbiota on cardiac regeneration, a process requiring well-orchestrated immune cell activities, is very likely (Fig. 4). However, potential pro-regenerative or pro-fibrotic roles of gut microflora/metabolites await experimental evidence. Moreover, since functional properties of immune cell populations are influenced by their origin and local tissue environment [134–138], it would be interesting to know whether cardiac resident and recruited immune cell subsets respond in different ways to microbial metabolites (Fig. 4).

**Fig. 4.** Scheme of representative pathways mediating the modulatory effects of the gut, liver, and kidneys on cardiac disease and repair. The gut harbors a rich diversity of microbes and microbial metabolites that can have different effects of cardiac health. For example, bacteria produce SCFAs, which were shown to influence cardiac repair by promoting recruitment of CX3CR1+ monocytes to the peri-infarct zone. It is still not known if SCFAs can influence recruitment and functions of other immune cells in the heart. On the other hand, the gut microbiota can have negative effects on cardiac function through production of TMA, which is converted into TMAO by hepatocytes. The gut microbiota has been shown to participate in a variety of immunomodulatory functions in several organs, and it is likely that it has important roles also in immune–cardiac interactions. Damage to the liver and kidneys can also negatively affect the heart by inducing myocardial inflammation.



### The liver and kidney

The overview of the current state of knowledge summarized so far focuses on the extra-cardiac organ systems whose roles in development and regeneration of the heart has been studied most extensively. The list is clearly not exhaustive. Other organs are also known to participate in the maintenance of cardiac homeostasis and influence cardiovascular health often by systemic alterations including immune and endocrine functions. For example, in a mouse model of hepatic cardiomyopathy, bile duct ligation-induced liver fibrosis caused myocardial inflammation, characterized by leukocyte infiltration and elevated expression of inflammatory markers that was accompanied by arterial hypotension and myocardial systolic and diastolic dysfunction [139]

(Fig. 4). Dampening the liver–heart inflammatory axis with a selective cannabinoid-2 receptor (CB2-R) agonist ameliorated both hepatic and cardiac pathologies [139]. Impairment of peroxisomal import and induction of IL6-like pro-inflammatory cytokine expression in hepatocyte-like cells have been reported to underlie age-related cardiac arrhythmia in fruit flies [140]. Another mode of liver–heart interaction is through hepatokines.  $\alpha$ 1-microglobulin, an Akt-NF $\kappa$ B-acting hepatokine, augmented macrophages infiltration and inflammatory activation in infarcted myocardium, ultimately leading to impairment of fibrotic repair and cardiac rupture in a mouse model [141]. On the other hand, hepatocyte-derived fibroblast growth factor (FGF21) induction downstream of interleukin 22

(IL22)-mediated STAT3 activation may serve as a survival cue for cardiomyocytes following MI [142].

An interdependent relationship between the kidney and the heart, particularly in relation to pathophysiology of heart failure and chronic kidney disorder, has been reported [143,144] (Fig. 4). Adding another level of complexity, a brain–kidney–heart network mediated by macrophages has been proposed to control myocardial adaptation to cardiac stress [145]. In a mouse model of transverse aortic constriction-induced pressure overload, sympathetic activation of renal collecting duct cells induced secretion of S100 calcium-binding proteins A8 and A9 (S100A8 and S100A9) that stimulated TNF expression in kidney macrophages. TNF, in turn, induced endothelial secretion of colony-stimulating factor 2 (CSF2, also known as GM-CSF) into the circulation. In the heart, CSF2 stimulated proliferation of a resident population of Ly6C<sup>low</sup> macrophages, thereby leading to cardiomyocyte hypertrophy by producing amphiregulin (AREG) [145], a critical driver of wound repair and fibrosis [146]. Deteriorating cardiac remodeling post-MI in the presence of renal failure is at least in part attributed to excessive renin–angiotensin system (RAS) activation [147]. In a rat model of MI and subtotal nephrectomy, administration of an Angiotensin II receptor blocker suppressed adverse structural remodeling and functional decline in the left ventricle, effects associated with attenuation of macrophage infiltration, cardiac oxidative stress, and inflammation [148].

## Concluding remarks

The study of inter-organ communications involved in heart development, physiology, and disease states is still in its infancy, and more work is required to fully understand the implications and molecular grammar of this crosstalk. The information about the impact of extra-cardiac organs on cardiac morphogenesis and reparative processes uncovered thus far delivers the unequivocal message that building a heart and mending a broken one are tasks achievable only by a multi-organ collaborative effort. It is likely that prognosis and preventive treatment of congenital heart diseases will be benefitted not only from knowledge about genetic predisposition and perturbation of intra-cardiac signaling but also by elucidating the multifaceted network of signaling pathways regulating cardiac development at a systemic level. Similarly, we have learned from different lines of compelling evidence that novel therapeutic strategies to achieve the seemingly unattainable goal of regenerating cardiac muscles in the infarcted myocardium may lie in the control of extra-cardiac organ perturbations and the

ability to modulate their regulatory signals. An innovative bioelectronic-based approach enabling spatial and temporal control of autonomic nerve activity, for example, [149], might permit regulation of immune responses in the heart as well as in lymphoid organs to prevent exacerbated inflammation and concomitantly harvest tissue regenerative functions of immune cells. Likewise, pro-biotic- or metabolite-based approaches that harness immune and/or neuronal modulatory roles of commensal microbiota present new attractive strategies to help stimulating cardiomyocyte replenishment in the infarcted myocardium. Such approaches could rely on naturally occurring products and personalized medicine to overcome variability of treatment responsiveness among individuals [150–152].

Elucidation of systemic perturbations and identification of paracrine factors and bioactive metabolites mediating multi-organ crosstalk will certainly be benefitted from recent advancements of omic approaches. Metabolomic profiling of plasma and the myocardium following MI has already been applied to identify changes in metabolites and metabolic pathways associated with different stages of responses to injury [153–156]. Likewise, proteomic analyses of cardiac and non-cardiac cell secretory factors (secretome) have uncovered novel players involved in myocardial infarct repair and fibrosis [157–159]. Further testing the potential of these candidate metabolites and secretory molecules to mimic the multi-organ coordination of cardiac regenerative processes of young mammals or non-mammalian vertebrates would be critical for clinical translation.

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## Conflict of interest

The authors declare no conflict of interest.

## Author contributions

SS and FA wrote the review.



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