Myocardial infarction and the immune response - Scarring or regeneration? A comparative look at mammals and popular regenerating animal models

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Abstract

Objectives: It has been well established that the survival and long-term outcome for patients suffering a myocardial infarction in part depends on the resulting immune response to injury. These processes are complex, and a clear path to useful immunotherapies for the treatment of cardiovascular damage in humans remains elusive. Mammals hold a great potential for repair of cardiac tissue during fetal and early neonatal life, an ability that is lost in the adult, coinciding with a maturation of the immune system. Unlike mammals, the axolotl and zebrafish, which are popular model organisms in regenerative medicine, successfully recover functionally and anatomically following infarction injury. In this review, we present an in-depth comparative look at the immune response to cardiac infarction damage in adult and fetal/early neonatal mammals as well as axolotls and zebrafish, with an emphasis on the role of macrophages. This current knowledge is instrumental for transferring new findings in regenerative animal models to the development of novel immune-modulating treatments. These could improve the rate of survival and quality of life after injury for the millions of people suffering from a myocardial infarction every year.

Key findings: The regenerative process in axolotls and zebrafish has been found to rely on the actions of key immune cells. Macrophages in particular are essential to cardiac regeneration in axolotls and zebrafish as well as mammalian fetuses and neonates. There is great interest in the heterogeneity of macrophage populations, as mammalian embryonic macrophages appear to be facilitators of regeneration, while monocyte-derived macrophages in adults chiefly promote fibrosis. Monocyte derived macrophages also exist in a spectrum of phenotypes grossly divided into pro-inflammatory M1 and immune-resolving M2 cells, with divergent roles following tissue damage. The phenotypes of axolotl macrophages remain uncharacterized, but early studies suggest that the macrophages recruited to the infarction site are primarily similar to embryonic or M2-type macrophages.

Conclusions: Findings in animal models as well as humans, indicates that the inflammatory response and especially the action of macrophages should be examined further, which requires a detailed understanding of these processes in models both capable and incapable of cardiac regeneration. Immunotherapies aimed at improving outcomes in mammals, should not eliminate the inflammatory response, but rather modulate it to resemble that of competent regenerators.

Abbreviations: ATP, adenosine triphosphate; CCR2, C-C chemokine receptor type 2; CM, cardiomyocyte; CI, cryoinfarction; CRP, c-reactive protein; CSF-1/-2, colony-stimulating factor 1/-2; CVD, cardiovascular disease; CXCL, chemokine (C-X-C) motif ligand; CXCR, C-X-C chemokine receptor; DAMP, damage-associated molecular pattern; FGF, fibroblast growth factor; HIF-1α, hypoxia-inducible factor a-alpha; I-CAM, intercellular adhesion molecule 1; IFN-γ, interferon gamma; IGF-1, insulin like growth factor 1; IL, interleukin; LOX, lysyl oxidase; MAMK, mitogen-activated protein kinase; MHC, major histocompatibility complex; MMP, matrix metallopeptidase; NGAL, neutrophil gelatinase-associated lipocalin; NK cell, natural killer cell (lymphocyte); NOD-like receptor, nucleotide-binding oligomerization domain-like receptor; PDGF, platelet-derived growth factor; PI3K/Akt, phosphoinositide 3-kinase/ alpha serine/threonine protein kinase; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; V-CAM, vascular adhesion protein 1

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1. Setting the stage: the immune response is vital for cardiac regeneration in regeneration competent models

Tissue regeneration occurs to some degree in all living organisms, but mammals are generally not particularly gifted in this regard.1 Adult mammalian tissues vary in their ability to be restored, as liver tissue for instance can be readily replaced while muscle tissue including cardiac muscle has very limited regenerative capability.2 Interestingly, this is quite another matter in utero and during the early neonatal stages. The mammalian fetus can regenerate cardiac tissue without scar formation.3,4 It is not the protected environment of the uterus giving mammalian fetuses this transient regenerative edge, as the transition towards fibrotic scarring generally begins prior to birth in most mammalian species. Skin from an adult mammal transplanted to a fetus heals through scarring, while fetal skin grafted onto an adult animal heals without scar formation, indicating that there is an intrinsic genetic basis in the cells of the fetal and adult tissue accounting for these differing responses.5 The immune response to tissue damage differs in mammals during fetal versus adult life, and is believed to play a central role in facilitating repair at this early stage of life.6,7 Other key cellular alterations involving the cell cycle of cardiomyocytes (CMs) and cellular metabolism also undergoes changes at this time, and the complete explanation as to why regenerative capacity is lost likely involves all of these factors. Importantly, immune cells convey some degree of control over the metabolism and proliferation of cardiac tissue cells during an injury response.8,9 The immune response is critical to regenerative processes in animal models capable of cardiac regeneration, such as the axolotl (Ambystoma mexicanum) and zebrafish (Danio rerio). A comparison of key physiological and immunological properties among adult and fetal/neonatal mammals, axolotls and zebrafish is shown in Fig. 1.

The axolotl is a urodele salamander and a popular model organism within the field of regenerative medicine, due to its ability to regenerate entire limbs, tail, spinal cord, retina as well as a host of other tissues, including substantial amounts of cardiac tissue.10 Similarly to the apparent intrinsic cellular potential for regeneration in injured mammalian fetal tissues, this seems to also be the case for injured axolotl tissues. This has been elegantly demonstrated with limb blastemas, which refers to the mass of cells that initially form at the stump of an amputated axolotl limb. When this blastema is removed and transplanted to another location an accessory limb forms here, provided there is also nerve innervation.11,12,13

The development of axolotls differs from most other salamanders as it is neotenic, which in short entails going through life and reproducing without undergoing anatomical metamorphosis. It can however, be stimulated to metamorphose by exposure to thyroid hormone, and will in rare cases metamorphose in nature.14

When a cryoinfarction (CI) damaging up to 50% of the ventricle is inflicted on an axolotl, it makes a full anatomical and functional recovery in a few months. The damaged tissue is gradually expelled while surrounding CMs dedifferentiate, proliferate and migrate into the damaged area where fibroblasts have deposited new extracellular matrix components.15–18

The zebrafish is the most popular model organism of regeneration, and has many advantages in terms of available molecular tools and cost. When conducting a study where a large sample size is needed, the zebrafish may be a more practical model due to space and cost. The zebrafish larva has the advantage of being see-through which allows researchers to for instance easily detect the expression of fluorescent proteins non-invasively with whole-animal time lapse and time course imaging+. This fact has made the zebrafish popular in for instance developmental studies and studying cell-to-cell interactions utilizing cell tracking.19 The zebrafish is however small, which comes with its own set of limitation for instance in terms of the amount of tissue available from each animal. The axolotl on the other hand requires more space, is more costly and the availability of species specific antibodies and molecular tools is more limited, at least at this point in time. On the plus side, as a tetrapod, it is more comparable to humans in terms of body composition, and its larger size allows for more intricate surgical procedures and larger samples to be collected. Its size also allows for a number of imaging modalities like micro-CT, MRI and PET systems.

| Capable of substantial cardiac regeneration | No | Yes | Yes | Yes |
| Cardiomyocytes capable of proliferation | Limited | Yes | Yes | Yes |
| Cardiomyocytes capable of dedifferentiation | Limited however, successfully induced in vitro | Yes | Yes | Yes |
| Cardiomyocyte binucleation | Widespread binucleation | Mononucleated until shortly before birth | Mostly mononucleated | Mostly mononucleated |
| Cardiomyocyte metabolism | Mainly fatty acid beta oxidation | Mainly glycolysis | Not well described | Mainly glycolysis |
| Thermoregulation | Endothermic | Transition from endothermic to endothermic during development | Exothermic | Exothermic |
| Hemodynamic forces on cardiovascular system | High | Moderate | Low | Low |
| Immune response to cardiac injury | Mainly pro-inflammatory followed by a pro-fibrotic response | Both pro- and anti-inflammatory followed by a pro-regenerative response | Both pro- and anti-inflammatory followed by a pro-regenerative response | Both pro- and anti-inflammatory followed by a pro-regenerative response |
| Subpopulations of macrophages recognized | M1 and M2 with several additional sub classifications (M2a, b, c, d and others) as well as distinct embryonically derived | At least two types of embryonically derived and one type of monocyte derived macrophage | No distinct subpopulations described at this time | Distinct M1 and M2 like subpopulations described |

Fig. 1. Key physiological factors that influence regenerative capacity in adult and fetal/early neonatal mammals, axolotls and zebrafish.
typically used for rodents, to quite easily be transferred to axolotls. With its genome fully sequenced in the beginning of 2018, modern molecular tools are more applicable than ever. Another type of salamander used for regenerative research are newts, which also have a high capacity for regeneration. However, the regenerative process is generally slower in newts compared to axolotls, which can be a challenge for the practicality of an experiment. Additionally, newts undergo spontaneous metamorphosis with important impacts on the regenerative process. They are also more challenging to keep, and require a semi-aquatic terrarium.

To further the understanding of the interplay between the immune response and regeneration it is of great interest to examine immune processes comparatively in non-regenerating and regenerating species. Identifying differences in the immune response that are of importance for functional repair could identify new therapeutic strategies for humans. Much remains to be discovered about the connection between regeneration and the immune response, and the specific signaling events and factors involved in instigating regeneration in axolotls and zebrafish. Here, we hope to inspire others to use these models in regenerative medicine, by providing a detailed introduction to the immune response to myocardial infarction (MI) in humans along with findings about the injury- and immune response in these popular regenerative animal models.

2. The immune response to cardiac injury in mammals – a double-edged sword

2.1. Cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of death globally, making up an estimated 31% of all deaths according the World Health Organization. Thanks to advances in acute care, an increasing number of patients survive initially, but are at risk of developing heart failure later on due to long-term complications caused by fibrosis and adverse remodeling of the surrounding tissue. Unlike that of an axolotl or zebrafish, a mammalian heart will never quite be the same following an infarction.

A MI leads to the death of a significant amount of cardiac tissue that cannot be replaced by the modest proliferative capacity of human CMs. As another strategy to compensate for lost cardiac tissue, CMs often undergo some degree of hypertrophy, a process that can negatively affect the functionality of the heart. Mammalian CMs have traditionally been considered to be completely devoid of proliferative ability in adults, but this view has been challenged as CMs have been shown to have a turnover rate of about 0.5–1%. In the last decade, a large number of studies have centered around the presence of endogenous cardiac stem cells in adult mammalian hearts, which has naturally fostered a great deal of enthusiasm for their possible usefulness in regenerative therapies, however, the existence of such a cell population has remained controversial. Unfortunately, many of the papers in support of cardiac stem cells published by a prominent researcher in the field, Piero Anversa at the Harvard Medical School has in October of 2018 been accused of publishing fraudulent scientific results and many of his landmark papers are now being retracted. In November of 2018, Professor Hans Clevers and his group published their work with single-cell mRNA sequencing of all proliferating cells in healthy as well as damaged murine hearts, which strongly supports that there are in fact no such thing as proliferating adult cardiac stem cells.

Others remain hopeful that CM dedifferentiation may represent a possible mechanism of cardiac repair, as some studies suggest mammalian cardiomyocytes do possess such potential. Still, this is obviously not enough to mend a damaged mammalian heart on its own. Perhaps because non of these paths to new cells are activated in an adequate number of cells, or because there are other mechanisms at play counteracting such efforts. Dedifferentiation appears to be the main source of new functional tissue in animal models capable of regeneration, a process ultimately linked to the immune system, as key factors released by immune cells have been found to induce dedifferentiation of salamander and mammalian CMs in vitro. For instance, embryonic macrophage conditioned media, as well as the interleukin oncostatin-M produced by salamander macrophages has been shown capable of promoting adult CM proliferation. Curiously, stem cells also appear to play a key role in axolotls and in newts after metamorphosis.

The formation of tough scar tissue does have an important purpose in human hearts. Unlike the axolotl heart, which experiences relatively mild hemodynamic forces, and can suffice with closing of an injury by clotting, the human heart must withstand substantial pressure and mechanical force. Compromised myocardium must quickly be reinforced with what is at best a non-contractile, but stable collagen based fibrous substitution to avoid rupture of the myocardial wall. Adverse remodeling of the surrounding tissue can lead to dilation of the ventricle, increased wall thickness, systolic dysfunction and increased risk of heart failure in the future. For any therapeutic strategy aiming to regenerate a human heart, avoiding excessive fibrosis and adverse remodeling, as well as promoting the formation of new functional tissue is of equal importance. Both of which may come about through modulating the immune response.

It is critical to consider, when comparing the responses to a MI in human patients to that of animal models that patients suffering an acute cardiac event have almost certainly suffered from a compromised cardiovascular system for years beforehand. In the majority of MI cases, the patient displays a substantial degree of arthrosclerosis, and often also suffers from hypertension (Fig. 2).

Arthrosclerosis is associated with a chronic pro-inflammatory state in the affected regions of the circulatory system. Hypertension has been linked to increased plasma levels of inflammatory markers. Cardiovascular disease also has a high degree of comorbidity with type-II diabetes, which is also associated with a systemic pro-inflammatory state and impaired healing. This means that the patient may at the time of infarction, have been developing an inflamed and dysfunctional vascular system for years, affecting the ability to mount a balanced immune response and initiate a regenerative program in the heart. Importantly however, adult mammalian animal models like rats and mice, which are otherwise perfectly healthy, are also incapable of substantially regenerating cardiac tissue.

Aging in murine models has been found to impact their ability to resolve inflammation following myocardial injury due to dysregulation of resolvins and metabolic pathways. With the majority of patients that need to be treated following a MI found among the aging population, such studies may suggest that aging murine models represent an interesting model system when researching cardiac repair and regeneration in mammals.

In addition to the before mentioned, there are a number of other factors that may affect the ability to mount a favorable immune response following MI. For instance, the use of common pain relieving medicines has been shown to have unfavorable effects for MI sufferers.

2.2. The immune response orchestrates the response to injury

Inflammation controls key aspects of tissue repair through complex signaling pathways between leukocytes and other cells of the injured tissue. Immune cells participate in removing dead and damaged tissue and can promote repair in the form of regeneration in competent organisms or fibrous scar tissue formation, meaning that the actions of the immune system are critical in determining the outcome following MI. Although a number of comprehensive studies have investigated the inner workings of the immune response following MI in mammals, our current understanding remains a simplified view of an incredibly intricate process.
The MI inflammatory response is referred to as sterile inflammation, and proceeds through three somewhat overlapping phases (Fig. 3). An inflammatory phase is first initiated, followed by a proliferative phase, and finally a maturation phase, with a resulting fibrous scar. Macrophages are involved during all stages of the immune response, with distinct phenotypes making different contributions to inflammation, fibrosis and repair.\textsuperscript{4,40,41} Monocyte derived macrophages are traditionally broadly divided into two main types, the classically activated and pro-inflammatory M1-type and the alternatively activated resolving M2-type.\textsuperscript{42} In addition, embryonically derived macrophages display distinct differences from monocyte-derived cells that make them favor a regenerative response.\textsuperscript{43} The majority of studies on mammalian macrophage heterogeneity have been conducted in murine models. In a 2018 paper,\textsuperscript{40} Bajpai and colleagues demonstrated that human cardiac tissue indeed contains two distinct macrophage subsets. One maintained through migrating monocytes which are CCR2 positive and a CCR2 negative population maintained through local proliferation, similarly to what has been shown in mice.

Today, it is recognized that the M1/M2 paradigm is a gross oversimplification and the source and phenotypes of macrophages still remains to be fully elucidated, however the M1/M2 nomenclature is still widely used. The M1 macrophage is characterized by high expression levels of pro-inflammatory cytokines, strong antibacterial properties, high production of reactive nitrogen and reactive oxygen species and promotion of the Th1 response. The M2 macrophages have also been further divided into subpopulations (M2\textsubscript{a}, M2\textsubscript{b}, M2\textsubscript{c} and M2\textsubscript{d}) based on differences in gene expression as detailed in a widely cited review from 2015.\textsuperscript{44} Overall, M2 macrophages are activated to a greater extent by tissue damage and produces less pro-inflammatory factors in favor of anti-inflammatory and pro-fibrotic factors. It is not fully established if M1 macrophages can shift to a M2 type, take on a mixed phenotype or if these macrophage subsets are derived from entirely different sources.

The inflammatory phase is characterized by the migration of leukocytes including neutrophils and monocytes into the damaged tissue, activation of local vasculatory cells and fibroblasts and the release of pro-inflammatory mediators. The inflammatory phase is rolled back as neutrophils undergo apoptosis, macrophages are found with an M2-phenotype and participate in resolving inflammation and remove apoptotic cells through phagocytosis.\textsuperscript{44,45} The cytokine milieu changes from being pro-inflammatory to immune-resolving and lymphocytes enter the site of injury in increasing numbers. The proliferative phase is marked by the proliferation and differentiation of fibroblasts to myofibroblasts, producing collagen and other key extracellular matrix proteins and rich neo-vascularization, all resulting in the formation of a dense collagenous scar surrounded by granulation tissue. During the maturation phase the myofibroblasts undergo apoptosis and the newly formed collagenous and granulation tissue is gradually replaced by a stronger, cross-linked fibrous matrix.

2.3. The inflammatory phase

As blood flow is restricted to the infarcted area, affected CMs become oxygen starved and die primarily by apoptosis or necrosis. The dead and dying cells set the alarm as they leak and rupture, allowing intracellular elements normally restricted to the cells interior to enter the extracellular compartment alerting resident immune cells. These damage-associated molecular patterns (DAMPs), will alongside other effectors like heat-shock proteins, fragmented extracellular matrix proteins and ATP (adenosine triphosphate) activate the complement system, extracellular TLRs (toll-like receptors) and intracellular NOD-like (nucleotide-binding oligomerization domain-like) receptors, setting of an inflammatory cascade as further detailed in previous reviews.\textsuperscript{46,47} The activated immune cells as well as CMs and supporting cells like fibroblasts produce ROS and pro-inflammatory cytokines, which in turn recruits additional leukocytes to the site.

Neutrophils first migrate to the injury site followed by monocytes differentiating into M1-type macrophages, while different classes of lymphocytes and dendritic cells follow at different time points.\textsuperscript{48} Vascular endothelial cells activated in response to MI increase their expression of adhesion molecules like I-CAM (intercellular adhesion molecule 1), V-CAM (vascular adhesion molecule 1) and selectins\textsuperscript{49} while the vessel becomes more permeable, facilitating the trans-migration of leukocytes across the vessel wall and into the damaged tissue. Some cytokines also have systemic effects, such as IL-6 (interleukin-6) that mediates the release of CRP (C-reactive protein) from the liver. Elevated plasma levels of both IL-6 and CRP as well as TNF-\alpha (tumor necrosis factor alpha) after a MI are associated with increased mortality, indicating that excessive inflammation does more harm than good.\textsuperscript{50-52}

Necrotic and damaged cells activate the complement system, which marks damaged cells for destruction and phagocytosis. Complement factors will also engage directly in cell destruction through the membrane attack complex, composed of a number of complement factors acting in concert to lyse and kill cells.\textsuperscript{53} Complement plays an important role in clearing damaged cells, however, the amplification cascade of complement can cause devastating tissue damage if it gets out of hand. Neutralization of complement has been shown to reduce myocardial injury and mortality in MI patients.\textsuperscript{54-56}

Neutrophils are drawn to the site of injury by pro-inflammatory cytokines, DAMPs, complement factors and ROS.\textsuperscript{57} Neutrophil numbers peak a couple of days post-MI, and then gradually decrease with very
few neutrophils remaining once the inflammatory phase subsides.\textsuperscript{48} They are activated through TLR and NOD signaling, inducing the release of ROS and pro-inflammatory cytokines.\textsuperscript{58,59} The cytokines expressed by neutrophils during the inflammatory phase are pro-inflammatory, and promote the infiltration and proliferation of monocytes, dendritic cells, natural killer cells, Th1 (T helper cell type 1), Th17 (T helper cell type 17) and B-lymphocytes as well as additional neutrophils.\textsuperscript{60–64} Neutrophils are also a source of matrix degrading enzymes, such as MMP-8 and MMP-9 (matrix metallopeptidase 8- and 9), collagenase and neutrophil elastase.\textsuperscript{65} Neutrophils phagocytose the degraded matrix components and cells coated in complement oposins. Complex signaling network controls the expression pattern of neutrophils. For instance, IFN-\(\gamma\) (interferon gamma) promotes the release of pro-inflammatory cytokines, while IL-10 does the

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**Fig. 3. Events of the inflammatory response to myocardial injury in adult mammals during the inflammatory, proliferative and maturation phases.**

Solid arrows indicate the cell produces the resulting factors. Dashed arrows indicate the cell is recruited via chemotaxis by the given factors. Factors represented by a circle are cytokines, factors represented by a triangle are growth factors, those represented by a hexagon are other types of signaling molecules. The status of vessels in and around the site of injury is displayed to the far left in each panel. Abbreviations used in the figure are as follows: CXCL = chemokine (C-X-C) motif ligand, DAMP = damage-associated molecular pattern, FGF = fibroblast growth factor, IFN-\(\gamma\) = interferon gamma, IGF-1 = insulin like growth factor 1, IL = interleukin, MCP-1 = monocyte chemoattractant protein 1, MMP = matrix metallopeptidase, TGF-\(\beta\) = transforming growth factor beta, Th1 = T helper cell type 1 (lymphocyte), Th17 = T helper cell type 17 (lymphocyte), TLR = toll-like receptor, TNF-\(\alpha\) = tumor necrosis factor alpha, Treg = T regulatory cell (lymphocyte), PDGF = platelet-derived growth factor, ROS = reactive oxygen species, VEGF = vascular endothelial growth factor.
Neutrophils play a critical role in shaping the inflammatory re- sponse, but excessive neutrophil activation leads to uncontrolled pro-inflammatory and enzymatic activity. Failure to attenuate the neu- trophils response at an appropriate time can cause devastating tissue damage. Inhibition of some of the neutrophil-sourced enzymes, and reducing the infiltration of neutrophils by antibody mediated blocking of certain adhesion molecules has been proven to reduce the extent of tissue damage following MI and reperfusion.

Monocytes first arrive at the site of injury shortly after neutrophils in response to chemotactic agents like MCP-1 (monocyte chemotactic protein 1) produced by neutrophils and endothelial cells. Histamine released by mast cells causes the local vasculature to dilate and increases the permeability of the vessels, which appears critical for the entry of monocyte-derived macrophages as experimental knock-out of histamine in a murine model showed dramatically reduced number of macrophages at the infarction site, and an exacerbation of cardiac injury.

The infiltrating monocytes differentiate into M1-type macrophages in response to high levels of IFN-γ, TNF-α and DAMPs at the site of injury, and play a critical role in orchestrating the inflammatory phase. M1-macrophages are first detected about 24 h post MI, and typically peak four days later. The gradual shift towards M2 polarization starts around five days post-MI.

M1-macrophages release the pro-inflammatory cytokines TNF-α, IL-1β, CXCL10 (chemokine C-X-C motif ligand 10), IL-6, IL-12 and IL-23. Plasma levels of TNF-α following a MI is an indication of the severity of MI damage and higher levels are associated with increased risk of heart failure. IL-1β is one of the most potent pro-inflammatory cytokines produced, and has wide reaching effects promoting the recruitment of additional inflammatory cells, stimulating the degradation of extra- cellular material and inhibiting the formation of myofibroblasts, which is why IL-1β must be down-regulated for the proliferative phase to begin. CXCL10 is a chemoattractant of other macrophages, dendritic cells, NK cells (natural killer cells) and Th1 lymphocytes, that also enhances the entry of Th1-lymphocytes. IL-12 and IL-23 influence the development of Th1 and Th17 lymphocytes respectively. Th17 cells are pro-inflammatory and characterized by their production of IL-17, which is why IL-17 is a key player in the inflammatory phase. At this time, neutrophil numbers are declining. Neutrophils at the end of their life perform important functions as they release in-flammatory and enzymatic activity. Failure to attenuate the neutrophil response at an appropriate time can cause devastating tissue damage.

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following MI. Treg cell depleted mice display increased and prolonged infiltration of neutrophils, increased numbers of M1-type macrophages, and decreased numbers of M2-macrophages accompanied by decreased levels of the M2 anti-inflammatory cytokines IL-10 and TGF-β. Treg cells themselves also secrete IL-10 and TGF-β. The newly formed myofibroblasts are stimulated to secrete collagen (mostly type-III), fibronectin and other extracellular fibers in response to TGF-β. Myofibroblast are characterized by their expression of smooth-muscle alpha actin, allowing the cells to contract much like a smooth muscle cell. In the context of tissue injury, the myofibroblasts utilize their contractile ability to pull the outer edges of the injury site together, in essence closing the wound. This can however, also create abnormalities in the surrounding tissue.

Matrix turnover is continuously regulated by macrophages and possibly other cells through the regulation of MMPs and their inhibitor TIMPs (metalloproteinase inhibitors). Through carefully controlled matrix deposition and augmentation, a temporary collagenous matrix rich in fibrin and fibronectin is formed.

As the proliferative phase is completed, a highly interactive collagenous scar is evident that still contains active myofibroblasts. Highly vascularized granulation tissue surrounding the scar still holds lymphocytes and macrophages that are believed to play an active role initiating the maturation phase.

2.5. The maturation phase

Following the proliferative phase, the newly laid type-III collagen is gradually exchanged with type-I that becomes cross-linked, increasing the tensile strength of the scar. There is an ongoing remodeling process during the maturation phase as shifting levels of different MMPs and TIMPs are produced and degraded. The matrix becomes mostly devoid of cells as most of the remaining leukocytes undergo apoptosis, however, some myofibroblasts may persist. The continued presence of myofibroblasts can have disadvantageous effects on cardiac function, as they, as well as the scar itself, displays different electrical properties than CMs. Most of the newly formed vessels disappear, as the new fibrous tissue no longer requires the same degree of oxygen delivery.

It is unclear exactly how the events of maturation are set in motion. M2-type macrophages are likely involved in inducing the apoptosis of myofibroblasts, and it has been suggested that this works in part through an expression of the enzyme arginase that depletes an essential amino acid from the local environment, that myofibroblasts as well as T-lymphocytes need for continued survival. Expression and secretion of anti-fibrotic mediators likely play a key role during this time, however, the exact nature of these effectors is largely unknown.

The mature scar ideally remains stable with minimal effect on cardiac function for years to come, however for unknown reasons it is not uncommon for a gradual diffuse fibrotic process to continue where the surrounding tissue over time becomes increasingly fibrotic. Disturbed flow and mechanical stress surrounding the damaged area may induce the continued expression of pro-fibrotic mediators like TGF-β. It is critical that the transition from the proliferative to the maturation phase occurs at an appropriate time. If the maturation phase begins to early the scar matrix may still be inadequate, and collagen deposition will cease as myofibroblasts disappear, resulting in an unstable scar prone to rupture. Conversely, a delay in the events of maturation, allows for prolonged tissue degradation and collagen formation, creating a larger scar area more likely to cause cardiac failure.

3. The fetal and neonatal immune response and cardiac regeneration

It has been hypothesized that the complexity of adaptive immunity that follows ontogeny is strongly associated with decreased regenerative capacity. Retained regenerative ability is seen in humans in certain tissues like the liver and endometrium. As before mentioned, mammals hold a much greater capacity for regeneration during fetal and early neonatal life. Murine fetuses have been shown to fully regenerate cardiac tissue following different models of injury, including coronary artery ligation and CL. Around postnatal day 7, the ability to heal without scarring and substantially regenerate lost cardiac tissue is more or less completely lost. This time correlates with when rodent CMs are no longer able to extensively proliferate, and when drastic alterations to the immune system occur as it begins to mature. The immune system also undergoes further alterations with age, corresponding to a simultaneous gradual decline in regenerative ability. The immature nature of the mammalian neonate immune system leaves the neonate more prone to infection and allergies, but may convey a regenerative advantage.

Some significant general differences in the immune response in neonates compared to adult mammals have been identified mostly using murine animal models as described in other reviews. Following cardiac injury in neonates, the infiltration of neutrophils, monocytes and macrophages is modest compared to adults. The macrophages that are present within the cardiac tissue, are mostly embryonically derived cells not differentiated from monocytes. These cells have a far better ability to phagocytose damaged cells and debris, compared to adult M1-type macrophages. These embryonic macrophages do not produce pro-inflammatory cytokines to the same extent as the macrophages that infiltrate site of injury in adults. Instead, these embryonic cells release IL-10, IL-6 and IL-23. IL-10 acts as a potent anti-inflammatory cytokine in a similar fashion in both adults and neonates, while IL-6 has been shown to inhibit the recruitment of neutrophils in neonates, while doing the opposite in adults. In one study, media conditioned by isolated embryonic macrophages could stimulate tube formation of endothelial cells in culture, indicating a strong pro-angiogenic potential, and induce proliferation of cultured rat CMs, while adult monocyte derived macrophage cultured media did not have the same potential to stimulate regenerative processes. However, others have observed adult macrophages successfully induce endothelial cell tube formation.

Endothelial cells have been shown to express less adhesion molecules during injury in neonates, reducing the number of leukocytes migrating into the tissue. As mentioned previously, embryonic macrophages also exist in the adult myocardium, albeit in more modest numbers, and are far outnumbered by infiltrating monocytes differentiating into M1-type macrophages following an injury. They also appear to gradually be replaced by different resident macrophages with age. Ablation of macrophages in neonates significantly hinders the regenerative process, as a fibrotic scar forms much like what happens in adults while neo-angiogenesis is impaired. As before mentioned, the same thing happens in axolotls in response to macrophage depletion.

The complement system is also somewhat different in neonates. Complement factors appear to be less cytotoxic, less haemolytic and found in smaller amounts in neonates. The lymphocyte response also differs. Antigen presenting cells express far lower levels of TLRs and MHCs (major histocompatibility complexes), and TLR stimulation of fetal blood has indicated that TLR activation doesn’t set in motion a pro-inflammatory cascade, but rather curiously increases the inflammatory cytokines to the same extent. The immune system also undergoes references in the immune response in neonates, reducing the number of infiltrating monocytes.

Mast cells secrete much more histamine in neonates, which favors M2-macrophage polarization and the differentiation of Th2 rather than Th1 lymphocytes. Th2 lymphocytes are not cytotoxic and express IL-4, IL-5 and IL-13 while counteracting IFN-γ induced tissue damage. It appears that the default pathway for naive neonatal lymphocytes, is to become Treg cells, which, as mentioned previously have been found to promote M2-macrophage polarization and convey cardio-protective and pro-regenerative signals in adults. There is generally a higher level of circulating Treg cells in neonates.

There is convincing evidence, that the neonatal immune response
and especially embryonic macrophages plays an important role in allowing mammals to regenerate tissues at this stage in life. Importantly, if these pathways exist in neonates it could be possible to reactivate them in adult mammals.

In a 2019 study, researchers injected isolated and decellularized cardiac extracellular matrix from neonatal mice around an infarcted area of adult hearts in vivo, resulting in improved outcomes and reduced fibrosis. These treatments also resulted in reduced number of macrophages present at the site of injury six weeks post injury, indicating that the neonatal extracellular material is able to convey an inflammation-resolving effect. Such results show that the enhanced ability to resolve injury and inflammation in neonates, is perhaps not solely facilitated by cell-dependent signaling. The same research group previously performed a similar study using decellularized zebrafish extracellular matrix, which was able to promote repair following infarction in a mouse model.

4. The axolotl immune system

In the context of axolotl research, the previous sections on the mammalian immune response and regenerative abilities of adult versus neonatal mammals begs the question, does the regenerative ability of the axolotl simply come down to a preserved neonate-like immune system?

The urodele axolotl is believed to have a rather immature immune system compared to mammals and anurans. Axolotls display a highly limited repertoire of MHC-II molecules, a fact believed to be of importance for their week humoral immune response. However, their immune system is certainly not non-existent. The majority of the components of a human immune system are also present in axolotls. They produce three subsets of granulocytes; neutrophils, eosinophils and basophils.

Morphologically highly similar to human granulocytes (Fig. 4). These cells are primarily found in the cortical layer of the liver in the axolotl, and while neutrophils are the dominant granulocyte in human blood, the eosinophil is more plentiful in neotenic axolotls. Monocytes virtually indistinguishable from human monocytes are present in the blood, and likely differentiate into macrophages through similar signaling pathways as in mammals as MCP-1 as well as CSF-1 and CSF-2 (macrophage colony-stimulating factor – 1/2) have been detected in axolotls and coincide with the presence of macrophages in the tissue. We have characterized the systemic cellular immune response following a surgical intervention and cryoinfarction in the axolotl, and found a clear up-regulation of eosinophils and neutrophils as well as monocytes, lymphocytes and plasma cells (Fig. 4a-e). Only a limited number of papers contain base values of leukocyte numbers in axolotls and it is not described at this time, how axolotl leukocyte numbers may be affected by the mode of blood collection, the specific strain or sex of the animals, rearing temperature etc. This would however, be an interesting subject for future studies. We have here provided novel data displaying the cellular inflammatory response in axolotls. This may aid other researchers in identifying leukocytes of interest for future studies and serve as an indication of what type of immune response one might expect to see in this model organism.

Macrophages play a critical role during regeneration in axolotls, but it is not characterized at this point, to which degree axolotl macrophages differentiate into subsets and how this may be relevant in the context of cardiac regeneration. In 2013, Goodwin and colleagues reported that after ablation of macrophages in axolotls prior to amputation, limb regeneration was completely blocked, and the animals instead displayed fibrotic scarring similar to wound healing in mammals. They have since shown that cardiac regeneration similarly fails as a result of macrophage ablation.

In their study on limb regeneration they found that following amputation the animals mount an immune response within 24 h that unlike that of adult mammals already at this early time consists of a reciprocal launch of both pro- and anti-inflammatory signaling, much like neonatal mammals as discussed previously. Neutrophils appeared at the site of injury within 24 h and where present for 4–6 days.

![Fig. 4. Cellular immune response to surgical intervention and cryoinfarction.](image-url)
Macrophages were also seen at these early time points with the expression of MCP-1 at its highest level at the site of injury already within the first day. These early arrivals where actively phagocytosing and peaked in numbers around 4–6 days post injury, slightly earlier than in adult mammals.

Following limb amputation, axolotls typically form a blastema rather than fibrous tissue, from which a new limb bud develops that gradually forms into a normal limb. The blastema is rich in collagen-III characterized by thin flexible fibers and fibronectin. Macrophage ablated animals instead formed a fibrous cap rich in thick collagen-I fibers and reduced levels of fibronectin. Interestingly, an ablated animal could later regenerate the limb successfully upon removal of the fibrous tissue after macrophage levels had been restored.

Macrophage depletion generally led to a higher degree of pro-inflammatory markers in the blastema, with simultaneously decreased levels of anti-inflammatory cytokines. TGF-β failed to be expressed and MMP-9 and MMP-3 were both reduced along with fibronectin. Furthermore, α-smooth muscle actin positive myofibroblasts were present in significantly higher levels than normal, all pointing towards a dysregulation of the remodeling process of the extracellular matrix as a key component of the failure to mount a regenerative response. TGF-β inhibition alone has also previously been found to inhibit limb regeneration.

Macrophage depletion had similar effects on cardiac regeneration following a CI with the formation of fibrous tissue in the axolotl heart, leading to abnormal cardiac function and increased mortality among the animals, apparently due to heart failure. The fibrous tissue formed in the heart was as the limb stump, found to be rich in collagen-I and lacking collagen-III and fibronectin, with myofibroblasts present. Furthermore, cardiac tissue in the macrophage-ablated animals displayed dysregulation of several LOX (l-lysyl oxidase)- enzymes, a family of proteins known to be associated with maturation of extracellular matrix components. Overexpression of LOX has been shown to induce cardiac hypertrophy and adverse remodeling in mice.

Neovascularization also appeared to be inhibited by a lack of macrophages while the proliferation of CMs was not affected. This shows that CM proliferation is independent of macrophage signaling, but also that the production of new CMs is not on its own sufficient for successful regeneration, a fact that may be critical to consider when developing new human therapies. The researchers also attempted to ablate macrophages at a later time point, 10 days after CI. They found that this prolonged the healing period, but could not block regeneration, suggesting that the regenerative program which is at least in part set in motion by macrophage signaling is mainly active during the early stages of regeneration as full recovery following a CI is only completed after at least 60 days.

These recent studies highlight the importance of the immune response in the context of regenerative medicine. It also clearly shows that the axolotl immune response to cardiac damage is perhaps not so much a case of a lesser response, but rather a different one. This hypothesis is further supported by findings in the zebrafish as discussed in the next section.

5. Important clues about the immune response and cardiac regeneration from zebrafish

The zebrafish immune system has been extensively studied, especially in terms of the innate immune system because the adaptive immune system develops after a well-defined lag phase, allowing innate processes to be studied in isolation. In recent years, the involvement of the immune response during fin and cardiac regeneration has also gained attention. In a 2017 study, large-scale transcriptome data from the regenerating zebrafish and another teleost fish, the medaka (Oryzias latipes) largely devoid of regenerative potential, were compared following cardiac injury. This demonstrated that a vast number of differentially expressed genes could be identified, related to the immune response, as well as metabolic processes, cell cycle control, oxidative reduction, DNA replication and stress response. Processes like the immune response, phagocytosis, angiogenesis and cell proliferation happen at a slower pace in the non-regenerating medaka compared to the zebrafish, a highly interesting observation supporting the idea that a different rather than a tempered immune response is required to minimize damage caused by inflammation and instigate reparative processes. In zebrafish, early activation of NFkB as well as the PI3K/Akt (phosphoinositide 3-kinase/alpha serine-threonine protein kinase) pathway and TLR signaling appear to be instrumental in facilitating cardiac regeneration. Following cardiac injury the non-regenerating medaka displayed a delayed and reduced macrophage response compared to zebrafish, resulting in slower clearance of neutrophils and defective neo-vascularization. When macrophage recruitment is delayed in zebrafish cardiac regeneration is compromised, while stimulating toll-like receptor signaling in the medaka enhanced the immune response resulting in enhanced injury resolution. Zebrafish have been shown to produce at least two distinct macrophage populations, a M1-type and an M2-type macrophage, with M1s transitioning to M2s during the course of the inflammatory response. The M1 type macrophages of zebrafish have been shown to express high levels of TNF-α, TNF-β, IL-1β and IL-6, while the M2 type express predominantly TGF-β1, CCR2 (C-C chemokine receptor type 2) and CXCR4b (C-X-C chemokine receptor 4b). This illustrates how cell surface markers can not be assumed to be shared between a certain subpopulation of cells in mammalian models and comparable cells in for instance zebra fish, as CCR2 is associated with M1-type macrophages in mammals.

Neutrophils quickly enter sites of injury in the zebrafish, but are more effectively cleared compared to the medaka. This again suggests that a swift and sufficient, yet appropriate immune response is required for successful cardiac regeneration in the zebrafish, which also appears to be the case in the axolotl.

Zebrafish also appeared to mount a stronger complement activation along with more pronounced B- and T-cell recruitment. The medaka on the other hand displayed a stronger and more prolonged recruitment of neutrophils and monocytes as well as an enhancement other metabolic pathways known to be associated with a pro-inflammatory milieu, such as steroid hormone response and ROS biosynthesis.

The immune response in axolotls and zebrafish is at the very least one of the important factors that makes them such gifted regenerators. Lessons from the immune system of such animal models could reveal key differences between theirs and the human immune responses following comparative cardiac damage. These discrepancies could prove instrumental in discovering how human hearts may heal more successfully through new forms of immunotherapy, aimed at mimicking the response of successful regenerators.

6. Discussion and perspectives: what can we learn from the immune/regeneration interplay in regeneration competent models?

The immune response plays an integral role in determining whether an infarction injury results in regeneration or fibrotic scarring. Especially the role of macrophages has been illustrated in both adult and fetal/early neonatal mammals as well as regenerating species like axolotls and zebrafish. In order to understand how these cells orchestrate such different responses to similar injuries it will be highly relevant to investigate more closely the phenotypes of axolotl and zebrafish macrophages (as well as those of other cardiac regeneration competent organisms). In the future, it would be of great value to verify which subpopulations of macrophages exist in the axolotl and zebrafish, as well as distinct surface markers. To date this is very limited in the axolotl and zebrafish beyond what is mentioned previously. Mapping out the mechanisms that are under the control of macrophage mediated signaling during regeneration in these animal models would provide novel therapeutic strategies for human immunotherapies.
The idea of immunotherapy for cardiac injury is not new, but non have resulted in true cardiac regeneration thus far. There are a number of strategies being explored to provide relief to the millions of people suffering from complications of a failing heart. These span a broad spectrum of approaches including different strategies for promoting endogenous cardiac repair, stem cell treatments as well as ex vivo techniques such as mechanical hearts, in vitro culture or even whole-organ 3D printing. In any case, these ideas in some way involve the immune system, or in the case of organ transplant – the circumscription of the immune response.

Here we would like to note that in studies utilizing cardiac stem cells in order to heal cardiac injury (which have provided some promising results), it has been found that while these introduced stem cells do not actually repopulate the myocardium per se, they produce a beneficial signaling environment that allows the hearts intrinsic repair mechanisms to gain footing. This appears to involve polarizing macrophages towards a more favorable phenotype.

Endogenous approaches are clearly the least invasive and likely more cost effective, however these ultimately require us to overcome the biological roadblocks that hinder adult mammalian cardiac regeneration in the first place. There is a need to determine to what extent pro-regenerative macrophages or the factors produced by such cells is capable of promoting true regeneration of adult mammalian myocardium in vivo rather than in cell culture alone, and if not, what inhibiting effects stunts these efforts - and can they be overcome? Comparative studies involving animal models both capable and incapable of cardiac regeneration such as those covered in this review will surely be instrumental in answering these questions about how exactly the immune system can promote cardiac regeneration, and if these findings can be transferred to actual human patients.

Author contributions

A. Dittrich drafted the manuscript in collaboration with H. Lauridsen. A. Dittrich performed the experiments referred to in Fig. 4.

Declaration of interest

None.

Ethical statement

All animal experiments as referred to in Fig. 4 was performed in accordance with local legislation and approved by the Danish National Animal Experiments Inspectorate/Dyreforsøgstilsynet under protocol 2015-0201-00615.

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