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Immune Modulation of Stem Cells and Regeneration

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Abstract

The immune system, best known as the first line of defense against invading pathogens, is integral to tissue development, homeostasis and wound repair. In recent years, there has been a growing appreciation that cellular and humoral components of the immune system also contribute to regeneration of damaged tissues, including limbs, skeletal muscle, heart and the nervous system. Here, we discuss key findings that implicate inflammatory cells and their secreted factors in tissue replacement following injury via stem cells and other reparative mechanisms. We highlight clinical conditions that are amenable to immune-mediated regeneration and suggest immune targeting strategies for tissue regeneration.

Keywords

regeneration; inflammation; macrophage; regulatory T cell

Introduction

For centuries, biologists have marveled at the ability of organisms such as salamanders to re-grow near perfect copies of amputated body parts through a precisely orchestrated process called epimorphic regeneration. Epimorphic regeneration occurs via the formation of a blastema, a mass of undifferentiated and differentiated cells containing a heterogeneous pool of progenitor cells. Instead of forming a blastema, the few mammalian tissues that are capable of regenerating, such as blood, skeletal muscle and epithelium, renew predominantly through stem cells. However, stem cell based regeneration has not proven broadly effective for most tissues plagued by degenerative processes such as the heart and nervous system. Here, we suggest that immune-mediated mechanisms of regeneration and repair may complement existing stem cell therapies or may be a viable alternative to using stem cells as a way to promote functional regrowth of vital tissues.

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Regenerative capacity and the development of a mammalian immune system: an inverse relationship

Following injury, immune cell activation is among the first responses detectable at the site of damage (Figure 1A). Whether immune activation results in tissue regeneration or scarring is determined by a variety of factors including age, species, and the availability of a stem or progenitor cell pool. Evolutionary and developmental advances in the immune system have been inversely correlated with the capacity to fully regenerate damaged tissues (Figure 1B) (Fukazawa et al., 2009; Mescher and Neff, 2005; Mescher et al., 2013). The more phylogenetically primitive urodele amphibians (salamanders), the only vertebrates with the ability to completely regenerate limbs as adults, have a “weak” immune response in terms of specificity, speed of onset, and memory compared to their anuran amphibian (frog) relatives. Selective pressure to seal wounds rapidly with impermeable scars may have increased as vertebrates left aquatic environments and became homeothermic, driving evolution of a robust inflammatory response and refined adaptive immune system at the expense of epimorphic regeneration. Likewise, Xenopus larvae, which start with an ancestral-like immune system, can regenerate hindlimbs and tails. After the peak of metamorphosis, when the immune system matures into a highly evolved, mammalian-like system, regenerative capacity is lost (King et al., 2012). In mammals, wounds penetrating the dermis undergo scarring postnally. During fetal development, however, dermal injuries regenerate in a distinct process of scarless wound healing (Table 1). A number of factors likely enable scarless healing (Larson et al., 2010), including varied composition of the extracellular matrix (ECM), intrinsic differences in fibroblasts, and a transient inflammatory response characterized by significant decreases in platelet aggregation, leukocyte infiltration, and cytokine production. In depth understanding of the unique immune systems of fully regenerating organisms or developmental stages may provide clues to therapeutic avenues to restore damaged tissues in mammals.

Developmental and homeostatic functions of the immune system

The immune system is integral to the initial development of an organism as well as the continuous replacement of differentiated cell types to maintain homeostasis (Figure 1A) (reviewed in (Pollard, 2009; Wynn et al., 2013)). Branching morphogenesis and remodeling in the kidney, pancreas, mammary gland, retina and vascular system are regulated by leukocytes and soluble inflammatory factors. Myeloid cells, for example, modulate vascularity (Nucera et al., 2011) by mediating angiogenic branching (Kubota et al., 2009) and anastomosis (Fantin et al., 2010). Mice deficient in the primary regulator of mononuclear phagocyte production, colony-stimulating factor 1 (Csf1), or its receptor Csf1R lack the majority of functional myeloid cells and display numerous developmental abnormalities due to disrupted ECM remodeling (Banaei-Bouchareb et al., 2004; Dai et al., 2002; Pollard JW, 1996; Rae et al., 2007). Branching morphogenesis in the mammary gland depends on eosinophils and mast cells (Gouon-Evans et al., 2000; Lilla and Werb, 2010), illustrating that numerous immune cells coordinate development. Immune cells also influence morphogenesis by acting directly on mammary stem cells (Gyorki et al., 2009) and phagocytizing both apoptotic and senescent cell debris (Dai et al., 2002; Munoz-Espin et al., 2013). Microglia phagocytize synaptic debris and are essential for the pruning of synapses during normal postnatal brain development (Paolicelli et al., 2011). Synapse pruning in the

developing retina relies on complement proteins C1q and C3 to tag CNS synapses for destruction (Stevens et al., 2007). Conversely, C1q and C3 are up-regulated in retinal synapses during glaucoma, suggesting aberrant reactivation of this developmental pathway promotes CNS degenerative disease.

Gene expression profiling, lineage tracing, and genetic models have been increasingly used to identify novel tissue-specific, subsets of immune cells that shape development and regeneration (King et al., 2012; Wynn et al., 2013). The recent discoveries discussed below begin to shed light on how these specialized or polarized populations of immune cells may be integral to promoting regeneration in mammalian tissues. The nature and efficiency of the reactivation of developmental functions during injury may be critical to the ability of an organism to completely regenerate injured tissue or not (Figure 1A). In this review, we focus on recent advances showing a proactive role of the immune system and its response to injury as a central mediator of tissue regeneration. By drawing from different systems, common mechanisms and themes occurring in injury and disease that may be relevant to new therapeutic avenues are highlighted.

Immune mechanisms of tissue regeneration

Mammals respond to organ damage through either compensatory growth of the remaining tissue, by activating resident precursor cell proliferation, or by the formation of a scar. Successful mammalian regeneration requires precise coordination of multiple processes, which include scavenging cellular debris, proliferation and activation of progenitor cells, immune modulation, angiogenesis and innervation of the newly forming tissue. While the involvement of immune cells in tissue repair has been appreciated since Metchnikoff observed that macrophages play a role in tissue repair in the late 1800’s, recent advances highlight new mechanisms that the immune system employs to regulate regeneration.

Debris clearance

Efficient clearance of cellular debris prevents the persistence of potentially toxic or immunogenic material in the tissue environment and also activates phagocytes to secrete immunomodulatory factors that perform downstream effector functions (Figure 2). Recent findings indicate that defects in debris clearance can prevent effective regeneration. Macrophages, the professional phagocytes of the immune system, mount a polarized, biphasic response to tissue injury. Macrophages and the monocytes from which they are derived exhibit considerable heterogeneity that is not yet fully understood. Following conditioning by the inflammatory milieu including local growth factors and cytokines, macrophages polarize into classically activated (M1) or alternatively activated (M2) subtypes based on their markers, function, and cytokine profiles (Gordon and Martinez, 2010). Typically, M1 cells produce high levels of pro-inflammatory cytokines and nitric oxides that aid in host defense but can also damage healthy tissue while M2 macrophages mediate wound healing, tissue repair and the resolution of inflammation. However, M1 cells can also play a positive role in tissue regeneration. In the heart and skeletal muscle, early infiltration by M1 macrophages facilitates clearance of necrotic tissue (Arnold et al., 2007; Nahrendorf et al., 2007), and disrupting macrophage polarization impairs healing and regeneration, respectively (Perdiguero et al., 2011). While macrophages influence multiple

facets of muscle regeneration, it appears that in some instances, debris clearance may supersede their roles in satellite cell proliferation, myofiber growth, and endothelial cell activation. For example, Hif-1a, the master transcriptional regulator of the hypoxia response, is dispensable in satellite cells during skeletal muscle regeneration. Surprisingly, myeloid-specific deletion of Hif-1a leads to decreased activation of Cox-2, decreased macrophage phagocytosis, and a subsequent delay in skeletal muscle regeneration (Scheerer et al., 2013).

Perhaps the strongest link between immune-mediated debris clearance and regenerative capacity has been documented in the CNS and demyelinating diseases (Figure 2). The sensitivity of the CNS to debris clearance may be attributed to the numerous inhibitory properties of myelin, the membrane sheath that insulates axons, when deposited in damaged tissue. While remyelination is robust in the CNS of young, healthy mice, the ability to restore the myelin sheath declines with age or in disease. Clearance of myelin debris depends on macrophages (Kotter et al., 2006) and recent data suggest that the decline in efficient CNS regeneration is linked to the immune system. Specifically, parabiosis experiments indicate that young macrophages recruited as monocytes from the blood have a greater capacity to efficiently clear myelin debris than old macrophages (Ruckh et al., 2012). Furthermore, chronic degenerative disease occurs when phagocytosis is compromised by loss-of-function mutations in microglial Triggering Receptor Expressed On Myeloid Cells 2 (TREM2) or DAP12 (also known as TYRO protein tyrosine kinase-binding protein), its transmembrane adaptor and signaling molecule. (Neumann and Takahashi, 2007). Conversely, transplantation of myeloid cells that over-express TREM2 into experimental autoimmune encephalomyelitis (EAE) mice, a model of multiple sclerosis, improved myelin removal and facilitated regeneration of the spinal cord (Takahashi et al., 2007). Given the inhibitory effects of myelin on oligodendrocyte differentiation, these data suggest a model in which augmenting the clearance of debris by immune cells can enhance CNS regeneration through efficient remyelination. Elegant genetic studies such as those in skeletal muscle injury models, discussed below, are beginning to reveal the significance of non-macrophage cell types in debris clearance and regeneration (Figure 2). For example, debris clearing fibro/adipocyte progenitors have recently been shown to be pivotal mediators of skeletal muscle regeneration and are discussed in greater detail below (Heredia et al., 2013). Whether varied types of debris and their key phagocytic cell types can influence regeneration in other tissues will be an important topic for future investigation

Progenitor cell activation and stem cell function

Perhaps the most astonishing discoveries in immune-mediated regeneration have been made in skeletal muscle, a well-studied model for adult mammalian regeneration that employs activation of satellite cells, the resident progenitors of the muscle. While the importance of macrophages in skeletal muscle regeneration have long been appreciated (Arnold et al., 2007), both eosinophils and regulatory T cells (T_{regs}) have now been shown to be necessary for activation of satellite cells, which give rise to newly formed myofibers following injury (Burzyn et al., 2013; Heredia et al., 2013; Wynn et al., 2013) (Figure 3A). Using a number of genetic models to trace and delete soluble factors and their receptors in a cell-type specific fashion, Heredia et al. showed that IL-4-secreting eosinophils mediate skeletal muscle regeneration by activating fibro/adipocyte progenitors (FAPs) which, as mentioned

previously, mediate necessary debris clearance. In the absence of IL-4, FAPs do not clear debris and instead differentiate into adipocytes, which contribute to muscle degeneration (Figure 2). T_{regs} modulate the activity of not only lymphocytes but also other immune cells such as macrophages, and thereby can indirectly influence the regenerative process. However, genetic ablation of T_{regs} has shown that muscle T_{regs} directly enhance satellite cell activation and differentiation by secreting amphiregulin (Burzyn et al., 2013) (Figure 3A).

Neurons do not efficiently regenerate in mammals and several studies suggest that the inflammatory response to injury impedes neurogenesis (Carpentier and Palmer, 2009; Ekdahl et al., 2003; Monje et al., 2003). In the zebrafish brain, which has the capacity to regenerate and replace neurogenic activity, recent work shows that inflammation is necessary and sufficient to initiate neurogenesis via progenitor cell activation. Following injury, microglial cells and other leukocytes activate radial glial cell proliferation and neurogenesis by secreting Leukotriene C4 (LTC4)(Kyritsis et al., 2012). Even in the absence of injury, LTC4 alone increases proliferation of progenitor glial cells and the production of newborn neurons. Furthermore, inflammation alone initiated production of S100β, an EF-hand type Ca^{2+}-binding protein, by radial glia and activation of the Gata3 transcription factor. These factors represent molecular switches that could be potentially targeted to promote neuronal differentiation and survival (Stella, 2012). Similarly, in rats, promoting inflammation in the eye through injury or pharmacological treatment can stimulate axon outgrowth in the normally non-regenerative retinal ganglion cell (RGC) axons of the primary visual pathway (Leon et al., 2000; Yin et al., 2003). Recent evidence suggests that in mice both neutrophils and macrophages are major sources of oncomodulin (Ocm), a key soluble factor in inflammation-induced regeneration, and are essential for neurite outgrowth in the CNS (Kurimoto et al., 2013; Kwon et al., 2013; Yin et al., 2006).

While the liver is a unique example of adult mammalian organ regeneration, the capacity for regeneration is compromised in chronic disease (Table 1). The presence of bipotent hepatic progenitor cells (HPCs) that can be activated to regenerate both cell types involved in bile synthesis, cholangiocytes and hepatocytes, leaves a window of therapeutic opportunity open. Recent studies in mice and humans have shown that macrophages control signaling pathways that regulate hepatic progenitor cell fate (Boulter et al., 2012). By studying divergent disease patterns in human samples and inhibiting Notch signaling in mouse models, the authors showed that Notch is required for adult biliary specification during chronic liver injury. During hepatocyte regeneration, the Notch signaling pathway is repressed though the ubiquitin ligase Numb, such that the loss of Notch signaling mediates exit from a biliary fate, and the acquisition of a hepatocyte phenotype in HPCs. Furthermore, phagocytosis of hepatic debris activates Wnt3a on macrophages, which, in turn, drives the Numb-activated hepatocyte program. In kidney regeneration, macrophage-derived Wnt7b is required for renal tubular epithelial regeneration (Lin et al., 2010). Cross-talk between the immune system and progenitor cell populations mediated by critical modulators of cell-to-cell signaling such as Notch and Wnt therefore seems to be a common regenerative signaling pathway in different tissues.

The immune system also affects progenitor and stem cells by creating the appropriate microenvironment for their development and function. The idea that macrophages create a

niche for newly forming blood cells during erythropoiesis, one component of the robustly regenerating hematopoietic system, was suggested half a century ago with the identification of erythroblastic islands (a central macrophage surrounded by developing erythroblasts) in the bone marrow. Only recently, *in vivo* studies using genetic and chemical models of macrophage depletion confirmed a supportive role for macrophages during red blood cell development and diseases affecting erythropoiesis such as polycythemia vera (Chow et al., 2013(Ramos et al., 2013). As noted in the introduction, ductal morphogenesis in mammary gland development depends on immune cells. Mammary stem/progenitor cells also rely on the continued presence of macrophages evidenced by their diminished repopulating activity in macrophage deficient (*Csf-1*$^{op/op}$) mice or following chemical ablation of macrophages (Gyorki et al., 2009), suggesting macrophages may constitute part of the normal mammary stem/progenitor cell niche. While macrophages are not required for intestinal development and normal crypt morphology, injury-activated macrophages in the colonic epithelial progenitor cell niche express a number of factors that promote proliferation and survival of epithelial progenitors. Furthermore, intestinal macrophages recruited to the site of injury and activated by the microbiota in a TLR-dependent manner support and promote proliferation of colonic epithelial progenitors (Pull et al., 2005). Therefore, the immune system is a crucial element for shaping the crypt progenitor cell niche in the injured intestinal epithelium.

Immunomodulation and immune cell heterogeneity

Appropriate spatial and temporal regulation of the immune response to injury or disease determines the soluble factor milieu and therefore the future fate of the tissue. Resolution of the inflammatory response leads to regeneration or chronic inflammatory cell activation and soluble factor production perpetuates tissue damage and hampers repair (Figure 1A). Commonly, a temporal shift or polarization occurs in the immune response that is typically driven by M1, or pro-inflammatory macrophages, and M2, or anti-inflammatory and reparative macrophages. Both arms of the immune response are required for repair in many systems such as heart, skeletal muscle, and the CNS. If initial, pro-inflammatory signals are not controlled, for example, excessive tissue damage can occur and block repair. Conversely, premature initiation of the anti-inflammatory program can also disrupt efficient tissue healing; for example, skeletal muscle regeneration is impaired when macrophages are prematurely skewed by treatment with IL-10 or genetic loss of MKP-1 (Perdiguero et al., 2011). Also, in both skeletal muscle regeneration and remyelination, M1 macrophages recruit and stimulate progenitor proliferation while M2 macrophages mediate differentiation, dispelling the common view that M1 macrophage responses are overall bad while M2 are good.

A host of M1 or M2 soluble factors are implicated in skeletal muscle regeneration. While M1 macrophages activate the proliferative stage of myogenesis and satellite cell proliferation through production of IL6, TNFα, IL1β, and G-CSF; IGF1 and TGFβ production by M2 macrophages supports myogenic differentiation and growth (Arnold et al., 2007; Lu et al., 2011; Saclier et al., 2013) (Figure 3A). Furthermore, the impact of M1/M2 macrophage skewing on modulating the inflammatory response and skeletal muscle regeneration has recently been highlighted with the identification of a new regulator of

M1/M2 balance (Figure 3A). AMP-activated protein kinase (AMPK), which regulates energy homeostasis by sensing ADP:ATP and AMP:ATP ratios, mediates the switch in macrophage polarization from M1 to M2 and is necessary for regeneration following skeletal muscle injury (Mounier et al., 2013). In wild-type mice, the phagocytosis of muscle debris triggers M1 macrophages to skew towards M2 (Figure 2). Mice with macrophages deficient in AMPK have impaired skeletal muscle regeneration due to the inability of AMPK-deficient macrophages to skew towards M2 following phagocytosis. While the lack of AMPK does not impede myoblast proliferation *in vivo* or *in vitro*, myogenesis is impaired by a defect in differentiation and myotube formation. The potential importance of AMPK signaling in other cell types and the signal from M2 macrophages that mediates myogenic differentiation and tissue regeneration remain to be identified and may hold promise as therapeutic targets for degenerative muscle diseases.

Dual roles of immune cells in regenerating tissue are an emerging theme. In addition to their direct role in satellite cell activation described above, T_{regs} are central immunomodulators of skeletal muscle regeneration by controlling T cell numbers and the bi-phasic, sequential recruitment of pro- and anti- inflammatory macrophages (Burzyn et al., 2013) (Figure 3A). Oligodendrocyte differentiation efficiently forms new myelin sheets around axons in young, healthy mammals. Peripherally derived macrophages and resident microglia are important for clearing debris but also have been recently shown to directly drive remyelination by promoting oligodendrocyte differentiation through secretion of activin-A (Figure 2). Importantly, a switch from M1 to M2 polarization was critical for effective remyelination (Miron et al., 2013).

Another emerging theme is that tissue-specific varieties of leukocytes and lymphocytes perform multiple functions beyond their roles in theimmune system that are instrumental for tissue homeostasis and disease. In addition to the muscle T_{reg} described previously, recent genetic fate-mapping and deletion studies reveal four different cardiac macrophage populations, the origins from which they arise, and the mechanisms that maintain macrophage homeostasis and expansion in response to cardiac stress (Epelman et al., 2014) (Figure 3B). Not only do their developmental origins differ, but also transcriptional profiling and functional assays show that each of the four subsets, tracked by expression of CCR2, Ly-6C, and MHC class II, have specialized functions. The resident cardiac macrophages that were MHC-II^{lo} contribute to homeostasis by phagocytosis of cardiomyocyte debris. Furthermore, cardiac stress led to upregulation of inflammasome and IL-1β related genes from monocyte-derived macrophage subsets that were CCR2^{+}. Finally, the macrophages that expressed high levels of MHC-II efficiently processed and presented antigen to T cells, suggesting a role in immunosurveillance. These findings shed light on the paradox raised by previous data that upon injury, blocking CCR2 can be cardioprotective while depleting macrophages by other strategies further increases injury and hampers cardiac function (Kaikita et al., 2004; van Amerongen et al., 2007). Together, these data suggest that preserving resident cardiac macrophage expansion via proliferation, while targeting peripheral monocyte recruitment, might lead to improved myocardial recovery after injury. This landmark study (Epelman et al., 2014) highlights the need to further delineate phenotypic and functional differences among immune cells within specific tissues during

homeostasis and following injury so that therapies can be developed to preserve subsets that are cytoprotective while targeting the activation or recruitment of immune cells, in a subset-specific way, that contribute to damage. Furthermore, gene expression profiling comparing cardiac macrophages to splenic and brain macrophages illustrates that in addition to heterogeneity within the same organ, significant variation in resident macrophages from tissue to tissue warrants consideration (Pinto et al., 2012).

Angiogenesis

The re-establishment of adequate blood flow to injured and newly forming tissue is a key aspect of regeneration. Immune cells support developmental angiogenesis by secreting soluble factors, remodeling matrix and physically pruning and supporting the vasculature during the maturation process. In the developing mouse retina, vessel remodeling is under the control of macrophages (Stefater et al., 2011) via expression of the Wnt ligands Wnt5a and Wnt11, which enhance the expression of the VEGF inhibitory receptor Flt1. Interestingly, genetic disruption of the myeloid non-canonical Wnt pathway can enhance wound angiogenesis and repair, suggesting the same signaling pathway found in the retinal vasculature could have therapeutic applications for modulating myeloid cell signaling to treat wounds (Stefater et al., 2013).

The wound healing response in adult mice and humans relies on immune cells for secreting pro-angiogenic factors. Interestingly, skin wounds in macrophage-deficient PU.1$^{-/-}$ mice heal normally and show minimal scar formation (Martin et al., 2003). However, temporal deletion of the myeloid lineage through diphtheria toxin-mediated cell death reveals that macrophages have distinct functions in different phases of skin wound repair. When deleted early, the loss of pro-inflammatory macrophages minimized scar formation due to reduced keratinocyte cell death and other damage while later deletion resulted in hemorrhage and lack of closure due to defects in angiogenesis, vascular maturation, and stabilization (Lucas et al., 2010). Similar conditional depletion of myeloid cells in a model of sciatic nerve injury resulted in a decrease in vascular density and delayed neural cell proliferation, implicating the immune system in the regenerative angiogenesis of neural tissue (Barrette et al., 2008).

The adult mammalian heart is notoriously resistant to regeneration following injury, due primarily to the irreversible withdrawal of postnatal cardiomyocytes from the cell cycle. Following injury of the adult heart, the inflammatory response and specifically the monocyte/macrophage response has a dual function in scar formation. During the later phase, Ly6C^{lo} and M2 macrophages are necessary for mediating angiogenesis concomitantly with fibrosis to form a scar (Nahrendorf et al., 2007). In contrast, before postnatal day 7, neonatal mice efficiently regenerate up to 20% of the mass of the heart following surgical ablation or myocardial infarction (Porrello et al., 2011; Porrello et al., 2013). Recent studies showed that the immune response to cardiac injury differs quantitatively and qualitatively during regeneration in comparison to the pro-fibrotic response mentioned (Aurora et al., 2014). Also, depletion of macrophages in P1 mice subjected to myocardial infarction impaired heart regeneration, at least in part, due to a lack of neoangiogenesis (Figure 3B). Given that the neonatal mouse does not mount a robust fibrotic response following ischemic cardiac injury, the data suggests that in mammalian heart regeneration, macrophages have

the potential to promote angiogenesis without activating fibroblasts. The relative distribution in the neonate of the four cardiac macrophage populations found in the adult and the potential contribution of each population to the process of neonatal heart regeneration and angiogenesis remain to be defined (Figure 3B). The unique gene expression profile of early neonatal macrophages is of future interest for developing therapies that target the immune system to promote angiogenesis and tissue regeneration.

Adult teleost fish, such as zebrafish, are highly regenerative and equipped to regrow fins, retinae, spinal cord, and heart muscle following amputation or injury. Zebrafish cardiomyocytes are small, mononucleated and have underdeveloped sarcomeric structure, similar to embryonic and early postnatal cardiomyocytes, and their proliferative capacity is integral to the heart's regenerative capacity. In both zebrafish and neonatal mice, the regenerated myocardium is derived from pre-existing cardiomyocytes (Jopling et al., 2010; Kikuchi et al., 2010). The epicardium provides progenitors important for angiogenesis during embryonic development and injury-induced heart repair or regeneration in both organisms (Bock-Marquette et al., 2009; Kikuchi et al., 2011; Smart et al., 2010) and recently has also been shown to regulate the inflammatory response and neutrophil infiltration after injury (Huang et al., 2012). Thymosin β4 (Tβ4) is a key activator of the epicardial progenitors that participate in neovascularization and its natural derivative β4-sulfoxide (Tβ4-SO) functions to prevent chronic inflammation and promote wound healing (Evans et al., 2013), further suggesting a link between regulation of the immune response and regenerative angiogenesis.

Regenerating skeletal muscle also relies on monocytes/macrophages for neoangiogenesis. Sterile injury models using transgenic lineage tracing of endothelial progenitors have shown that macrophage depletion compromises the differentiation of endothelial progenitors through the secretion of growth factors (Zordan et al., 2014). In place of compromised progenitor differentiation and angiogenesis, collagen accumulates and the injured muscle becomes fibrotic.

Finally, ischemic disorders of the CNS, such as retinopathies, are strongly associated with deficient or aberrant angiogenesis. Recent studies in an ischemic retinopathy model show that ER stress in ischemic neurons leads to down-regulation of netrin-1, which suppresses vascular regeneration in the hypoxic CNS. Neuronal netrin-1 triggers an angiogenic switch in macrophages. Depleting retinal macrophages or antibody-mediated blockade of VEGF hinders vascular regeneration, suggesting that neuronal-derived netrin-1 is a potent mediator of myeloid-cell-induced CNS vascular regeneration (Binet et al., 2013).

The significance of immune regulation of angiogenesis, progenitor cell activity, debris removal, and appropriate polarization of subsequent immune responses and soluble factor secretion suggests that a closer look at pro-regenerative therapeutics targeted at immunity is warranted. Below, we discuss some of the diseases that lend themselves to such therapies and examine potential ways to harness the immune system to promote regeneration.

Clinical Perspectives and challenges

Disorders susceptible to regenerative immunity

Multiple sclerosis—Multiple sclerosis (MS) is an autoimmune disease caused by inflammatory damage to the myelin sheath that protects the nerve cells, leading to progressive degeneration of the demyelinated neurons (Table 1). Research has largely focused on therapies that could replace the myelinating cells, called oligodendrocytes, which derive from neural stem cells or further restricted cells called oligodendrocyte progenitor cells. Inflammation plays a complex role in the disease course of MS. While inflammation had been generally thought to inhibit regeneration in the CNS, several recent studies suggest that promoting inflammation acutely, for example with zymosan injection, can stimulate oligodendrocyte production (Foote and Blakemore, 2005; Setzu et al., 2006). The positive influence of acute inflammation on oligodendrocytes has been confirmed by zebrafish studies showing that inflammation was sufficient for regeneration (Kyritsis et al., 2012). How these effects manifest during the chronic and oligodendrocyte-specific inflammatory response of MS remains to be investigated further. It appears that shifting or changing the polarization of the inflammatory response may represent an alternative approach to simply blocking or inducing inflammation in the context of demyelinating disease. As an example, the recent findings showing that activin-A, solely expressed from M2 macrophages, can support remyelination (Miron et al., 2013) suggests that immunomodulatory therapies that induce a M2 macrophage shift may be candidates for clinical use in MS (Weber et al., 2007).

Muscular dystrophy—Muscular dystrophies refer a group of muscle wasting diseases characterized by progressive skeletal muscle weakness, defects in muscle proteins, and the death of muscle cells and tissues. Despite the ability of skeletal muscle to regenerate from satellite cells, necrosis of myofibers persists in the context of inflammation and changes in the muscle environment that eventually weaken the muscle system and hamper function (Table 1).

The immune system plays a pivotal but complex role in the pathogenesis of muscular dystrophies. Through their synthesis of TGFβ, macrophages in MDX mice, the mouse model for Duchenne's muscular dystrophy, directly induce collagen production in fibroblasts and further amplify collagen accumulation through activation of profibrotic alternatively activated macrophages (Vidal et al., 2008). Furthermore, treating MDX mice with a peptide that blocks the interaction between leukocyte expressed integrin $\alpha_M\beta_2$ and fibrinogen dampens muscle inflammation and ameliorates disease. Myeloid cell infiltration and its temporal shift from M1 to M2 are essential to regeneration but recent discoveries highlight the significance of other cell types like T_{regs} and eosinophils (Burzyn et al., 2013; Heredia et al., 2013). Not only do they modulate polarization of the immune response but they also have autonomous effects on satellite cell and progenitor cell differentiation, respectively. Given that glucocorticoids are currently the only therapy for muscular dystrophy, this disease may benefit from development of specific immunomodulatory therapies directed at T_{regs} and/or eosinophils or the soluble factors they produce.

Heart failure—A myocardial infarction (MI) occurs every 25 seconds in the United States and often leads to heart failure, the leading cause of death in the developed world (Minino et al., 2011). Given that the adult mammalian heart lacks the inherent ability to regenerate, ischemic loss of tissue is accompanied by replacement of myocytes with a fibrous, noncontractile scar tissue which compromises cardiac function and ultimately leads to heart failure. Although some studies suggest that mammalian cardiomyocytes have measurable capacity for turnover (reviewed in (Porrello and Olson, 2010)), the presence of a true cardiac stem cell is controversial and the response of the heart is not sufficient to recover functional myocardium following significant cellular loss. Failure of several stem cell therapies suggests that providing progenitors may not be adequate and that approaches aimed at the immune response may be required (Santini and Rosenthal, 2012). Furthermore, while a general wound healing response occurs, complete with an inflammatory reaction, no therapeutic measures have been developed to modulate the immune system to prevent heart failure. Starting more than 30 years ago, clinical trials aimed at general blocking inflammation to more target approaches such as anti- CD18 integrin and complement inhibition have had detrimental or little effect respectively on outcome following MI (reviewed in (Frangogiannis, 2012)).

Experimental models of MI and data from patients suggests that while an inflammatory response is required for infarct healing, defects in resolution, and containment of the response result in adverse remodeling of the infarcted heart (Table 1). A number of recent advances in understanding the immune response to MI, in particular monocytes and macrophages, suggest that the heart harbors a unique spectrum of myeloid cells and elicits specialized immune responses in response to injury (Figure 3B). Greater understanding of the monocyte subsets and the kinetics by which they are recruited to the injured heart (Nahrendorf et al., 2007) has led to a number of additional studies that are increasing our understanding of how current therapies might affect cardiac immunity. For example, angiotensin-converting-enzyme (ACE) inhibitors, a standard treatment for MI and heart failure, may be beneficial to infarct healing by having anti-inflammatory properties and influencing specific monocyte migration to the heart (Leuschner et al., 2010). Furthermore, Ly-6C^{hi} monocytes recruited in the initial inflammatory phase of the response to myocardial infarction also dictate the reparative phase by differentiating into Ly-6C^{lo} macrophages (Figure 3B) (Hilgendorf et al., 2014).

In spite of these advancements, the focus of therapeutic strategies has been to stop uncontrolled or prolonged inflammation in order to prevent adverse remodeling and progression to heart failure. Studies have yet to shift to promoting specific components of immunity to stimulate regeneration of the myocardium, instead of modulating scar formation. Until recently, the concept of mammalian heart regeneration was not tractable due to lack of *in vivo* models. The capacity for neonatal mice to regenerate their hearts depends on a unique population of macrophages, suggesting a therapeutic opportunity may exist to promote heart regeneration by modulating the immune response either alone or in combination with therapies that stimulate cardiomyocyte proliferation. Lessons from skeletal muscle and remyelination suggest efficient debris clearance is key to successful regeneration, by both eliminating inhibitory factors from the tissue milieu and by triggering

signaling cascades in phagocytes that are necessary for downstream soluble factor release or effector functions that promote regeneration. Careful genetic dissection of the key regulators of debris clearance and the consequences following cardiac injury may provide molecular targets for new therapies

Liver fibrosis—The liver continues to be a unique example of adult mammalian solid organ regeneration. However, chronic liver disease, caused by viral infection, autoimmunity, toxic injury or steatosis, remains a major cause of morbidity and mortality worldwide (Table 1). Recent efforts have uncovered opposing roles for the immune system in controlling regeneration during chronic liver disease. Pro-inflammatory Kupffer cells and infiltrating macrophages initiate and promote fibrosis by stimulation of stellate cells. In contrast, M2-like restorative macrophages and NK cells drive the resolution of fibrosis by inducing stellate apoptosis and senescence and also provide Wnt signals to drive hepatocyte regeneration (Boulter et al., 2012; Krizhanovsky et al., 2008). In addition, Arg1-expressing M2 macrophages protect the liver during schistosomiasis, not by battling the infection, but by suppressing liver fibrosis and chronic inflammation (Pesce et al., 2009). Current therapies focus on deterring fibrosis by dampening inflammation and fibroblast activation but have yet to enhance the positive, M2-like cells.

The underlying mechanisms of degenerating diseases in the heart, CNS, skeletal muscle, or other organs clearly differ by the presence or absence of a functional progenitor cell population that has the capacity to repopulate the damaged tissue. However, whether the regenerative strategy is to stimulate endogenous progenitors, reprogram other cell types to replenish the tissue, or to transplant exogenous cells, the native inhibition of these processes that is present within the diseased tissue will have to be appropriately modulated. The research highlighted here suggests that understanding and having the tools to fine-tune inflammation will be key to promoting a permissive environment for regeneration.

Inflammation: Harnessing the good and halting the bad

Within minutes of injury, infiltrating neutrophils and other inflammatory cells release reactive molecules that can further damage the tissue. Furthermore, chronic inflammation perpetuates tissue remodeling and functional impairment in a number of diseases. As a consequence, drug development and therapy has historically emphasized anti-inflammatories by way of steroids, NSAIDS, and even more targeted approaches such as anti-TNF monoclonal antibodies. The studies we highlight suggest that new developments require a more fine-tuned approach that allows specific blockade of the negative effects of inflammation in an environment that is permissive for the positive effects of the immune response. Systematic dissection of these components will aid in defining the threshold for which immunity is strong but regeneration is permitted (Figure 1B) and allow for precise therapeutic modulation to promote regeneration.

One emerging theme is to modulate the polarization of the immune response and recent data suggests modulating the port of entry for inflammatory cells may be one strategy. Similar to repair of the heart or skeletal muscle (Arnold et al., 2007; Nahrendorf et al., 2007), recovery from spinal cord injury requires a biphasic monocyte/macrophage response, in which M1

and M2 cells enter the injured tissue through distinct routes (Shechter et al., 2013). M1 macrophages penetrate the injured spinal cord through the leptomeninges while the cerebrospinal-fluid filled choroid plexus provides a permissive environment for M2 macrophages to repair the spinal cord. These results suggest that therapeutic modulation at distinct ports of entry to the injured CNS could be a novel approach to promote repair and regeneration.

Scrutiny of the immune responses in animal models of efficient regeneration will be essential for clues to therapeutically modulate the disease environment. Recent studies showed direct evidence for immunological control of complete regeneration in the adult vertebrate and neonatal mammal. Axolotls, aquatic salamanders, deploy a rapid and robust inflammatory response in the amputated limb that includes nearly immediate and early up-regulation of anti-inflammatory cytokines and macrophage dynamics. Systemic macrophage depletion of macrophages blocks axolotl limb regeneration, which can be restored upon re-amputation and replenishment of the macrophage population (Godwin et al., 2013). This dynamic and simultaneous induction of inflammatory processes in regenerating axolotl limbs is reminiscent of the rapid and concurrent fibroblast activity and cytokine secretion noted during scarless wound healing in the mammalian fetus (Larson et al., 2010). In addition, macrophages are necessary for heart regeneration in the neonatal mouse (Aurora et al., 2014).

The development of treatments targeting the immune system is currently hindered by the lack of markers to discriminate amongst subpopulations of immune cells, creating a gap in our understanding of how various subsets behave in normal and diseased tissues. An additional strategy to identify novel immunomodulatory targets for regenerative therapies is to thoroughly dissect the diversity and function of resident tissue-specific immune cells under conditions of homeostasis and injury, similar to the recent discovery of four cardiac macrophage subsets discussed above (Figure 3B) (Epelman et al., 2014). Therapies targeted at individual immune cell populations or soluble factors are being tested, though they primarily aim to curtail inflammation and affiliated fibrosis. Small molecules or monoclonal antibody inhibitors to CSF1R target macrophages, by blocking its ligand, ligand binding, or activation signaling (Patel and Player, 2009). While targeting inflammation alone could be sufficient to promote regeneration, a more plausible scenario will be the need to create an immunologically permissive environment in the context of other regenerative therapies. As an example, a recent study showed that acinar cell to beta-like cell conversion occurs in response to treatment with cytokines (Baeyens et al., 2014). In addition, recent findings suggest that efficient iPS formation depends on chromatin remodeling changes that are mediated by TLR signaling (Lee et al., 2012).

Evolution clearly selected fast healing and containment of injury or infection at the expense of the ability to reform a completely functional tissue. In order to selectively undo the loss of regenerative capacity without compromising the specificity and strength of the mammalian immune system, much remains to be learned about the functions of immunity, good and bad, in development, homeostasis and injury. The recent advances highlighted here improve our understanding of the cells and signals involved and underscore the potential of immunotherapies for tissue regeneration.

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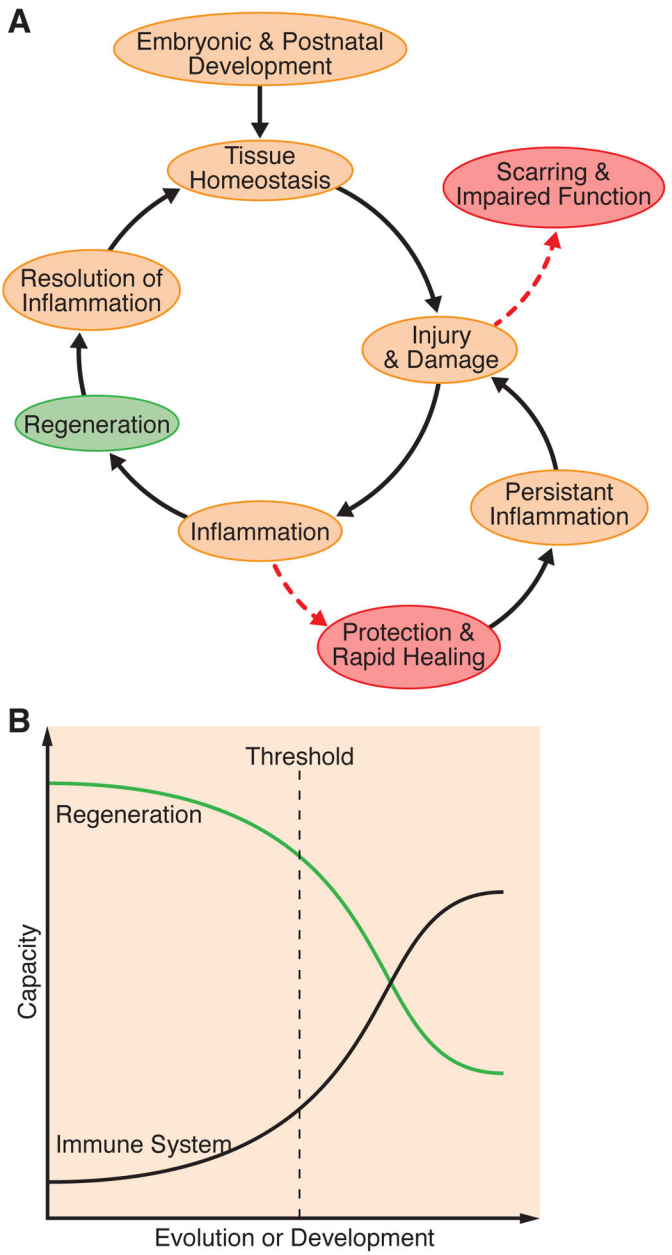

Figure 1. The influence of the immune system on development, homeostasis and disease

(**A**) During embryonic and postnatal development, the immune system regulates processes such as branching morphogenesis, ductal formation and angiogenesis. Similar functions are maintained in some adult tissues to maintain normal homeostasis. Injury or disease elicits an inflammatory response that can either promote functional restoration of the tissue (regeneration) or a rapid healing response that may protect the organism at the expense of preserving structure and function. Inflammation usually resolves in the regenerative response while inflammation often persists in wound healing and scar formation, ultimately impairing the normal function of the tissue. (**B**) Inverse relationship between the capacity to regenerate and the strength and intricacy of the immune system during development or

evolution. The threshold indicated on the graph conceptualizes the balance point at which the pro-regenerative components of the immune response are maintained within the context of a more advanced immune system. Identifying this threshold will be a key step towards the development of regenerative therapies targeted at immunity.

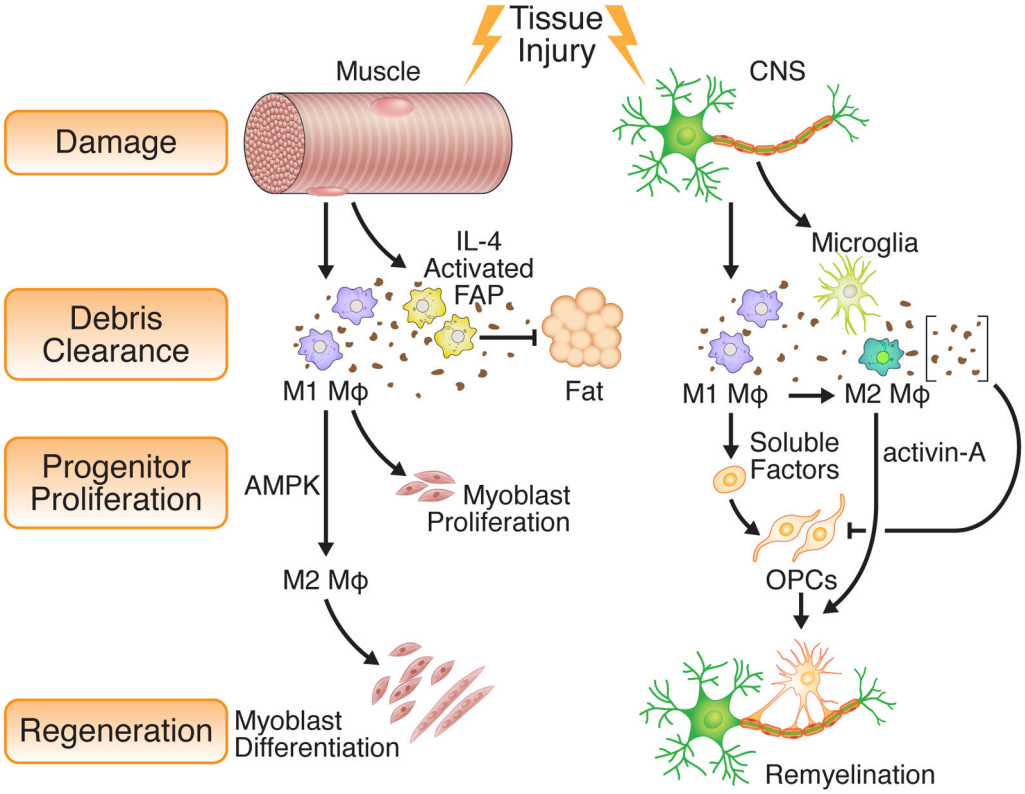

Figure 2. Debris clearance as a coordinator of regeneration

Debris clearance orchestrated by the immune system is a key activator of subsequent steps in regeneration, including progenitor cell activation, differentiation, and immune polarization. The comparison shown between skeletal muscle and the CNS highlights the key cell types and soluble factors involved. Following skeletal muscle injury, both M1 macrophages and fibro/adipocyte progenitors (FAPs) clear cellular debris. FAP phagocytic activity depends on eosinophil derived IL-4; in its absence, the progenitors differentiate into fat, causing muscle dysfunction. Phagocytic M1 macrophages promote myoblast proliferation and polarize to an M2 phenotype via AMPK-mediated signaling. M2 polarization is required for appropriate myoblast differentiation. In the CNS, mature neurons lack robust regenerative potential. However, remyelination occurs in young, healthy adults by activation of oligodendrocyte progenitor cells (OPCs). Activation of OPC proliferation depends on efficient clearance of myelin debris, which contains inhibitory factors, and also macrophage -derived soluble factors. Similar to myoblasts, the proliferation and recruitment of OPCs depends on M1 macrophages while differentiation of OPCs and remyelination relies on M2 macrophage-secreted activin-A.

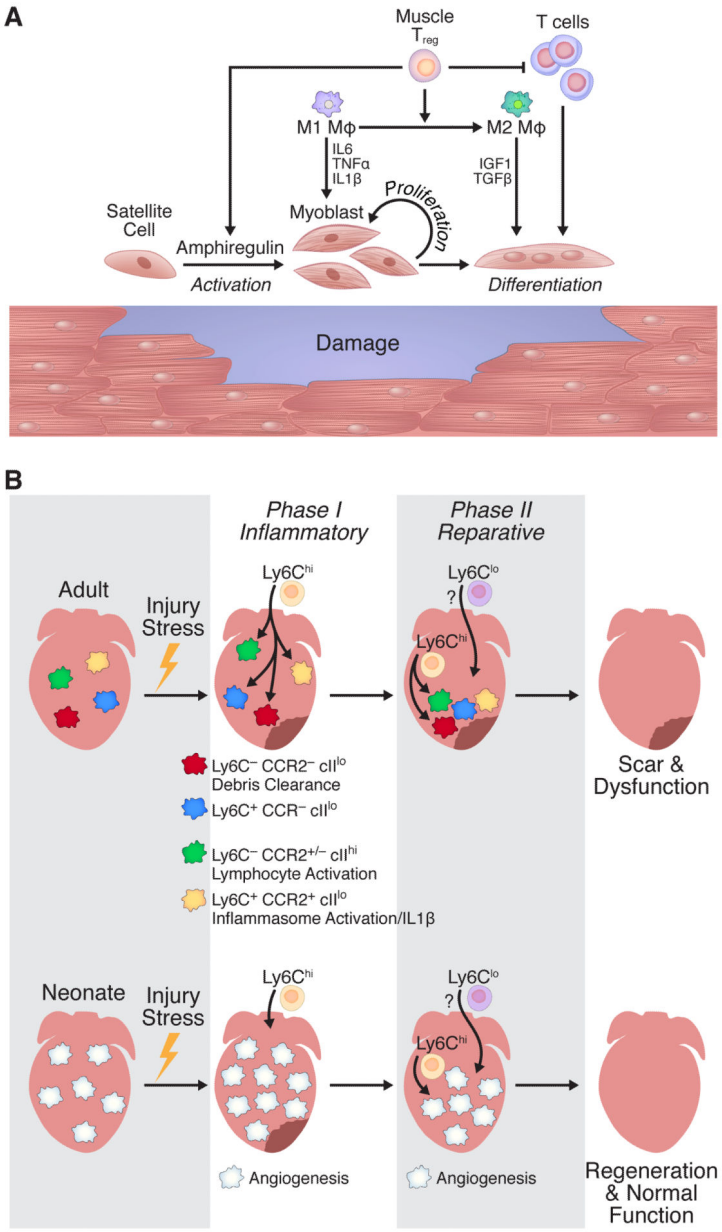

Figure 3. Immune cell polarization and heterogeneity are key components of tissue repair or regeneration

(**A**) The regenerative capacity of skeletal muscle is driven by satellite cell activation, proliferation and differentiation. M1 macrophages are early responders that secrete cytokines with proliferative effects on myoblasts. The next phase of the response involves myotube differentiation driven by M2 macrophage-secreted IGF1 and TGFβ. Specialized muscle T_{reg} cells influence all stages of regeneration: early secretion of amphiregulin activates myoblast proliferation, while in subsequent phases of regeneration muscle T_{reg} are necessary for myotube differentiation, M1 to M2 polarization and attenuation of excessive T lymphocyte responses. (**B**) The adult mammalian heart, which lacks regenerative capacity, contains different cardiac macrophage subsets, with diverse functions, developmental

origins, and mechanisms of homeostasis. The four populations, segregated by expression levels of CCR2, Ly-6C, and MHC class II, perform different functions as indicated. Upon injury such as a MI, a biphasic monocyte response occurs to promote an initial inflammatory phase followed by a reparative phase mediated by Ly-6C^{hi} or Ly-6C^{lo} splenic monocytes respectively. The two systems are linked by the ability of splenic Ly-6C^{hi} monocytes to replenish all four subsets in Phase I. In the neonatal mouse heart, which can fully regenerate following MI, there is also a biphasic splenic monocyte response though the characterization of subsets of resident cardiac macrophages has yet to be investigated. Interestingly, neonatal cardiac macrophages differ from adult in their localization, abundance, and gene expression profile following injury and are required for regeneration by promoting angiogenesis. The regenerative subtype in the neonate has yet to be defined.

Table 1

Summary of the regenerative capacity, mechanisms, and disease states of representative mammalian tissues

The process of regeneration is summarized for six different tissues, each with varying regenerative capacities: skin, heart, skeletal muscle, CNS, liver and bone. For each, steps that have the potential to be influenced by immunity are listed as well as the disease state and summary of immune targeted therapeutics.

TISSUE	DEBRIS CLEARNACE MECHANISM	PROGENITOR CELL POOL	IMMUNE-DERIVED STIMULI	POLARIZATION & HETEROGENITY	DISEASE STATE DUE TO FAILED REGENERATION	DEFECT	DRUGS TARGETING IMMUNITY
Skin (dermis)	Macrophages, fibroblasts, keratinocytes	Fibroblasts, endothelial cells	TGFβ, EGF, VEGF, IGF	Early and late macrophages (similar to M1/M2)	Full-thickness wound scarring in adults	Excessive inflammation Decreased migration Collagen accumulation	IFN-α, IFN-β, IFN-γ; steroids
Heart	M1 or Ly-6C^{hi} monocytes/macrophages	Resident cardiomyocytes	n/a	Four pools of cardiac macrophages; sequential Ly6C^{hi}, Ly6C^{lo}	Myocardial infarction Heart failure	Lack of progenitors; Insufficient immune polarization	ACE inhibitors
Skeletal muscle	Macrophages and FAPs	Satellite cells	T_{reg} amphiregulin, IL-6, IL-1β, TNFα, IGF1, TGFβ	Muscle T_{reg}; M1/M2	Muscular dystrophy	Myofiber defect; satellite cell exhaustion; chronic inflammation	Glucocorticoids
CNS (myelination)	Macrophages and microglia	Oligodendrocyte progenitor cells	IGF1	M1/M2	Multiple sclerosis	Impaired clearance of myelin debris	anti-GM-CSF; Pioglitazone (PPARg agonist); Glatiramer acetate
Liver	Kupffer cell and liver macrophages	Hepatocytes and Hepatic Progenitor Cells	Eosinophil IL-4; Kupffer IL-6, TNFα	Inflammatory and restorative macrophages; T_h1, T_h2, T_h17 lymphocytes	Chronic liver disease (viral, toxic, autoimmune, metabolic)	Unresolved fibrosis	TLR4, CCR2 inhibitors; PPAR-α/β agonists
Bone	Osteoclast	Monocyte progenitor	Csf1	n/a	Osteopetrosis or osteoperosis	Lack of or excessive bone resorption	IFN-γ1b, corticosteroid; RANKL inhibitor (mAb)