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Current Opinion in Immunology

Metabolic regulation of innate immune cell phenotypes during wound repair and regeneration

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Metabolism regulates an array of cellular processes from embryonic development through adulthood. These include proliferation, differentiation and the effector functions of adult cells to maintain homeostasis and repair. It is becoming clear that bioenergetic shifts can control how cells respond to environmental disruptions during tissue injury to initiate a healing response. Specifically, innate immune cells shift their phenotypes to initiate and resolve inflammation, and there is intense interest to understand how these responses might regulate healing outcomes. Here, we review recent literature describing how cellular metabolism and metabolic byproducts regulate phenotype conversions among innate immune cells. Although most studies of this kind do not focus on tissue damage, we discuss how metabolic regulation of these phenotypes promotes tissue repair. In particular, we provide a framework for considering the extent to which altering the innate immune response might shift fibrotic repair towards regenerative healing.

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Current Opinion in Immunology 2021, **68**:72–82

This review comes from a themed issue on **Innate immunity**

Edited by **Carla Rothlin** and **Vijay Rathinam**

https://doi.org/10.1016/j.coi.2020.10.012

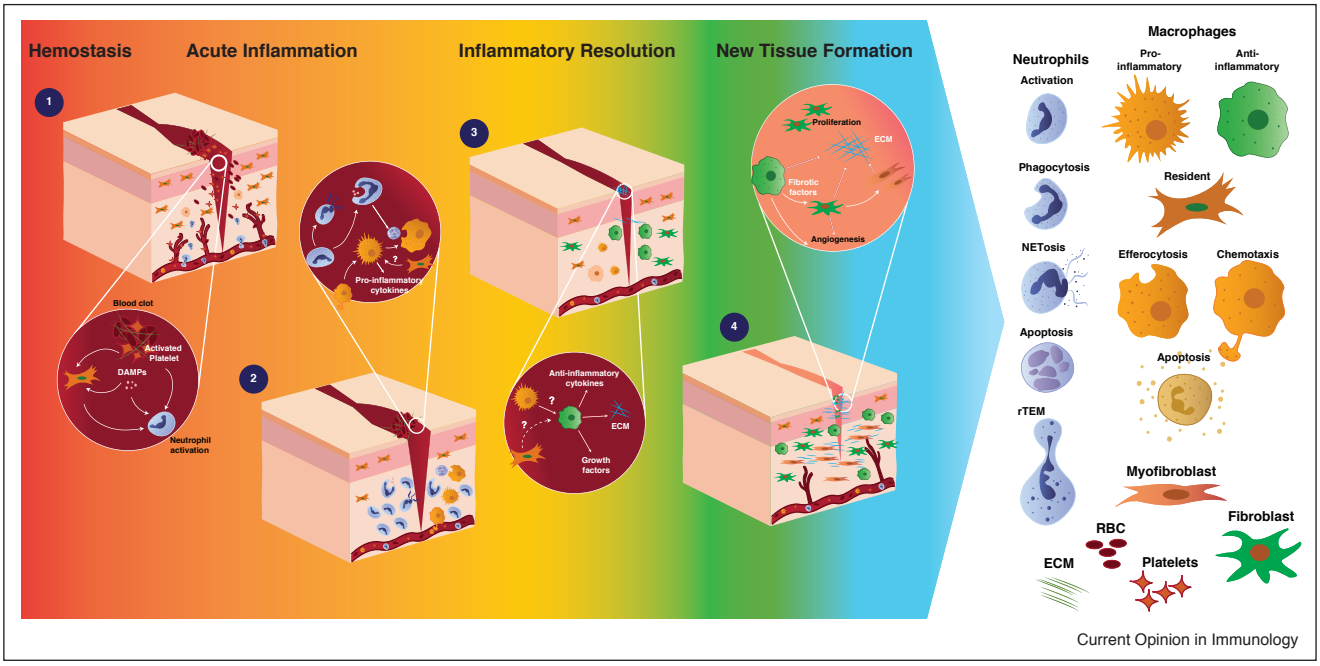
Introduction

Among vertebrates, either fibrotic repair or regeneration is the end product of nonpathological wound healing. While the innate immune response to tissue damage defines the early stages of repair and regeneration [1,2], the degree to which specific immune cell behaviors and phenotypes can explain one healing trajectory or the other remains poorly understood. In fact, the early phases of these distinct healing types are quite similar [3••,4] and studies in a diverse array of regenerative organisms has reinforced our notion that innate immunity (germline encoded, non-specific cellular defense plus humoral killing capacity) is an ancient response to tissue injury. For instance, prior to regenerative healing in many invertebrates, immune defense is mediated by a variety of specialized phagocytic cells concomitant with humoral killing activity by antimicrobial molecules [5]. Professional immune cells with phagocytic activity (neutrophils, dendritic cells and macrophages) emerged during vertebrate evolution, and while an independent, adaptive class of immune cells developed as well (e.g. B and T cells), the innate immune response has remained the first line of defense to tissue damage [6]. Although studies in amphibians and fetal mammals suggested to some extent that the evolution of a strong inflammatory and adaptive immune response occurred at the expense of regenerative healing [7,8], complex tissue regeneration in lizards and mammals supports a more nuanced view of how the immune response to tissue damage regulates healing outcomes [9,10].

Generally speaking, injuries to mammalian skin and musculoskeletal tissue result in fibrotic repair brought about through a stepwise progression of overlapping processes [rev. in Ref. 11] (Figure 1). In certain species where injuries to these systems result in tissue regeneration (e.g. ear pinna and skin in spiny mice and rabbits, rodent digit tips, etc.) a similar progression occurs except that new tissue production transitions to morphogenesis instead of fibrosis [12]. Thus, when considering fibrotic repair and regeneration side-by-side, healing can generally be divided into three major periods. The first period comprises a common wound healing phase including hemostasis (clotting and platelet activation), an inflammatory response initiated and resolved by innate immune cells, re-epithelialization and fibroblast/progenitor cell accumulation. The second period is defined by new extracellular matrix production, cell proliferation and the emergence of myofibroblasts. A divergence between fibrotic and regenerative healing which began during the second period continues during the third period where scar remodeling or morphogenesis occurs [4,13].

In response to tissue injury, the behavior of neutrophils and macrophages is thought to play a major role in coordinating the innate immune response (Figure 1). During hemostasis, a fibrin matrix is produced that is infiltrated by erythrocytes to form a blood clot in the wound bed. This process attenuates blood loss, creates a temporary hypoxic environment and enhances the migration of circulating leukocytes to the injury site. Factors released from activated platelets, tissue resident macrophages and damaged

Figure 1



Early tissue healing progresses similarly during fibrotic repair and regeneration. Injured tissue heals in a stereotypical fashion, ultimately diverging as new tissue is produced to form a scar (fibrotic repair) or to faithfully replace the damaged tissue (regeneration). (1) Immediately after injury, hemostasis serves to stem blood loss and platelets initiate blood clotting by activating coagulation factors. Damage associated molecular patterns (DAMPs) released into the wound attract neutrophils and signal to tissue resident macrophages. (2) During the acute inflammatory response, neutrophils become activated and phagocytose cell debris and microbes. Activated macrophages infiltrate the wound and are polarized towards a pro-inflammatory phenotype where they secrete inflammatory cytokines that initiate an inflammatory cascade. Pro-inflammatory macrophages also phagocytose neutrophils and cellular debris. (3) Inflammation resolves as macrophages adopt an anti-inflammatory phenotype characterized by the release of anti-inflammatory cytokines and growth factors that stimulate the tissue healing/repair process. Most remaining neutrophils are removed through apoptosis and neutrophil extracellular traps and neutrophil death (NETosis), although some re-enter the vasculature. (4) Inflammation gives way to new tissue formation as anti-inflammatory macrophages secrete chemokines that further stimulate angiogenesis, and extracellular matrix (ECM) deposition. Wound-edge keratinocytes, that were stimulated to migrate during inflammation, complete re-epithelialization as inflammation resolves and progenitor cells and connective tissue cells begin accumulating in the wound microenvironment. The cellular response during this phase of healing drives the composition and tempo of new tissue formation. During fibrotic repair, fibroblasts differentiate into myofibroblasts and deposit densely packed bundles of collagen into the wound bed. In contrast, connective tissue fibroblasts produce a pro-regenerative matrix rich in fibronectin and matrix metalloproteinases (MMPs) that support tissue morphogenesis during regeneration.

cells (e.g. damage associated molecular patterns - DAMPs), trigger neutrophil and monocyte recruitment (chemotaxis). Neutrophils, which help trigger inflammation, entrap and kill pathogens while also debriding the wound area [rev in Ref. 14]. Macrophages arrive from circulation and adopt a pro-inflammatory phenotype that amplifies inflammation while they phagocytose neutrophils and cellular debris. Pro-angiogenic factors secreted by the first wave of macrophages stimulate angiogenesis and axonal sprouting occurs in concert. As healing proceeds, macrophages begin to express an alternative phenotype associated with anti-inflammatory activity which serves to resolve inflammation [rev. in Refs. 15,16]. Wound-edge keratinocytes, that were stimulated to migrate during inflammation, complete re-epithelialization as inflammation resolves and progenitor cells and connective tissue cells begin accumulating in the wound microenvironment. As fibroblasts produce new extracellular matrix molecules, a key transition to the rebuilding phase occurs [12] and resident progenitor and connective tissue cells will either generate scar tissue or instead, undergo proliferation and morphogenesis to accurately replace the damaged tissue during regeneration (Box 1).

In the context of fibrotic repair, the cellular and molecular underpinnings of these events have been reviewed extensively [13,17]. What has received less attention is the degree to which metabolic changes regulate how immune cells initially respond to tissue damage and then later shape the duration and magnitude of inflammation [18]. Moreover, it is becoming clear that cellular metabolism directly effects the phenotype of innate immune cells, which in turn have the capacity to regulate fibrosis [19]. Innate immune cells endure metabolic reprogramming to

Box 1 xxx

Fibrotic repair or regeneration are the end products of normal wound healing. Although both seemingly represent two extremes of the final healing process, the degree to which the innate immune response and inflammation regulates regeneration remains poorly understood. To understand tissue repair against the backdrop of innate immunity and inflammation, we envision three general models that can explain how the early responses to tissue damage might differentially affect healing outcomes. First, if the endpoints of fibrotic repair and regeneration lie along a smooth continuum, where local cells have the potential to heal by either means, then identical injuries could either trigger a response that is unique enough for each outcome that the two paths immediately diverge towards fibrosis or regeneration or injury could trigger a response that is generally similar and instead differs primarily in its magnitude. In either scenario, affecting the early response could, in principle, shift the healing outcome. A third possibility is that fibrotic repair and regeneration represent two totally different responses that occur in a tissue-specific or species-specific manner and are relatively independent of the local innate immune response. Thus, upon the resolution of inflammation each healing outcome proceeds according to cell autonomous mechanisms regulated by local responding cell types (e.g. endothelial, keratinocyte, fibroblast, etc.). Although an answer to this conundrum continues to elude biology, wound healing and regeneration studies where the innate immune and inflammatory response has been characterized can provide important clues.

perform diverse functions and these modulations are crucial for adapting to the tissue environment, sensing extracellular signals, generating excess reactive oxygen species (ROS) and synthesizing and secreting cytokines and growth factors. Understanding how metabolic shifts drive phenotypic changes in innate immune cells has important implications for understanding how these cells regulate different types of tissue healing. Here, we review how metabolic changes act as part of the innate immune response and contribute to innate immune cell polarization and phenotype switching. Although most research on this topic has been conducted in the context of fibrotic repair, we discuss how these mechanisms might also act in the context of tissue regeneration.

Cellular damage and inflammation during tissue repair

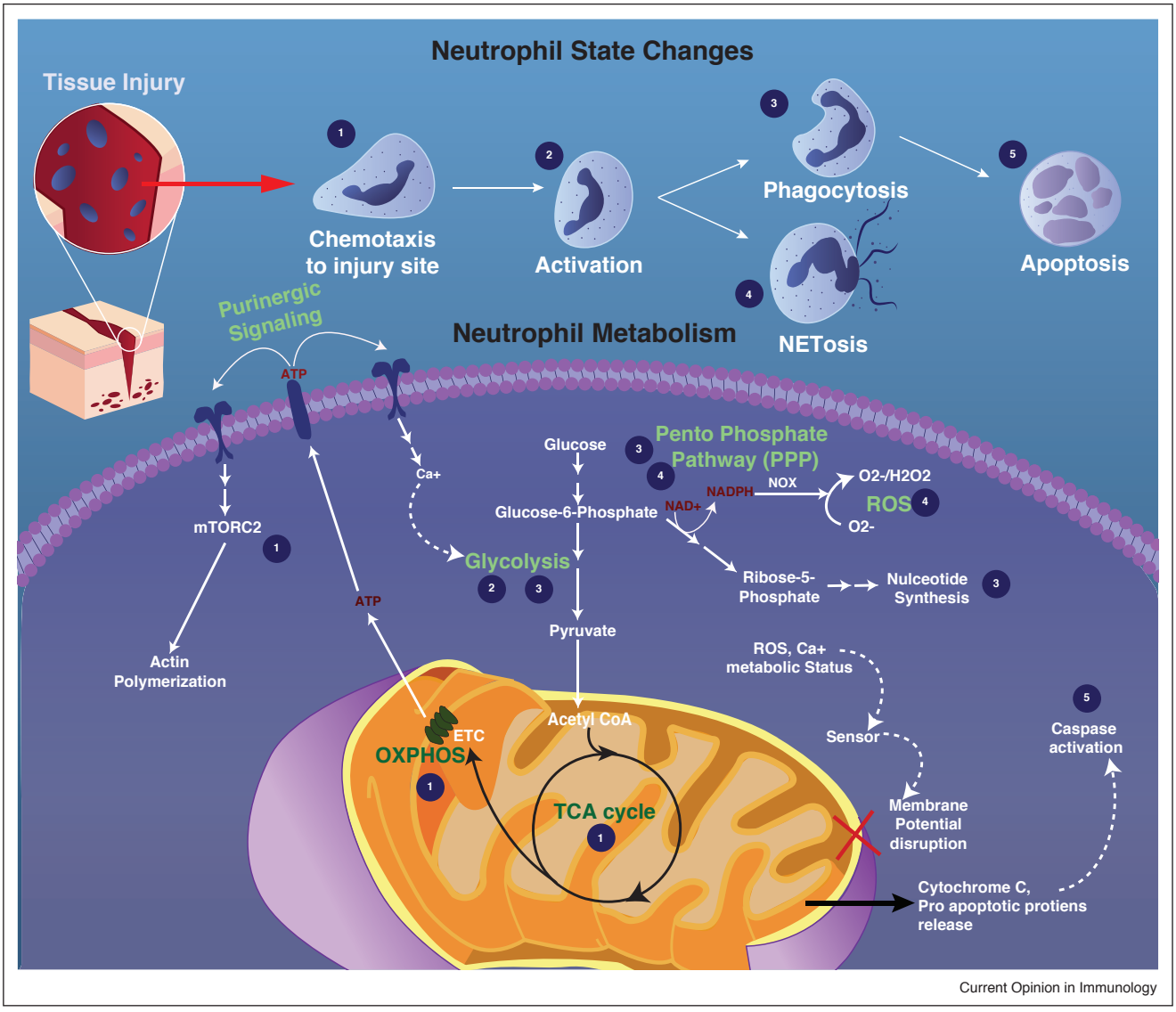
Individually and collectively, cells work to maintain tissue and organismal homeostasis in the face of injury, disease, infection and pathogen invasion. As such, cells act as frontline sensors to detect and mitigate extrinsic stressors. The sensing capacity of cells depends, in part, on a baseline metabolic state partially regulated by mitochondrial phenotype which is highly sensitive to deviations in biomolecules within and between the various cell compartments [20]. Tissue injury immediately disrupts the local microenvironment by flooding the area with the cytoplasmic contents of ruptured and dying cells (e.g. DNA, mtDNA, RNA, ATP, metabolic products, positive and negative ions, etc.) and by altering oxygen levels, pH, and ion flux in surrounding cells. These alterations upset cellular equilibrium and activate the cell damage response (CDR) which triggers changes in cellular behavior [21,22]. Once tissue damage is detected, the innate immune response is activated.

In most organisms, inflammation is the stereotypical response to severe tissue damage and is a direct consequence of the innate immune response [23,24]. Hemostasis, platelet degranulation, and histamine release amplify pro-inflammatory signals that are detected by resident macrophages and serve to attract innate immune cells (circulating neutrophils and macrophages). The infiltration, activity, and clearance of innate immune cells, contributes significantly to progression through the first two phases of wound healing, including inflammatory initiation and resolution (Figure 1) [25]. Neutrophils are recruited to injury sites through the release of signaling molecules from injured tissue such as DAMPs and chemokines. Mitochondrial DAMPs (formyl peptides and mitochondrial DNA) released from injured tissue potentially activate the inflammatory signaling cascade in neutrophils, triggering their migration and degranulation [26]. Activated neutrophils contribute to inflammation (by releasing cytokines and ROS), tissue debridement (by phagocytosis and neutrophil extracellular trap-NET) and angiogenesis (by secreted growth factors) [14]. Subsequently, circulating macrophages are recruited to the wound and become polarized to a pro-inflammatory phenotype during the initial phase of inflammation. These macrophages adopt a phagocytic phenotype and secrete pro-inflammatory cytokines such as TNF-α, IL-1β and additional ROS while also eliminating neutrophils, apoptotic cells and bacteria. This activity serves to 'clean-up' the injury site as the wound healing process progresses to resolve inflammation. In fact, a phenotypic switch among recruited macrophage from pro-inflammatory to anti-inflammatory is one of the key events that signals inflammatory resolution [16]. Anti-inflammatory macrophages initiate extracellular matrix (ECM) production, angiogenesis and tissue remodeling by releasing anti-inflammatory cytokines and growth factors [15]. *In vitro* and *in vivo* studies have established that a critical first step in the maturation of innate immune cell phenotypes is a reprogramming of their cellular metabolism.

Neutrophils: first wave responders to tissue injury

In the first wave of the innate immune response, neutrophils are quickly recruited to injured tissue where they infiltrate the wound site and protect against infection. As part of their defensive repertoire, neutrophils perform diverse functions including phagocytosis, ROS production, cytokine release, NET formation and apoptosis, all of which are important for normal tissue repair (Figure 2). Altering these functions can lead to inappropriate inflammation which in turn can exacerbate fibrosis and scaring

Figure 2



Metabolic changes are tied to neutrophil phenotype. In response to tissue damage, neutrophils perform diverse functions in the wound bed including phagocytosis, ROS production, cytokine release, NET formation (neutrophil extracellular trap) and apoptosis. Discrete metabolic changes are tied to these different functions. (1) Damage associated molecular patterns (DAMPs) released from cells (e.g. DNA, histones, HMGB1, N-formyl peptides, ATP, etc.), chemokines and lipid mediators act as powerful neutrophil chemoattractant. ATP released from mitochondria into the extracellular space activates purinergic signaling and mTOR which are required for neutrophil chemotaxis. mTOR signaling and extracellular Ca^{2+} influx downstream of purinergic signaling reinforce chemotaxis and lead to neutrophil activation. (2) Following activation, neutrophils rely heavily on glycolysis to meet their energy demands. (3) NADPH oxidase (NOX)-generated reactive oxygen species (ROS) are crucial for the phagocytic function of neutrophils. NOX uses excess NADPH produced via shunting of the glycolytic intermediate (Glucose-6-P) through the pentose phosphate pathway (PPP). Nucleotide synthesis through ribose-5-P is also activated in phagocytic neutrophils. (4) Neutrophil extracellular traps (NETs) and neutrophil death (NETosis) also rely on glycolytic by-products from the PPP to control inflammation and bacterial killing. (5) Rising intracellular levels of ROS and Ca^{2+} efflux signal to mitochondria which can disrupt the mitochondrial membrane potential (MMP). Disruption of MMP leads to release of cytochrome C which activates caspases leading to apoptosis.

[14]. Importantly, metabolic pathways appear to play a significant role in all of these functions [14,27].

Historically, mitochondrial energetics were considered dispensable for neutrophil activity until recent work demonstrated that mitochondrial-produced ATP was crucial for neutrophil chemotaxis [28–30]. Although glycolysis is the major ATP source for neutrophils (discussed below), mitochondrial-produced ATP released into the extracellular space is crucial for neutrophil chemotaxis

and activation via purinergic P2Y2 nucleotide and A3-type adenosine receptors [28,31]. Purinergic signaling in response to mitochondrial ATP activates the mTOR pathway inducing neutrophil chemotaxis [32]. Calcium signaling reinforces mitochondrial ATP production until glycolytic-mediated ATP synthesis stimulates a second round of purinergic signaling which triggers extracellular Ca^{2+} influx and neutrophil activation (Figure 2) [28]. Inhibiting mitochondrial ATP synthesis by uncoupling mitochondrial oxidative phosphorylation with carbonyl cyanide 4 (trifluoromethoxy)phenylhydrazone (FCCP) in neutrophils stimulated with leukocyte chemotactic factor, *N*-Formylmethionyl-leucyl-phenylalanine (fMLP) or lipopolysaccharide (LPS)-activated serum inhibits chemotaxis as a function of decreasing mitochondrial membrane potential [29]. Alternative inhibitors of mitochondrial ATP production (e.g. potassium cyanide, rotenone, carbonyl cyanide m-chlorophenyl hydrazine [CCCP]) similarly inhibit neutrophil chemotaxis as does inhibiting mTOR signaling using rapamycin or the potent mTOR inhibitor (PP242) [32]. Although it is assumed that these principles operate during tissue repair, *in vivo* data is almost non-existent. Recent work in zebrafish showed that disrupting *DNA polymerase gamma* (*Polg*), components of the electron transport chain (ETC), or enzymes that reduce mitochondrial ROS (mtROS), reduced neutrophil motility thus providing a crucial role for mitochondrial function during neutrophil migration *in vivo* [33••]. Similarly, disrupting the TCA cycle enzyme *isocitrate dehydrogenase1/2* (IDH1/2) led to reduced neutrophil migration in a glioblastoma model [34]. Mitochondria-mediated apoptosis is also vital for neutrophil clearance. Mitochondria act as an internal sensor for ROS where they regulate Ca^{2+} release to control the activation of neutrophil apoptosis [30]. Disrupting mitochondrial membrane potential leads to cytochrome C release along with other pro-apoptotic proteins, both of which activate caspase proteins to induce apoptosis. Compromising these mitochondrial functions can induce neutropenia (reduced inflammation) or prolonged neutrophil survival (chronic inflammation) which are deleterious to the proper wound healing process [35]. Thus, although *in vivo* data is limited, these studies support a key role for mitochondrial function during neutrophil activation, chemotaxis and clearance.

A major function of neutrophils as they accumulate at an injury site is to phagocytose bacteria and cellular debris. The early wound microenvironment is hypoxic and activated neutrophils rely heavily on glycolysis-generated ATP to remain active [36]. Glycolysis plays a major role in the phagocytic function of neutrophils where the inhibition of glycolysis, but not mitochondrial function, hinders this behavior [29,37,38]. In addition to ATP generated by glycolysis, shunting of the glycolytic intermediate (Glucose-6-P) through the pentose phosphate pathway (PPP) generates additional NADPH used for ROS production by NADPH oxidase (NOX) which is crucial for neutrophil phagocytic activity (Figure 2) [39]. In addition to phagocytosis and ROS production, neutrophil extracellular traps (NETs) and neutrophil death (NETosis) regulate inflammation and tissue damage [40]. These particular neutrophil functions also appear to be heavily reliant on glycolysis, and PPP generated ROS [41,42]. Persistent NET formation can increase fibrosis [43,44] and disrupted glucose processing in blood from diabetic patients causes retinal damage due to chronic inflammation induced by NOX-dependent NETosis [45,46]. Thus, it appears that the efficiency with which aerobic glycolysis operates in neutrophils can dampen or enhance their protective function to kill bacteria which if disrupted can impede the normal repair process.

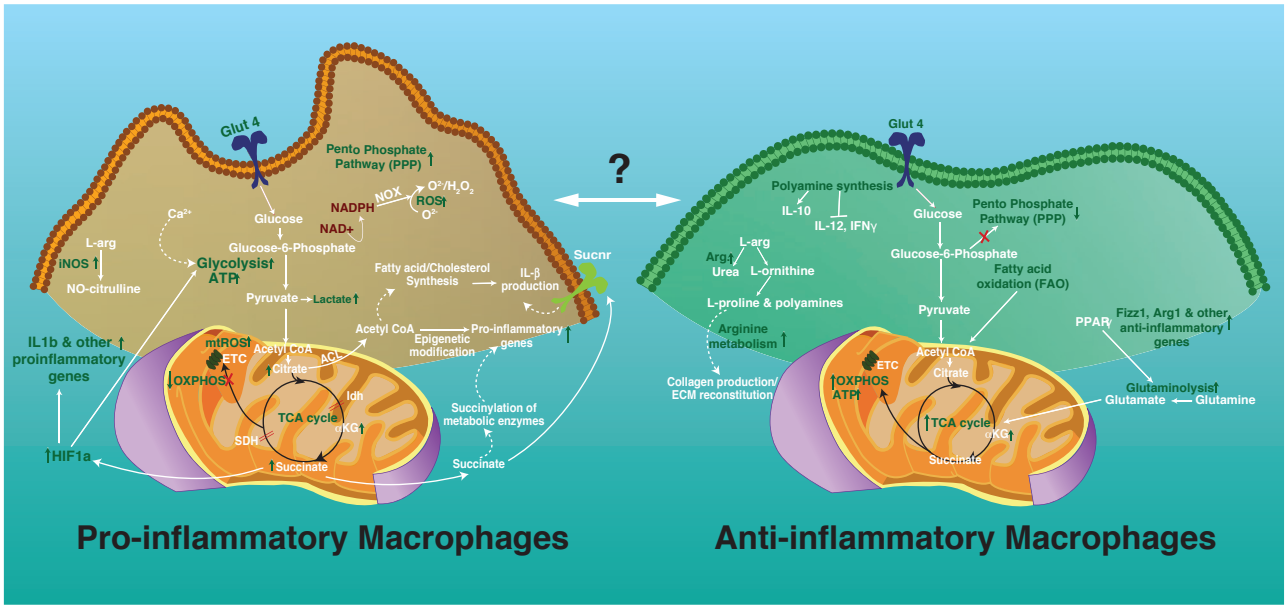
Recent work comparing neutrophils and humoral killing ability from regenerating and non-regenerating rodents suggests that altering the balance between cellular and non-cellular protection can reduce inflammation in regenerating species. Comparing neutrophils from spiny mice (*Acomys* spp.) to neutrophils from non-regenerating rodents (lab and wild-caught *Mus*) showed no difference in migration or ROS production [46]. However, whereas bacterial killing ability was strongly dependent on neutrophils and serum in non-regenerating species, bacterial killing in spiny mice was almost entirely dependent on serum. A bias towards humoral killing in spiny mice is consistent with the observations that neutrophils were recruited more slowly to wounded tissue during regeneration and that peak neutrophil numbers during fibrotic repair coincided with significantly higher myeloperoxidase activity compared to regenerating tissue [47]. Similarly, assessing the inflammatory status of healing ear tissue between regenerating and non-regenerating rodents, a recent study documented a similar inflammatory duration, but with dampened production of pro-inflammatory cytokines during acute inflammation (e.g. CCL2, CXCL1) [3••]. These data suggest that reducing, but not abrogating, neutrophil-driven inflammation may be an important feature of regenerative healing. Although ROS produced by neutrophils is required for subsequent cell proliferation and fin and tail regeneration in zebrafish and *Xenopus* respectively [48,49], too much may do more harm than good. For instance, tipping the balance of neutrophil ROS production can lead to increased tissue damage and delayed wound healing [50]. Although experimentally induced neutropenia demonstrates that neutrophils are not essential for tissue repair [51,52], these collective data support that a careful balance between neutrophil activity and bacterial killing are crucial factors that might influence later healing outcomes.

Macrophage phenotypes during complex tissue repair and regeneration

Although tissue resident macrophages maintain homeostasis and serve as sentinels to detect cellular damage,

their exact contribution during the course of tissue healing is poorly defined. Moreover, a role for tissue resident macrophages during regeneration is completely unknown. In contrast, circulating macrophages recruited to injured tissue are thought to play a central role in perpetuating, regulating and resolving inflammation along with instructing cells through the later stages of tissue repair [16]. Importantly, emerging evidence suggests that circulating macrophages are not static and instead are present as a highly dynamic population that is sensitive to microenvironmental cues [53,54]. Although multiple macrophage phenotypes likely co-exist in the healing microenvironment, they are generally classified as pro-inflammatory and anti-inflammatory during the time course of tissue repair (Figure 3). Pro-inflammatory macrophages are stimulated by damage signals and secrete pro-inflammatory cytokines such as TNF-α, IL-1β and ROS which serve to amplify inflammation. Reparative macrophages secrete anti-inflammatory cytokines that help resolve inflammation, and growth factors that induce angiogenesis, ECM remodelling, and proliferation of resident fibroblasts [55]. Numerous studies have demonstrated a general requirement for macrophages during fibrotic repair [56–58] while others have begun narrowing down how various subpopulations present in the wound microenvironment regulate specific aspects of healing [59,60]. More recently, macrophages have emerged as a key regulator of complex tissue

Figure 3



Current Opinion in Immunology

Metabolic alterations reinforce pro-inflammatory and anti-inflammatory macrophage phenotypes. During the time course of tissue repair, macrophages are generally classified as pro-inflammatory or anti-inflammatory. During the acute inflammation, pro-inflammatory macrophages amplify inflammation and these macrophages rely on glycolysis for their energy requirements. The glycolytic intermediates are diverted through the pentose phosphate pathway (PPP) and produce NADPH. NADPH generated via the PPP is used by NAPDH-oxidase (NOX) to produce ROS and also acts as a co-substrate for the production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS). This NO mediated ROS production can act negatively on mitochondrial oxidative phosphorylation (OXPHOS). Pro-inflammatory cytokine production is amplified via several components of altered metabolism. The tri-carboxylic acid (TCA) cycle exhibits two breaks in pro-inflammatory macrophages which leads to production of excess succinate and citrate. The cytosolic citrate is converted into acetyl-CoA by ATP-citrate lyase (ACL) which amplifies the production of pro-inflammatory cytokines. This acetyl-CoA will also increase fatty acid oxidation (FAO) and cholesterol synthesis which are crucial for production of IL1-β. This assists during the phagocytosis of microbes which is an important property of pro-inflammatory macrophages. Similarly, excess succinate is able to further amplify pro-inflammatory genes through the succinylation of metabolic enzymes. Succinate also helps to stabilize hypoxia inducible transcription factor-1 (HIF-1α) which further produces the pro-inflammatory genes. Importantly, regulating the balance of TCA intermediates such as citrate and succinate is key to resolving inflammation through the polarization of macrophages to an anti-inflammatory phenotype. The major energy source for anti-inflammatory macrophages is mitochondrial OXPHOS, as the glycolysis feeds directly to TCA cycle (PPP is blocked). This is further boosted by increased production of acetyl co-A via fatty acid oxidation (FAO). Because of glutaminolysis, glutamine increases the α-ketoglutarate (αKG)/succinate ratio which eventually increases OXPHOS. The peroxisome proliferator-activated receptor γ (PPARγ) also triggers glutaminolysis to enhance mitochondrial respiration in anti-inflammatory macrophages. Arginine metabolism also plays an important role in the production of L-proline and polyamines that are crucial for collagen production and ECM remodelling. The degree to which macrophages observed at different stages of tissue repair occur from phenotype switching (i.e. from pro-inflammatory to an anti-inflammatory state) or represent new macrophages migrating into healing tissue remains unresolved.

regeneration and depletion studies have demonstrated a functional role for these cells during regeneration of salamander limbs, zebrafish fins, spiny mouse ear tissue, and mouse digit tips [47,61,62]. Given the apparent plasticity of macrophage phenotypes *in vitro*, an open question is how to accurately define phenotype classes that temporally exist during tissue repair *in vivo*. Although cell surface markers and single-cell sequencing approaches are one way to skin the cat, metabolic profiling can provide useful indicators of macrophage populations that regulate different types of healing [63].

Metabolic regulation of pro-inflammatory macrophages

Early metabolic observations of resting versus immune-primed murine peritoneal macrophages demonstrated a bias towards lower oxygen consumption and less lactate production in resting macrophages [64]. Subsequent work revealed that inflammatory macrophages burn through glucose while shunting byproducts through the PPP to produce excess NAPDH, while also exhibiting two breaks in the tricarboxylic acid (TCA) cycle that compromises oxidative phosphorylation (OXPHOS). As circulating macrophages infiltrate damaged tissue, the hypoxic microenvironment stabilizes hypoxia inducible transcription factor-1 (HIF-1α). When stabilized in macrophages, HIF-1α directs the production of pro-inflammatory cytokines and key enzymes necessary for glycolytic metabolism [65,66]. Loss of *Hif-1α* in peritoneal macrophages substantially reduces tissue inflammation and macrophage recruitment in part through a disruption in Warburg (glycolytic) activity. *In vitro* studies have suggested that mitochondrial ROS (mtROS) is an important trigger for conversion of macrophages to a pro-inflammatory phenotype and for maintaining glycolysis [67]. mtROS appears to induce DNA damage and downstream PARP activity which depletes NAD+ pools [67]. This cascade activates NAD+ salvage which is required to replenish NAD+ to support glycolysis and maintain the pro-inflammatory macrophage phenotype. As activated macrophages consume glucose and glutamine, glycolytic intermediates are shunted through the PPP to produce excess NADPH. NAPDH-oxidase (NOX2) uses excess NAPDH to generate superoxide (O_2^-) that in turn dismutates into hydrogen peroxide. Although the early ROS burst associated with wound healing is required for the later proliferative phase of regeneration [49,68], it remains unclear how ROS produced solely from pro-inflammatory macrophages contributes to regenerative healing [47]. One clue may rest with the synthesis of nitric oxide (NO) where inducible nitric oxide synthase (iNOS) uses NADPH as a co-substrate in pro-inflammatory macrophages. ROS produced by NO acts as a double edge sword during inflammation. On the one hand, while NO can inhibit OXPHOS preventing macrophage conversion to an anti-inflammatory phenotype, excess NO attenuates the inflammatory phenotype [69,70••]. For instance, NO produced during the early stages of skeletal muscle repair has been shown regulate the balance between fibrosis and regeneration [71]. Triglyceride metabolism is important for the phagocytic function of macrophages which is crucial for wound debridement and apoptotic neutrophil clearance (efferocytosis) [72,73]. Mitochondrial fission and subsequent Ca^{2+} signaling in macrophages appears to be crucial for efferocytosis [74] and mitochondrial fragmentation can accelerate wound closure by inducing ROS signalling [75•]. This suggests that the proper tuning of metabolic pathways may be important for tissue repair outcomes.

The mitochondrial TCA cycle and OXPHOS are repurposed in pro-inflammatory macrophages to produce secondary metabolites resulting from breaks in the TCA cycle [76]. The reduced level of isocitrate dehydpprogenase and succinate dehydrogenase (SDH) create two break points in the TCA cycle which lead to the accumulation of succinate and citrate (Figure 3). When citrate is transferred to the cytoplasm it is converted to acetyl-CoA by ATP-citrate lyase (ACL) which increases histone acetylation and the expression of target genes linked to pro-inflammatory signals that prevent polarization to an anti-inflammatory phenotype [77•]. Acetyl-CoA also feeds fatty acid and cholesterol synthesis, both of which are crucial for pro-inflammatory macrophage activation signalling and production of IL1-β [78]. Succinate acts both as an effector protein (succinylation of metabolic enzymes) and an extracellular signalling molecule (through succinate receptor 1) to balance pro-inflammatory and anti-inflammatory phenotypes [79–81]. The inhibition of succinate oxidation can polarize macrophages towards an anti-inflammatory phenotype [82]. Itaconate synthesised from citrate inhibits succinate-mediated ROS production and also activates antioxidant and anti-inflammatory gene expression [83,84•,85]. Thus, production of these TCA intermediates (citrate and succinate) appears to play a crucial role in balancing the pro-inflammatory macrophage phenotype which ultimately may control the tempo for how inflammation is resolved.

Metabolic regulation of anti-inflammatory macrophages

Anti-inflammatory macrophages, in contrast to pro-inflammatory macrophages, rely to a greater degree on mitochondrial OXPHOS and an intact TCA cycle [86]. High mitochondrial membrane potential and elevated ATP production are hallmarks of a resolving macrophage phenotype and altering these properties can direct macrophages to a pro-inflammatory phenotype [69,87]. Unlike in pro-inflammatory macrophages where glycolytic byproducts feed into the PPP and lactate is produced from pyruvate, in anti-inflammatory macrophages glycolysis primarily contributes to OXPHOS either directly through an intact TCA cycle or through fatty acid oxidation (FAO) [69,88]. FAO is critical for alternative macrophage

activation where in addition to the endogenous triglyceride pool, CD36 mediated uptake and lysis of triglyceride by liposomal acid lipase helps supply fatty acid oxidation [89]. In the absence of glucose, OXPHOS activity can also be maintained by glutaminolysis where glutamine increases the α-ketoglutarate (αKG)/succinate ratio which is crucial for suppressing a pro-inflammatory phenotype [90]. αKG via glutaminolysis acts to epigenetically re-program macrophage genes necessary for alternative activation [91•] while peroxisome proliferator-activated receptor γ (PPARγ) activates glutaminolysis to enhance respiration in macrophages [92]. Arginine metabolism is another crucial pathway that specifies macrophage polarization. While arginine is converted to NO via iNOS in pro-inflammatory macrophages, in anti-inflammatory macrophages arginine is converted to ornithine by arginase-1 which itself is a well-established marker of the alternative polarization state. Recent work has shown that downstream conversion of ornithine to spermidine enhances mitochondrial function via translational modification of enzymes important for TCA and OXPHOS [93••,94]. In addition, spermidine can suppress the production of pro-inflammatory (IL-12 and IFN-γ) cytokines while also activating IL-10 [95]. Proline produced by arginase is necessary for collagen production and ECM reconstitution, but excessive proline production can exacerbate fibrosis [94]. These studies underscore how an intact TCA cycle and OXPHOS act to promote and sustain anti-inflammatory macrophage phenotype [63]. Recent work examining macrophages during musculoskeletal regeneration in spiny mice found important spatiotemporal differences in anti-inflammatory (CD206+) macrophages compared to fibrotic repair in mice [47]. A more detailed analysis of macrophage phenotypes in this comparative system should yield important insight into how different macrophage states can affect healing outcomes. While metabolic state may be useful in identifying key macrophage phenotypes that participate during tissue repair, future studies testing the functional requirements for these populations will be essential for determining the extent to which they can be manipulated to alter healing outcomes.

Conclusion and outlook

Innate immunity and inflammation are crucial for fibrotic and regenerative tissue repair and we know that basic functional modules of innate immunity are conserved across species irrespective of their regenerative and non-regenerative outcomes. Neutrophils and macrophages play a pivotal role in regulating inflammation and as such, express a spectrum of phenotypes that can be defined by aspects of cellular metabolism. As outlined above, specific features associated with distinct metabolic states and bioenergetic flux regulate not only the secretomes of these cells (pro-inflammatory versus anti-inflammatory cytokines, growth factors, etc.), but also behaviors necessary for defense (bacterial killing) and tissue clearance (phagocytosis). Moreover, the resilience of the metabolic state to environmental perturbations also affects the re-programming ability from one phenotype to another. Furthermore, altering components of cellular metabolism during the normal course of healing or disease progression can lead to poor immunological performance or pathological outcomes. Understanding the plasticity of macrophages that reside in healing tissue will shed light on the degree to which they can be manipulated.

In the last decade, it has become clear that a specific subset of macrophages reside in the dermis and epidermis where they act as sensors to detect infection, inflammation, and tissue damage. Although we have a good appreciation for how these cells respond to pathogens, evidence suggests they are dispensable for wound repair [60]. This suggests that circulating macrophages recruited to injury sites adopt all the phenotypes characterized during tissue repair and alone are responsible for the phenotypes observed using various methods [96]. Recent work depleting macrophage populations at various stages underscores that specific populations regulate specific phases of tissue healing [55,58,59] and the degree to which heterogeneity within these populations exists will surely shed light on how they affect specific responding cell types. In the context of complex tissue regeneration, the picture remains far more obscure.

For instance, although progress has been made in understanding the cellular and molecular basis for how vertebrates either repair or regenerate complex tissue injuries, how innate immune cells functionally regulate healing outcomes is far less understood. This stems in part from methodological hurdles and a lack of certain molecular tools, but also from a disconnect across the many disciplines that contribute to our understanding of tissue repair. Most investigators who study wound healing or tissue regeneration attempt to understand the overall process by breaking it and determining how a particular molecule or gene or cell type contributes (positively or negatively). While this deconstructive approach contributes to a blueprint for the natural progression of each process (similar to classic analysis of tissue development), it is restricted in what it can tell us if we are interested in altering the course of healing from repair to regeneration; a primary goal of regenerative medicine. To advance the field of regenerative medicine, a comparative approach between the two types of healing can reveal shared properties between healing modalities and key differences at the genetic, molecular and cellular level that could be exploited to shift fibrotic repair to regeneration. In this regard, there is intense interest to understand how innate immune cells may differentially regulate repair or regeneration. To this last point, it is entirely possible that the key to regenerative healing lies in the ability of resident connective tissue cells to respond to appropriate

cues [97] or to the presence of specific progenitor populations that can carry out a regeneration program [98].

Conflict of interest statement

Nothing declared.

Acknowledgements

We would like to thank Emily Johnson for designing figures. We apologize to those authors whose work we could not include due to space limitations. AWS's lab is supported by grants from NSF (IOS-1353713) and NIH (NIAMS – R01AR070313 and NIDCR – R21DE028070).

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- • of special interest
- •• of outstanding interest

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