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Macrophage regulation of angiogenesis in health and disease

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Abstract

Macrophages are primarily known as phagocytic innate immune cells, but are, in fact, highly dynamic multi-taskers that interact with many different tissue types and have regulatory roles in development, homeostasis, tissue repair, and disease. In all of these scenarios angiogenesis is pivotal and macrophages appear to play a key role in guiding both blood vessel sprouting and remodelling wherever that occurs. Recent studies have explored these processes in a diverse range of models utilising the complementary strengths of rodent, fish and tissue culture studies to unravel the mechanisms underlying these interactions and regulatory functions. Here we discuss how macrophages regulate angiogenesis and its resolution as embryonic tissues grow, as well as their parallel and different functions in repairing wounds and in pathologies including chronic wounds and cancer.

1. Introduction

Since their discovery by Metchnikoff and colleagues over one hundred and thirty years ago, macrophages have been considered important players in the body's immune system, using their phagocytotic abilities to provide defence against invading pathogens [1]. More recent work has revealed that macrophages are exceptionally diverse and phenotypically plastic cells, with the capacity to dynamically engage with and modify their local environment via production of cytokines, growth factors and proteolytic enzymes [2]. These tools allow macrophages to regulate a broad range of processes that occur during embryogenesis, tissue growth and homeostasis, as well as tissue repair [2]. However, in the context of disease, macrophages assume phenotypes that express mediators and display behaviours that impede normal tissue processes and contribute to chronic pathologies such as non-healing wounds, tissue scarring and cancer [3, 4].

Angiogenesis – the formation, growth and remodeling of new blood vessels from pre-existing vasculature [5] – is of course essential for building the vascular network that facilitates transport of oxygen and nutrients to all tissues in the body. This becomes particularly important in highly metabolically active tissues such as newly developing

tissues in embryos, healing wounds or cancers, since generally all cells need to be within a few hundred micrometres of a blood capillary for survival [6]. Angiogenesis is a complex process that involves remodeling of extracellular matrix, together with the activation of quiescent endothelial cells (ECs). Subsequently, endothelial sprouts form, extend and sometimes undergo a fusion process called anastomosis, until they mature into stable vessels capable of sustaining blood flow, or they remodel and regress, depending on the balance of pro- and anti-angiogenic signals [7]. Angiogenesis is thereby a highly integrated process, relying on interaction with and regulation by different cell types within the vascular microenvironment: immune cells in general, and macrophages in particular, are major players here [8, 9]. In this review we will focus on what has been learnt about the many contexts and ways that macrophages can influence angiogenesis, from studies in model organisms like zebrafish and mice through to human tissue culture and clinical observations, and how pathologies can result when these interactions go awry.

2. The many faces and phenotypes of macrophages

2.1. Macrophage ontogeny

Understanding the origins of macrophages provides an appreciation of their incredible diversity. Researchers in the late 1960s developed a linear cellular hierarchy known as the mononuclear phagocytic system [10]. This theory postulated that hematopoietic stem cells in the bone marrow would differentiate through a series of intermediate progenitor cells to constantly replenish all macrophages found in peripheral tissues, delivered systemically via the circulation. More recently, breakthroughs in next generation sequencing and other fate mapping approaches using in vivo imaging, lineage tracing and flow cytometry have transformed our ability to interrogate macrophage populations. Using these tools, investigators have demonstrated in rodent models that some macrophages are indeed generated by exactly this differentiation process, with certain subsets in the intestine being derived from circulating adult monocytes [11]. However, other studies have shown that some tissue-resident macrophages populations are largely descended from waves of embryonic precursors that colonise tissues and replenish themselves locally. Resident macrophages in the mouse brain are derived almost entirely from embryonic yolk-sac progenitors [12], while subsequent waves of foetal progenitors contribute extensively to macrophage subpopulations in other tissues such as the lung [13]. The same appears to be true in other models such as the zebrafish where various populations of macrophages are derived from multiple waves of progenitor cells throughout embryonic development [14]. Moreover, examination of dermal macrophages in patients following bone marrow transplants demonstrated that while some macrophages are replaced by circulating donor cells, resident host macrophages persist, indicating that both mechanisms are relevant in humans [15]. However, while this ontogenetic heterogeneity results in some differences in gene expression profiles, macrophages from various origins can still populate most organs and fulfil locally relevant functions based on the niche they ultimately colonise [16]. This has driven an alternative approach whereby macrophages are classified into phenotypic subtypes that describe their subsequent behaviours and functions including their cytokine expression profiles and localisation within specific microenvironments.

2.2. Pro-inflammatory and anti-inflammatory macrophages – the M1/M2 paradigm

Highlighting the plasticity of macrophages is their capacity to 'polarise' or become activated by external stimuli. These stimuli are present in the surrounding tissue and can be broadly classified as either pro- or anti-inflammatory. Historically, it was considered that activated macrophages came in two discrete populations, classically activated macrophages (M1) that had been stimulated by pro-inflammatory conditions, and alternatively activated macrophages (M2) stimulated by anti-inflammatory conditions [17]. In recent times this dichotomy has been extensively challenged and it is now believed that in vivo macrophages exist in a continuum of phenotypes between stereotypical M1 and M2 populations [18]. Studies have shown that macrophages in certain contexts – such as human decidual macrophages in the first trimester – express both typically pro- and anti-inflammatory cytokines simultaneously, therefore not conforming to conventional M1/M2 categorisation [19]. Furthermore, these polarisation states are not fixed, as macrophages constantly interact with their environment and are capable of adjusting cytokine expression and switching their phenotype in response to changing conditions within tissues [20].

Within the M1-M2 paradigm, reviewed in [21], macrophages assume the pro-inflammatory M1 phenotype upon exposure to lipopolysaccharide (LPS) and/or inflammatory mediators such as interferon (IFNγ), tumour necrosis factor (TNFα) or damage associated molecular patterns (DAMPs). These macrophages secrete pro-inflammatory cytokines such as interleukins (IL-1, IL-6), nitric oxide (NO) and TNFα. They acquire a highly phagocytic behaviour, resulting in a greater capacity for phagocytosis of debris, pathogens and spent cells, such as apoptosed neutrophils. This phagocytosis of neutrophils or exposure to cytokines such as IL-4 or IL-13 stimulates macrophages to adopt an anti-inflammatory M2 phenotype, secreting anti-inflammatory cytokines such as IL-10 and mediators associated with tissue remodeling including epidermal growth factor (EGF) and transforming growth factor β (TGF-β). The generic M2 macrophage phenotype has further been divided into four discrete subtypes (M2a, M2b, M2c and M2d) based on the stimuli used to derive them in tissue culture experiments as well as their subsequent cytokine expression profiles and presumed functions, although it is currently not clear whether all four populations exist in vivo [22]. How M2 macrophages are derived – whether recruited in from distinct monocyte subsets or local, but switched by cues from the microenvironment – also remains poorly understood, reviewed in [23]. Importantly, the identity of the 'pro-angiogenic' macrophage remains contentious. Angiogenic signals were previous ascribed to M2 macrophages, given their presence in actively growing and vascularising cancers, together with their reparative roles during tissue repair [24, 25]. However, recent evidence from our lab and others challenges this idea, and suggests a far greater complexity in macrophage-vessel interactions, as discussed below.

3. What function for macrophages in developmental and homeostatic angiogenesis?

3.1. Macrophages in developmental angiogenesis – sprouting and remodeling.

Macrophages assume numerous roles that co-ordinate the various phases of developmental angiogenesis. The earliest phases of embryonic vessel development, termed vasculogenesis, whereby vessels derive de novo from mesoderm, occurs prior to the birth of the first macrophages and so these steps must be macrophage

independent. However, they later become essential for supporting embryonic vessel anastomosis by directly interacting with endothelial tip cells, providing guidance to nascent vessels as they sprout and then fuse to form a functional, lumenised vessel with blood flow [8] (Figure 1A). This 'cellular chaperone' function was identified in embryonic mouse hindbrain and observed by live imaging in the developing zebrafish trunk musculature [8]. A similar "nurturing" role was described for developing mouse retinas, although in this study macrophages were also able to induce angiogenesis in an aortic ring tissue culture model prior to any direct vessel contact, suggesting a pro-angiogenic soluble macrophage-derived stimuli [26]. Vascular endothelial growth factor A (VEGFA), was the most likely candidate since it can potently induce endothelial tip cell formation and has been shown to be expressed by stimulated human macrophages in culture [27], but surprisingly macrophages appeared not to express VEGFA in these contexts, and addition of VEGFA in tissue culture promoted vessels that differed in morphology to those stimulated by macrophage conditioned media [8, 26]. The nature of this soluable mediator therefore remains unclear, although the specific subset of macrophages directly interacting with sprouts was characterised by their expression of tyrosine kinase with immunoglobulin-like and EGF-like domains (TIE2) and neuropilin 1 (NRP1), drawing comparisons to anti-inflammatory M2 macrophages normally associated with tumours [8]. More recently, TIE2 [28] has been shown to mark classically activated pro-inflammatory M1 macrophages also, raising questions as to the true polarisation and phenotype of these pro-angiogenic cellssuggesting that TIE2 may be a marker of macrophage angiogenic capacity and that this function may be separate from the macrophage polarization state (reviewed in [29]).

Macrophages have also been implicated in remodeling of some embryonic vascular plexi leading to regression of superfluous vessels to establish mature vascular networks (Figure 1B). Studies of the developing mouse testes showed that macrophages engulf cells labelled with endothelial cell markers, and that depletion of macrophages resulted in extensive, but poorly organised vascular networks [30]. These remodeling macrophages were labeled with NRP1 and TIE2 as well as other typically M2 markers such as arginase 1, but also expressed very low levels of key endothelial growth factors such as VEGFA [30]. Post-natally, macrophages have been shown to be critical in remodeling the vasculature in mouse retinas. Examinations of the transient hyaloid vascular plexus soon after birth reveal that these embryonic vessels fail to undergo pruning in mice genetically depleted of macrophages [31]. This macrophage-mediated vascular regression is dependent on an angiopoietin 2 (ANG2) signaling pathway that stimulates macrophage WNT7B production to drive ECs towards apoptosis, unless a counterbalancing survival signal is received from neighbouring perivascular support cells such as pericytes [32]. Importantly, it appears that macrophage vessel pruning by active phagocytosis may be pivotal only in certain situations where large scale remodelling is required, for example whole networks of vessels require remodelling. Indeed, studies of zebrafish developmental angiogenesis suggest that this is not the primary mechanism deployed when smaller scale, more selective pruning is required [33]. Macrophages also have a secondary capacity to fine-tune vascular complexity, for example by secretion of anti-angiogenic factors such as soluable VEGF receptor 1 (VEGFR1) through autocrine WNT5A and WNT11 signaling, thereby resulting in sequestering of VEGFA, thus inhibiting angiogenesis-inducing VEGF signals during later retinal development in the mouse [34].

3.2. Macrophages in maintenance of vascular homeostasis

Beyond developmental stages, macrophages maintain their capacity to interact with ECs and regulate vessel maturation, maintenance, and survival [35]. For example, human macrophages stimulated towards an M1 phenotype secrete a number of pro-angiogenic growth factors including VEGFA and fibroblast growth factor 2 (FGF2) [36], while M2a macrophages express high levels of platelet-derived growth factor B (PDGFB), which is key for recruitment of pericytes [36]. Together with other perivascular cells, pericytes take up position around vessels to stabilise the surrounding extracellular matrix as well as strengthening junctions between endothelial cells, thereby limiting permeability across the endothelial monolayer and providing structural support [37]. Mouse models have shown that macrophages can themselves directly contribute to this population of vessel supporting cells by transdifferentiating into perivascular cells such as smooth muscle cells [38] or into pericytes in early cerebrovascular development [39]. Other studies in mice have further identified particular populations of macrophages that control vascular permeability indirectly, possibly via regulation of key EC junctional adhesion molecules such as VE-cadherin[40]. Macrophages can also facilitate vessel sprouting and regression by manipulating the surrounding environment, secreting proteases such as matrix metallopeptidase 9 (MMP9) that cleave and remodel the surrounding extracellular matrix to provide conduits for tip cells [41]. Finally, macrophages can also act as anti-angiogenic regulators, with recent in vitro studies on mouse macrophages showing secretion of endostatin, which impairs EC proliferation and survival by inhibiting VEGFR2 signaling [42].

Most, if not all, of these various macrophage-EC interactions and regulatory mechanisms can be seen throughout vascular homeostasis as tissues are maintained. A good example of this is the human (non-pregnant) menstrual cycle, where cyclical fluctuations in the sex steroids progesterone and estrogen result in repeated proliferation, differentiation and shedding of the endometrial lining [43] (Figure 1C). These phases are supported by, and dependent upon, tightly regulated changes in the local vascular architecture. Initially, the endometrium begins its regeneration in the estrogen dependent proliferative phase, accompanied by a hypoxic environment that stimulates local macrophages to secrete VEGF and encourage vessel sprouting [44]. Subsequently, these newly formed vessels mature into spiral arterioles as the progesterone dependent secretory phase induces remodeling of the endometrium in preparation for embryo implantation. Macrophages are believed to mediate the maturation and remodeling of these vessels [45], with the secretory phase being accompanied by an influx of macrophages that double their numbers within the endometrium during the proliferative phase [46]. In the absence of a pregnancy the ensuing decrease in progesterone levels leads to vascular constriction – which must precede subsequent tissue degradation to limit ischemic hemorrhaging – and an inflammatory cascade, which leads to the shedding of the luminal endometrial layer [47]. An important element of this inflammatory cascade is a further influx of macrophages during the menstrual phase [46]. This increase is associated with a surge of macrophage-derived proteases and cytokines, suggesting that macrophages may play key roles in initiating menstruation, and then clearing the resultant damaged tissue and subsequent repair of the endometrium [45]. Specifically, macrophages have been shown to express high levels of anti-angiogenic factors such as angiopoietin-2

(ANG2) and thrombospondin-1 (THSP1) during this phase, and switch back to a pro-angiogenic expression profile once the tissue re-enters the proliferative phase [48]. These macrophages are in part derived from resident endometrial macrophages but are also supplemented by chemotaxis-based recruitment of monocytes from the circulation [49], highlighting the contextual importance of the local environment in regulating macrophage behaviour and their capacity to influence endothelial cells.

4. Role of macrophages in wound angiogenesis

Macrophages have a very well characterised series of roles during the inflammatory and resolution phases of wound repair (Figure 2). Generally, they are considered later arrivers at the site of any tissue damage than the very rapidly responding wound neutrophils. Wound macrophages comprise both tissue resident macrophages and migrating monocytes that differentiate into wound macrophages en route to sites of damage; they are present throughout inflammation, proliferation and remodeling stages of wound healing [50]. Macrophage is greek for "big eater" and at a wound site these cells are vital for phagocytosis of debris, dead cells, pathogens and spent neutrophils in a process called efferocytosis: moreover, they secrete a wide range of cytokines and growth factors to orchestrate a variety of processes including wound re-epithelialisation, extracellular matrix deposition and remodeling, establishment of granulation tissue and re-vascularisation [51]. Here, we will focus on their roles in regulating wound angiogenesis and its resolution.

4.1. Macrophage recruitment

Tightly controlled inflammation and monocyte/macrophage recruitment is the typical response of an organism to injury or infection. Following the formation of a clot to seal the wound site and stem blood loss, mouse wound models have shown that circulating monocytes appear within hours of tissue damage [52]. Several damage-associated signals at the wound site trigger macrophage recruitment. Tissue damage studies in zebrafish brains show how the rapid burst of ATP released from injured cells subsequently acts as an early activator of tissue resident macrophages [53]. Other rapidly released damage signals include ROS, such as hydrogen peroxide, and these too have been shown to be attractants for local leukocytes [54]. However, the majority of wound macrophages are recruited from the circulation and first need to transmigrate through EC lined capillaries to access damaged tissues. Activated monocytes disrupt EC cell-cell adhesions to cause the formation of intercellular gaps via release of inflammatory mediators such as TNF-α and IL-6 [55]. Tissue culture experiments with human cells have shown how the endothelial cells lining vessels in the wound proximity become activated and adhesive via a nuclear factor kappa-B (NF-κB)-dependent pathway [56]. These factors enable monocytes/macrophages to dock onto the endothelium and extravasate through the vessel wall to reach the site of damage [57].

4.2. Vessel sprouting

Macrophages are known to facilitate vessel sprouting in contexts of injury repair using a plurality of interrelated mechanisms. Macrophages infiltrate tissues by secretion of proteolytic enzymes that cleave and remodel the ECM, such as MMP-2 and MMP-9; this also releases pro-angiogenic growth factors linked to the ECM [58].

Macrophages are also sources of molecules such as semaphorins that provide direction guidance cues and survival signals to new vessels as they sprout [59]. Importantly, wound macrophages themselves are potent sources of VEGFA, and so can directly drive vessel sprouting and vascularisation. Studies in wounded mouse skin have demonstrated that macrophage-secreted VEGF is critical for vessel formation during wound angiogenesis [9, 60]. These macrophages express high levels of typically pro-inflammatory M1 markers, IL6 and iNOS, but also expressed the more anti-inflammatory M2 related TIE2 receptor [9]. More recent studies in zebrafish have revealed the specific roles and interactions that macrophages have during wound vessel sprouting, but raise some questions about the exact phenotype of these macrophages. Our live imaging experiments following tissue damage showed TNFα expressing, early responding macrophages interacting extensively with sprouting vessel tips and expressing high levels of VEGFA [61]. This VEGFA secreting pro-angiogenic role for pro-inflammatoryM1 macrophages was also conserved in human primary macrophages [61]. These experiments also revealed an additional pro-angiogenic role for early wound macrophages, where we observed them dismissing anti-angiogenic neutrophils that arrive before macrophages and interact directly with vessel tips while expressing repressive VEGF-inhibiting soluable VEGFR1 [61]. Together, these studies emphasise the multifaceted roles that macrophages play in establishing nascent vessel sprouting in wound healing. However, they also highlight that the specific macrophage phenotype(s) driving this process remain to be entirely defined.

4.3. Anastomosis

Following their role in driving vessel sprouting, macrophages also appear to be key in subsequent vessel anastomosis, just as in embryonic development; ablation of macrophages in mouse wounds during the proliferation phase of wound healing results in failure of this step and severe hemorrhage [60]. Further studies performed on mice have shown that implanted Matrigel plugs enriched specifically with M2 macrophages increased subsequent vessel formation and stabilisation at 14 days post implantation [62]. Our observations showed that these anastomosing wound vessels appear to have contributions from both arterial and venous EC lineages, occasionally resulting in chimeric vessels [61].

One key aspect regulating how the nascent, tortuous and leaky wound vascular networks achieve patency is by becoming wrapped in a pericyte layer. In a model of mouse lung tissue damage, macrophages express Amphiregulin (AREG), which induced pericyte activation and differentiation to restore the vascular barrier function [63]. More recent studies have shown that macrophages expressing both M1 and M2 markers can physically interact with 3D engineered vessels implanted into mice to facilitate anastomosis and integration [64]. Live imaging experiments in zebrafish revealed that macrophages responding to laser injury of blood vessels in the brain express adhesion molecules such as Cadherin 5 (CDH5) that accumulated at macrophage-EC adhesion sites. This allowed macrophages to adhere to two opposing sprouts and exert mechanical traction to pull them together [53]. Our wound healing experiments in zebrafish further indicate that pro-inflammatoryM1 macrophages switch off TNFα expression during this phase, transitioning towards a more M2 anti-inflammatory polarised phenotype as they interact with anastomosing vessels [61]. However, again the precise phenotype(s) of these anastomosis-assisting macrophages remains elusive.

4.4. Regression/remodeling

As the wound matures and enters the remodeling phase, the tissue reorganises and restructures to resemble its form prior to injury. Blood vessels undergo extensive pruning during this period, with many of the new vessels undergoing apoptosis until the vascular density returns to normal levels and pattern [65]. Macrophages play key roles in this process too, with our recent work providing evidence in both mouse and zebrafish wounds that ECs are phagocytosed by wound macrophages [61]. This process is in part due to a reduction of pro-angiogenic stimulus, with our zebrafish experiments showing that stimulation of wound macrophages to remain in a pro-inflammatory, VEGFA expressing phenotype at later stages of wound healing results in a failure of vessel regression [61]. However, while TNF-negative, (presumably anti-inflammatoryM2) macrophages are essential for inducing vessel apoptosis, they did not recapitulate the WNT signaling used in development to mediate this remodeling response. One possible mechanism for macrophage-induced clearance may be associated with the partial and selective coverage of newly formed capillaries in the wound site by pericytes, which play a key role in shielding ECs from anti-angiogenic signals [66]. Given the macrophage's capacity to influence pericyte migration and differentiation [63], this signaling axis may be crucial in ultimately determining vessel fate in the remodeling wound. Further investigation is required to identify the specific macrophage phenotype(s) involved in this process, and the mechanisms used to mediate vessel regression.

5. Role of macrophages in pathological angiogenesis

While tightly regulated inflammation is critical for normal angiogenesis, dysregulated inflammation is of considerable clinical significance because it causes angiogenesis to go awry and drive vascular dysfunction. Understanding the interface between macrophages and ECs is therefore crucial for identifying new therapeutic avenues for numerous diseases. For example, inflammatory bowel disease (IBD) is largely driven by macrophage involvement in chronic inflammation of the intestinal mucosa, driving continual growth of new vessels to increase local vascular density and support the constant regeneration of the damaged tissue [67]. These vessels lack pericyte coverage and as a consequence are poorly perfused and leaky, which contributes to the progression of IBD [68]. Atherosclerosis is another vascular disease driven by chronic inflammation, which in this instance is due to dysregulated lipid metabolism [69]. Macrophages play a key role in driving subsequent plaque formation, migrating to the sub-endothelial spaces within artery walls and sequestering low-density lipoproteins (LDLs) to become lipid-laden "foam cells" [70]. The Foreign Body Response (FBR) – the process triggered in tissues exposed to implanted biomaterials – is also a consequence of unresolved inflammation, with deranged macrophages fusing with each other to form multinucleated giant cells along the material/tissue interface which results in aberrant, leaky vessels and extensive fibrosis [71, 72]. Furthermore, failure to resolve inflammation and appropriately remodel wound vessels is linked to the development of keloid scars of the skin that extend beyond the boundaries of the initial wound [73]. Below, we will examine cancer and chronic wounds, where dysregulated macrophage-EC interactions result in clinically significant pathologies.

5.1. Tumour inflammation and angiogenesis

Tumours have long been considered 'wounds that do not heal', sharing many molecular mechanisms and cellular interactions with repairing tissues, with the exception that while these mechanisms are finely regulated in repair, this is not the case in cancer [74]. Angiogenesis is indispensable for tumours to grow beyond 1mm in diameter, and one of the key roles of the tumour microenvironment is to co-opt the surrounding vasculature to fuel subsequent tumour expansion [75]. These tumour-associated vessels resemble early wound healing vessels, being highly tortuous, leaky and presenting low pericyte coverage, which may be partially enabling for cancer cell metastasis.

Tumour associated macrophages (TAMs) have been shown to be important drivers for cancer neovascularisation to enable tumour expansion (reviewed in [76]), with numerous mouse models demonstrating their pro-angiogenic capacity [77]. Occasionally, TAMs even engage in the process of vascular mimicry, forming non-endothelial "vessels" in response to the tumour microenvironment [78]. The primary population of pro-angiogenic TAMs are the extensively characterised TIE2-expressing macrophages [77, 79] which, at least in vitro, exhibit increased secretion of proteases (e.g. MMP9), and pro-angiogenic factors (e.g. VEGFA) [79]. Pro-angiogenic responses of these TAMs were enhanced further by exposure to ANG2, a key TIE2 ligand that both induces macrophage recruitment and promotes extravasation by increasing vascular leakage, and is secreted by activated tumour-localised ECs in several mouse tumour models [80]. Further profiling of TAMs recruited to mouse tumours revealed an enhanced expression of typically M2 markers such as mannose receptor C-type 1 (MRC1) and haemoglobin scavenger receptor (CD163), suggesting that, unlike in a wound scenario, anti-inflammatoryM2 macrophages are the primary drivers of angiogenesis [81]. Indeed, biopsies from human ovarian tumours indicate a correlation between numbers of infiltrated anti-inflammatoryM2 macrophages and levels of vascularisation, leading to increased metastasis and overall poorer prognosis [82]. However, M1 pro-inflammatory macrophages are known to be crucial in promoting tumour initiation by inducing chronic inflammation that contributes to the establishment of the hypoxic and vessel-stimulating tumour microenvironment [83]. Together, these findings highlight the many remaining unknowns in defining the precise function and phenotype of macrophages within the complex and context dependent process of tumour angiogenesis.

One strand of cancer therapy investigations, led by Judah Folkman and colleagues, has focused on the perceived "Achilles heel" of a tumour's requirement for an angiogenic supply. Most of these therapeutics have been developed around blocking VEGF signaling in various ways, for example by infusion of the anti-VEGF monoclonal antibody Avastin [84]. However, these anti-VEGF treatments can trigger the starved cancer to metastasise. Although these treatments continue to be used as part of some cancer combinatorial treatments, new, counter approaches are being developed which focus on improving tumour perfusion to enhance the efficacy of existing chemotherapeutic approaches [85].

5.2. Macrophage dysfunction in diabetic/chronic wounds, and how this impacts on revascularisation

Over 2 million compromised or chronic wounds are treated annually in the UK alone [86], incurring significant reduction in quality of life, increased morbidity and mortality rates, and are a huge burden on health services [87]. These wounds include diabetic ulcers, vascular ulcers and pressure ulcers, and are frequently related to chronic arterial disease, venous insufficiency or prolonged pressure [88]. Diabetic wounds account for a major and growing subset of chronic wounds, with 15-25% of nearly 500 million diabetes sufferers worldwide developing non-healing wounds, particularly diabetic foot ulcers, which can ultimately lead to limb amputation [89]. This impairment of tissue repair affects sufferers of both Type 1 Diabetes Mellitus (T1DM) [90] caused by complete insulin deficiency due to destruction of pancreatic β-cells, or Type 2 Diabetes Mellitus (T2DM) due to insulin resistance in peripheral tissues, with the resultant high blood and tissue glucose in both conditions believed to be a key driver of chronic wounds [91]. Other important risk factors for developing compromised wounds are obesity (affecting over 650 million people worldwide [92]) and ageing (becoming clinically significant after 60 years of age, affecting over 700 million worldwide [93]. All of these conditions have complex pathogenesis but broadly share the same causative mechanisms of stalled inflammation and angiogenesis [88]. Indeed, recent clinical studies have identified a panel of key genes that distinguish healing from chronic wounds, with dysregulated inflammation and angiogenesis genes making up over half of this chronic wound 'gene signature' [94].

This prolonged inflammatory stage is associated with a changed composition in cell number and phenotype compared to acute wounds [95]. In diabetic mice, the hyperglycemic environment impairs macrophage ability to efferocytose neutrophils, resulting in increased and persistent neutrophil numbers at chronic wound sites [96]. Furthermore, anti-inflammatoryM2 macrophages numbers are decreased with a consequent reduction in key growth factors involved in later proliferative phases of wound healing such as TGF-β and insulin-like growth factor-1 (IGF-1) [97]. Conversely, pro-inflammatoryM1 macrophages make up 80% of the total cell number at the wound margins of mouse diabetic wounds and chronic venous ulcers seen in humans [98], resulting in excessive reactive oxygen species (ROS) [99], and pro-inflammatory cytokine production such as Il-1β, IL-17 and TNFα within these wounds [97, 100]. Chronic wound patients in the clinic also present with high levels of pro-inflammatory cytokines such as TNFα in their wound exudate [101], which is linked to changes in expression of MMPs that increase ECM proteolysis, as well as increases in apoptotic load, both of which feedback to elevate inflammation and hence contribute to wound chronicity [102, 103]. However, despite the increased number of macrophages in chronic wounds, angiogenesis is shown to be insufficient, with decreased capillary density and vascularity in diabetic wounds [104]. By contrast to healthy wounds in which pro-inflammatory macrophages drive vessel sprouting, the increased pro-inflammatoryM1 population in diabetic mouse wounds leads to a decrease in pro-angiogenic signals for sprouting (e.g. VEGF [105]). Unsurprisingly, these wounds also have decreased levels of pro-angiogenic signals for vessel maturation (ANG1 [106]) and pericyte recruitment (PDGF [107]), corresponding to key angiogenic processes at later wound repair stages. Furthermore, diabetic mouse wound models also have increased levels of some anti-angiogenic signals, such as ANG2 [108]. Together, these observations offer some explanation as to why wound angiogenesis is impaired in chronic wounds, leading to fewer vessels, with many of these remaining immature, leaky and poorly perfused. Ultimately, these deficits in

inflammation and angiogenesis lead to persistent tissue hypoxia and failure of wound closure due to defective epithelial behaviour, decreases in fibroblast migration and proliferation, and increased necrosis [109].

Numerous treatment strategies have been deployed to rescue these wound healing defects, with TNFα being a prime target for regulating the inflammatory response. Paradoxically, both suppression of TNFα in mouse models of excessive inflammation [110] and early treatment of healthy mouse wounds with exogenous TNFα [111] promoted improved healing. These results shed light on why the strategy of surgical debridement that resets the acute inflammatory response continues to be a mainstay in managing chronic wounds in the clinic [112]. Other macrophage-centric therapeutic approaches have shown promise, such as isolation of macrophages from young, healthy donors, stimulating these cells via hypo-osmotic shock, and administering these activated macrophages directly to pressure ulcers in elderly patients [113]. However, while these macrophages were characterised as having increased VEGF expression, they also displayed markers for both classically M1 and M2 macrophages, and their mechanism for action within the wound generally or driving angiogenesis specifically is less than well characterized [114]. Further study is required to unravel how our growing knowledge of macrophage control of healthy angiogenesis can guide the next generation of therapeutics to ameliorate deficiencies in chronic wound healing.

6. Conclusions and unanswered questions

Macrophage involvement in directly and indirectly controlling angiogenesis in healthy and diseased tissues is extensively supported, as highlighted in this review. Our understanding has been greatly improved by the use of complementary models such as zebrafish, mouse, human tissue culture and clinical studies. However, there are many outstanding questions with regards to the precise interactions involved. Historically, the paradigm of M1/M2 (or pro-/anti-inflammatory) was used to define the differing roles that macrophages play in angiogenic control, but now we can see how this binary concept is too simplistic given the staggering diversity of macrophage phenotypes, their functional plasticity, and the context-dependent nature of their behaviours and functions in healthy and pathological tissues. Moreover, recent experiments comparing in vivo and in vitro stimulation of macrophages show that more genes are regulated in opposite or unrelated ways than genes that clearly overlap for pro-inflammatoryM1 or M2 anti-inflammatory phenotypes, highlighting why so many markers translate poorly between models and investigations [115]. It also remains crucially important to understand how these dynamic phenotypes correlate with macrophage angiogenic capacity, influence macrophage interactions with ECs, and how these can be harnessed to generate novel therapeutic approaches that ameliorate chronic wounds or combat tumour growth. Recent technological breakthroughs such as tissue specific CRISPR gene editing and single cell sequencing promise to drive these next steps towards a comprehensive understanding of macrophage phenotypes and how they regulate angiogenesis.

Declaration of Competing Interest

None.

Figure legends

Figure 1. Macrophage control of developmental and homeostatic angiogenesis. (A) Schematic showing angiogenesis during murine embryonic hindbrain development (approx. 10.5-12.5 days post fertilisation). Following the establishment of new vascular sprouts in response to VEGF signaling, pro-angiogenic macrophages (green) are critical in expanding the vascular plexus, initially interacting with endothelial tip cells to enable bridging and establish links with neighbouring tip cells and thus facilitate vessel anastomosis. (B) Schematic of remodeling transient hyaloid vessels in the post-natal murine eye (approx. 8 days postpartum). Anti-angiogenic macrophages (blue) use WNT signaling to induce endothelial cell death, which these macrophages subsequently phagocytose. (C) Schematic showing cyclical vessel sprouting and regression during menstruation. Macrophages express both M1 and M2 markers, increase in numbers towards the shedding phase, and are key in driving sprouting and regression via VEGF signaling and MMP secretion.

Figure 2. Roles of macrophages in wound angiogenesis. (A) Low and high-magnification confocal views showing macrophages (red) interacting with new vessel sprouts (green) within a zebrafish needle stab wound at 3 days post injury (DPI) – see schematic for site of injury. (B) A time series of confocal microscopy views illustrating zebrafish wound angiogenesis following laser wound injury at 4 days post fertilisation (DPF), indicating the key timepoints for vessel sprouting (1-3 DPI), anastomosis (3-6 DPI) and remodeling back to the unwounded pattern (6-10 DPI). (C) Schematic of the key macrophage-endothelial cell interactions occurring during the wound healing timecourse, corresponding to timepoints shown in (B). Following injury, hemostasis and inflammation occur, with early anti-angiogenic wound neutrophils associating with endothelial tip cells first and secreting soluable VEGF receptor (sFlt) that suppresses vessel sprouting. Early, pro-angiogenic, pro-inflammatory wound macrophages (green) are recruited by damage signals and drive sprouting by displacing neutrophils from tip cells, and act as point sources of VEGF. Macrophages then mediate anastomosis by direct vessel interactions, allowing the formation of a patent vascular plexus. In later stage wounds, anti-inflammatory macrophages (blue) drive vessel remodeling and regression/clearance by phagocytosing those endothelial cells that are not maintained in the mature vascular bed. Scale bars: A = 20μm, B = 40μm.

Figure 3. Dysregulated macrophage-endothelial cell interactions can drive pathological angiogenesis. In acute wounds (top right panel), pro-angiogenic macrophages (green) drive vessel sprouting and dismiss anti-angiogenic neutrophils. By contrast, chronic wounds (bottom left panel) exhibit excessive and stalled inflammation with persistent neutrophil infiltration, where anti-angiogenic macrophages (blue) lack capacity to induce appropriate vessel sprouting and maturation for healthy tissue repair. In tumour angiogenesis (bottom right panel),

macrophages (many displaying anti-inflammatory markers) are induced via interaction with the tumour microenvironment to subvert normal angiogenic cues and drive vascularisation of tumours, resulting in vessels which fail to mature and don't subsequently resolve as in a wound.

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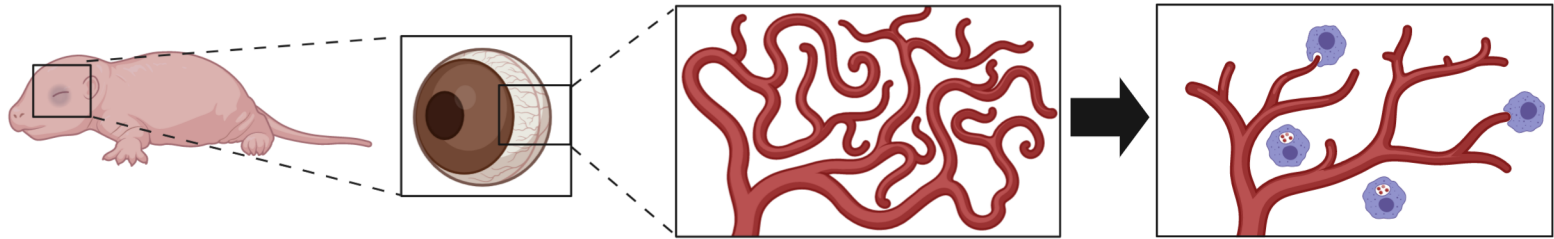
M2(LPS–) vs. Alternatively Activated Macrophages. Front. Immunol., 2019. **10**: p. 1-14.

Figure 1

A. Sprouting angiogenesis
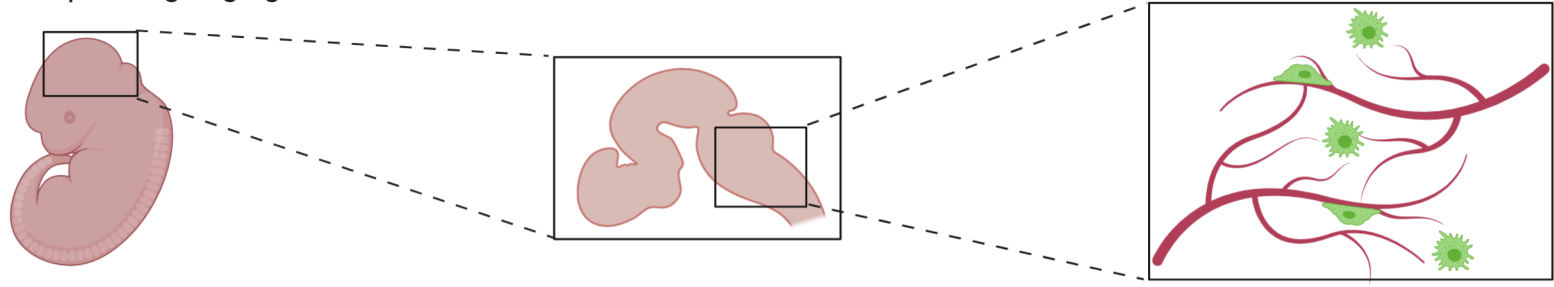
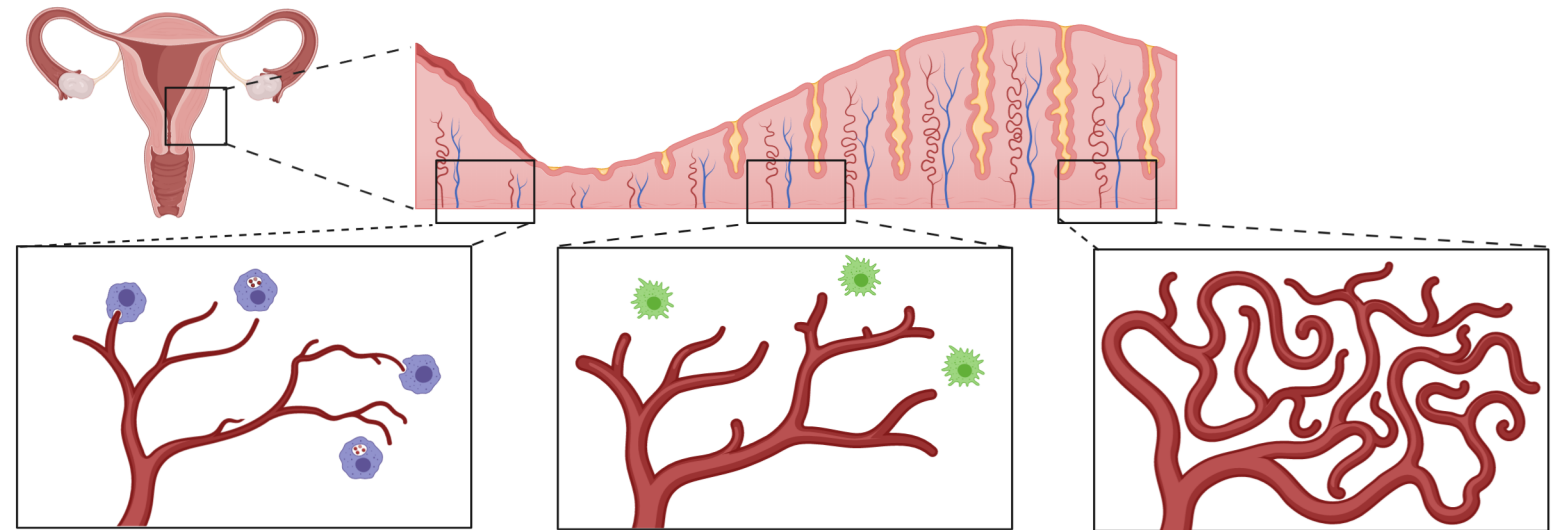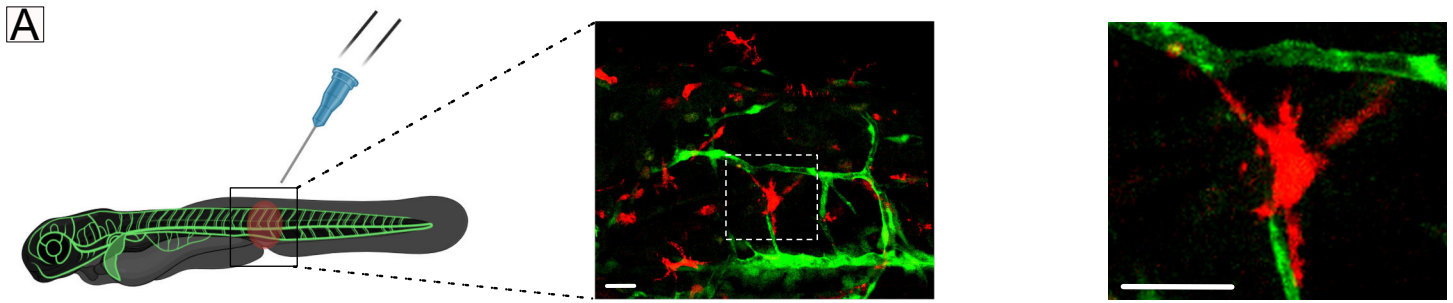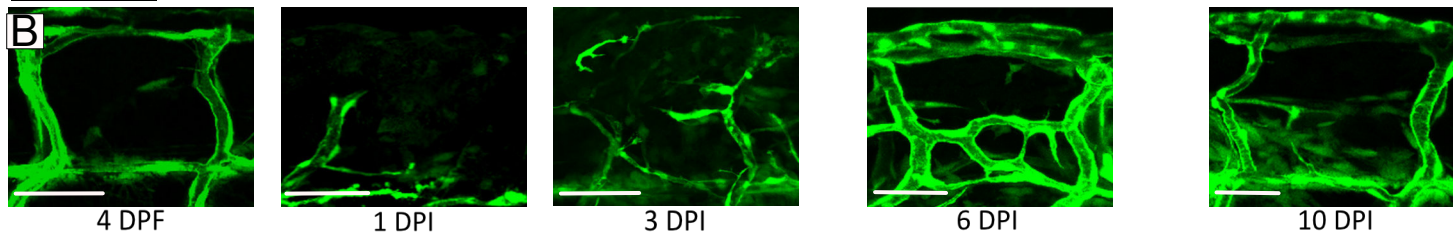## B. Vessel regression/remodeling

C. Angiogenesis during menstrual cycle
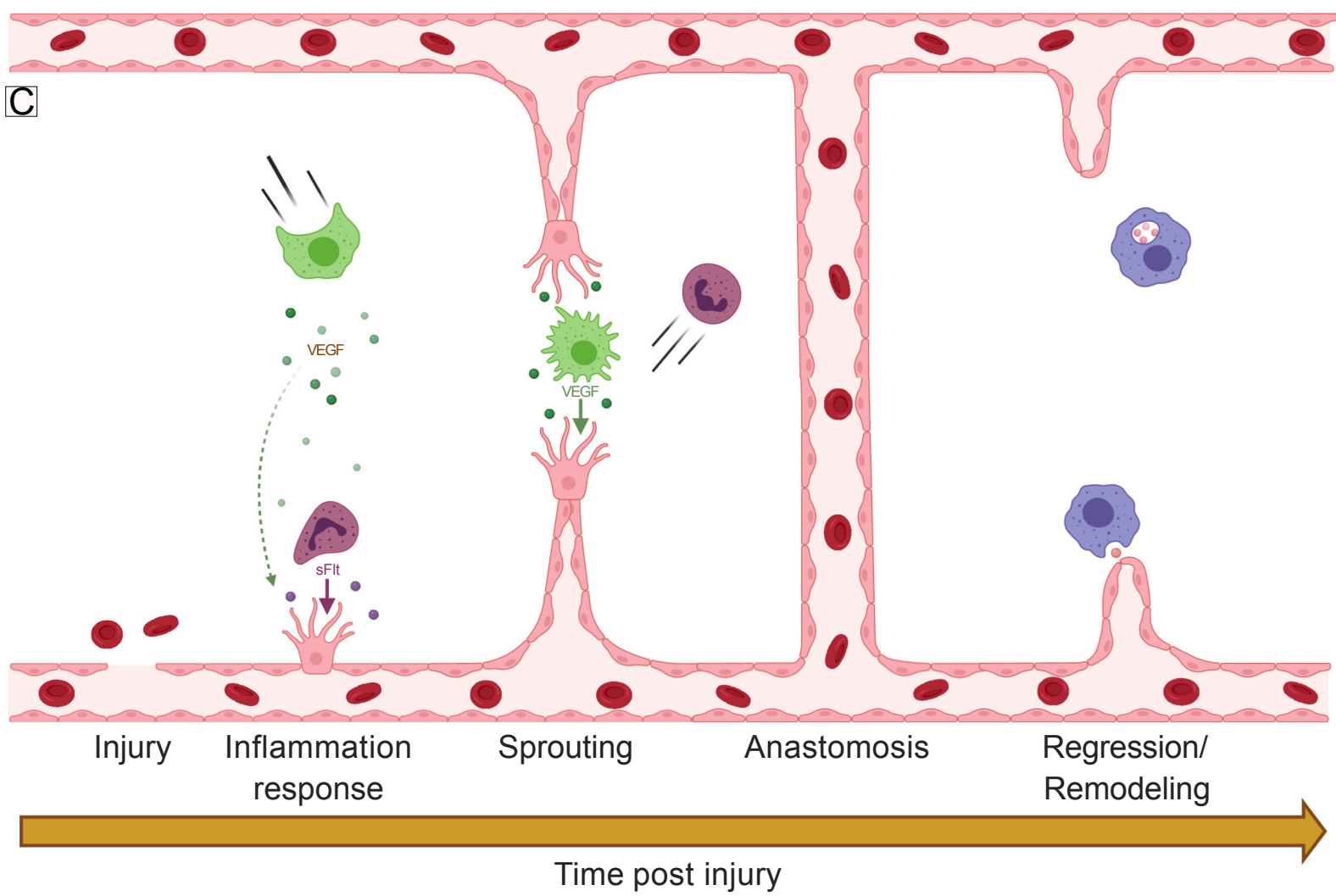
Figure 2

A

B
Endothelial cells

4 DPF   1 DPI   3 DPI   6 DPI   10 DPI

C

VEGF

sFlt

VEGF

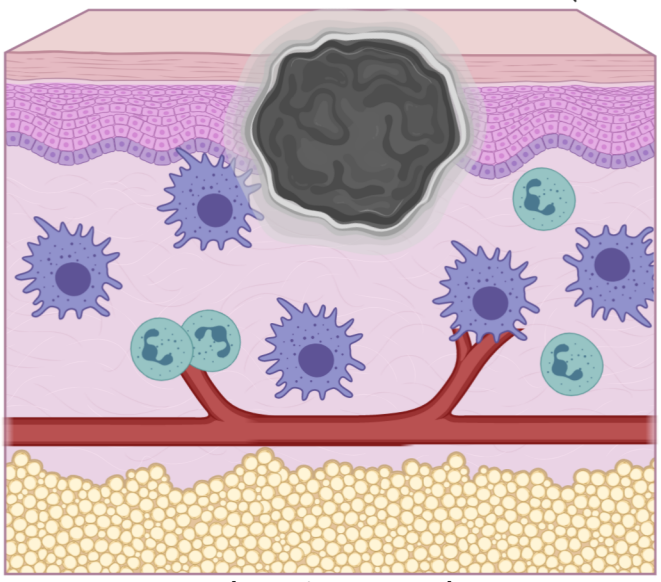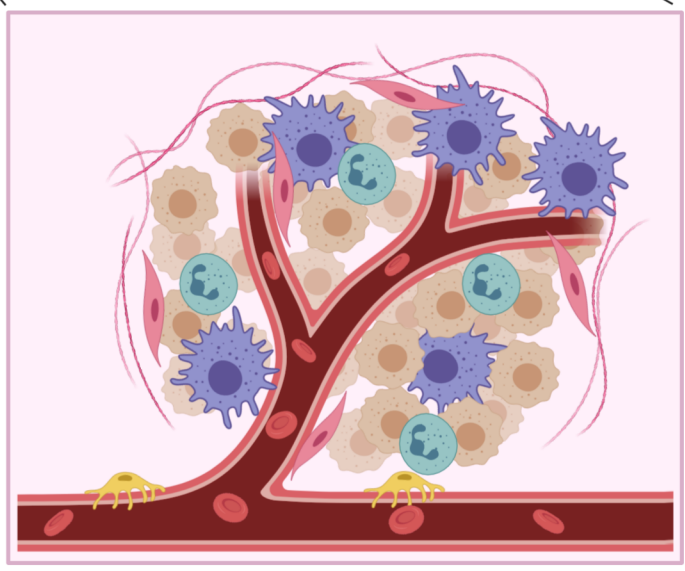Injury   Inflammation response   Sprouting   Anastomosis   Regression/ Remodeling

Time post injury

Figure 3
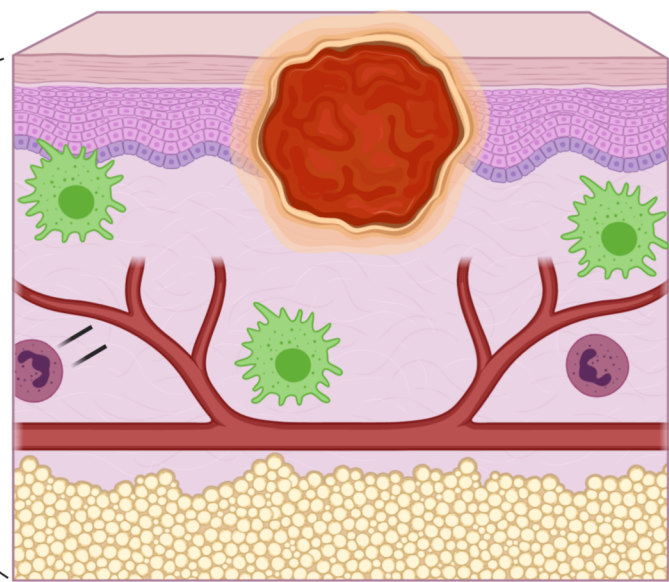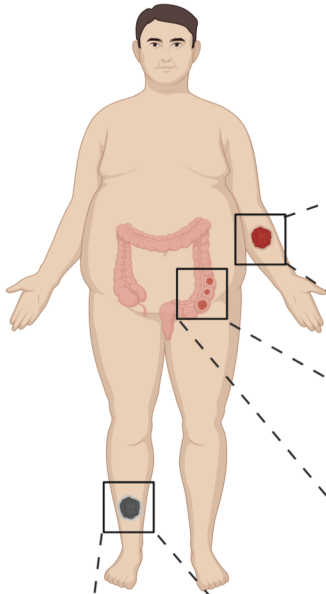
Acute wound

Chronic wound

Cancer