Inflammation and Immunity in Organ Regeneration

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Abstract

The ability of vertebrates to regenerate amputated appendages is increasingly well-understood at the cellular level. Cells mediating an innate immune response and inflammation in the injured tissues are a prominent feature of the limb prior to formation of a regeneration blastema, with macrophage activity necessary for blastema growth and successful development of the new limb. Studies involving either anti-inflammatory or pro-inflammatory agents suggest that the local inflammation produced by injury and its timely resolution are both important for regeneration, with blastema patterning inhibited in the presence of unresolved inflammation. Various experiments with *Xenopus* larvae at stages where regenerative competence is declining show improved digit formation after treatment with certain immunosuppressive, anti-inflammatory, or antioxidant agents. Similar work with the larval *Xenopus* tail has implicated adaptive immunity with regenerative competence and suggests a requirement for regulatory T cells in regeneration, which also occurs in many systems of tissue regeneration. Recent analyses of the human nail organ indicate a capacity for local immune tolerance, suggesting roles for adaptive immunity in the capacity for mammalian appendage regeneration. New information and better understanding regarding the neuroendocrine-immune axis in the response to stressors, including amputation, suggest additional approaches useful for investigating effects of the immune system during repair and regeneration.

Keywords: regeneration, inflammation, amphibian, zebrafish, appendage, digit

1. Introduction

Wounds or other injuries in multicellular animals rapidly elicit a series of events involving immune cells which not only defend the organism against invasion by pathogens at the damaged site, but also stimulate processes that restore serviceable if not completely normal structure and function to the injured tissue or organ. These early events comprise the acute inflammatory response, a complex reaction to warning signals from microbes and damaged or necrotic cells which includes vascular responses, recruitment of leukocytes, and localized activation of innate immune components which together activate cells of adaptive immunity both locally and systemically (Kumar et al., 2005). Throughout history inflammation has been recognized as central to the study of wound healing as well as deleterious repair-like processes, such as fibrotic disorders and atherosclerosis, but until recently was largely ignored by most biologists attempting to understand regeneration of limbs or other organs. In the 21st century the importance of inflammation and immune cells for organ regeneration has become more widely understood and the interest of such biologists in immunity has now begun to grow rapidly. Without injury and ensuing inflammation, regeneration or repair does not occur, and greater appreciation of this has helped shift studies of such processes from developmental biology to the more appropriate field of “regenerative biology.”

Two areas of research were of major importance in leading developmental biologists to investigate inflammation. First, new murine models of organ regeneration,

such as scar-free wound healing in fetal skin (Martin, 1997) and closure of ear holes in immunodeficient animals (Heber-Katz, 1999) showed that regeneration of normal skin, with inductive signaling and patterning of new hair follicles and glands, may be inhibited by inflammation involving fully developed adaptive immunity (reviewed by (Harty et al., 2003). At the same time microarray, proteomic, and other global analyses of classic regeneration models revealed prominent expression of immune- and inflammation-related genes and proteins seldom considered previously by developmental biologists (Grow et al., 2006; Ishino et al., 2003; Katogi et al., 2004; King et al., 2009; King et al., 2003; Pearl et al., 2008; Santosh et al., 2011).

Other work stimulating the interest of developmental biologists in immune cells during tissue regeneration includes new insights into the active nature of the resolution of inflammation, which re-establishes tissue homeostasis and precedes tissue repair, and the key role of macrophages in this process. New details of the transition from inflammation to resolution, including the roles of cellular communication by both protein and lipid-derived factors (Gilroy and De Maeyer, 2015) and regulation of gene activity, including the importance of micro-RNA-21 (Sheedy, 2015), in promoting resolution are steadily being uncovered. Assimilation of this knowledge into studies of organ regeneration in animals moves well beyond classic developmental biology and is essential for current understanding and future work in the field of regenerative biology.

Here we provide an overview of experimental models particularly useful for examining the interplay of inflammation and developmental patterning in animal organ regeneration, followed by a review of recent experiments showing the importance of inflammatory cells and immunity in determining the eventual outcome of the

regenerative process. The importance of neuroendocrine-immune system interactions in repair and regeneration of vertebrate tissues, including pro-and anti-inflammatory aspects of this interaction, is then also considered in light of new, broader concepts on the role of stress in both immune defense and tissue repair.

2. Different modes of organ regeneration in vertebrates and invertebrates are variations on common themes.

Pathologists define two normal kinds of repair in response to injury: *healing* and *regeneration* (Kumar et al., 2005). Healing is a tissue response to a wound or to inflammatory processes in internal organs due to necrosis or infection, and consists of variable proportions of cellular regrowth and fibrous tissue production or scar formation. Regeneration refers to the growth of cells or tissues that replace both form and function in damaged organs of mammals or amputated appendages of amphibians or teleosts (Goss, 1992). Unlike the normal healing response, functional organ regeneration typically involves minimal scarring and requires an intact or a reconstituted connective tissue scaffold (Kumar et al., 2005).

Innate immune mechanisms, driving the early inflammatory phase of both wound healing and organ regeneration, help activate signaling pathways required for nuclear programming of local cells (Antonio et al., 2015). Various receptors that sense cellular damage or microorganisms, including toll-like receptors (TLRs) expressed by fibroblasts and keratinocytes (Portou et al., 2015), stimulate signaling pathways that increase epigenetic plasticity and render cells more responsive to microenvironmental cues capable of changing the cells' phenotype (Cooke et al., 2014; Lee et al., 2012).

Phenotypic change and reprogramming of gene expression in cells of injured tissues are generally more important during organ regeneration than in tissue repair (Jessen et al., 2015; Knapp and Tanaka, 2012). Other cues that impinge on cells undergoing reprogramming in regenerating systems direct these cells in forming new tissues and include factors from signaling centers which establish the pattern of the regenerated organ.

As indicated in Figure 1 regeneration of amputated vertebrate appendages involves both stem cells of limb tissues and differentiated cells that are triggered by the injury to undergo partial nuclear reprogramming often called “dedifferentiation”, which give rise to proliferating populations of progenitor cells comprising a “blastema,” in which growth is accompanied by patterning and proximal differentiation of new tissues (Knapp and Tanaka, 2012). Morgan (1901) termed this process *epimorphic regeneration*, in which new morphological structures develop from proliferating mesenchymal cells, as in an embryonic organ rudiment. Regeneration in invertebrates, in which regenerative mechanisms are more widespread and diverse than among vertebrates, does not generally involve overt cell dedifferentiation or blastema formation (Brockes and Kumar, 2008; Sanchez Alvarado and Tsonis, 2006). Studies with hydra and planarians, however, suggest that in all animals regeneration depends on inflammation (Mastellos et al., 2013), followed by variable cell reprogramming and growth, leading to re-establishment of an organ pattern, all of which involve many of the same families of cytokines, growth or signaling factors, and major signaling pathways (Agata et al., 2007; Rinkevich and Rinkevich, 2012; Sanchez Alvarado and Tsonis, 2006).

The most widely studied models of epimorphic regeneration include amputated fins of zebrafish (*Danio rerio*) and other teleosts; limbs and tails of various newt genera, particularly *Notophthalmus*, and the neotenic salamander, the axolotl (*Ambystoma mexicanum*); larval anuran limbs and tails, most commonly with *Xenopus*; and the distal phalanges of mice or rats. The capacity for limb regeneration in amphibians is optimal during larval stages and in anurans is lost as the hindlimbs complete development (Dent, 1962; Mescher et al., 2013).

Why epimorphic regeneration is restricted largely to urodeles, larval anurans, and certain fishes among the vertebrates is unknown. There is some evidence for the local evolution of taxon-specific mechanisms required for regeneration (Garza-Garcia et al., 2010), but regenerative ability is usually regarded as a primordial metazoan property, based on its phylogenetic distribution and ecological considerations (Bely and Nyberg, 2010). Recently published fossil evidence suggests a well-developed capacity for limb and tail regeneration in a 300-million-year-old amphibian ancestor, *Micromelerpeton*, considered by most authors the most basal member of the clade comprising modern salamanders, frogs and caecilians (Frobisch et al., 2015; Frobisch et al., 2014). From this point of view one can ask whether there are differences between vertebrates capable and incapable of regeneration with regard to cells or systems important for the regenerative process.

Comparative immunologists have examined the same amphibians models used to study regeneration and shown that many aspects of adaptive or acquired immunity remain poorly developed in newts, salamanders, and larval anurans, but that anuran metamorphosis produces an adaptive immune system much like that of mammals

(Robert and Ohta, 2009). Regenerative biologists have recently shown increased interest in immunology, in part to test the hypothesis that the development of efficient adaptive immunity during anuran larval ontogeny (and perhaps more generally during vertebrate phylogeny) causes changes in the systemic response to injuries that lead to attenuation or loss of regenerative capacity. Only an overview of epimorphic regeneration will be given here, with the focus on how inflammation and immunity has been studied to date in these systems. Several recent reviews of this and other models of regeneration are also available (Godwin, 2014; Poss, 2010; Simon and Tanaka, 2013; Wehner and Weidinger, 2015)

The inflammatory response provoked rapidly by injury appears to be essentially similar in all of these systems. Very rapid coagulation and vasoconstriction greatly reduce blood loss in amputated amphibian limbs. Erythema and edema are present but variable during and after wound closure. Histological analyses of amputated newt limbs, reviewed by Schmidt (1968), showed that immigration of various leukocytes to the injured site is directly comparable to that in mammalian wounds, a result recently confirmed with quantitative studies in axolotls (Godwin et al., 2013). Analyses of selected inflammation-related genes by qRT-PCR in larval *Xenopus* limbs (Grow et al., 2006) and a large array of cytokines by immunoassay in axolotl limbs (Godwin et al., 2013) reveal that amputation triggers expression of the major cytokines, chemokines and other factors which characterize acute wounds in mammals. Factors revealed by these studies included proteins promoting inflammation as well as those triggering active resolution of inflammation (Figure 1). Although expression patterns of specific eicosanoids, resolvins, and other lipid mediators of inflammation and resolution have

not been studied during epimorphic regeneration, all evidence currently available suggests that protein factors mediating the immediate events triggered by traumatic injury are similar in both wound healing and organ regeneration (Kizil et al., 2015).

In appendages of fish and amphibians epidermal keratinocytes begin to migrate on a fibrin substrate within hours of amputation, closing the wound rapidly with little tissue contraction. The resulting wound epithelium directly contacts the underlying injured tissues undergoing inflammation, and the edge of the cut skin's dermal layer usually remains to mark the limit of the amputation wound. Proliferation of basal keratinocytes in the proximal skin and continued centripetal migration gradually thicken the wound epithelium into a stratified structure sometimes called the apical epithelial cap (AEC). Blocking epidermal migration across the amputation site with grafts of complete skin or other methods inhibits epimorphic regeneration, without affecting repair of individual tissues in the limb stump (Mescher, 1976). The AEC seems to function during limb regeneration like the apical ectodermal ridge during embryonic limb development, establishing a positive feedback loop of fibroblast growth factor (FGF) signaling between this epithelium and the mesenchymal cells below (Murawala et al., 2012). In regenerating axolotl limbs, basal cells of the AEC express *fgf8* and the underlying mesenchymal cells express both *fgf10* and *fgf8* (Christensen et al., 2002; Han et al., 2001; Yokoyama et al., 2000). FGF10, like FGF7, is not mitogenic for fibroblasts or other mesenchymal cells, signaling only through an isoform of the FGF receptor expressed specifically by epithelial cells (Igarashi et al., 1998), which led to the alternate designations of FGF7 and 10 as keratinocyte growth factor (KGF) 1 and 2 respectively. This work suggests that in regenerating amphibian limbs cells of the

wound epithelium produce FGF8 (and perhaps other mitogens) promoting mesenchymal cell proliferation, and are in turn stimulated to grow and remain active by KGF2 from the mesenchymal cells. Supporting this are observations that expression of both *fgf8* and *fgf10* is diminished in *Xenopus* limbs amputated at stage 56 (Nieuwkoop and Faber, 1994) *Xenopus* limbs, during early metamorphosis, when regenerative capacity is being lost (Yokoyama et al., 2000). Introduction of exogenous FGF10 into amputated stage 56 limbs induces *fgf8* expression and AEC formation, mesenchymal expression of *fgf10* and genes related to limb patterning, and formation of relatively complete limbs (Yokoyama et al., 2001).

What is the origin of the mesenchymal cells during regeneration? While regeneration in planarians depends primarily on a preexisting population of stem cells called neoblasts, which are activated by injury, vertebrate limb regeneration relies much more heavily on reprogramming and release of differentiated cells (Poss, 2010). Among the activities of macrophages in injured limb tissues is secretion of various matrix metalloproteinases, most abundantly MMP9 (Godwin et al., 2013; Yang and Bryant, 1994), and other degradative enzymes which digest most extracellular matrix (ECM) components in and immediately adjacent to these tissues (Stocum and Cameron, 2011). Histolysis releases from skeletal muscle, dermis and other connective tissues, including cartilage or bone, viable mesenchymal cells that contribute to the blastema and produce a provisional ECM rich in hyaluronate (Mescher and Munaim, 1986; Smith et al., 1975). Although details vary in different genera, such as the source of myogenic cells in blastemas of newt and axolotl limbs (Sandoval-Guzman et al., 2014), this process of dedifferentiation gives rise to most cells of the regeneration blastema in

amphibians (Stocum and Cameron, 2011). Cellular dedifferentiation involves genetic reprogramming, with suppression of genes for cell specialization and activation of genes characteristic of stem or progenitor cells, including *msx* (Koshiba et al., 1998), *id* (Shimizu-Nishikawa et al., 1999), *sal14* (Christen et al., 2010; Neff et al., 2011), and others (Christen et al., 2010; Stocum and Cameron, 2011). Despite these dynamic changes in gene expression, dedifferentiating cells of the limb retain a heritable, tissue-specific memory of their origin (Kragl et al., 2009), mediated at least in part by stably maintained histone modifications (Hayashi et al., 2015). The blastema produced by the mesenchymal cells undergoes rapid growth and distal elongation, during which inflammation in the limb undergoes resolution (Godwin et al., 2013) and redifferentiation of tissues begins proximally, largely in continuity with existing tissues in the limb stump (Stocum and Cameron, 2011).

Proliferating and migrating “fibroblasts” from connective tissues in the limb stump constitute most of the early blastema in amphibians (Schmidt, 1968). As reviewed elsewhere (Bryant et al., 2002; Mescher, 1996; Muneoka and Sassoon, 1992) dermal fibroblasts, though less than 20% of cells in the limb stump, give rise to roughly half of all blastema cells, with the remainder likely derived from other connective tissue fibroblasts. Various experimental approaches have shown the importance of the fibroblasts for determining the pattern of tissues in the limb regenerate and indicate that all limb tissues except skeletal muscle still regenerate with essentially normal organization when limb skin is the sole initial source of blastema cells (Bryant et al., 2002; Holder, 1989). Tools for better analyzing the function of fibroblasts in simpler models of limb regeneration are under development (Sato et al., 2008), but specific

roles of these cells and the importance of their contributions to the ECM during blastema patterning remain largely unexplored (Godwin et al., 2014).

During limb regeneration fibroblasts in dermis and internal limb tissues, along with mesenchymal stem cells contained within those populations, are likely to have key roles similar to those of connective tissue progenitors in the embryonic limb bud, where growth of these cells is coordinated with limb patterning by varied activities of extracellular mitogenic and morphogenic factors (ten Berge et al., 2008; Yakushiji et al., 2009). Studies of vertebrate limb development have shown that the mesenchymal cells producing skeletal elements, tendons, ligaments, dermis, and the connective tissue components of muscle arise from lateral plate mesoderm. These cells produce the initial limb bud and provide a subtle prepatter which cues placement of subsequently arriving cells of the myogenic and other lineages (reviewed by (Hasson, 2011). Evidence from a variety of studies indicates that the morphogenic prepatter in limb buds includes regional differences in the structures of heparan sulfate (HS) within the mesenchymal cell proteoglycans, which localize and highly influence the activities of HS-binding factors such as most FGFs, hedgehog, and other factors (Ashikari-Hada et al., 2009; Kobayashi et al., 2010; Nogami et al., 2004). Recently reported work, including experiments with artificial ECM, has shown that HS and FGFs also mediate positional information during axolotl limb regeneration (Phan et al., 2015).

One feature besides the need for cellular dedifferentiation which distinguishes many regenerating vertebrate organs from their counterparts in the embryo is the dependence of cell proliferation on factors released in a paracrine manner from regenerating nerves. Best studied in regenerating limbs, the neural effect is required

during the early phase of regeneration until the blastema is well-formed; regeneration after that point, including additional growth and morphogenesis of the new limb, can occur independently of nerves (Brockes and Kumar, 2008; Singer, 1952, 1974). The molecular basis of the nerves' influence remains an active field of investigation (Kumar et al., 2007), but most of the evidence supports a model in which axons, together with FGF from the AEC, are required for survival and sustained proliferation of early blastema cells in both amphibian limbs (Mescher, 1996) and zebrafish pectoral fins (Simoes et al., 2014). During the initial phase of blastema growth in urodeles nerves also promote synthesis and accumulation of hyaluronate, which significantly changes the local microenvironment, increases tissue volume, and facilitates cell movements (Mescher and Cox, 1988; Mescher and Munaim, 1986). Mesenchymal stem cells of mammals exert multiple well-known immunomodulatory properties (Gharibi et al., 2015), including many anti-inflammatory effects mediated by their production of cross-linked hyaluronate chains, which can then inhibit activation of both local monocytes and T cells (Kota et al., 2014). Similar effects may be expected of the hyaluronate synthesized by mesenchymal cells of the regeneration blastema, which may play a major role in the resolution of inflammatory activity that is so important for successful patterning in regeneration, as discussed below.

Scar-free healing of full-thickness wounds in vertebrate skin, often referred to as *skin regeneration*, reproduces structurally normal skin with functional epidermal appendages, unlike typically healed skin wounds which have poorly organized, subepidermal scar tissue and lack hair follicles in mammals and skin glands derived from epidermis (Garg and Longaker, 2000; Martin, 1997). Initially discovered and

studied in fetal skin during the first half of gestation, this experimental model has in recent years contributed greatly to better understanding the molecular basis of regeneration in other organs as well, including the importance of the developing immune system for scarring (Eming et al., 2014).

3. Fine control of inflammation is important for organ regeneration.

As discussed in earlier reviews, studies of regenerative capacity in embryonic skin and its loss during fetal development indicate that maturation of the immune system and subsequent changes in the local inflammatory response of skin wounds may underlie the increased fibrosis and reduced ability to regenerate the functional architecture of this organ after wounding in late gestation (Harty et al., 2003; Mescher and Neff, 2005). We suggested that in larval anurans similar development of the lymphoid organs and the dispersed system of interacting immune cells could be involved in the gradual decline of the developing hindlimbs' regenerative ability during the pro-metamorphic period. Recent work has confirmed the importance of inflammatory regulation for a variety of regenerating systems, including amphibian limbs, and specific molecular and cellular components of inflammation important for subsequent regeneration have begun to be elucidated. Various studies will be discussed here from an evolutionary perspective; other aspects of the interplay between inflammation and organ regeneration have also been reviewed recently (Forbes and Rosenthal, 2014; Godwin and Rosenthal, 2014; Kawasumi et al., 2013).

Studies of scarring in amphibian skin

Experiments with wounds of dorsal skin in larval and adult bullfrogs (*Rana catesbeiana*) indicate that changes occurring during metamorphosis cause a transition from normal skin regeneration to scar formation similar to the gradual loss of the capacity for hindlimb regeneration (Yannas et al., 1996). Full-thickness excisions of larval skin regenerated with essentially normal epidermal and dermal structures, while similar wounds in adult frogs formed skin with no epidermal pigmentation, epidermal glands that were smaller, fewer in number, and abnormal compared to those of intact skin, and dense fibrous tissue in place of normally organized dermis. With *Xenopus*, a phylogenically more basal group than *Rana*, full-thickness incisional wounds on the dorsal hindlimb skin of 8- and 15-month-old postmetamorphic animals were filled by skin with normal epidermis and epidermal glands like those of intact skin, although dermis was replaced by fibrotic scar-like tissue, with involvement of myofibroblasts (Bertolotti et al., 2013). Excisional skin wounds of *Xenopus* froglets (of unreported age) were found to heal completely without scarring, but these experiments involved skin flaps in which the dermis but not the epidermis was excised (Yokoyama et al., 2011). Although the different nature of the wounds in these studies make comparisons difficult, (Bertolotti et al., 2013) point to refinements in *Xenopus* immune competence which develop in the year following metamorphosis, particularly the components of adaptive immunity (Robert and Ohta, 2009), to explain the wound healing differences in postmetamorphic frogs.

Among urodele amphibians larval axolotls (*Ambystoma mexicanum*), 7-10 cm in length, heal full-thickness excisional wounds without scarring or myofibroblast formation (Levesque et al., 2010). In this neotenic species, adults 12-15 cm long and >9 months

old, also regenerate the complete skin of large excisional wounds, forming normal dermis, normal epidermis with mucous-secreting Leydig cells, and epidermally derived skin glands (Seifert et al., 2012). Axolotls experimentally induced to undergo metamorphosis also closed wounds with complete, scar-free skin, although in these animals regeneration was much slower and the result less perfect, with significantly higher leukocyte infiltration (Seifert et al., 2012). A more detailed discussion of skin regeneration in developing anurans and urodeles is also available (Godwin and Rosenthal, 2014).

As mentioned the role of innate and acquired immune cells in the loss of scarless skin regeneration in developing mammals is well-established (Gawronska-Kozak et al., 2006). Interestingly development of the mammary gland, a derivative of the integument and the only organ developing predominantly after birth, involves almost the full range of cells in the peripheral immune system. Formation of the mammary duct system at puberty requires both positive regulation by macrophages, eosinophils, and other innate immune cells (Gouon-Evans et al., 2000) and complex negative regulation by antigen-presenting cells (APCs) and CD4⁺ Th1 cells (Plaks et al., 2015). Postnatal organ regeneration in mammals can be expected to require, like pubertal expansion of the mammary ductal system, regulatory interactions between the newly developing tissues and cells of the immune-competent environment.

Studies of amphibian and teleost appendage regeneration

Comparing forelimb regeneration in axolotls after induced metamorphosis with that in normal age-matched paedomorphic axolotls, it was found that the rate of

regeneration in metamorphs was half that of the paedomorphs and that 100% of the regenerated metamorph limbs had moderate to severe skeletal defects compared to only 7% of the paedomorph limbs which had only slight defects (Monaghan et al., 2014). Although the etiology of these dramatic changes in axolotl limb regenerative capacity after induced metamorphosis was not examined, it is noteworthy that metamorphosis appears to heighten aspects of adaptive immunity in this species, as indicated by the significantly enhanced humoral response (5-fold increase in circulating plasma cells) to an immune challenge compared to paedomorphic animals (Ussing and Rosenkilde, 1995).

It was recently found that modulation of the inflammatory response to amputation is critically important for limb regeneration in axolotls (Godwin et al., 2013). Using cross-reactive mouse cytokine protein detection arrays these investigators found induction of 40 specific chemokines and cytokines, both proinflammatory and anti-inflammatory, within injured limb tissues, usually within 1 day of amputation, with most factors remaining elevated through the early blastema phase of regeneration (Figure 1). The patterns of induction for most cytokines after amputation, as well as the local accumulation of neutrophils and monocytes/macrophages in regenerating limbs, were similar to patterns seen in mammalian wounds. Simon and coworkers also found that expression of many other immunomodulatory genes, including those for annexins, galectins, CXC chemokines, and thymosin β 4, in addition to genes involved in tissue remodeling, was regulated similarly during regeneration of adult newt limbs, tail, CNS, and heart (Mercer et al., 2012). In larval *Xenopus* the migratory response of neutrophils

and macrophages to wounding also was very similar to that of mammals, and was not affected by seeding of the wounds with *E. coli* (Paredes et al., 2015).

In a significant advance Godwin (Godwin et al., 2013) also showed for the first time the central importance of macrophages among the immune cells active in amputated axolotl limbs. For this experiment circulating monocytes and tissue-resident macrophages were temporarily deleted by intravenous injections of liposome-encapsulated clodronate. The absence of macrophages during the first week after amputation led to increased levels of the inflammatory cytokines IL-1 β and TNF α locally and lower levels of various anti-inflammatory cytokines compared to untreated controls. Levels of TGF- β 1, synthesis of which is upregulated in the axolotl limb beginning within hours of amputation and lasting through blastema formation and which is required for blastema cell proliferation (Levesque et al., 2007), remain very low in macrophage-depleted limbs (Godwin et al., 2013). In such limbs the significant alterations of local cytokine levels were accompanied by a lack of mesenchymal proliferation, followed by distal scarring and the complete, permanent inhibition of limb regeneration. The amputation wound underwent epithelial closure in the absence of macrophages, but an AEC supportive for regeneration did not form. Regeneration was not blocked if macrophage depletion was delayed until after a blastema had formed or upon reamputation of fibrotic limb stumps in animals treated months earlier.

Godwin et al. (2013) also found that regenerative failure in macrophage-ablated axolotls included greatly reduced activation of *MMP9* and *MMP3*, the matrix metalloproteinases produced most abundantly during urodele limb regeneration (Vinarsky et al., 2005; Yang and Bryant, 1994; Yang et al., 1999). Inhibition of these

enzymes in adult newts leads to abortive limb regeneration and distal scarring (Vinarsky et al., 2005). The requirement of macrophages for limb regeneration therefore, seems to include adequate proteolytic activity that promotes tissue dedifferentiation and formation of the progenitor cell populations (Godwin et al., 2013).

Recent studies of macrophage activity in various mammalian models of tissue repair and regeneration make clear that this cell population includes cells with a range of distinct functional phenotypes, with proinflammatory M1 macrophages associated with the first phases of acute inflammation gradually being replaced under the control of complex environmental stimuli by anti-inflammatory M2 cells (Chazaud, 2014; Das et al., 2015). The emerging M2 macrophages not only promote the resolution of inflammation, but also exert many salutary effects on the parenchymal cells of damaged organs, promoting recovery of tissue homeostasis, stimulating stem and progenitor cell development, and contributing to conditions necessary for tissue repair and regeneration (Chazaud, 2014). Since macrophage ablation in axolotl limbs led to the complete dysregulation of local cytokine expression as well as the inhibition of tissue dedifferentiation and cell proliferation (Godwin et al., 2013), successful epimorphic regeneration in amphibians likely also depends on complex interactions between this subset of immune cells and the emerging progenitor cell lineages which form the new limb (Godwin and Rosenthal, 2014).

The requirement of macrophages for limb regeneration may seem counterintuitive in light of studies on skin wound healing in *PU.1* null “macrophageless” mice, in which skin wounds heal not only more quickly but with less scarring than in control mice (Cooper et al., 2005; Martin et al., 2003). However, in clodronate-treated

axolotls immigration of neutrophils to the injured tissues was relatively unaffected, while *PU.1* null mice completely lack both macrophages and functional neutrophils. Such mice are genetically incapable of raising most components of a normal inflammatory response and must be maintained with daily antibiotic injections after wounding (Cooper et al., 2005). Many aspects of inflammation involve neutrophils, principally removal of the bacteria from wounds. Recent studies of wound repair in germfree mice show that in the absence of commensal skin bacteria there was decreased neutrophil accumulation in wounds, but increased macrophage accumulation, increased angiogenesis, increased expression of anti-inflammatory cytokines and other genes related to the M2 macrophage phenotype, and accelerated wound healing with greatly reduced scarring (Canesso et al., 2014). The authors attribute the improved wound healing in germfree mice to the predominance of M2 or healing macrophages in their wounds. As suggested one reason macrophages are required in urodele limb regeneration may be the need for the M2 phenotype to counteract the various proinflammatory effects, including those mediated by neutrophils, although the latter cells may themselves also exert pro-resolution effects in certain regenerative models (Keightley et al., 2014). Clearly the roles played by both sets of immune cells will be of interest in further investigations on inflammation during epimorphic regeneration.

Other investigations into the nature of the inflammatory response underlying epimorphic regeneration have elucidated the innate responses to injury which elicit leukocyte accumulation and the acute inflammation. To test the importance of signaling by hydrogen peroxide (H_2O_2) and other diffusible reactive oxygen species (ROS) during the onset of regeneration, as suggested by transcriptome analyses in several

experimental systems, new detection techniques are being applied to classic models of epimorphic regeneration. The thin and transparent nature of the tail tip in larval zebrafish and *Xenopus* allows *in vivo* microscopic analysis of leukocyte recruitment and other events in response to amputation (Paredes et al., 2015). Expression of the H₂O₂-sensitive ratiometric fluorescent probe Hyper in these larvae allows intracellular levels of H₂O₂ and closely related ROS to be monitored dynamically and quantitatively. Within minutes of tail amputation in both models, cells at the wound margin begin sustained production of H₂O₂, which leads to formation of concentration gradients of ROS that extend 100-200 μ m (Love et al., 2013; Niethammer et al., 2009). In both species such gradients were required for leukocyte recruitment to the wound. Moreover, inhibiting ROS production in the *Xenopus* tadpoles with pharmacological or genetic approaches reduced levels of Wnt/ β -catenin signaling and cell proliferation, and blocked tail regeneration (Love et al., 2013).

As discussed below with the neuroendocrine-immune stress axis, effective tissue repair or organ regeneration requires a fine balance of factors regulating acute inflammation. While the inflammatory response is required to initiate repair and regeneration, excessive or prolonged inflammation interferes with successful patterning of a new organ, often promoting fibrosis (Eming et al., 2009; Stramer et al., 2007; Wilgus, 2008). Beclomethasone, a synthetic anti-inflammatory corticosteroid, has a significant inhibitory effect on hindlimb regeneration in stage 53 *Xenopus laevis* if present from the time of amputation, but not if treatment is delayed one day (King et al., 2012). Regeneration of caudal fin primordia in zebrafish larvae, which is complete within 3 days of amputation, is also blocked by beclomethasone present only during the first 4

hours post-amputation but not if treatment begins at that time (Mathew et al., 2007). Similar effects of glucocorticoids on the process of wound healing in mammals have been known for many years (Levenson and Demetriou, 1992). Like axolotl limbs, adult zebrafish caudal fins require macrophages for normal regeneration (Petrie et al., 2014). It is noteworthy that larval zebrafish caudal fin primordia regenerated completely in PU.1 morphants lacking myeloid cells (Mathew et al., 2007), suggesting that macrophages promoting inflammatory resolution and tissue homeostasis permissive for epimorphic regeneration may not be necessary when other immune cells are missing or immature.

As mentioned in *Xenopus* and other anurans the capacity for complete hindlimb regeneration declines during prometamorphosis, manifested by increasingly poor patterning of distal structures and fewer digits the longer amputation is delayed during larval development (Dent, 1962). Regeneration of these distal limb structures was improved in stage 54/55 *Xenopus* larvae (but not in later stages) by treatment with the COX-2 inhibitor celecoxib or celastrol, an inhibitor of NF- κ B activation (King et al., 2012). Dramatically improved hindlimb regeneration, including formation of 5 normal digits, was also reported after treatment with cyclosporine A, an inhibitor of allograft rejection, using stage 54 of another anuran, the Saudi toad (*Bufo arabicus*) (Fahmy, 2014). Such results suggest that persistent inflammation or other immune-related activity such as autoimmunity in the regeneration blastema, produced perhaps by delayed resolution of the acute response to injury, interferes with signaling or other cellular interactions required for normal limb patterning in larval anurans (King et al., 2012).

This hypothesis is supported by RT-PCR data showing that expression of factors involved in resolving inflammation, including annexin-1, fibrinogen-like protein 2 (FGL-2), and suppressors of cytokine signaling 1 and 3, persisted and increased in amputated *Xenopus* limbs at stage 55 or 57, in which regenerative capacity is reduced, compared to regeneration-competent stage 53 (Mescher et al., 2013). Expression of galectin-1 was also rapidly upregulated and remained high through 5 days post-amputation in stage 57 limbs. FGL-2 and galectin-1 are both secreted immunomodulatory factors important for establishing allograft tolerance (Xie et al., 2012). Persistent expression of these factors in limb stumps during the larval period when limb regenerative ability is lost supports the proposal that developmental changes in cells mediating aspects of the acute inflammatory response are important in this loss. Low level expression of these factors was also seen in limbs at stage 55 and/or 57, but not stage 53, *before* amputation, indicating that cells with immunoregulatory activity become resident in limb tissues during development (Mescher et al., 2013).

In regenerating stage 53 hindlimbs downregulation of these proresolution factors 1 to 4 days after amputation is accompanied by upregulation of several genes required for limb patterning (*msx1*, *shh*, *sall1*, *hoxa13*) (Mescher et al., 2013). However at later larval stages expression of the proresolution factors in amputated limbs persists, suggesting prolonged inflammation, and expression of these pattern-related genes is inhibited or attenuated. Together these data suggest that inflammation triggered by amputation must be resolved before signaling required for blastema formation and patterning can occur. Given the quantitative importance of fibroblasts derived from dermis and other limb connective tissues during initial formation of the blastema

discussed earlier (Bryant et al., 2002) and that excessive fibrosis and scarring commonly occur with dysregulated inflammation, such cells are a likely target for the disruptive effects of unresolved inflammation on patterning in the limb stump. For example, fibrosis is likely to interfere with the distinctive regional expression of specific HS-sulfotransferases important for normal limb patterning.

Dent (1962) showed that the decline of regenerative capacity in prometamorphic *Xenopus* hindlimbs occurs first at proximal levels and then at increasingly more distal levels. The experiments indicating increasingly persistent inflammation in amputated limbs at stages 53, 55, and 57 used limbs all amputated just proximal to the developing ankle, the tarsus/shank junction. Whole-mount immunohistochemistry of such limbs using antibodies against a marker for immature dendritic cells indicates that epidermal Langerhans-like cells first appear proximally in the limb, with a few such cells scattered in skin at the developing ankle level by stage 53 but abundant in skin of the foot and digits by stage 57 (Mescher et al., 2007). Studies of dendritic cells and other immune cells in the skin of larval and adult urodeles, and comparison of the results with further work on anuran skin, will be important for clarifying a role for such cells in skin or limb regeneration. The preliminary observations are consistent with the view that loss of regenerative ability in these limbs may be due to the proximo-distal development of cutaneous immunity in the limbs and the activation of local APC s after amputation.

It should be noted that regeneration studies involving *Xenopus* hindlimbs and tails (discussed below) at prometamorphic stages may be affected by the fact that immunological maturation can proceed independently from external morphological development at these stages (Ruben, 1970; Ruben et al., 1972). Sibling tadpoles were

found to reach different morphological stages (46-50) at the same chronological age, but activity of cells mediating the immune response to tissue allografts was always equivalent to that of the most morphologically advanced members of the population (Ruben et al., 1972). Similar results with differentiation of lymphocytes in age-matched larvae have also been reported during the period when hindlimb regeneration declines (stages 53-58), even following experimental delay of metamorphosis (Rollins-Smith et al., 1997). Nye and Cameron (2005) have called attention to the variable outcomes among animals in sibling populations and between different laboratories in larval *Xenopus* hindlimb regeneration studies, which often complicate interpretation of such work, as discussed for example by Slack et al. (2004). The continued maturation of lymphocytes and possibly other immune cells when external morphological development is delayed by suboptimal nutrition, overcrowding, and other types of stress may be contributing factor in the variably defective regenerative response seen in some larvae compared to age-matched controls. As Ruben et al. (1972) point out, it would seem important that investigators concerned with regeneration or other aspects of normal *Xenopus* larval development should particularly select individuals showing rapid developmental progress.

The ability of beryllium ions to inhibit regeneration and generate fibrosis locally in urodele limbs when applied briefly in solution to the wound surface has been known for decades (Scheuing and Singer, 1957; Thornton, 1951), but the strong and persistent immunoadjuvant effects of beryllium in animal tissues have only been recognized more recently (Cummings et al., 2009; Sawyer et al., 2005). As shown in Table 1 regeneration of stage 53/54 *Xenopus* hindlimbs was inhibited in a dose-dependent

manner following brief (<1 minute), local treatment of the amputation surface with BeSO₄. Analysis of gene activity associated with specific aspects of regeneration by qRT-PCR provided insight into the mechanism of the beryllium effect. Expression of *IL-1β*, which peaked at 6 hours post-amputation in untreated limbs, increased continuously during the week following amputation and beryllium treatment (Mescher et al., 2013). Expression of immunosuppressive *FGL-2*, which diminished by day 5 of regeneration in controls, also remained maximal on day 7. Similar expression patterns were found for *mmp-9* and *C3* (complement component 3), with the latter of special interest because of its synthesis in cells of the blastema and wound epithelium rather than in leukocytes, unlike the other immunomodulatory factors examined (Del Rio-Tsonis et al., 1998; Mescher et al., 2013).

Expression of the genetic reprogramming marker *SALL4* was largely unaffected by Be, but *Shh*, *TBX3*, and *SALL1* expression for patterning was strongly inhibited (Mescher et al., 2013). These data indicate that in the presence of strong, unresolved inflammation produced by beryllium, cellular dedifferentiation and reprogramming still can occur locally but that the tissue interactions and signaling gradients that normally promote limb patterning cannot be established. Resolution of the acute response that initiates repair or regeneration must occur if the developmental mechanisms underlying regenerative growth are to be reestablished.

It is of interest that larval limbs of *Xenopus* are more sensitive to beryllium than similarly sized axolotl limbs. Comparing Tables 1 and 2 shows that 10 mM BeSO₄ caused visible inflammation locally in *Xenopus* limb stumps and blocked regeneration, but had no effect on the axolotl limb stumps, which all regenerated normally. With larval

urodele limbs only a 10-fold higher dose of beryllium consistently inhibited regeneration (Table 2). Application of that BeSO_4 concentration to the amputation surface of the anuran larvae produced systemic inflammation and death within 2 or 3 days (Table 1). No axolotls died after exposure to BeSO_4 at any concentration. These differences between anuran and axolotl larvae in sensitivity to beryllium may reflect the weaker capacity for cell-mediated immunity in urodeles (Barlow et al., 1981; Cohen, 1971; Tournefier et al., 1998). Chronic beryllium disease in human, caused by hypersensitivity to this metal, is characterized by accumulation in the lungs of Be-specific CD4^+ T cells producing granulomatous inflammation (Dai et al., 2013; Fontenot and Kotzin, 2003). Tolerance of beryllium by axolotls, like their relative tolerance of allografts, may be due to the highly restricted MHC II diversity and weak T helper cell activity in this species (Sammut et al., 1999).

The key importance of anti-inflammatory factors for limb patterning during anuran regeneration also clearly emerges from studies demonstrating the ability of postmetamorphic *Xenopus* limbs to form digit-like structures after receiving transplants of larval limb progenitor cells expressing a constitutively active form of β -catenin and supplemented with FGF10 and Shh (Lin et al., 2013). Such cell grafts survived and produced multidigit regenerates with a substantial contribution of host cells, but only if the hosts had undergone thymectomy at stage 48/49. Since functional T cells still occur after thymectomy at that stage (Horton and Manning, 1972), grafted cells were further treated with the anti-inflammatory peptide thymosin $\beta 4$ ($\text{T}\beta 4$), which reduced apoptosis, doubled the proliferation rate of donor cells, and permitted further improvements in the quality of limbs regenerated (Lin et al., 2013).

Released from platelets and macrophages during wound repair and expressed early during limb regeneration in anuran and axolotls (King et al., 2003; Monaghan et al., 2012), T β 4 is known to down-regulate inflammation and promote several aspects of repair in many systems (Philp and Kleinman, 2010). The single methionine of T β 4 is rapidly oxidized by ROS to produce T β 4-sulfoxide, a derivative with much greater anti-inflammatory activity than T β 4 in various *in vitro* and *in vivo* assays (Young et al., 1999). Using the larval zebrafish tail model, Evans and coworkers (Evans et al., 2013) showed that the rise in H₂O₂ triggered by amputation converts T β 4 to T β 4-sulfoxide, which then promoted inflammatory resolution. Similar effects of T β 4-sulfoxide were found with zebrafish and mouse heart injuries (Evans et al., 2013). Thus roles for endogenously produced T β 4-sulfoxide are also likely important during successful epimorphic regeneration.

Studies with regenerating larval anuran tails

An early indication that regulatory T cells (T_{regs}) were also involved in regeneration came from work in the lab of Takeo Kubo on *Xenopus* tadpole tail regeneration, which occurs following amputation at any prometamorphic stage except for a refractory period during stages 45-47 (Beck et al., 2003). These investigators found distinct differences in local, amputation-triggered expression of several immune-related genes following amputation during the refractory period, which was also shown to coincide with the onset of rapidly increased expression of T cell markers in the tadpoles (Fukazawa et al., 2009). Expression of *foxp3*, a marker for T_{regs}, is strongly up-regulated within hours of tail amputation at regeneration-competent stage 52/53, but not during the earlier refractory stages, suggesting that the restoration of tail regenerative

ability occurs as T_{regs} suppress immune cell functions that impair regeneration during the refractory period. Consistent with this view, tail regenerative ability was significantly greater at stages 45-47 in PU.1-morphant tadpoles, which were effectively depleted of leukocytes. Moreover, tail regeneration during the refractory period of normal larvae was improved by treatment with the immunosuppressants celastrol, cyclosporine A, IKK inhibitor VII, or FK506 (Fukazawa et al., 2009). The authors proposed that autoreactive immune cells developing during the refractory period recognize tail blastema cells as 'non-self' and inhibit regeneration at this time, with the development of T_{regs} and the establishment of a local immune regulatory system permitting regeneration again at later stages. Significantly improved patterning of larval anuran hindlimb regenerates following treatment with celastrol (King et al., 2012) or cyclosporine A (Fahmy, 2014) support such a hypothesis and suggest that improving autoimmune responsiveness may also play a role in the prometamorphic decline of limb regenerative capacity.

Local activity of T_{regs} has been found to promote regeneration in several mammalian tissues, including skeletal muscle (Castiglioni et al., 2015), bone (Liu et al., 2015), kidney tubules (Gandolfo et al., 2009), pancreatic islet β cells (Zhao et al., 2012), and liver (Lu et al., 2013). Although in most of these studies the anti-inflammatory or immunosuppressive effects of T_{regs} were either directly implicated or assumed to be part of their proregenerative activity, recent work from different groups has revealed unique populations of T_{regs} capable of stimulating repair directly, at least in part through the production of amphiregulin, an epidermal growth factor family member, in both skeletal muscle (Burzyn et al., 2013) and lung (Arpaia et al., 2015). In *Xenopus* hindlimbs expression of *foxp3* was up-regulated within a few hours of amputation (persistently in

Be-treated limb stumps) at both regeneration-competent and -incompetent stages, and like the other markers of immunomodulation was also prominent in unamputated limbs at stage 57 (Mescher et al., 2013).

Further work on regulation of regeneration in the tadpole tail showed that FK506 can rescue regenerative capacity during the refractory period if given for just the first 12 hours post-amputation, and that this period is characterized by expression of a gene similar to *PhyH*, which is down-regulated by FK506 and encodes an enzyme (phytanoyl-CoA hydroxylase) that associates with an FK506 binding protein, potentially a useful marker for autoimmune cells inhibiting *Xenopus* tail regeneration (Naora et al., 2013). The most recent report on this work identifies immune-related genes specifically expressed by proliferating *Xenopus* tail blastema cells, including *interleukin-11* and “*cd200like-related*” (Tsujioka et al., 2015). CD200, an Ig-type cell surface protein, is a well-characterized tolerance-signaling molecule produced in murine and human trophoblast and decidua which promotes the local generation of T_{reg} subsets, suppresses natural killer (NK) cell activity, and induces indoleamine 2,3-dioxygenase 1 (IDO1) activity in macrophages, helping to prevent spontaneous abortions (Clark et al., 2015). CD200 is expressed on many other mammalian cell types, where it interacts with the inhibitory receptor CD200R1 on myeloid cells, including T_{regs}, to down-regulate immune cell functions in a wide range of inflammatory diseases (Vaine and Soberman, 2014).

Studies of mammalian digit tip regeneration

In addition to regeneration of fetal skin, another example of regeneration in mammals that includes patterning is the regrowth of amputated digit tips of mice and humans (Han et al., 2008). New work with this murine model has clarified the requirement that a proximal portion of the nail organ remains on the digit stump after amputation for successful regeneration, and indirectly raises the possibility of immune activity in this regeneration. The Ito lab recently showed that nail stem cells (NSCs) reside in the proximal nail matrix and moreover found, by conditionally deleting β -catenin in epidermal cells, that NSC maintenance and differentiation, as well as nail formation and digit regeneration depend on Wnt signaling activity in the nail epithelium (Takeo et al., 2013). Wnt proteins also control effector T cell development, peripheral T_{reg} activation, and maturation of dendritic cells and other APCs (Staal et al., 2008), and recent studies of Wnt signaling in developing murine epidermis using another conditionally expressed allele show that impaired epidermal Wnt secretion disrupts cutaneous immunity and causes failure of the skin to develop and function normally as well as chronic inflammatory skin disease (Augustin et al., 2013).

Lymphocytes and APCs within the nail epithelium were not examined in the mouse studies, but detailed analyses of the human nail apparatus, which is very similar to that of mice anatomically and in keratin expression patterns (Fleckman et al., 2013), reveal many differences in cutaneous immunity between the nail organ and other skin of digits (Ito et al., 2005). Unique features of the nail organ include: reduced density of both helper and cytotoxic T cells in the nail epithelium and underlying mesenchyme; nearly complete downregulation of HLA-A, -B, and -C expression on keratinocytes and melanocytes of the proximal nail matrix, with expression instead of HLA-G, which can

inhibit NK and cytotoxic T cell functions; remarkably down-regulated expression of MHC class II and CD209 (DC-SIGN) by Langerhans cells; low numbers in the mesenchyme of NK and mast cells, both key players in innate immunity; and prominent local expression of macrophage inhibitory factor and several other anti-inflammatory or immunosuppressive proteins in the proximal nail matrix (Ito et al., 2005; Saito et al., 2015).

These observations strongly suggest the presence of a specialized nail immune system (NIS), a site of relative immune privilege functioning to suppress inflammatory and/or autoimmune damage to this critically important structure. Ito et al. speculate that the NIS evolved because it promoted species survival by protecting individuals from loss of claws (and later nails) after proinflammatory mechanical or environmental insults and by accelerating restored homeostasis, repair, and use of these appendages and limiting pain and swelling after claw trauma or infection (Ito et al., 2005). Despite the well-established requirement for the nail organ during digit tip regeneration, the potential role of the NIS in this regenerating system has been ignored. Studies of immune system components in the murine nail apparatus, their dependence on local Wnt signaling and their possible function in modulating the acute inflammation produced by amputation will likely provide new insights into the importance of the nail organ for digit regeneration, which has become a clinically important model of epimorphic regeneration in mammals (Rinkevich et al., 2015).

4. Neuroendocrine-immune interactions in repair and regeneration

The phases of wound repair in mammals are regulated by the secretion or activation of cytokines, lymphokines, and growth factors by immune cells which also

bear major responsibilities for host defense, most notably macrophages, neutrophils and T lymphocytes (Park and Barbul, 2004). Many of the factors that regulate the early events of wound healing and inflammation, both polypeptides and small molecules, are also produced by various neurons and other cells and include many hormones, neurotransmitters, and neuromodulatory peptides with fundamental roles within the endocrine and nervous systems (Shepherd et al., 2005) and appear to be essentially the same in both mammals and ectotherms (Verburg-van Kemenade et al., 2013). As indicated by other papers in this volume, research on the interactions between the neuroendocrine and immune systems now encompasses many aspects of inflammation and host defense.

Best understood among the categories of neuroendocrine-immune interactions are those mediated by the hypothalamus-pituitary-adrenal (HPA) axis, which is not only central to an animal's response to stress, but also helps to regulate early events of tissue repair and regeneration. The key proinflammatory cytokine IL-1 β activates the HPA axis by stimulating corticotropin-releasing factor (CRF) synthesis in hypothalamic neurons and release of CRF into the portal circulation (Rivier and Rivest, 1993; Shepherd et al., 2005). In the anterior pituitary CRF triggers production and release of adrenocorticotrophic hormone (ACTH), stimulating secretion of adrenal corticosteroid hormones. Given the sensitivity of immune cell activity to corticosteroids, the HPA axis appears to couple immunological status to homeostatic endocrine control via levels of circulating IL-1 and other cytokines.

In amphibians the hypothalamic-pituitary-interrenal gland axis is essentially similar to the mammalian HPA axis, with corticosterone and aldosterone the principal

corticosteroids induced (Rollins-Smith, 2001). Lymphocyte functions in amphibians are also highly sensitive to the inhibitory effects of these corticosteroids. Physiologically relevant concentrations (1-10 nM) of either hormone inhibit proliferation and induce apoptosis in cultured lymphocytes from *Xenopus* adults or tadpoles (Rollins-Smith, 2001). During metamorphic climax in *Xenopus* (stages 61-63), when the immune system shifts from tolerance of larval to adult cells and antigens, circulating plasma levels of both corticosteroids rise rapidly and transiently to concentrations inhibitory for lymphocyte activity and growth (Jolivet Jaudet and Leloup Hatey, 1984; Kikuyama et al., 1993).

The importance of the neuroendocrine-immune system axis for limb regeneration in newts was suggested by a classic series of experiments by Schotté (1961). Initial experiments showed that when the pituitary gland was removed around the time of limb amputation, regeneration of the limb was inhibited due to aberrant events early in the wound healing process which led rapidly to intense fibrosis within the distal limb stumps and failure of blastema formation. Hypophysectomies performed five days after amputation allowed some degree of limb regeneration and if delayed until after blastemas were well-established or later hypophysectomy had no effect on subsequent limb regeneration. Transplantation of interrenal gland tissue or replacement therapy with ACTH or cortisone was reported to restore normal limb regeneration in animals that underwent concomitant hypophysectomy and amputation. These studies are consistent with the more recent work demonstrating the importance of a well-regulated inflammatory response for successful limb regeneration, and clearly support a role for a central neuroendocrine-immune interaction in the early events of limb regeneration.

However the results from Schotté's group and other investigators showed considerable variation in the effects of early hypophysectomy on limb regeneration, ranging from no effect to delayed regeneration, with or without treatments with ACTH, cortisol or other corticosteroids (Liversage et al., 1985; Schmidt, 1968; Schotte, 1961).

Recent work on the biological basis of the immunosuppressive effects of stress in humans and animals may help clarify some inconsistencies regarding the reported effects of hypophysectomy and hormone replacements on urodele regeneration. All components of the HPA axis (CRF, ACTH, and enzymes for glucocorticoid synthesis) are now known to be wide-spread in cells of the skin and gastrointestinal tract, where they enhance immunoprotection and repair in these organs after exposure to pathogens or injury (Lin et al., 2014; Talaber et al., 2013; Vukelic et al., 2011; Zmijewski and Slominski, 2011). Although the anti-inflammatory, immunosuppressive effects of corticosteroids are well-studied and widely used clinically, considerable evidence also exists for potent modulating or enhancing effects of glucocorticoids on lymphocytes (Jozic et al., 2014; Plaut, 1987; Webster et al., 2002; Wiegers and Reul, 1998). In light of the harmful effects of prolonged stress, it is important to appreciate that natural selection produced the biological stress response as a survival mechanism. An important part of the fight-or-flight reaction involves immune activation, and a large body of work indicates that acute or short-term stress, lasting from minutes to hours, significantly enhances innate immunity as well as adaptive, primary and secondary immune responses (Dhabhar, 2014).

Numerous studies with both human subjects and a wide range of mammals have shown that systemic effects on the immune system accompanying acute stress, such as

that typically experienced with wounding, increase the efficacy of tissue repair (Dhabhar, 2013). Both glucocorticoids and catecholamines released with acute stress rapidly induce significant changes in the distribution of leukocytes from the lymphatic organs, first into the circulation and then to the skin and other peripheral sites to fight infection and facilitate tissue repair (Fauci and Dale, 1975; Mills et al., 1998). Similar responses have been demonstrated following cold-shock in teleosts (Pickford et al., 1971) and limb amputation in newts (Sicard, 1982; Sicard, 1985). While acute stress has an immunoprotective and beneficial effect on tissue repair, chronic stress lasting several hours per day for weeks or months, can produce neuroendocrine and immune changes that interfere with wound healing, an effect well-documented in both humans and animal models (Christian et al., 2006; Dhabhar, 2013), including tree lizards (French et al., 2006).

The effects of acute stress on immune cells themselves are also now recognized as clearly biphasic. Although low levels of glucocorticoids produce effects beneficial for wound healing, synthetic corticosteroids and pharmacologic doses of natural glucocorticoids can retard wound healing, an effect well-known clinically and from studies with other mammals (Ehrlich and Hunt, 1968) and recently shown to occur in salamanders (Thomas and Woodley, 2015). Exogenous synthetic glucocorticoids inhibit regeneration of embryonic and adult zebrafish fins (Mathew et al., 2007) and larval *Xenopus* limbs (King et al., 2012), but with newts daily injections of cortisone following amputation produced only a slight delay of limb regeneration (Manner, 1955; Williams, 1959). At all stages of regeneration newt forelimbs accumulate ^3H -corticosterone in distal tissues following intraperitoneal injection of the compound, a result not affected by

ablation of the pituitary or interrenal tissue (Bromley, 1977). Future studies on neuroendocrine-immune system interactions during epimorphic regeneration, using an experimental system in which inherent variation can be minimized, must take into account the likelihood of ACTH and glucocorticoid sources outside the HPA and the biphasic nature of glucocorticoid effects.

5. Summary and concluding remarks

Unlike the simpler process of tissue repair, organ regeneration includes both patterning and proliferation of new cells, re-establishing the interactive system of developing tissues that forms a functional new organ. Nonregenerative repair or healing usually involves scarring or fibrosis as a major component, which may preclude the patterning required for complete regeneration. Studies of regeneration in vertebrate appendages, and more recently skin, indicate the centrality of immune-related cells in determining how successfully a functional organ is reproduced after loss or injury.

Recent work has shown that cells of the adaptive immune system are required to regulate organogenesis of the mammary gland during puberty (Plaks et al., 2015). No other organ characteristic of mammals develops predominantly in an immune-competent environment. However organ regeneration in animals with highly developed adaptive immunity can be expected to have mechanisms providing local tolerance of re-expressed embryonic antigens or other means of evading immune interference with successful patterning of new organ components. Among amphibians adaptive immunity appears to be rather basic in urodeles and in evolutionary terms first becomes well-developed and more like that of mammals in post-metamorphic anurans. An animal's capacity for both limb regeneration and scar-free skin regeneration seems to correlate

inversely with the phylogenetic development of its adaptive immune system. This generalization can be readily investigated in anurans during larval development and metamorphosis, and after induced metamorphosis in the axolotl, a neotenic urodele amphibian. Recent work with these and related model systems has revealed several aspects of inflammation and immune system involvement that appear to be fundamental for successful organ regeneration.

(1) Most aspects of the inflammatory response, including both cytokine and chemokine expression, as well as the schedule of leukocyte arrival and activation, appear to be similar during the initial phase of both repair and regeneration. Dampening inflammation with glucocorticoids at the time of amputation can completely inhibit regeneration.

(2) During the tissue response to injury, inflammation is concomitant with cellular reprogramming, which is especially important during regeneration. The response to amputation of fully developed limbs in many salamander species includes histolysis and cellular dedifferentiation within injured limb tissues which release first fibroblasts, then other cells to produce a small mesenchymal blastema. Interactions between these cells and the distal wound epithelium lead to growth and the formation of signaling centers that establish an initial pattern for development of the new limb.

(3) Resolution of inflammation, an active process involving changes in the local population of macrophages and in gene expression in other immune cells, must occur in a timely manner for successful regeneration. Studies with larval anuran (*Xenopus*) hindlimbs indicate that resolution of local inflammation is delayed after amputation at prometamorphic stages, during which regenerative capacity declines, and that early in

prometamorphosis this decline can be partially reversed by treatment with anti-inflammatory agents. Conversely limb regeneration at earlier stages is blocked by brief local treatment of the amputation wound with beryllium, a persistent proinflammatory adjuvant, an effect seen with larval urodele limbs only at much higher doses.

(4) Macrophages are key players in several aspects of inflammation as well as resolution, and their participation is required during the inflammatory response leading to regeneration, likely owing to their importance for resolution.

(5) Preliminary results of work on larval frog tail and limb regeneration has also implicated components of the adaptive immune system, including professional APCs and regulatory T cells, in the cellular response that precedes regeneration, suggesting that autoimmunity against new epitopes required for pattern formation may be involved in the regenerative decline that accompanies development of these cells and their deployment in peripheral tissues.

Studies of the response to amputation in larval anuran hindlimbs at different developmental stages suggest that persistent inflammation interferes primarily with the establishment of signaling centers in the injured tissues and the patterning required for formation of organ-specific features during regeneration. The basis for this interference is not known, but given the importance of fibroblastic cells both in the signaling centers and morphogen gradients of the embryonic limb bud (ten Berge et al., 2008) and for patterning muscles and other internal tissues during limb development (Hasson, 2011), it is likely that prolonged or excessive proliferation of such cells in the persistent post-amputation inflammatory environment resulting from a more developed adaptive immune response disrupts patterning, leading to increasingly defective limb

regeneration. Immunosuppression improves larval tail regeneration during its refractive period and skeletal patterning during hindlimb regeneration in premetamorphic *Xenopus*.

Localized immunomodulation of the response to amputation or other injury may also be involved in the clinically relevant regeneration of mammalian digits, in which participation of the proximal nail organ is required for successful regeneration. Like hair follicles the human nail organ is an immune-privileged site which apparently provides protection for stem cells in these structures against damage both from pathogens and from autoimmune or inflammatory reactions during growth and repair. As with the other regenerative models reviewed here, investigation of immune activities and potentially immunosuppressive properties of cells within the murine nail organ will likely provide new insights into digit regeneration.

Finally, although classic studies with amphibians indicated the importance of the HPA axis and neuroendocrine-immune interactions for successful limb regeneration, the work became controversial with variable results and apparent inconsistencies in follow-up studies. Reconsideration of this topic in light of new information on the peripheral synthesis of HPA agents, the biology of glucocorticoids, and beneficial role of stress in tissue repair will provide additional useful insight on the roles of neuroendocrine factors in regulating the early phase of organ regeneration.

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Table 1. Effects of post-amputation limb treatment with beryllium in *Xenopus*, stage 53/54

mM Be	n limbs	% mortality	regeneration	inflammation
0	13	0	9/13 (70%)	none
10	10	0	3/10* (30%)	local
20	14	5/14 (36%)	0	local
40	6	5/6 (83%)	0	systemic
100	5	5/5 (100%)	----	systemic
				*all 3 unpatterned "spikes"

Table 2. Effects of post-amputation limb treatment with beryllium in axolotl larvae, 4 cm

mM Be	n limbs	% mortality	regeneration	inflammation
0	16	0	100%	none
10	8	0	100%	none
20	8	0	100%	none
40	14	0	7/14 (50%)	slight, local
100	8	0	1/8 (13%)	local

Figure 1. Expression profiles after amputation (arrow) of inflammation-related factors (chemokines, cytokines, annexins, etc.), the major types of leukocytes involved, and major regeneration-specific events during the course of limb regeneration in larval amphibians.

(Text in this figure is sized to accommodate a single-column wide figure.)

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