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Scar-free wound healing and regeneration in amphibians: Immunological influences on regenerative success

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ABSTRACT

Salamanders and frogs are distinct orders of Amphibians with very different immune systems during adult life, exhibiting varying potential for scar free repair and regeneration. While salamanders can regenerate a range of body parts throughout all stages of life, regeneration is restricted to early stages of frog development. Comparison of these two closely related amphibian orders provides insights into the immunological influences on wound repair, and the different strategies that have evolved either to limit infection or to facilitate efficient regeneration. After injury, cells of the immune system are responsible for the removal of damaged cells and providing a cohort of important growth factors and signaling molecules. Immune cells not only regulate new vessel growth important for supplying essential nutrients to damaged tissue but, modulate the extracellular matrix environment by regulating fibroblasts and the scarring response. The profile of immune cell infiltration and their interaction with local tissue immune cells directly influences many aspects of the wound healing outcomes and can facilitate or prevent regeneration. Evidence is emerging that the transition from wound healing to regeneration is reliant on immune cell engagement and that the success of regeneration in amphibians may depend on complex interactions between stem cell progenitors and immune cell subsets. The potential immunological barriers to mammalian regeneration are discussed with implications for the successful delivery of stem cell therapeutic strategies in patients.

1. Introduction

Amphibians comprise a large group of animals that includes the anurans (frogs, toads) and "tailed" urodeles (salamanders such as newts and axolotls). This animal group exhibits a range of diverse species-specific life cycles, during which some species undergo dramatic changes in appearance. Because amphibian eggs develop *ex vivo*, both frog and salamander species were used extensively from the 1900s to study embryonic development. The observation that frog tadpoles could regenerate their tails and salamanders could regenerate whole limbs and tails during adult life stages was first formally described by Spallanzani in 1768 (Tsonis and Fox, 2009). Since that time, perfect adult regeneration of many other important tissues and structures as diverse as heart, brain, spinal cord, jaws, retina and lens, has also been described (reviewed in Brockes and Kumar (2008), Carlson (2007), Stocum (2012), Tanaka and Reddien (2011)). Salamanders maintain their ability to repair wounds without scarring, and regenerate a range of organs and tissues throughout their lives. In stark contrast, frogs fail to maintain regenerative capacity past metamorphosis and show a progressive loss of scar-free repair with development, concomitant with maturation of the immune system (Bertolotti et al., 2013).

Recent advances in scientific tools have allowed investigation of the underlying mechanisms that regulate the regenerative process in amphibians with greater molecular resolution. Although both frogs and salamanders share closely related biology, dramatic differences in adaptive immunity are observed between these two distinct groups of amphibians. Regenerative capacity is inversely correlated with the maturation of the immune system (reviewed in Godwin and Brockes (2006), Mescher and Neff (2005)). The immunological regulation of regeneration in salamanders has been implicated by a number of studies where immunosuppressant therapies or irradiation have interfered with normal adult regeneration (references therein Fahmy and Sicard (2002)). However, salamanders have remarkably different immune systems to that of their frog and toad counterparts in the amphibian world (Chen and Robert, 2011). It is possible that these differences may confer the permissive signaling required for regeneration in the adult life of the salamander.

The xenopus frog and the axolotl salamander have emerged as the most popular models to study amphibian immunology (Fig. 1). The success of regeneration in these species relies on both intrinsic and extrinsic inputs to coordinate a concerted gene program

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Fig. 1. *Xenopus laevis* (African clawed frog) and *Ambystoma mexicanum* (Axolotl, or Mexican salamander) are common laboratory model amphibians with unusual appearance. A post-metamorphic xenopus is shown on the left and neotenic adult wild type axolotl shown right. *Xenopus* image courtesy of LHG creative Photography Under licence CC BY-NC-ND 2.0.

integrating wound healing with *in vivo* reprogramming of cell identity, cellular proliferation and redevelopment in an adult context. Understanding the influence of immune function on scar-free wound healing and regeneration may provide opportunities to identify individual components that may restrict regeneration in frogs, and factors that may be required for successful completion for the program in salamanders.

Comparing different amphibian models is likely to result in a broader appreciation of the underlying mechanisms of scar-free repair and regeneration in adult tissues. This review will discuss what is currently known about the role of the immune system in scar-free repair and regeneration in amphibians, with a view towards translating this knowledge to extending the regenerative capacity in mammals. Defining the factors that influence the loss of regenerative capacity in frogs and the maintenance of this trait in salamanders has important consequences for understanding how to improve healing outcomes in human patients.

2. Scar-free repair and regeneration is progressively lost with frog development

Anuran amphibians such as the xenopus frog show a loss of regenerative capacity with development (see Fig. 2A). In particular, xenopus larvae show reduced regeneration competence with age and a decreased efficiency to form new and correctly patterned tail structure after amputation. A metamorphic change in body plan whereby the tadpole tail-like structure is remodeled and a tail-less frog body plan emerges coincides with a dramatic restriction in regenerative capacity. Amputation of developing limb at early stages before metamorphosis (stages 50–53) results in perfect regeneration of a complete limb however amputation at progressively later stages during development results in regeneration imperfectly patterned or incomplete limbs. Amputation after the events of metamorphosis have commenced, (stages 57–60 onwards) is followed by pattern-deficient regeneration of a cartilaginous spike lacking muscle and featuring excessive collagen production and connective tissue (Mescher and Neff, 2006).

The gradual loss of regenerative ability in the transition towards and through metamorphosis, along with the appearance of fibrotic wound healing in older anurans, mirrors the changes during developing mammals from fetal scar-free repair to adult scar-based repair of skin. Both scenarios involve a dynamic developmental landscape and major changes in the ECM, with immune signaling identified as a major regulator. In mammals, fetal scar-free wound healing is associated with an immature immune system a muted inflammatory microenvironment in which scar-less healing is progressively lost with development (reviewed in Kishi et al. (2012), Wilgus (2007)). Modulation of the inflammatory landscape during the scar-less healing phase, by genetic deletion of the potent anti-inflammatory cytokine IL-10, induces scar tissue formation (Liechty et al., 2000). This strongly suggests that in the regenerative context, the inflammatory landscape is the critical regulator of the fibrotic response rather than any particular cell-intrinsic property.

3. Regeneration is maintained throughout all life stages in salamanders

Salamanders such as the newts undergo complex life cycles that are comparable with frog development but maintain their tail appendages after metamorphosis. Unlike frogs, newts can regenerate a range of structures such as the lens, the heart, the tail and even the limb, throughout any stage of development. Analysis of repeated lens regeneration annually over a 16-year period in adult newts showed no decline in regenerative capacity or molecular changes in the regenerative tissue over this period (Eguchi et al., 2011). By the time of the last tissue collection, these newts were at least 30 years old, which strongly suggests that regeneration is not age dependent in this species. Other salamanders such as the axolotl rarely naturally transition through metamorphosis but can be artificially pushed through this process by hormonal treatment. Metamorphosis induces changes to the body plan but regeneration and scar-free healing is maintained in axolotls post metamorphosis (Seifert et al., 2012b; Young et al., 1983, 1985) (see Fig. 2B). In comparison to the dramatic changes observed in xenopus immune system, induced metamorphosis in both young and adult axolotls causes relatively subtle changes to the immune system. These changes include minor alterations in pattern of circulating leukocytes and in the responsiveness in some aspects of humoral immunity (Ussing et al., 1995). Additional experiments have indicated that the programs for anatomical metamorphosis and some aspects of hematopoietic development (acquisition of MHC class II) appear to be entirely separate (Völk et al., 1998).

4. Phases of regeneration and scar-free wound healing

Regeneration of whole adult limbs in the salamander is a dramatic example of complex tissue repair involving many different cell types with distinct embryonic origins. Regeneration of a limb after amputation can conceptually be divided into 3 distinct phases: the initial wound healing phase, the progenitor cell activation phase and finally, the re-development phase. This last phase is thought to be a recapitulation of embryonic development on a larger scale, in an adult context. Reactivation of embryonic gene programs in the presence of an adult immune system may present distinct challenges. One complication may arise from the dual function of chemokine guidance cues. Several chemokines used during development can also function as immune cell guidance molecules in adult tissues (Raz and Mahabaleshwar,

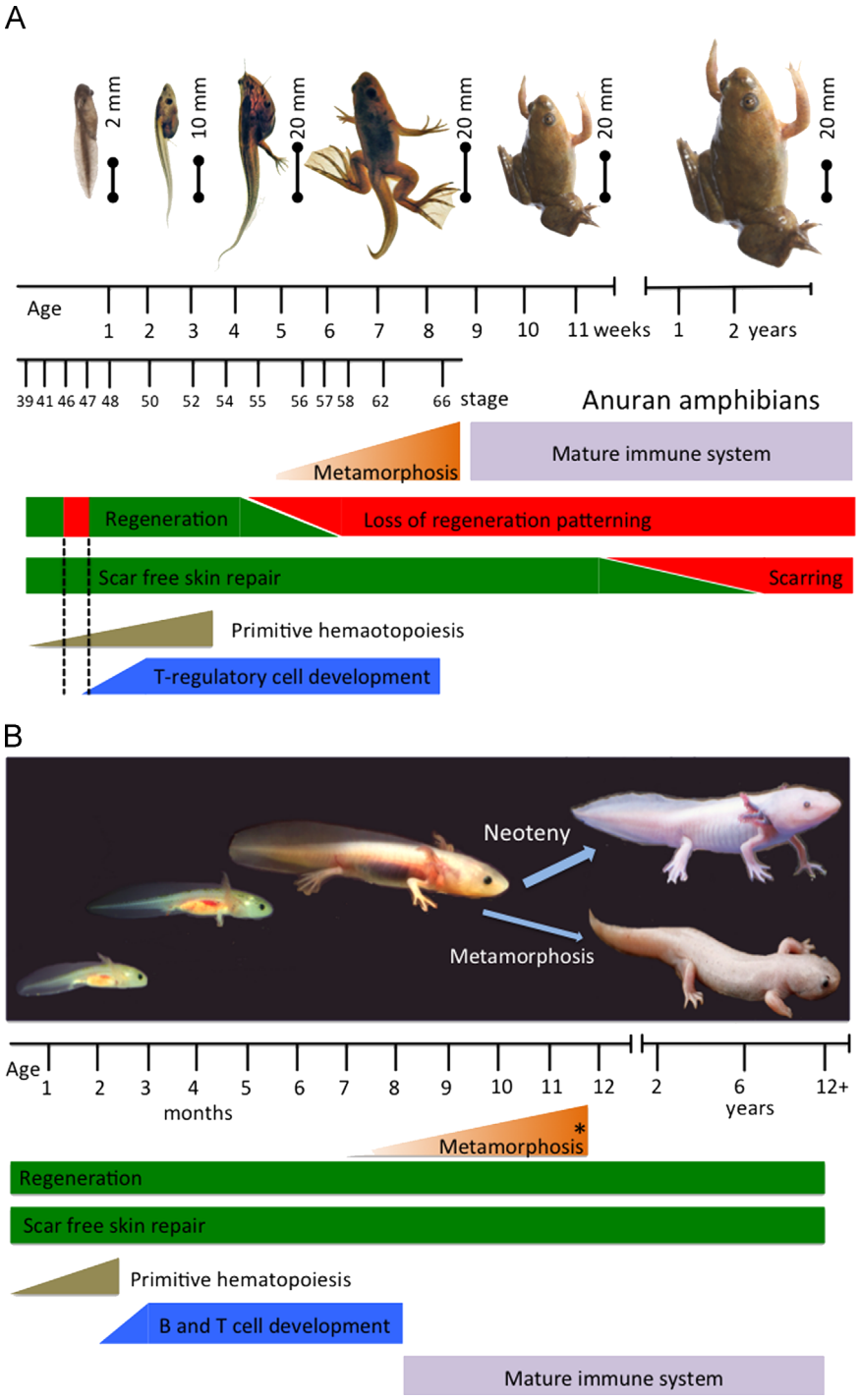

Fig. 2. Scar-free healing and regeneration is inversely correlated with development in anuran amphibians but is maintained in adult life in urodeles. (A) The development of sophisticated adult adaptive immunity begins at the onset of frog metamorphosis and is associated with a progressive loss of patterned regeneration where the number of digits that can be regenerated gradually declines between stage 55 to stage 60. While amputation at stage 53 results in a perfectly patterned limb, at stage 60 regeneration results in a "non-patterned spike". Tail regeneration is transiently lost during a "refractory period" (stage 45–47, marked with dotted line), which coincides with early immune cell development in the absence of regulatory T cells (T-regs). Restoration of functional regenerative potential in the tail is correlated with T-reg cell development. Scar-free wound healing is lost in adult anuran amphibians. (B) Scar free wound healing and regeneration is maintained throughout all life stages in salamanders. Metamorphosis is linked to relatively minor changes in adaptive immunity and regenerative capacity is maintained. Most salamander species transition through metamorphosis; however some species like the axolotl may have neotenic life cycles that do not normally transition through metamorphosis but can be chemically induced to do so. Unlike frogs, scar-free healing is maintained in adult life in salamanders. Various axolotl natural pigment mutants are available. (Common laboratory "white mutant" shown). *Xenopus* images courtesy of David Bay and the National Science Foundation.

2009) and may require regeneration-specific regulation. These potential conflicts are yet to be formally tested.

The progenitor activation phase is context dependent. Lens regeneration is mediated through transdifferentiation (Henry and Tsonis, 2010), whereas in the case of the heart, direct cardiomyocyte dedifferentiation and cell cycle re-entry may be the main source of new contractile tissue (Bettencourt-Dias et al., 2003). In the case of a complex structure such as the limb, both local dedifferentiation and tissue resident stem cell activation contribute to the progenitor cell pool (reviewed in Tanaka and Reddien (2011)).

The initial wound healing phase of limb regeneration shares many features with scar-free repair of wounds outside of a regenerative field (*i.e.* flank or back of the animal). Transcriptomic analysis in the salamander has revealed that many of the gene signatures are common to both injury contexts in this initial phase before a divergence in gene signatures associated with the regenerative program (Knapp et al., 2013). As is the case for many regeneration contexts, the success of limb regeneration is dependent on signaling from damaged nerve during the progenitor activation phase (Kumar and Brockes, 2012). The divergence of the gene expression profiles observed between scar-free repair of wound to the flank and regeneration of whole limbs is thought to be a result of different injury context inputs, such as functional innervation and coordinate interactions between cells with different positional identities (reviewed in Makanae and Satoh (2012)).

5. Reprogramming events during regeneration may be linked with immunological regulation

In both salamanders and frogs, the wound-healing phases in both skin repair and regeneration models exhibit strong up-regulation of genes involved in cellular reprogramming (Sall4, Oct4, Klf4, c-Myc) (Knapp et al., 2013; Looso et al., 2013; Maki et al., 2009; Monaghan et al., 2009; Neff et al., 2011; Sousounis et al., 2013; Stewart et al., 2013). Specifically, Sall4 is a cytokine that plays a role in epigenetic modulation of chromatin structure and antagonizes differentiation, and is therefore a likely candidate for controlling de-differentiation of mature limb tissues during regeneration (Neff et al., 2011). The expression of Sall4 in xenopus limb regeneration shows dynamic temporal and spatial regulation where expression can be detected in both fore and hind-limbs during the fully and partially regeneration-competent stages (stages 52–53 and 54–55, respectively), whereas SallL4 is not expressed in developing xenopus limbs at the regeneration-incompetent stages (stage 57 and later) (Neff et al., 2005).

Cytokine signaling is regulated within cells by a negative feedback mechanism involving suppressors of cytokine signaling (SOCS) family members. SOCS3 is a known regulator of Sall4 expression and is transiently up-regulated in regeneration competent stages at much higher levels than regeneration incompetent stage amputated limbs. SOCS proteins are known to fine tune both innate and adaptive immune responses and inflammation (Tamiya et al., 2011). This regulation may be important for induction of immune tolerance of differentiating cells and the progression of regeneration (Grow et al., 2006). Activation of inflammatory signaling has also been implicated as an important regulator for nuclear reprogramming pathways by enhancing an open chromatin configuration and enhancing reprogramming efficiency (Lee et al., 2012). It is intriguing to speculate that the *in vivo* reprogramming events associated with formation of progenitor populations in certain contexts of regeneration may be directly regulated by immune cell recruitment and activation.

6. The precise regulation of inflammatory pathways may be critical for regenerative competence

Correct pattering during regeneration may also be under the influence of the inflammatory environment. Several studies modulating inflammatory signaling by various agents such as glucocorticoids, non steroidal anti-inflammatory agents and immunosuppressant molecules have shown to improve pattering of pattern deficient stage 54/55 *Xenopus* limb regeneration model (reviewed in King et al. (2012)). The light metal Beryllium (Be^{2+}) is a potent agonist of inflammation, causing a chronic insult that is not resolved by normal anti-inflammatory mechanisms. Treatment with this inflammatory agonist results in a complete failure of regeneration in both salamander and xenopus tissues normally capable of regeneration. Although Be^{2+} can cause some cytotoxicity, the reported changes in the inflammatory landscape and induction of fibrosis are likely to be important contributors for regenerative failure in these animals (King et al., 2012; Thornton, 1949) by interfering with the normal expression of genes required for patterning (Mescher et al., 2013).

In contrast to the salamander, the regenerative capacity of frog limbs is inversely correlated with development and appears to decline with progression through metamorphosis and the accompanying changes in ontogeny of the adaptive immune system (see Fig. 2). Notably, the most dramatic changes to the frog immune system occur during the developmental period of metamorphosis, as demonstrated by the rejection of allografts taken from regeneration competent early tadpole skin that has been cold preserved and re-grafted to the same host post metamorphosis (Izutsu and Yoshizato, 1993). In general, larval frog immune responses are much less developed and lower in efficiency than post metamorphic responses (Rollins-Smith, 1998).

Transcriptome analysis in frogs comparing regeneration incompetent stages versus regeneration competent stages revealed important differences in immunological signaling and the resolution of inflammation (Grow et al., 2006; Pearl et al., 2008). Proteomics performed on stage 53 regeneration competent limbs has supported this analysis (King et al., 2009). Similar proteomics analysis in axolotl limb regeneration (Rao et al., 2009), gene expression analysis (Knapp et al., 2013; Mercer et al., 2012; Monaghan et al., 2012; Stewart et al., 2013) and systems biology approaches (Jhamb et al., 2011) have confirmed the dynamic landscape of immunological signaling during amphibian regeneration. Experiments comparing the resolution of inflammation in both regeneration competent (stage 53) xenopus limbs and non-competent (stage 57) limbs show a perdurance of pro-inflammatory signaling within non competent limbs (King et al., 2009). These data support the resolution of inflammation as a critical factor in the progression of regeneration.

7. Regeneration-specific extracellular matrix is regulated by immune cell–fibroblast interactions

Scarring is a potential outcome of wound healing where aberrant molecular signaling precludes regeneration. Scarring and functional tissue replacement, appear to be mutually exclusive processes and it is likely that fibrosis is incompatible with tissue and organ regeneration. The profile of ECM production and remodeling appears to be very different between contexts of regeneration and non-regenerative contexts that form fibrotic scar tissue (Gassner and Tassava, 1997; Grounds, 2008; Mercer et al., 2013; Seifert et al., 2012a), and the regulation of fibrotic pathways appears to be a prerequisite for efficient regeneration in several contexts in both amphibians and mammals.

Immune cell infiltration directly determines the tissue microenvironment in wounds by producing a plethora of cytokines and soluble mediators that act directly on mesenchymal tissues and in particular tissue fibroblasts (Kovacs, 1991). Fibroblasts are the major source of ECM and their immunological regulation is a key factor in the development of fibrotic scar tissue or the ECM components of regenerating tissue (Moyer and Wagner, 2011). A requirement for early interactions within the wound to permit regeneration could be blocked by excessive fibroplasia and collagen production driven by inflammation, which underscores the

requirement for a regulated immune system during regeneration. Defining the regulation of ECM production during different regeneration contexts in amphibians is likely to be informative in the development of mammalian regenerative therapies.

8. Comparison of amphibian immune systems

Several lines of evidence have implicated both negative and positive roles for various immune system components in the success of amphibian regeneration. The adult frog immune system is complex, including a complete innate immune system and a diverse range of adaptive immune responses, and is fundamentally similar to that of mammals in terms of specificity, speed of onset and memory (reviewed in Du Pasquier et al. (1989), Mescher and Neff (2005)). In contrast, the salamander immune system has not been well characterized. Salamanders have a strong innate immune system but relative to frogs and mammals they are considered relatively immune-deficient as they appear to lack several key adaptive immune responses (Chen and Robert, 2011). Despite a large T-cell repertoire and a reasonable B-cell repertoire in salamanders, the humoral response is extremely slow (60 days), mediated by one unique IgM class of Immunoglobulins and fails to mount appropriate memory of previous challenges (Kaufman et al., 1995; Tournefier et al., 1998). Salamanders also fail to elicit detectable responses to soluble antigens (Charlemagne, 1979a)) and cellular co-operation (T-helper triggering of B cell function) has not been demonstrated during humoral responses; on the contrary, thymectomy, X-ray irradiation or corticosteroid treatment enhances the humoral response (Charlemagne, 1979b, 1981; Tournefier, 1982).

The cytotoxic immune response in salamanders is also very slow with poor mixed lymphocyte reactions (Kaufman et al., 1990; Koniski and Cohen, 1992) lacking acute xenograft rejection reactions. Chronic rejection does eventually occur and appears to be dependent on the thymus. Despite this observed state of immunodeficiency, the diversity of T and B cell antigen receptors is very high (Tournefier et al., 1998) and comparable with frogs. Although most of the data on salamander immunity has been obtained in axolotls, based on the chronic rejection of xenografts, weak immune responses appear to be generally applicable to many different species and genera of salamanders (Cohen, 1971). The real life implication for a weak adaptive immune response in the salamander is clearly demonstrated by their extremely high sensitivity to viral infection relative to other more resistant amphibians such as frogs and toads (Cotter et al., 2008). Transcriptional responses of axolotls to viral infection revealed that although a complex immune response is mounted, axolotls fail to induce an early T-cell proliferative response in the spleen. By comparison, xenopus is capable of clearing a closely related virus and are able to induce early T-cell responses in the spleen (Cotter et al., 2008).

9. The relationship between immune system maturation and regenerative capacity

In frogs, the age-related decrease in regeneration competence to form a new and correctly patterned tail appears to be related to the intensity of the inflammatory response and changes related to age-dependent structural modifications in the thymus (Franchini and Bertolotti, 2012). Interestingly, frogs transiently lose their regenerative ability between stage 45–47 (see Fig. 2). This developmental stage is termed the “refractory period” and coincides with the ontogeny of primitive immune cell development. Immunosuppressant therapy or immune cell depletion during this period restores regenerative capacity (Fukazawa et al., 2009). The natural restoration of regenerative capacity thereafter coincides with the emergence of T-regulatory cells (T-reg) and suggests that the unregulated population of immune cells in the early tadpole during the refractory period directly interferes with functional regeneration.

Likewise, skin wounding experiments have recently revealed an age-dependent loss of the scar-free repair and regeneration featured in young froglets, associated with a maturation of the immune system and an altered wound healing response (Bertolotti et al., 2013). Amphibians lack lymph nodes and as such antigen presentation and tolerization may occur entirely within the skin and other peripheral tissues (Mescher and Neff, 2006). In larval frog skin, rejection is very limited and cellular immunity is slower and weaker in nature: cells expressing MHC class I and II proteins including antigen presenting cells (APCs) are more restricted. Larval antibodies also display lower affinity and are less diverse compared with genetically identical adults with restricted repertoires, and NK cell activity toward allogeneic tumour cells is present in adults but not tadpoles (Rollins-Smith, 1998). Thus, two developmentally distinct immune systems are active in frogs: the larval (ancestral) system without classical MHC class I antigen presentation or efficient effector mechanisms (Robert and Cohen, 1998) and the post metamorphic (evolved) form of immune system with strong adaptive immunity similar to mammals associated with regenerative failure.

After metamorphosis, (stage 53–57 in xenopus) larval skin becomes heavily populated with both dendritic and Langerhans cells (Carrillo-Farga et al., 1990; Castell-Rodriguez et al., 1999; Du Pasquier and Flajnik, 1990; Mescher et al., 2007a). Putative epidermal T cells are also reported (Mescher et al., 2007b). These cells form an integral part of adult skin, and have all been implicated as regulators in mammalian tissue repair and their appearance in cutaneous niche may directly regulate the success of skin repair and regeneration in frogs. It is likely that changes in skin repair during late pro-metamorphic animals where regeneration-style closure is replaced by mammalian contraction-based closure are directed by these cells (Mescher and Neff, 2005).

The importance of immune timing is further underscored by experiments in which reciprocal transplantation of limb blastemas between larval (regeneration competent) and post metamorphic (regeneration incompetent) stages indicated that a failure in post-metamorphic regeneration may be an intrinsic property of mature xenopus cells (Sessions and Bryant, 1988). Although this experiment demonstrates that intrinsic changes to the cells may prevent adult regeneration in xenopus, the immunological landscape within local tissue may still impose important impediments to regeneration.

10. Adaptive immunity and regeneration

In mice, the adult adaptive immune system initiates development around birth (Chaouat and Voisin, 1979; Haynes et al., 1988), whereas in humans, adaptive immunity can be detected at gestational week 10 and the fetal immune system has the capacity to detect non-inherited antigens (*i.e.* from a dizygotic twin) and must induce tolerance (Trowsdale and Betz, 2006). Similar to the mechanism of fetal tolerance by maternal T-regs, fetal T-reg's mediate tolerance to developmental antigens. In humans, mice and birds, fetal and adult T cells constitute distinct populations that arise from different HSC's that are present in different stages of development (Mold et al., 2010). Hematopoiesis occurs in distinct waves generating distinct populations where the initial

wave of T cell differentiation appears to favor a population whose role is to promote tolerance to "self antigens" during development.

Loss of tolerance with the emergence of adult adaptive immune response may contribute to the loss of regeneration in later stages of vertebrate development. Indeed, xenopus features a high degree of tolerance for new antigens during early stages of development (Mescher and Neff, 2006; Robert and Ohta, 2009). In salamanders, immunosuppression using Cyclosporin A showed dose dependent inhibition of limb regeneration, which could be rescued using Interleukin 2 (IL-2), suggesting a specific role for T cell activation in a successful limb regeneration program. Acute responses are normally controlled by major histocompatibility complex (MHC) gene expression and the cooperation between a range of antigen presenting cells, CD4+ helper T cells and CD8+ effector or cytotoxic T cells. Notably, acute rejection of skin grafts in frogs occurs acutely in approximately 20 days whereas rejection in salamanders takes around 60 days and is considered a chronic rejection event that does not involve the expansion of cytotoxic T cells (Kinefuchi et al., 2013). However, whereas the axolotl shows limited diversity and deficient MHC architecture (Tournefier et al., 1998), other salamander species have MHC gene diversity similar to higher vertebrates (Bos and DeWoody, 2005). Although the capacity for MHC-class I mediated destruction of xenografts by natural killer (NK) cells is yet to be described in salamanders, the weak allograft rejection shared by these species is likely due to the poor generation of cytotoxic T lymphocytes or potentially some other mechanism of tolerance induction.

In salamanders, perfect regeneration of a new lens after surgical removal involves the transdifferentiation of pigmented epithelial cells (PEC) activated specifically on the dorsal margin of the iris. The loss of pigment during PEC dedifferentiation is accompanied by the invasion of macrophages that phagocytose the melanosomes discharged from pigmented cells (Reyer, 1982). The immune system has also been indirectly implicated in lens regeneration via local thrombin activation and the formation of a fibrin clot (Godwin et al., 2010). The fibrin clot attracts leukocytes and is thought to form a paracrine signaling center that participates in the activation of cellular proliferation of dorsal epithelial cells and their transdifferentiation into new lens cells. An alternative to surgical removal of the lens where the lens is damaged by pricking but not physically removed results in faster regeneration than surgical removal of the complete lens (Kanao and Miyachi, 2006). Damage to the newt lens by pricking recruits dendritic cells to the injury site resulting in the destruction and extrusion of the lens from the optic chamber and activation of lens regeneration from the dorsal margin or the iris. In both forms of lens regeneration, the location of new lens production is spatially distinct from the injured lens tissue. Importantly, the ectopic lens induction elicited by transfer of dendritic cells from newts with pricked lens tissue to naïve animals is abrogated by surgical removal of the spleen or inhibition of immune signaling by systemic nitric oxide inhibition (Kanao and Miyachi, 2006). Taken together, these experiments point to a predominately immune-mediated mechanism of the initiation of lens regeneration and implicate a role for peripheral tolerance (Godwin and Brockes, 2006). Dendritic cell activation of regeneration however may be specific to the lens context, as limb regeneration in newts appears to be independent of the spleen (Fini and Sicard, 1980).

11. Innate immunity and regeneration

The activation of regeneration and tight regulation of inflammatory resolution at various stages of regeneration are likely to influence the success and quality of vertebrate regeneration in a range of injury contexts. The innate immune system is responsible for orchestrating the progression of pro-and anti-inflammatory responses to injury. Key players in innate immune function are myeloid cells (monocytes, macrophages and granulocytes), which regulate many aspects of inflammation and injury resolution. Tissue resident macrophages and monocyte-derived macrophages comprise a diverse group of cells from varied developmental origins that form the ancient and evolutionarily conserved mononuclear phagocyte system.

A large body of macrophage literature has emerged, demonstrating a wide range of different phenotypes and functions for these cells outside their primary role in innate immunity. In vertebrates, myeloid cells are found in nearly every tissue from the early stages of development where they remain throughout the entire life of the organism (Stefater et al., 2011). It is now well established in several vertebrate models that macrophages regulate development, maintain homeostasis and have important roles in influencing the quality of repair and regeneration. Tissue specific macrophages directly regulate programmed developmental apoptosis pathways required in mouse brain, chick retina (Frade and Barde, 1998; Marin-Teva et al., 2004) and eye development via secretion of Wnt7b (Lang and Bishop, 1993; Lobov et al., 2005). Macrophages have also been demonstrated in a range of contexts to regulate development via mechanisms independent of apoptosis. These include roles in tissue patterning, morphogenesis and regulation of cell fate decisions. Genetic ablation of macrophage populations in mice have revealed a diverse role in the development and cell fate decisions within many different tissue types (reviewed in Stefater et al. (2011)).

Macrophages also directly regulate repair and regeneration by modulation of the local inflammatory microenvironment, the resolution of inflammation and local sources of growth factors during various phases of wound resolution (Delavary et al., 2011). After injury, circulating myeloid cells are recruited and the balance between invading and tissue resident myeloid populations may influence the type of immune response that ensues, in a context dependent manner. Macrophages produce a plethora of soluble effector molecules including but not limited to PDGFs (platelet-derived growth factors), IGFs (insulin-like growth factors), HGFs (hepatocyte growth factors), FGFs (fibroblast growth factors), TGFs (transforming growth factors), CSFs (colony-stimulating factors), Wnt ligands and many immune-related molecules (Stefater et al., 2011). These factors orchestrate the balance between inflammatory and regenerative responses: in damaged mouse muscle, inflammatory macrophages alter their phenotypic state and resolve primary inflammation, such that genetic silencing of the machinery required for anti-inflammatory signaling in macrophages results in failed regeneration and the induction of abnormal tissue fibrosis (Ruffell et al., 2009).

Although unregulated macrophages in the refractory period of tail regeneration in the developing xenopus may antagonize regeneration (Fukazawa et al., 2009) (Fig. 2A), in the presence of a regulated immune system macrophages appear to play a positive role in several contexts of amphibian development and regeneration. Ablation of macrophages in xenopus results in abnormal limb morphogenesis and death at metamorphosis indicating that embryonic macrophages are required to fulfill an essential function during embryogenesis (Smith et al., 2007).

Recent studies assessing immune signaling early in amphibian regeneration have revealed that both pro-inflammatory signaling and anti-inflammatory signaling are up-regulated simultaneously, suggesting that the balance between inflammatory mediators is essential in determining the success and quality of regeneration (Godwin et al., 2013; King et al., 2012). Systemic depletion of macrophages prior to limb amputation in axolotls completely blocked limb regeneration and transformed the amputated tissue to a fibrotic stump featuring

epithelial hyperplasia (Godwin et al., 2013). Depletion of macrophages after formation of the progenitor cell pool known as a "blastema" resulted in a reproducible delay in limb regeneration, implicating a role at multiple stages at regeneration. Alterations in the expression profile of several gene pathways with known roles in the regeneration program upon macrophage ablation implicates their engagement in regulation of key developmental programs (Godwin et al., 2013).

Macrophages also interact with the adaptive immune cells to modulate the function of fibroblasts and are considered the master regulators of fibrosis and scar tissue formation (Wynn and Barron, 2010; Lucas et al., 2010). Indeed, dramatic dysregulation of developmental gene programs, altered inflammatory signaling and the activation of fibrosis occurs in the absence of macrophages in early limb regeneration in the salamander (Godwin et al., 2013). The widespread engagement of macrophages in regenerative responses reinforces the importance of these cells in influencing injury resolution and the re-activation of developmental gene programs. The essential role for macrophages in regeneration has been confirmed in other regenerative animal models such as zebrafish: ablation of macrophages but not neutrophils severely impaired the inflammatory resolution and tissue regeneration, resulting in the formation of large vacuoles in the regenerated fins (Li et al., 2012).

Myeloid granulocytes such as neutrophils are also important in the resolution of infections and can provide important signals for recruitment and activation of monocyte/macrophage cell types. In mammalian contexts of optic nerve or thymic regeneration, neutrophil engagement is thought to be important for efficient repair (Kurimoto et al., 2013; Nakayama et al., 2011). In mouse skeletal muscle regeneration, depletion of neutrophils alters clearance of necrotic tissue in the early phase, whereas they are dispensable for overall regeneration (Teixeira et al., 2005). In some mammalian contexts of wound healing neutrophils are thought to play a role in activation of fibrosis, however they have also been reported to provide a source of matrix degrading enzymes in some wounds (Heissig et al., 2010). The types of wound healing responses elicited within different injury contexts are likely to be functionally linked to the initiation of specific programs operating in regenerative organisms. The exact role of granulocytes in regeneration appears to be context dependent and is yet to be determined in different amphibian regeneration models.

Another important component of innate immunity is the complement system that provides early activation of the healing response. Complement comprises a group of circulating proteins that forms part of the innate immune system and is primarily involved in the recognition and clearance of pathogens from an organism (reviewed in Ricklin et al. (2010), Sarma and Ward (2011)). In addition to functions in opsinisation, lysis and clumping of antigen bearing cells, complement activation can activate macrophage and neutrophil chemotaxis and amplify the cytokine cascade of an immune cell infiltrate. Complement proteins are generally produced by cells in the liver but are also produced by tissue macrophages, blood monocytes and some specialized epithelial cells. Protection from complement-mediated cell-lysis can be afforded by expression of complement regulatory membrane proteins such as CD59.

Inhibition of complement protein C3 and C5 have been shown to be specifically regulated during limb and lens regeneration in the salamander (Kimura et al., 2003). Recently C3a has been shown to induce complete regeneration of the embryonic chick retina from stem/progenitor cells present in the eye and is independent of fibroblast growth factor receptor signalling (Haynes et al., 2013). These experiments showed direct activation of stem/progenitor cells by injury and inflammatory signalling. Studies in xenopus have also revealed the upregulation of complement components during tail regeneration (Tazaki et al., 2005) and higher levels in regeneration competent versus non-competent limbs (Grow et al., 2006)). These experiments implicate complement as an important regulator of regeneration worthy of further investigation.

12. Immunological barriers limiting adult regeneration

Why are tissue regenerative pathways in amphibians so privileged, and what are the evolutionary pressures limiting effective regeneration in higher vertebrates? It has been suggested that cells undergoing dedifferentiation early in regeneration may be recognized and eliminated by cytotoxic T cells or NK cells if they display proteins recognized in the adult as non-self (King et al., 2012). The potential for dedifferentiated cells to be recognized in adult tissue, in a situation analogous to the identification of virally infected cells or neoplastic cells, could rely on an advanced adaptive immune system that fails to induce peripheral tolerance of regeneration specific self-antigens. In salamander spinal cord regeneration, introduction of foreign antigens slows or inhibits normal regeneration and features morphological immunocomplexes that parallel scar-based repair normally observed in the mammalian context (Margotta et al., 1989). Cell mediated killing of dedifferentiated cells may present a major impediment to adult regeneration but may provide opportunities to target the mechanisms of tolerance and the induction of regeneration in non-regeneration competent contexts (discussed in Mescher and Neff (2006)).

Further studies are required to examine the role of tolerance in limb regeneration. Although cytotoxic T cell mediated acute rejection of allografts has not been observed in salamanders, spontaneous tumour formation in urodele amphibians is very rare (Brockes, 1998). Moreover, regeneration competent tissues in salamanders are refractory to chemical carcinogenesis whereas regeneration-incompetent tissues are not (Delriotsonis and Tsonis, 1992). The regulatory mechanisms that regulate appropriate cell growth and tolerance of embryonic antigen re-expression in adult tissues is yet to be resolved. Given the known role for the regulation of NK cell-mediated lysis by monocyte/macrophage populations (Jewett et al., 2012; Kloss et al., 2008; Tseng et al., 2010) we propose a model in which the essential role of macrophages in the success of limb regeneration in the salamander could include a role for preventing NK cell-mediated lysis of progenitor populations in addition to regulating inflammation and developmental fate decisions (Fig. 3).

13. Conclusions

The maintenance of scar-free repair and regeneration during adult life seems to be unique to salamanders within vertebrate animals. Although there is some evidence for intrinsic cellular changes during development that may limit regenerative capacity, there is compelling evidence for the dramatic species variation in the extrinsic immunological environment of damaged tissues, which may be critical for allowing activation of the regeneration program during the wound-healing phase and altering intrinsic cellular potential within injured tissues.

What properties of the salamander immune system that allow for functional regeneration, or which inhibitory components emerge in frogs during development and maturation of the immune system, are yet to be defined. Both frogs and salamanders colonise similar aquatic environments and the forces driving the specific evolution of their respective immune systems poses interesting biological questions. Frog developmental stages are characterized by variable capacities for scar-free repair and regeneration. Different coping strategies for immunological challenges in frogs and salamanders may reside in expanded functions or

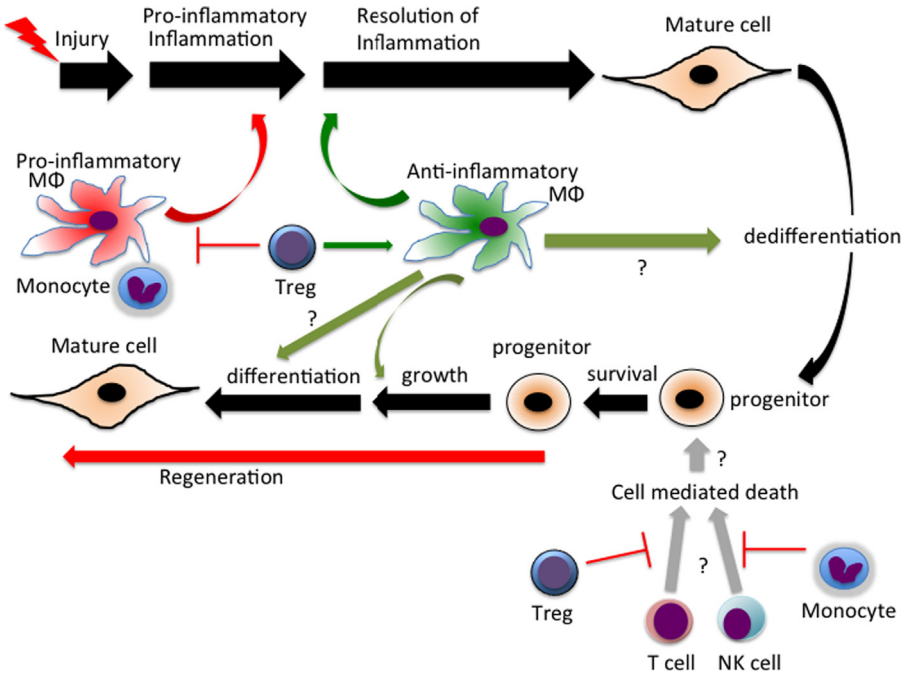

Fig. 3. Hypothetical model for monocyte/macrophage (*MΦ*) regulation of progenitor cell activation, survival, growth and differentiation in the salamander. Within the influx of various white blood cells types that invade the damaged tissue monocyte/macrophage cell types are critical for regenerative success. Monocyte/macrophage populations regulate the resolution of inflammation, provide growth factors and signaling molecules important for angiogenesis and may influence the differentiation of progenitor cells. Monocyte/macrophage populations may also play a role in facilitating dedifferentiation and the regulation of cell mediated lysis.

efficiencies in innate immune signaling. This may afford enhanced protection from some pathogens, but the weak adaptive immune responses of salamanders put them at great risk especially from viral pathogens. Molecular analysis of the immunological changes within these species should help to clarify the mechanisms that underlie the selective retention or loss of regenerative capacity. Further characterization of amphibian immune systems will be informative as to which immune components are compatible with perfect regeneration in adult tissues, and can be translated to a human context.

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