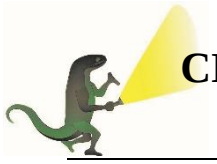

CHAPTER 3 Impact of blastema homogenate application on inflammation and wound healing in lizard limbs

INTRODUCTION

Appendage regeneration via epimorphosis visibly follows the same set of events, placed in a predetermined order of chronology. Across all phyla, the series of epimorphosis involves, epithelial wound closure, dedifferentiation of cells located at the site of injury followed by reformation of lost tissue (Alvarado, 2000). Covering exposed tissue is the first step towards formation of wound epithelium. The newly formed tissue bud then grows further in size and restores both structure and function of the original organ. This stage is followed by occurrence of blastema which primes the entire regenerative machinery.

Blastema: A key to successful epimorphosis

Wound epithelium is formed by all animals displaying appendage regeneration, but over a variable time scale. For an instance, amputated salamander appendage forms wound epithelium in first 24 hours (Tanaka 2016), while lizard tail takes as long as 4 days (Clause and Capaldi, 2006; Ranadive et al., 2018). In the next step, stratification of wound epithelium leads to apical epithelial cap (AEC) formation (Christensen and Tassava, 2000). Blastema is like an aggregate of cells which have migrated and gathered at the site of wound healing. The cluster comprises of various progenitors, de-differentiated cells arising from all the tissues injured by amputation. Both resident stem cells and the migrated ones give rise to a rich niche of progenitor cells known as ‘blastema’ (Muneoka et al., 1986a; Tweedell, 2010). This aggregated cell pool is a function of multiple molecular signalling cascades, which are regulated by immune modulators.

Blastema, being a group of progenitors receives and reciprocates to the signals arriving from the microenvironment. These cues are localised or arrive with the blood flow and allow progressive redifferentiation (Satoh et al., 2008). Extracellular matrix (ECM), in coherence with immune cells, seals the wound, clears out the debris and lays path for reparative machinery to work upon. As a preliminary response to injury, blood clot is formed, which ensures restricted blood loss to prevent haemorrhage. This builds up the stage for restoration action play and yet its presence and roles are debatable across the regenerative paradigms. In salamanders no visible clot is formed (Miller et al., 2019) while

in lizard tail and mice digits, clot formation cues successful regeneration (Lehoczky et al., 2011; Delorme et al., 2012). Blood clot is also a steppingstone for immune response which categorically plots the route of wound healing (Shiu et al., 2014). Both pro and antiinflammatory mediators coordinate the process inflammation and its resultant impact on the outcome as well (Filbin, 2006). Blastema, principally is a hub of signalling moieties, which reconstructs all tissues. This process, when dissected in further details turns out to be a result of successful crosstalk between external wound epithelium and the aggregated pool of progenitors. A mere presence of glandular cells and collagen mass can lead to hindered regeneration as it inhibits a fluent molecular crosstalk amongst the two parties (Mescher, 2017). All regeneration proficient clans exhibit collagen-less, explicit molecular exchange amongst AEC and cells below, especially highlighted in axolotls and salamanders (Tanaka, 2003), frogs (Kawakami et al., 2006), fishes (Nakatani et al., 2007) and lizard tail (Lozito and Tuan, 2016). A set of experiment was designed to underline these molecular trade-offs, which involved two main approaches. In first one, the amputated limb was sealed within the peritoneum after removing the skin region and secondly, growing epithelium was repeatedly peeled off the healing limb (Miller et al., 2019). Both of these basic experiments demonstrated the importance of traumatic environment and healthy wound epithelium in successful blastema formation. Further, multiple researchers have deciphered the vital roles of wound epithelium in both initiating and maintaining blastema based molecular signalling (Boily and Albert, 1988; Simkin et al., 2015).

When the wound is sutured close surgically, no regenerative outcome is achieved in mouse digit tips (Simkin et al., 2015), while in lizards, epithelialisation is much more delayed in time as compared to salamanders (Lozito and Tuan, 2017). Thus, wound epithelium has a wide range of roles in blastema formation and function. On the other hand, blastema stands indispensable for all realms of epimorphic regeneration ranging from regrowing amphibian limbs to reformed antlers (Li et al., 2005; McLean et al., 2011). All these various blastema types require a troop of signalling molecules such as WNTs (Buch et al., 2017), FGFs (Poss, 2000), TGFs (Hutchins and Kusumi, 2016) and immune modulators (King et al., 2012). The niche of progenitors, migrates, proliferates and dedifferentiates to reconstruct the lost body part (Love et al., 2013; Zhang et al., 2016). Remarkably, the niche is formed and brought into action, based on the triggers provided by injury (Tseng et al., 2007). This is a transient stage in the colossal event of regeneration as blastema is a temporary yet most critical for successful restorative regime (Vinarsky et al., 2005). Bulging up and flattening

of blastema are equally crucial and for regeneration, while in lizards, the latter job is performed by a consortium of MMPs. The matrix is modified as original cells placed here lose their identity to dedifferentiate into pool of progenitors (Figure 3.1).

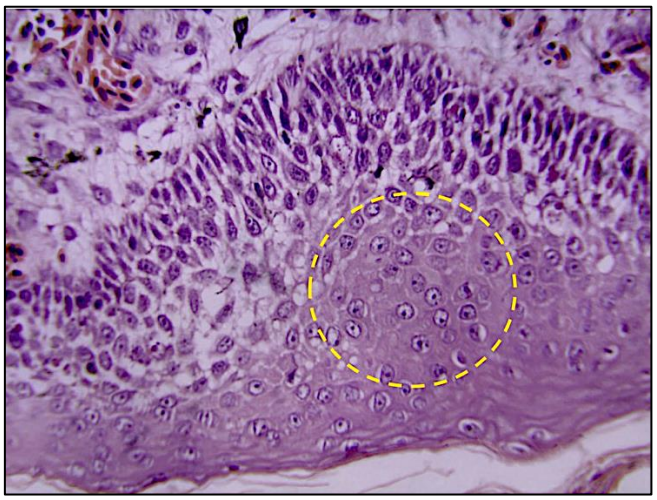

Figure 3.1 Blastema with stem cell like cells observed in H&E-stained tissue section of lizard tail at 5dpa (40X objective)

In lizards, the necessary 'dialogue' amongst the wound epithelium and blastema is mediated by molecules such as TGF-β, WNT family members (Delorme et al., 2012, Gilbert and Singerland, 2013), BMPs (Lozito and Tuan, 2016) and FGFs (Ranadive et al., 2018). All these factors are cued to the site of wound epithelium via immune modulators, which reach the regeneration locale in the beginning (Vitulo et al., 2017). All the well communicated signals relayed in this microenvironment govern the progenitor cells' re-entry into cell cycle and the process of dedifferentiation initiates. A regulatory entity orchestrating these events are speculated to be either wound epithelium, blastema itself or rather the unique signalling that occurs at the site of amputation. In spite of a plethora of studies conducted to characterise the blastema across various regenerative realms, its exact composition, pattern formation and signals relayed through it are debatable (Miller et al., 2019).

In lizards, blastema formation is confined to only one appendage, i.e., the tail. Limb on the other hand is deprived of any such development and elicits prominent scarring. This tissue specific disparity in post-injury response, could be the primary reason for the overall differential wound healing discourse and its outcomes observed in the two appendages of this reptile. By far we were convinced about the specific participation of immune modulators in directing the course of variable wound healing results in the appendages of

lizard. As mentioned in earlier chapters, the time frame of wound healing in tail (4dpa) and limb (9dpa) is also variable due to altering status of inflammation. Thus, there is a striking difference in the healing microenvironments, which plausibly leads to differential repair results as well. Considering all these observations recorded so far, we tried to comprehend this study by trying to develop pro-regenerative microenvironment in limb as well. In order to attain this objective, we first used healing tail tissue (3dpa) as it marks reduction in inflammation (Chapter 1 and 2) and applied its homogenate on the healing limb. This ectopic application showed no betterment in the morphology of healing limb.

Since the idea was to simulate the 'pro-regenerative' condition in the otherwise scar-prone limbs, we re-calibrated our approach and considered using tail blastema for this pilot study. Blastema is a proven mediator of regenerative outgrowth and seemed to be a promising candidate for this preliminary study. Additionally, from a previous report in lab, we knew that lizard limb forms multiple layers of collagen beneath the epithelial cover, culminating into thick scar formation (Ranadive et al., 2018) and possesses heightened inflammatory status for a prolonged time period. Lizard tail on the contrary, witnesses a brief inflammatory response followed by complete tissue restoration. Thus, applying blastema homogenate (with high antiinflammatory character) on healing limb (having elevated immune response), would necessarily combat the rising proinflammatory mediators and check the overall level of inflammation. Therefore, we covered the healing limb with blastema homogenate and checked the scarring limb for any morphological or molecular changes, to be precise, any alteration in the inflammatory status and/or scar formation. Figure 3.2 depicts the intact tail and blastema as pool of progenitors.

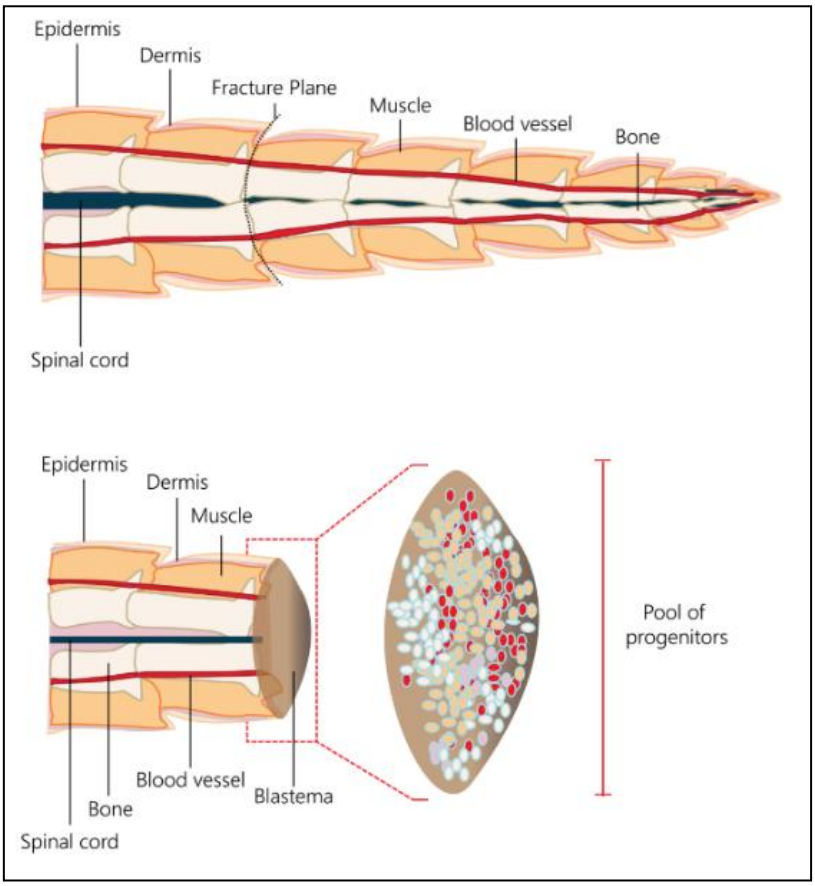

Figure 3.2: Schematic depiction of normal intact tail tissue and cell types constituting regeneration blastema

MATERIALS AND METHODS

Animals were categorised as per the time frames determined earlier. The Tail group consisted of 10 individuals, in which caudal autotomy was induced and the stumps were allowed to heal naturally until blastema stage is reached (5-6dpa). The blastema regions of regrowing tail were procured and homogenised in pre-cooled PBS (pH- 7.4).

On the other hand, limb group was divided into four sub-groups (0, 3, 6 and 9dpa) like earlier and the 'tissue homogenate of blastema' was applied aseptically using autoclaved round soft bristle paintbrush (Faber-Castell, India). on the scarring limb at an interval of 12 hours, repeatedly for 9 days. Image A in Materials and Methods section and figure 3.3 shown here, comprehend the procedure followed for this preliminary venture.

Figure 3.3: Blastema homogenate application on healing limb

The healing limb tissue was harvested at 0, 3, 6 and 9dpa to observe the phenotypic alterations along with the signalling variations caused due to ectopic application of blastema tissue homogenate on scarring limb. The blastema homogenate was first applied immediately after limb amputation and continued through the entire healing time of limb at an interval of 12 hours (i.e., throughout 9 days- twice daily). In order to achieve the said objective, harvested tissue chunks were processed for performing, Haematoxylin & Eosin (H&E) staining and western blot. The detailed protocol has been mentioned in Materials and Methods chapter.

RESULTS

Morphology: Blastema homogenate paces the healing process

On applying the blastema homogenate on healing limbs, the healing rate visibly increased. The healing frame of limbs throughout the 9 days, comparing the control (3.4A-D) and blastema treated groups (Figure 3.4E-H) are described here. A thick massive blood clot is observed at 3dpa stage in control group (Figure 3.4B) which is not found in the treated individuals (Figure 3.4F). The blood clot gets resolved within three days of amputation and a visible epithelial cover appears on the healing limbs under blastema treatment (Figure 3.4F-G). At 6dpa, the clot visibly shrinks and stiffens in control group (Figure 3.4C). Blastema treated limbs show thickened epithelium over the amputated region, while no

sign of blood clot is observed here (Figure 3.4G). At 9dpa, the clot starts to shed off and the 'healed' epithelium becomes visible, while in blastema homogenate treated individuals, the epithelium thickens and scales begin to reappear (Figure 3.4D; 3.4H). Overall, morphology of control and treated individuals clearly display the significant rise in rate of healing. Although the regenerative features are not visible at this preliminary stage, observing the histological details of these appendages provided leads to the internal alterations caused by the manual infestation of the blastema homogenate.

Histology: Blastema homogenate treatment alters collagen deposition in healing limb

Treating the healing limbs with whole blastema homogenate clearly reduces the time needed for healing of limb. Blastema homogenate also altered the structural organisation the healing microenvironment of treated limbs. This observation was made in the 9dpa stage limb tissue section, stained with H&E. As seen in figure 3.5A under 20X objective, thick collagen deposition is observed beneath the epidermis of control limb, while the treated limb shows thickened epidermis with numerous cells embedded the layers of collagen under 20X objective (Figure 3.5B). When observed closely, at higher magnification (40X objective), the firm collagen scar is observed in control section, with a thin layer of epithelium (Figure 3.5C), while treated tissue section has many cells in the apparently dynamic scar (Figure 3.5D).

Impact of blastema homogenate on immune profile of lizard limb

Present set of studies were planned to check the impact of immune mediators on the course of differential wound healing in tail and limb. In earlier chapters, role of chemokines and blood cells has been sited. Therefore, it was apparent to check the impact of blastema homogenate on the profile of immune mediators. Western blot analysis of a few chosen molecules was carried out in order to check the impact of blastema treatment on the protein profile of treated limbs microenvironment. We checked the trend of protein expression for COX-2, IL-10, TNF-α, IL-6, IL-22 and IL-17. Protein profile was scanned across the entire healing frame (0, 3, 6 and 9dpa) of limbs. Levels of COX-2 depleted significantly by 9dpa in blastema treated limb, while IL-10, an established antiinflammatory mediator visibly rose across the healing frame (Figure 3.6). TNF-α, levels increased at 3dpa stage as compared to resting (0dpa), while in the following 6dpa and 9dpa stage, the expression

reduced. IL-6 levels reduced continuously through the entire healing frame. IL-17 also followed the similar decreasing trend wherein at 9dpa the treated samples showed almost no band for molecule. IL-22, on the other hand showed a marked increase in protein expression at 6dpa, while its levels reduced significantly by 9dpa (Figure 3.6).

DISCUSSION

Blastema formation is the key feature for epimorphosis and its absence may lead to lack of regenerative forces (Alvarado and Tsonis, 2006). Apparently in the two appendages (tail and limb) in Lizard, presence and absence of blastema, dictates the outcome of wound healing. It may also be a prime causative entity responsible for differential outcome of wound healing in tail and limb. As per observations recorded in this primary series of experiments, it is clear that blastema has the 'healing power' which propelled the repair process in homogenate treated limbs.

Plausibly, blastema extract carries the 'scar-free programme', which shows its impact in the limb microenvironment too. Lizard limb lacks the power to regenerate, possibly because it does not provide a blastema friendly environment for it to develop. Miller and group (2019) have cited multiple reports, which state importance of progenitor cell survival to initiate any regenerative response. Traumatic injury such as limb amputation might cause rise in reactive oxygen species (ROS) levels as observed in *X. laevis* (Tseng et al., 2007), while it definitely shows hiked inflammation as observed in *H. flaviviridis* (Khaire et al., 2021). Both, ROS and injury can hinder blastema formation in this microenvironment, while they have been found to be extremely essential for tail regeneration in geckos (Zhang et al., 2016). Trauma induced death of important cells at the amputation site could be another reason leading to scar formation, instead of a creative process such as regrowth (Miller et al., 2019). On the other hand, in axolotls, caspase mediated cell death has been proven to be absolutely essential for successful appendage regeneration (Tseng et al., 2007). Providing blastema rich environment to the healing limb boosted the repair rate, probably due to presence of crucial progenitor cell extracts in the homogenate.

As reported by Ghayemi and group (2020), local transplantation of rabbit ear pinna blastema cells could promote tendon repair in the model. Scientific advancement has harboured new possibilities, such as cell therapies, surgical grafting (Gaspar et al., 2015),

but model-based limitations do not support such endeavours in lizards. Hence application of blastema extract on the healing limb was envisaged for recording its preliminary effect on scarring limb. The histology data obtained here, clearly show newly formed collagen being 'different' in the control and blastema treated individuals. Thickness of the collagen fibres has remarkably reduced in blastema homogenate treated limbs, along with the presence of various cell populations intercalating the collagen layers. Blastema homogenate might have helped in improving the matrix quality which can be easily seen in through the sections.

Reportedly, scar maturation proceeds post 9dpa in a normally healing lizard limb (Ranadive et al., 2018) while in case of blastema treated limbs, the stiffening and scar maturation began by as early as 6dpa, as observed in present results. This observation lucidly explains the participation of progenitors in early wound healing which are regularly observed in lizard tail repair. Although cues for regeneration arising from blastema are yet to be characterised, distinct improvement in healing routine was observed here. Further, major inflammatory cytokines have shown altered protein expression to confirm their regulatory contribution in the overall healing blastema treated appendage.

COX-2, a well-established regulator of inflammation showed reduction in protein levels on application of blastema homogenate. As proven by researchers in past and reiterated in our own results (Chapter 1), COX-2 triggers various proinflammatory mediators and causes rise in inflammation in the microenvironment (Kang et al., 2007, Kalinski 2012; Khaire et al, 2021). Ectopic application of blastema homogenate visibly curbs the levels of COX-2 expression and this might in turn reduce the resultant inflammation as well. This observation was backed up by the protein level of IL-10, a pivotal antiinflammatory mediator, which rose simultaneously throughout the healing stages of the treated limb. Heightened IL-10 expression suggests decrease in inflammatory status, which could be a result of receding COX-2 levels in the microenvironment. This interaction can be further explored by checking the status of prostaglandins and EP receptors active at this site of action.

Protein levels of other proinflammatory mediators such as TNF-α and IL-6 also reduced under blastema treatment reinforcing the idea of reduced inflammation being supportive for accelerated tissue healing. Also, it is well-known that TNF-α and IL-6 regulate

recruitment of multiple cellular and humoral inflammation boosters in the microenvironment (Hinson et al., 1996; Aoki and Narumiya, 2017). It would be interesting to study the overall impact of blastema treatment on complete immune profile of the treated appendage. We also observed IL-17 reducing gradually at every healing stage with simultaneous rise of IL-22 protein level. We have observed similar results in tail microenvironment, where successful tissue regeneration is observed (Chapter 1). These results suggest early tissue repair and reorganisation occurring at the limb healing site. Although regenerative outcome was not noted in treated limbs, massive reduction in the protein levels of major proinflammatory mediators, with simultaneous rise in antiinflammatory ones, clearly underlines the role of blastema homogenate in controlling inflammation.

CONCLUSION

Blastema holds the key mediators provoking scar-free wound healing in lizard tail and it can modulate and increase the healing rate in an otherwise slowly scarring limb. Even though this is a pilot study, its results open multiple avenues for future explorations. Transcriptional and proteomic characterisation of blastema might answer numerous queries regarding differential wound healing profile as observed in the appendages of lizard. The preliminary study conducted here, and the observations made are comprehended in the chapter summary (Figure 3.7).

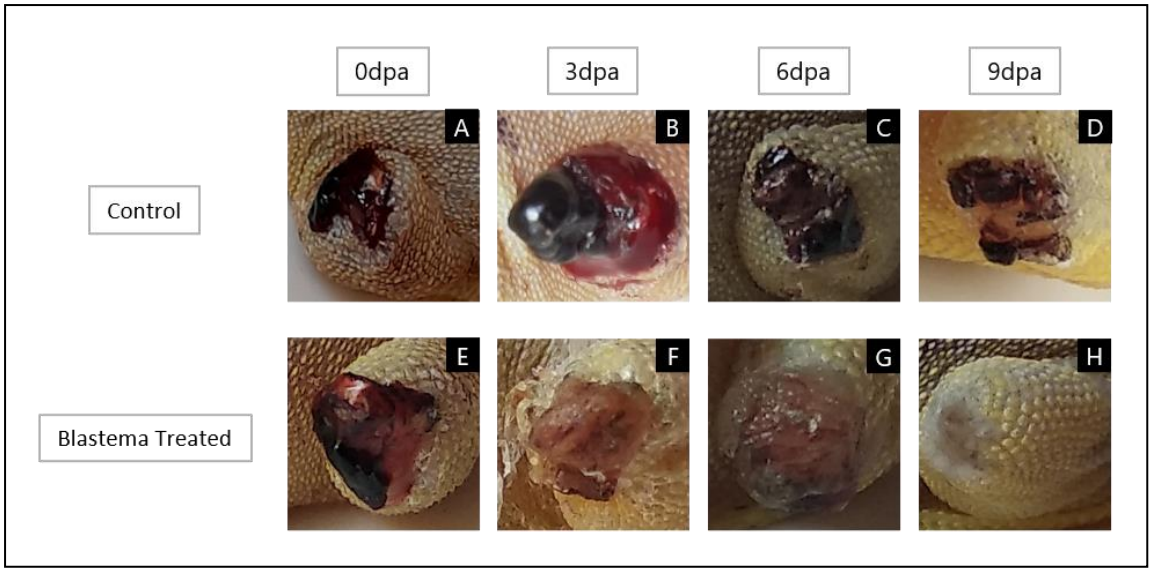

Figure 3.4: Morphology of healing limbs

A-D: Healing lizard limb morphology across the healing frame in control animals; E-H: Healing lizard limb morphology across the healing frame in treated animals. Blastema was harvested and pooled from 10 lizards. (n = 3).

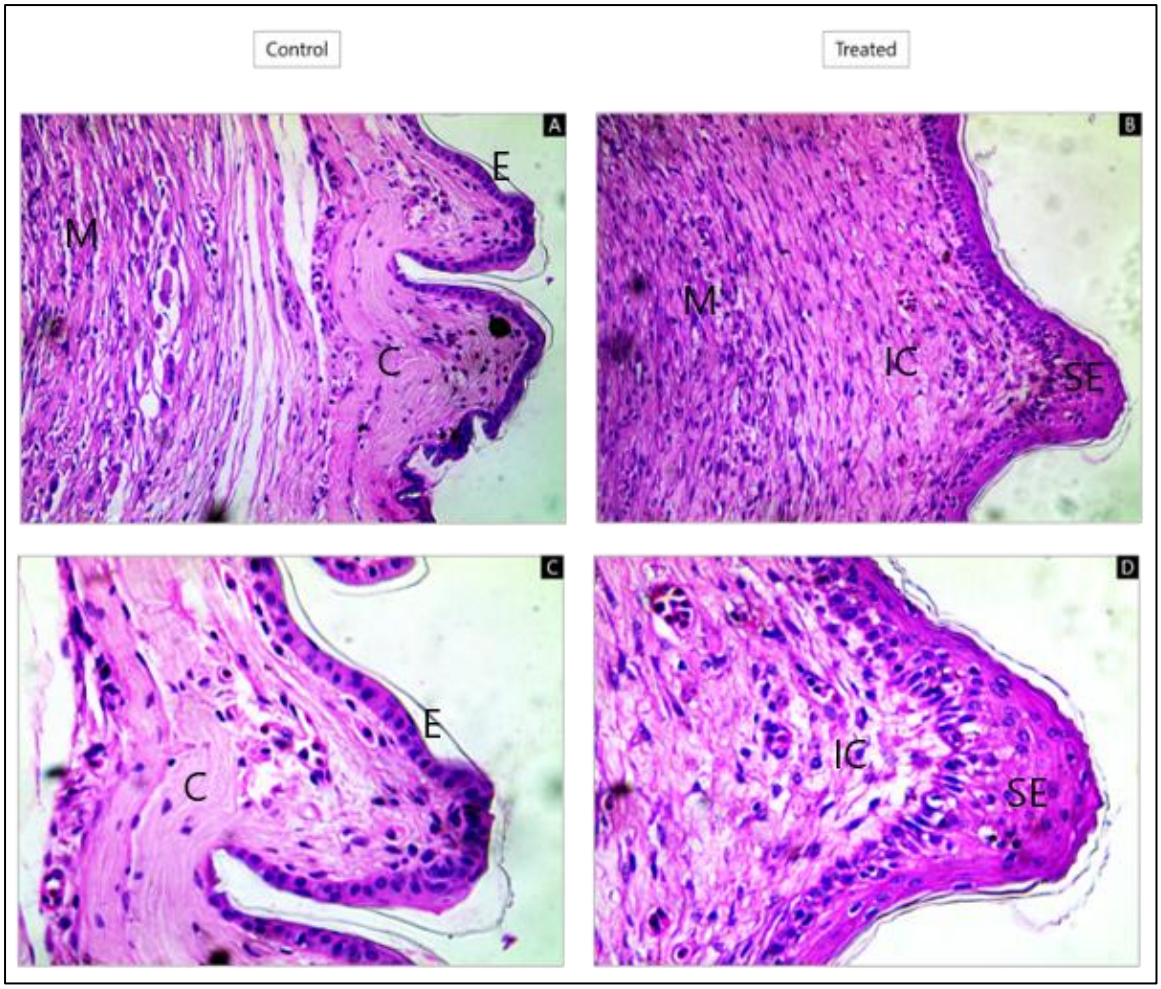

Figure 3.5: Limb histology panel

A: H&E stained 9dpa limb section of control lizard limb (20X objective); B: H&E stained 9dpa limb section of blastema treated lizard limb (20X objective); C: Thick collagen deposition as observed under 40X magnification in control limb (9dpa); D: Stratified epithelium and various cell types dispersed in collagen layers as observed under 40X magnification in treated limb (9dpa). E: Epithelium; C: Collagen; M: Mesenchyme; SE: Stratified Epithelium; IC: Interrupted collagen.

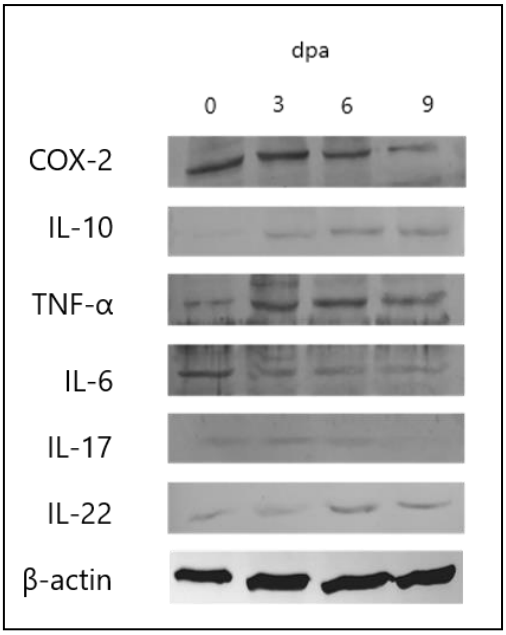

Figure 3.6: Western blot panel of blastema treated limb

Protein expression profile of COX-2, IL-10, TNF-α, Il-6, IL-17 and IL-22 across the healing frame of blastema treated limb (n = 3). β-actin was used as a loading control.

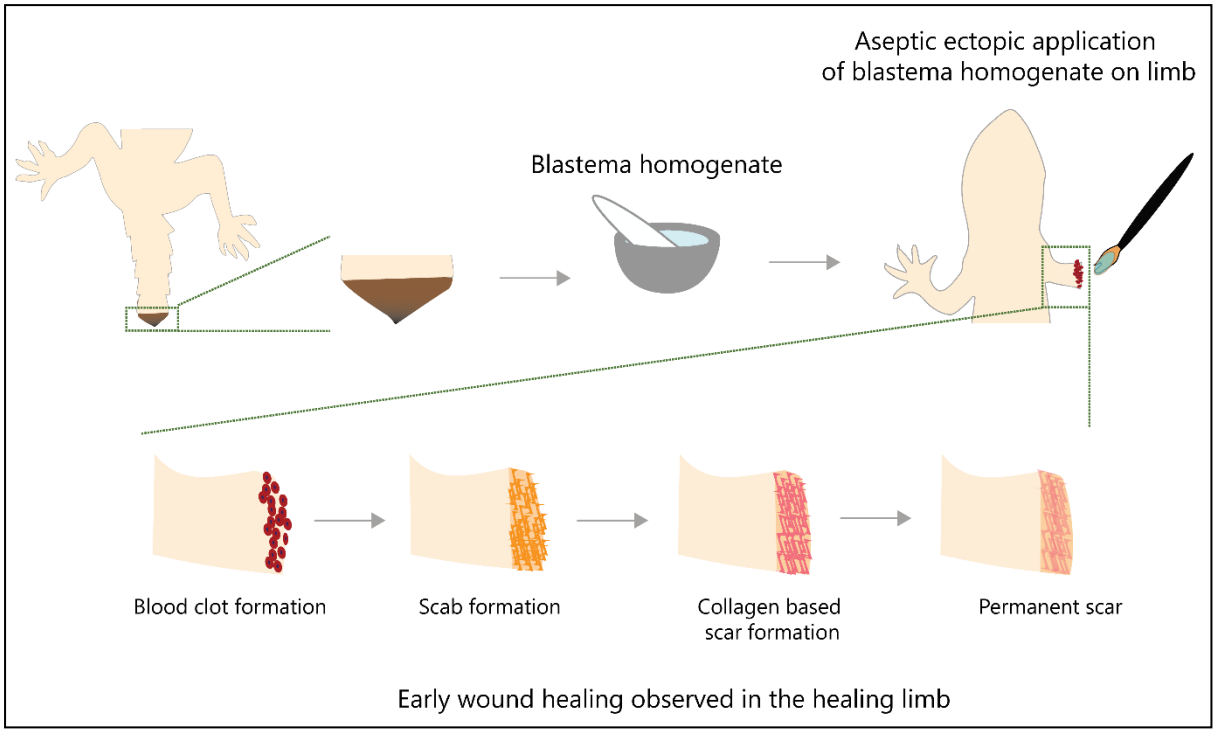

Figure 3.7: Chapter Summary