UNDERSTANDING THE ROLE OF CYCLOOXYGENASE IN THE TEMPORAL REGULATION OF INFLAMMATORY MEDIATORS DURING WOUND HEALING IN LIZARD

[EXECUTIVE SUMMARY OF Ph.D. THESIS]

October-2021

KASHMIRA SATISH KHAIRE

Department of Zoology, Faculty of Science,
The Maharaja Sayajirao University of Baroda,
Vadodara-390 002, India

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EXECUTIVE SUMMARY

INTRODUCTION

In the dynamic journey from a single cell to a highly complex, articulate living system, multicellular organisms have learnt to repair their respective physical system in an orderly fashion (Morgan, 1901; Brockes et al., 2001; Kowald et al., 2020). Organ repair provides the living forms a chance to suit the surrounding requirements and thrive to the best of their potential. It operates in multiple animal groups and at variable intensities (Suzuki and Mittler, 2012).

Animals regenerate the injured or lost tissue through four major modes (Alvarado and Tsonis, 2006; Gilbert, 2014).

a. Morphallaxis: Rearrangement of pre-existing tissues/cells
b. Stem cell-mediated regeneration: Activation of resident unipotent stem cells
c. Epimorphosis: De-differentiation, proliferation and re-differentiation
d. Compensatory Regeneration: Hypertrophy and Hyperplasia

These four routes contour to heal and repair the lost tissue, ideally by replacing the original part (Poss, 2010; Tanaka and Reddien, 2011). Bely and Nyberg (2010) discussed that the multifaceted event of regeneration is primarily classified in five subtypes, predominantly based on the degree and intensity of repair observed.

a. Whole-body regeneration, e.g.: Hydra and Planaria (Chera et al., 2009; Rink, 2018)
b. Structural regeneration, e.g.: Axolotl and Salamander appendages; Teleost fish tail (Tanaka et al., 2016; Patel et al., 2019; Arenas Gomez et al., 2020)
c. Organ regeneration: e.g.: Tail regeneration in lizards; Heart regeneration in Zebrafish; Lens regeneration in newts (Sousounis et al., 2015; González-Rosa et al., 2017)
d. Tissue regeneration: e.g.: Gut lining regeneration in Drosophila (Belacortu and Paricio 2011; Worley et al., 2012)
e. Cellular regeneration: e.g.: Axon regeneration in *C. elegans* (Ghosh-Roy and Chisholm, 2010; Basu et al., 2017)

Apparently, invertebrates seem to have won the restoration race despite being placed lower than the advanced groups such as reptiles and mammals. However, higher vertebrates also

have some representation on the regeneration front wherein members of the classes such as Pisces, Amphibia and Reptilia possess limited restorative ability. All these clans are endowed with epimorphic potentials to regrow their lost body parts. All organisms eliciting epimorphosis follow a definite path of events, wherein, post-injury, the exposed tissue forms a wound epithelium, which further leads to the formation of 'regenerative blastema' through de-differentiation. Eventually, blastemal cells undergo re-differentiation (after repeated cycles of proliferation) to reconstruct the structural and functional replica of the original tissue (Muneoka et al., 1986; Reddien and Alvarado, 2004; Agata et al., 2007; Kierdorf et al., 2007).

Epimorphosis holds the technical details of regeneration machinery, governed and guided by wound repair, a cardinal step directing the course and chronology of repair events (Roy and Gardiner., 2002). The process of wound repair is organised by a synchronised set of events, Inflammation, Proliferation and Remodelling. Ratio, proportion and time span of each sub-event constructs either pro-regenerative or scar making microenvironment.

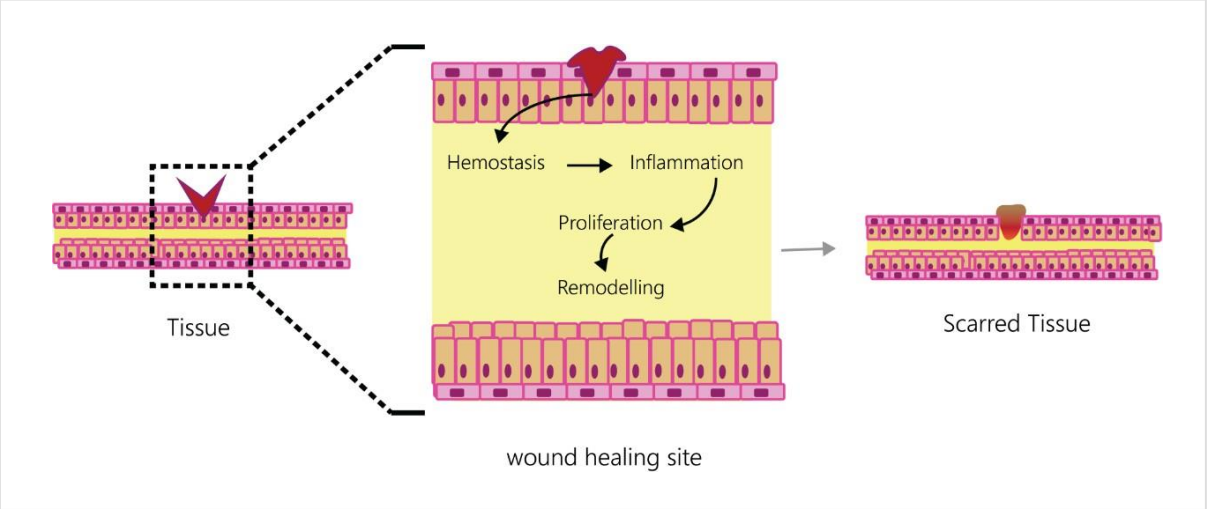


Figure I: Proceedings at the wound healing site

Of the events of wound repair, the first step is haemostasis, which registers the trauma of injury and prepares the wounded site for the following course of inflammation (Shaw and Martin, 2009). Inflammation is a consolidated product of multiple cellular and molecular annexed pathways participating in systemic immunity, a cardinal step to combat any pathogenic influx (Takeo et al., 2015). During this cellular warfare, the healing front is developed for the next steps in line, i.e., 'cell proliferation' and 'remodelling' as illustrated in figure I. Among all the cellular processes managing this repair hotspot, inflammation is the

first to initiate immediately after injury (Eming et al., 2007; Kyritsis et al., 2012; Karin and Clevers, 2016). It is a process designed to meet the emergencies with all forces diverted to chase and kill any foreign entity attempting to invade the body (Singer and Clark, 1999; Nourshargh and Alon, 2014). Being the composite outcome of a large number of molecular events, numerous accomplices modulate inflammatory pathway. Primarily they are either cells of immunity or secretory molecules released by these cells (Henry and Garner, 2003).

Hematopoietic stem cells (HSCs) originating from the bone marrow are made to reach all tissues of the living system, where they are specified as either myelo-erythroid progenitors or lymphoid progenitors. These progenitors are responsible for forming a huge consortium of immune cells, which are localised at the peripheral vasculature around the tissues or present in circulation. These cells create the microenvironment, which determines the level of inflammation locally and also the resultant effect on the healing process. Along with the cellular components, their secretions, termed cytokines drive inflammation and tissue repair mechanisms through various signalling pathways activated (Cameron and Kelvin, 2000). All these regulators function in coherence with each other to govern the signalling cascades of inflammation and based on their action and activity, they are categorised as:

i. Proinflammatory mediators
ii. Antiinflammatory mediators

As per their respective functions, molecules are either called proinflammatory, which promote and aid inflammation, while others who lead to its resolution are called antiinflammatory. It is equally noteworthy that the cellular players, once contributing to promote inflammation, can further evolve to abate it in context-specific fashion (Rosique et al., 2015; Ellis et al., 2018). Cells of immunity such as neutrophils and monocytes, derived macrophages, lymphoid cells (B lymphocytes and T lymphocytes), released cytokines, neuropeptides, leukotrienes, prostaglandins (PGs), proteases, hydrolases and Reactive oxygen species (ROS) - all are the primary proinflammatory mediators, introduced to the healing site depending on the stage of repair (Chow et al., 2005; Lawrence, 2009). Neutrophils are the first cluster of cells rushed to a trauma site. Early on the repair front, they function in accordance with interleukins (ILs) released locally and remove away the cell debris. Damage-associated molecular proteins (DAMPs) and Pathogen associated molecular proteins (PAMPs) bind to the neutrophil surface receptors and activate them. Neutrophils and chemokines produced by them are crucial for recruiting other inflammatory agents such

as macrophages, T cells, and ILs. Neutrophils also recruit cell proliferation and angiogenic factors like Vascular endothelial growth factor (VEGF), Membrane cofactor protein-1 (MCP-1) and Epidermal growth factor (EGF) (Engelhardt et al., 1998; Theilgaard-Mönch et al., 2004). The entire routine is swiftly performed and the resultant apoptotic neutrophils are cleared off by none other than macrophages. This cell clearance primarily works as a stop signal for tissue-specific inflammation (Ferrante and Leibovich, 2012).

Classically activated M1 macrophages lead to heightened inflammatory action and release an inundation of proinflammatory interleukins such as IL-1β (Zhou et al., 2014), IL-6 (Schafer et al., 2007) and Tumor necrosis factor- α (TNF-α) (Kroner et al., 2014). Once the cell debris and other potentially harmful entities have been cleared, the same macrophages are 'alternatively activated' into their antiinflammatory forms, named as M2 type (Yunna et al., 2020). They also release many interleukins, but of antiinflammatory in nature, which promote the reduction of local inflammation. This drastic shift of events prepares the cells of surrounding tissue to undergo both genotypic and phenotypic changes, thus driving them towards the regenerative phenomenon. Otherwise, the microenvironment remains arrested in the catastrophic state and does not allow any growth or healing (Mia et al., 2014; Da Silva et al., 2015). As shown in figure II, based on all characters attributed to this process, inflammation is either Acute/Reparative Inflammation or Chronic Inflammation (Ryan and Majno, 1977; Rankin, 2004).

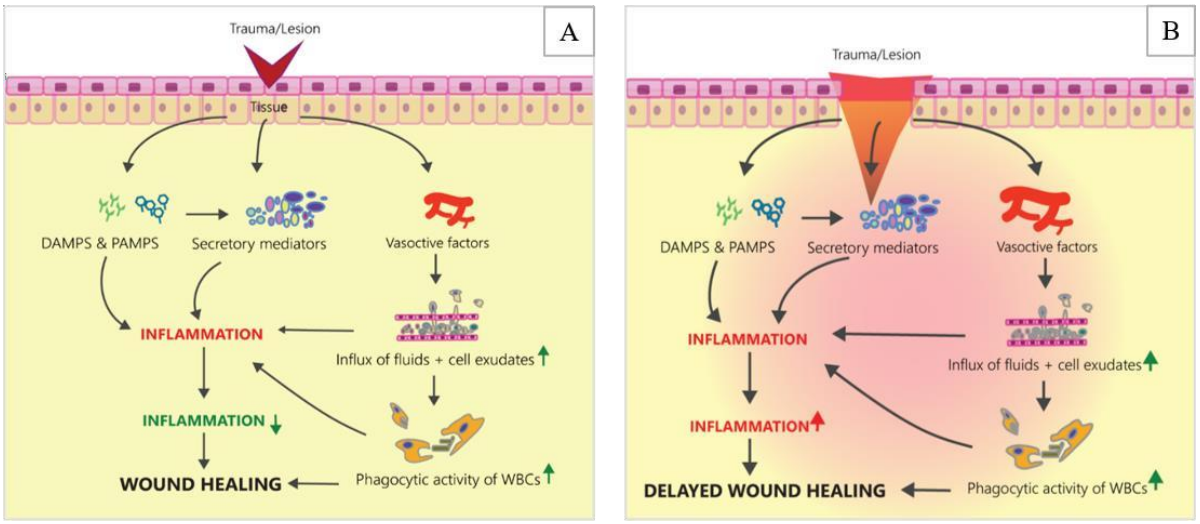


Figure II: Wound healing effect under A: Acute; and B: Chronic inflammation

Post-injury, an intensified blast of the immune system drives acute inflammation, predominantly short-lived and immensely effective (Ryan and Majno, 1977; Ariel and Serhan, 2007). On the other hand, prolonged stay of unresolved inflammation maintains a harsh, intolerable wound environment and, in the process, jeopardizes the very existence of the healthy tissues around. This unresolved immune response is termed 'chronic inflammation' (Tidball, 2011; Oishi et al., 2018).

Owing to tissue injury, along with interleukins and cells of the immune system, a troop of eicosanoids is activated parallelly. These are one of the primary mediators of inflammation, supporting both its initial rise and gradual resolution (Khanapure et al., 2007). Proinflammatory mediators released during the rigorous cellular events post-injury also induce phospholipases production, which in turn catalyses the release of membrane associated arachidonic acid (Langenbach et al., 1995). Injury to tissue leads to release of cyclooxygenase (COX) family of enzymes, which acts on the available arachidonic acid and converts it into active prostaglandins (Ricciotti and FitzGerald, 2011).

Animal models lacking early availability of prostaglandins have compromised immune response (Tilley et al., 2001; Liang et al., 2005). There occur to be three members in the cyclooxygenase family, COX-1, COX-2 and COX-3 (Simmons et al., 2004). The last one being a spliced gene variant of the first isoform (Willoughby et al., 2000; Simmons et al., 2004). COX-1 is a proven organiser of tissue homeostasis and its absence or inhibition causes a multitude of developmental anomalies. The COX-2, on the other hand, functions predominantly under the inflammatory milieu (Seibert et al., 1994; Kuwano et al., 2004). It is a cardinal regulator of inflammation and its expression profile can be altered via drugs such as NSAIDs (Lee et al., 2009). The present study is one such venture, wherein the focal point of the project was to observe the congruence in COX-2 derived inflammation and wound healing, and how the former governs the differential healing outcome in the same model organism.

Unravelling the molecular interplay of COX and inflammation has been well documented for decades (Mahajan and Sharma, 2005; Lu et al., 2017), however, we were keen to understand the regulation of inflammation through the COX-2 pathway, its timely effect on wound repair and its participation in the formation of both, scar-free regenerate tissues and permanent scars. For this venture, *Hemidctylus flaviviridis* was used, as regeneration potential is

confined to its tail. Induced tail autotomy results in wound epithelium formation (by 4dpa) that further leads to constructing blastema, owing to complex molecular crosstalk amongst epithelium and mesenchymal cells beneath. The blastema is an aggregate of de-differentiated progenitors, which eventually re-differentiate to form the structural and functional replica of the original tissue, through epimorphosis. On the contrary, lizard limb forms a thick collagenous scar, interrupting the crosstalk between epithelial and mesenchymal cells. Herein, the wound heals slowly with a remarkable deposition of collagen that matures to form a scar.

The variation in the extent of inflammation is long suspected to be a reason behind the biased wound healing and also the resulting differential regeneration potential exhibited by the lizard's tail and limb. Studies by Sharma and Suresh (2008) as well as Buch and coworkers (Buch et al., 2017; 2018) have shown that COX-2 derived PGE_2 is one of the early response inflammatory signals arriving at the wound micro-niche post autotomy. Any attempt to impede the activity of COX-2 resulted in delayed wound healing and loss of regeneration (Sharma and Suresh, 2008). Therefore, we hypothesised that the level of COX-2 induced PGE_2, a master regulator of the inflammatory mediator, could be different at the amputation site of tail and limb in lizard which may leads to differential expression of other inflammatory mediators in a context specific manner resulting in scarring of limb and 'superhealing' followed by regeneration of tail post-amputation.

RESEARCH METHODOLOGY

In order to check our hypothesis, a detailed study was devised with one primary objective, which was to study the temporal expression pattern of cyclooxygenase and ascertain its role in the regulation of inflammatory response during wound healing in the appendages of lizard *H. flaviviridis*. Specific aims were formulated to achieve this objective, results of which are comprehended in the three chapters of this thesis. The timepoints determined for the observation were based on the pivotal hallmarks of healing observed in the tissues - haemostasis, inflammation, granulation followed by proliferation and wound epithelium formation. For tail, 0 (resting), 1, 2, 3 and 4dpa (days post-autotomy) and for limb, 0, 3, 6 and 9dpa (days post-amputation) were considered for this study, owing to the difference in repair time needed for these appendages and based on the previous studies in the lab (Buch et al.,

2017; Ranadive et al., 2018). Techniques such as Enzymatic Analysis, Immunohistochemistry, SDS-PAGE, Western blot, q-RT-PCR, FACS and WBC counting were deployed at various instances.

KEY FINDINGS

Of the three specific aims, the first was to evaluate the roles of COX-2 driven inflammation in orchestrating differential wound healing in the tail and limb by observing the temporal status of humoral inflammatory mediators in the disparate microenvironments (Chapter 1). Enzymatic activity of COX-2, the concentration of PGE_2, followed by protein (Western blot analysis) and gene expression (q-RT-PCR analysis) status of various pro and antiinflammatory interleukins were evaluated. The COX-2 activity elevated from 2dpa onwards in lizard tail, while in limb it was relatively high and increased progressively at the following time points. As COX-2 belongs to the family of early response genes and is strongly induced by mitogenic and proinflammatory stimuli (Lasa et al., 2000), protein and transcript levels of COX-2 were checked in the next step. In resemblance to its hiked activity, COX-2 protein and gene levels were also found to be elevated till the 3dpa stage in tail. This suggests the participation of COX-2 in modulating early inflammation, which is reduced at 4dpa, during proliferation and epithelialisation. On the contrary, in limbs, COX-2 gene expression increased from the basal level till the terminal time point of 9dpa. This suggests mRNA stabilisation in limb tissue, due to elevated proinflammatory interleukins as found in human bones, macrophages and granulosa cells by Kang and coworkers (Kang et al., 2007).

COX-2 based PGE_2 formation, is boosted by proinflammatory cytokines, governing its transcriptional and post-transcriptional levels (Kang et al., 2007). PGE_2 expression followed a trend of COX-2 activity in tail, while in limb, it showed a significant decrease after 3dpa, until 9dpa. Interestingly, the basal level of PGE_2 in limb (0dpa) is higher than the terminal time point for tail (4dpa). As suggested previously by many research groups, this disparity could be an impact of differential and tissue specific COX-2 function (DuBois et al., 1998; Simmons et al., 2004; Tsatsanis et al., 2006). Also, other inflammation curbing prostanoids might participate in causing early resolution and resultant super healing in tail, while contrasting results are observed in limb (Bos et al., 2004; Korbecki et al., 2014).

Owing to prolonged inflammation as evident from elevated gene and protein levels of various proinflammatory mediators such as COX-2, PGE_2, EP2, TNF-α, iNOS, IL-6, IL-17 and IL-22, throughout its healing frame i.e., from 0dpa to 9dpa. In the tail, inflammation spiked immediately post-injury (from 0dpa to 2dpa) and resolved quickly (at 3dpa and 4dpa), allowing all the following formative processes. COX-2 and PGE_2 levels rose during the initial two days after tail amputation, but they were reduced from 3dpa onwards and so did all the proinflammatory mediators. EP4 increased gradually from 0dpa to 4dpa in tail, which supports the reduced inflammatory status observed in this appendage. IL-10, a pivotal antiinflammatory mediator, was found to be increased during the healing frames of the tail. In limbs, gene and protein levels of IL-10 gradually reduced at 3, 6 and 9dpa compared to the resting stage (0dpa). Interesting functional coherence was observed amongst COX-2 derived PGE_2, EP2 and EP4, which led to increased levels of inflammation in limb (PGE_2-EP2 based) and reduced inflammatory profile in the tail (PGE_2-EP4 based).

IL-17 and IL-22 also showed a peculiarly differential behaviour in the two appendages. IL-17 showed a significant reduction in gene expression traversing all the time points, for tail group, after the early inflammation (2dpa). This supports the idea that the reduction of chief proinflammatory mediators cause an overall decline of inflammation at tissue level in the tail and promotes regeneration supportive wound healing. As opined by Veldhoen and group (2006), reduction in IL-17 expression can be a coherent effect of another regulatory mediator like IL-6, which has shown a significant decline in tail. It could even be due to the specific signalling dictated by the EP receptors (Hinson et al., 1996). Nevertheless, in limb tissue, IL-17 was elevated, except when scar formation and collagen deposition started at the site of healing. IL-22 in the tail tissue showed a well-pronounced increase in its transcripts from 1dpa till 3dpa, after which its level reduced significantly. This ensures its participation in early epithelialisation, as achieved in tail (4dpa). IL-22 elicits a protective role when combined with IL-17, which induces explicitly anti-microbial peptides in human keratinocytes (Sabat et al., 2013).

In limb, IL-22 followed the trend of IL-17, with a noticeable rise in gene expression at the time of scab formation in limb tissue, in unity with other proinflammatory mediators like IL-6, TNF-α, iNOS and IL-17. It is thus proved that IL-22, in coherence with IL-17, reconstructs the framework for scar-free healing in tail, but supports scar formation under the prolonged inflammatory response in limb. Discovering this novel participation of IL-17 in the

regeneration model recommends further investigation, where the performance of this cardinal inflammatory mediator can be explored. Our observations, thus, clearly demonstrate the impact of early resolved or prolonged inflammation on the disparate wound healing outcomes as observed in tail and limb, respectively.

The second aim was to assess the presence and roles of cellular mediators of inflammation in the two healing microniches viz., the tail and limb. Both conventional (Total WBC count) and FACS methods were deployed to check the tissue-specific and systemic blood cell profile on autotomy of tail and amputation of limb. Further, we studied the expression status of various immune cells in the immediate microenvironment of both healing tail and limb. We also checked the trend of expression and recruitment of these cells on the systemic front. We attempted the contemporary technique of FACS, to decipher various blood cells' local and systemic roles in inflammation and wound healing. Although we did not receive any conclusive directive in this venture, our total WBC count method indeed clarified the image for us. We recorded an astounding rise in the levels of leukocytes during the elevated inflammatory period in the limb. Both tissue environment and systemic levels of WBCs were found high in the case of the amputated limb. At the same time, in the tail, these cell numbers increased immediately post-injury and settled close to basal level at the end of wound healing. All the cells identified in the lizard blood smear and total WBC count have been well recorded, analysed and presented in Chapter 2.

For the third aim we tried to simulate the 'tail-like' pro-regenerative conditions in the otherwise scarring limb. Since the regeneration promoting blastema formation is confined to the tail and does not occur in the scarring limb, we tried to bring these two facets of wound healing together and simulate the 'tail-like environment' in limbs of lizards. As a pilot step, we applied 3dpa tail homogenate on the healing limb ectopically throughout its healing time, i.e., 0 to 9dpa. We observed no significant change in the morphology or any improvement in the healing pattern of the treated limb. Further, we checked the impact of blastema homogenate on the healing limb, wherein treated limbs achieved wound healing and scarring earlier than the control ones.

We then attempted to study the molecular changes appearing in the treated appendages compared to the control ones. We received exciting results in this preliminary analysis, wherein applying blastema homogenate on the amputated limb has reduced the healing time

in the treated animals (Chapter 3). A comparative analysis of the Haematoxylin & Eosin (H&E) stained sections of control and blastema homogenate treated limbs clearly showed the interrupted collagen layers in the scar tissue. The epithelium of the healed limb was also multi-layered and many cells were found embedded in the collagen layers. We also checked the status of inflammatory mediators in the tissue chunks of limbs treated with blastema homogenate and found a drastic reduction in their levels of proinflammatory ones (COX-2, TNF-α, IL-6, Il-17) as compared to control samples (Chapter 3). Meanwhile, antiinflammatory mediators namely, IL-10 and IL-22, increased gradually from 0 to 9dpa stage progressively on blastema treatment.

CONCLUSION

Overall, our study proves the cardinal role of COX-2-PGE_2 derived inflammation in steering the differential wound healing episodes in the appendages of *H. flaviviridis*. Both cellular and humoral mediators of inflammation contribute to either early resolution of inflammation as observed in the tail of the gecko or its persistent elevated levels as found in the limbs. Further, blastema formation and its components seem to plausibly hold the cues for scar-free regeneration, as seen in the tail of the lizards. It possesses all the molecular signals of early repair, which visibly expedite the healing process in the limb, upon its ectopic application on healing limbs. Characterising blastema and observing its components can answer our queries regarding the loss of regenerative abilities in higher vertebrates.

Meanwhile, assessing the complete prostanoid profile in the different appendage microniches and evaluating their tissue-specific receptor expression can provide a holistic idea about the effector inflammation at the healing site. The promising results obtained here, such as the increased healing rate and interrupted scar formation, open up new research avenues to explore wound healing mediated regenerative mechanisms. A graphical summary of the entire work is presented in the following image (Figure 1).

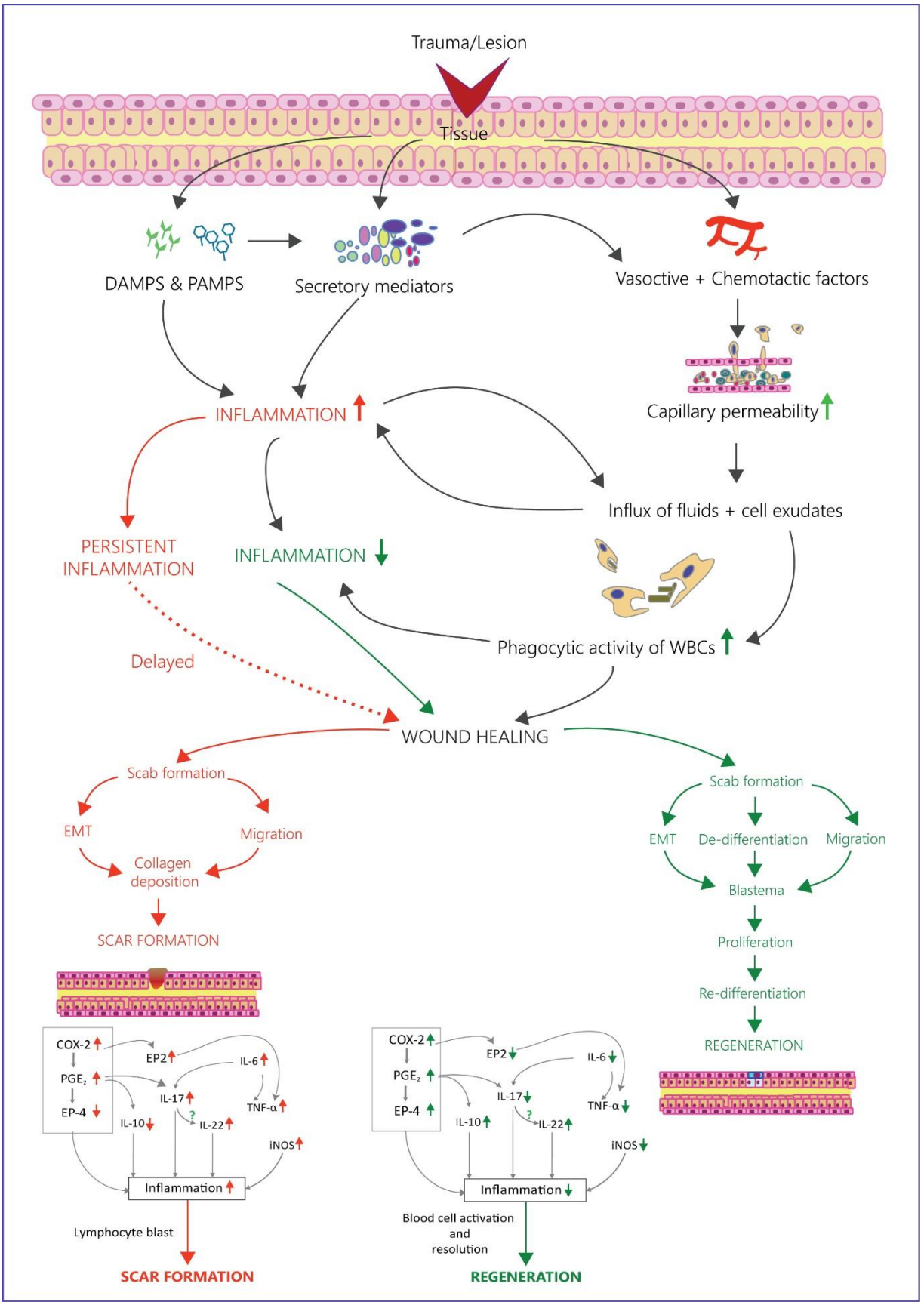
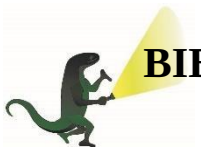


Figure 1: Summary of entire Ph.D. work



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