Discovering Which Macrophage Subtypes Are Involved in Regeneration

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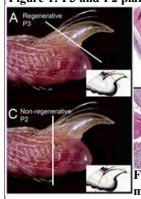
Introduction

Regeneration is a phenomenon noted in several different animal species. For example, certain lizards salamanders and fish, all share the ability to regenerate lost or damaged bone, tissue, nerves, muscle, tendons, skin, etc. Humans, however, do not share this ability. If a person loses a limb, a finger, or simply endures a deeper tissue injury, the lost or damaged tissue will not regrow. Instead, scar tissue forms over the area of injury.

However, humans are not entirely barred from regeneration. In both humans and mice, when the distal third of a digit tip is severely damaged or amputated entirely, the bones, ligaments, nerves, skin, nail all fully regenerate [3]. Thus, after this distal third is completely amputated, when given enough time, the digit tip regenerates completely, creating a new digit tip that mimics the amputated tip [3]. **Figure 1** details the two planes of amputation used to gather samples from regenerative and non-regenerative locations. **Figure 2** shows the complete regeneration of a mouse digit tip.

Figure 1: P3 and P2 planes of amputation [3]



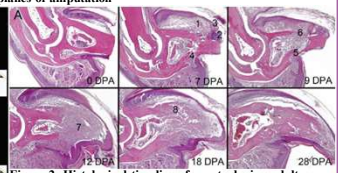
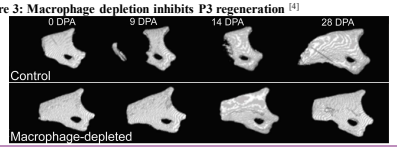


Figure 2: Histological timeline of events during adult mouse digit regeneration following a distal P3 amputation[3]

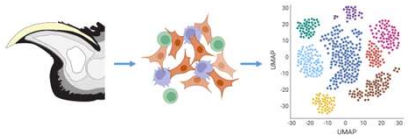
Following injury in this location, macrophages flood the damaged tissue, leading to inflammation, histolysis, re-epithelialization, revascularization, and cell-proliferation at the point of injury [4]. The exact role of each macrophage depends on its subtype; for example, whether it is pro-inflammatory, anti-inflammatory, etc. While we have shown that macrophages in general are responsible for regeneration, it is unknown which subtypes of macrophages are specifically responsible [4].

If macrophages are responsible for driving regeneration over scar-formation, we expect to find populations of macrophages that are unique to the regenerating digit compared to the non-regenerating digit amputation. Knowing which macrophage subtypes are present in the regenerating digit tip will help us identify which cells to target for future therapies.

Figure 3: Macrophage depletion inhibits P3 regeneration [4]



Methods



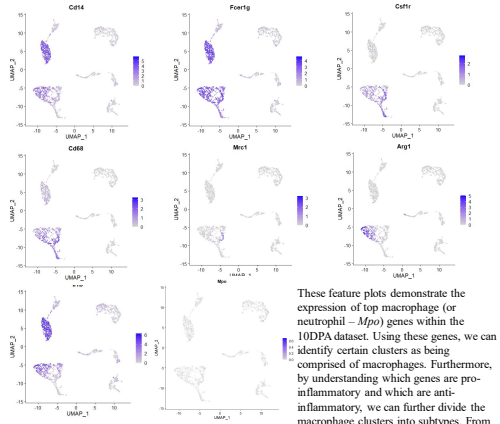
- First, cells were taken from both P3 and P2 amputations (see **Figure 1**) of mouse digit tips at 10 days post amputation (10DPA) and 14 days post amputation (14DPA).
- Next, we uploaded the single cell data from these samples into R Code, and analyzed the data using Seurat, a package of R Code that specializes in single-cell genomics.
- Using Seurat, we grouped all cells within each sample into clusters and plotted these clusters in a UMAP format. Cells are clustered by similar gene expression.
- Once plotted, we analyzed the UMAPs and used the top 50 differentially expressed genes to identify macrophage clusters. To compare more thoroughly, we also created feature plots of some of these genes to create a visual representation of the expression of each gene within the clusters.
- Then, we cross-referenced between the original UMAP clusters, UMAPs divided by cell source detailing whether each cell within the clusters is from the P3 regenerating amputation or the P2 non-regenerating amputation, and feature plots highlighting specific traits to discover which macrophage subtypes are more present in regenerating samples than non-regenerating samples.

Feature Plots

Figure 4: Table of macrophage gene expression, divided into inflammatory and anti-inflammatory subtypes [5][6][7]

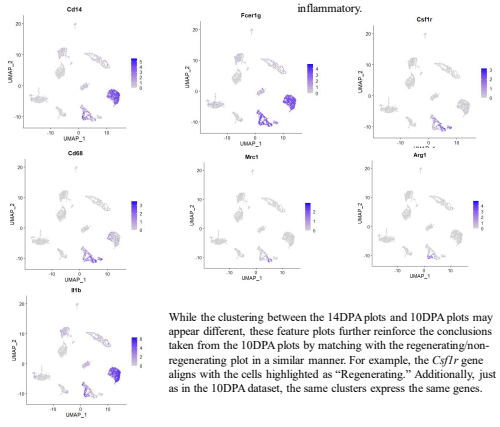
Macrophage Gene Expression			
	Inflammatory	Anti-inflammatory	Reference
Cd14	Hi	Hi	Dang 2020; Eichenfield 2016
Fcer1g	Hi	Hi	Dang 2020
Csf1r	Mid	Hi	Eichenfield 2016
Cd68	Mid	Hi	Eichenfield 2016
Mrc1	Lo	Hi	Gensel 2015
Arg1	Lo	Hi	Gensel 2015
IL1b	Hi	Lo	Gensel 2015

Figure 5: Feature plots of *Cd14*, *Fcer1g*, *Csf1r*, *Cd68*, *Mrc1*, *Arg1*, *Il1b*, and *Mpo* expression in the 10DPA dataset



These feature plots demonstrate the expression of top macrophage (or neutrophil – *Mpo*) genes within the 10DPA dataset. Using these genes, we can identify certain clusters as being comprised of macrophages. Furthermore, by understanding which genes are pro-inflammatory and which are anti-inflammatory, we can further divide the macrophage clusters into subtypes. From these plots, we can tell that clusters 0 and 1 are pro-inflammatory macrophages while clusters 4 and 8 are anti-inflammatory.

Figure 6: Feature plots of *Cd14*, *Fcer1g*, *Csf1r*, *Cd68*, *Mrc1*, *Arg1*, and *Il1b* expression in the 14DPA dataset



While the clustering between the 14DPA plots and 10DPA plots may appear different, these feature plots further reinforce the conclusions taken from the 10DPA plots by matching with the regenerating/non-regenerating plot in a similar manner. For example, the *Csf1r* gene aligns with the cells highlighted as "Regenerating." Additionally, just as in the 10DPA dataset, the same clusters express the same genes.

UMAPs

Figure 7: UMAP plot of the 10DPA dataset alongside the same UMAP specifying whether each cell originates from a regenerating or non-regenerating sample (key macrophage clusters marked with arrows)

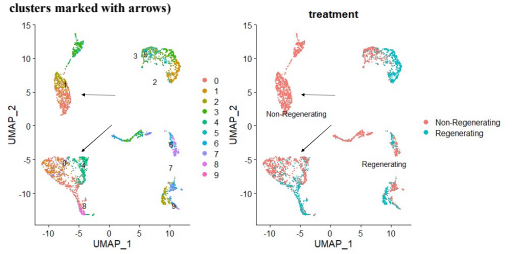
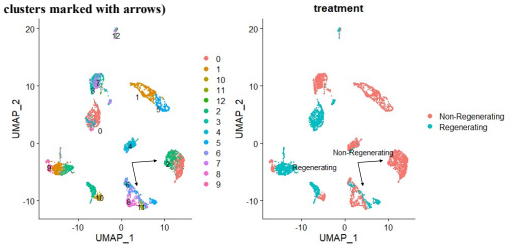


Figure 8: UMAP plot of the 10DPA dataset alongside the same UMAP specifying whether each cell originates from a regenerating or non-regenerating sample (key macrophage clusters marked with arrows)



Conclusions

From these UMAPS, we can state that by the distinct clustering of the cells, regenerating and non-regenerating samples do express extremely distinct genes. The UMAP clusters were formed based on the genes expressed in the entirety of the dataset, ignoring the samples' origins. However, upon plotting the data focusing on the origin (regenerative versus non-regenerative), we can see that the two attributes match with their own unique clusters, demonstrating that the regenerative and non-regenerative samples express different genes. As these two sample sets (regenerative and non-regenerative) came from the same animal in similar locations (distal third of the digit tip for regenerative, proximal to nail bed of the digit tip for non-regenerative), these data suggest that while these two locations are close proximally and functionally, there is a genetic distinction between these two points that correlates with regenerative or non-regenerative capabilities. Additionally, regenerative macrophages appear to express more anti-inflammatory genes than non-regenerative, due to the *Csf1r* and *Cd68* feature plots aligning with the regenerating cells in the regenerating/non-regenerating UMAPs.

Acknowledgements/References

[3]Simkin, J., Han, M., Yu, L., Yan, M., & Muneoka, K. (2013). The mouse digit tip: from wound healing to regeneration. In *Wound Regeneration and Repair* (pp. 419-435). Humana Press, Totowa, NJ.

[4]Simkin, J., Sammarco, M. C., Marrero, L., Dawson, L. A., Yan, M., Tucker, C., Cammack, A., & Muneoka, K. (2017). Macrophages are required to coordinate mouse digit tip regeneration. *Development (Cambridge, England)*, *144*(21), 3907–3916. https://doi.org/10.1242/dev.150086

[5]Gensel, J. C., & Zhang, B. (2015). Macrophage activation and its role in repair and pathology after spinal cord injury. *Brain research*, *1619*, 1-11.

[6]Eichenfield, D. Z., Troutman, T. D., Link, V. M., Lam, M. T., Cho, H., Gosselin, D., ... & Glass, C. K. (2016). Tissue damage drives co-localization of NF-κB, Smad3, and Nrf2 to direct Rev-erb sensitive wound repair in mouse macrophages. *elife*, *5*, e13024.

[7]Dang, D., Taheri, S., Das, S., Ghosh, P., Prince, L. S., & Sahoo, D. (2020). Computational approach to identifying universal macrophage biomarkers. *Frontiers in physiology*, *11*, 275.

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