Digit Amputation Level Influences Macrophage Polarization

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Introduction

Macrophages play a critical role in the body's healing process after physical trauma to skin, bone and muscle. Early after injury, macrophages exhibit an inflammatory activation state. These inflammatory macrophages (M1) create a sterile environment, clean up tissue debris, and recruit new blood vessels into the healing tissue [1-3]. Inflammatory macrophages then transition to a resolution or "tissue repair" state (M2) in which they reduce inflammation and promote collagen deposition by fibroblasts, bone mineralization by osteoblasts and myoblast differentiation in muscle [1-4]. Without the proper balance of inflammatory and tissue repair macrophages, skin, bone, and muscle injuries will not heal [2,4]. Thus, macrophages orchestrate the healing response across the body, and make promising therapeutic targets to improve wound healing in pathological conditions such as non-union fractures, volumetric muscle loss, and chronically open wounds. However, what controls the shift in macrophages from the M1 type to M2 is not well understood.

Objectives

To better understand what drives a shift in macrophage activation state, we leverage a model of traumatic injury in which amputation through the bone and skin of a mouse digit tip (P3) results in complete regeneration of the tissue. In comparison, a more proximal amputation through the mouse second phalangeal element (P2) results in bone callus and scar tissue formation. These two injuries give us a comparison of regeneration and scar formation within the same animal and is ideal for studies that investigate the impact of the local environment on macrophage activation states. Our previous studies suggest that M1 in a P2 injury are predominantly glycolytic and express high levels of the inflammatory cytokines TNF and IL1b. In contrast M1 in a P3 injury shows a metabolic preference for fatty acid oxidation and express lower levels of TNF and IL1b. Thus, activation states of macrophages between the two injuries are distinct. We hypothesize that factors within the two injury environments control the metabolic and transcriptomic state of the macrophage. To test this hypothesis, we expose unstimulated macrophages (M0) to the paracrine factors from the homogenized tissue in the injury environment and analyze changes in metabolic and transcriptomic state.

As we are interested in how different biochemical factors could influence macrophage polarization, we have chosen to use itaconate as an experimental factor. This metabolite both increases wound healing while also reducing macrophage polarization, which appears contradictory [10,11]. Thus, we will set aside a negative control (M0 plate with no tissue supplied), P3, and P2 dish which we will add itaconate to and observe if changes in polarization occur.

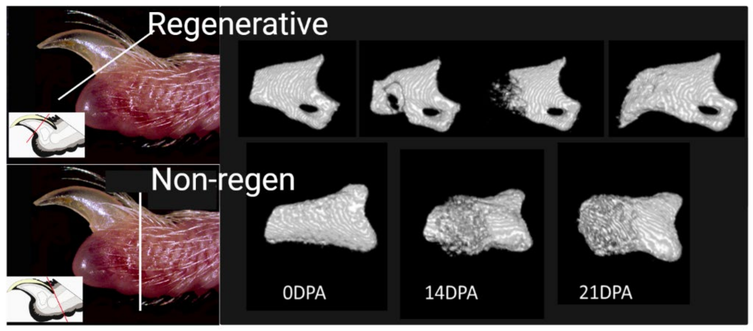
Methods

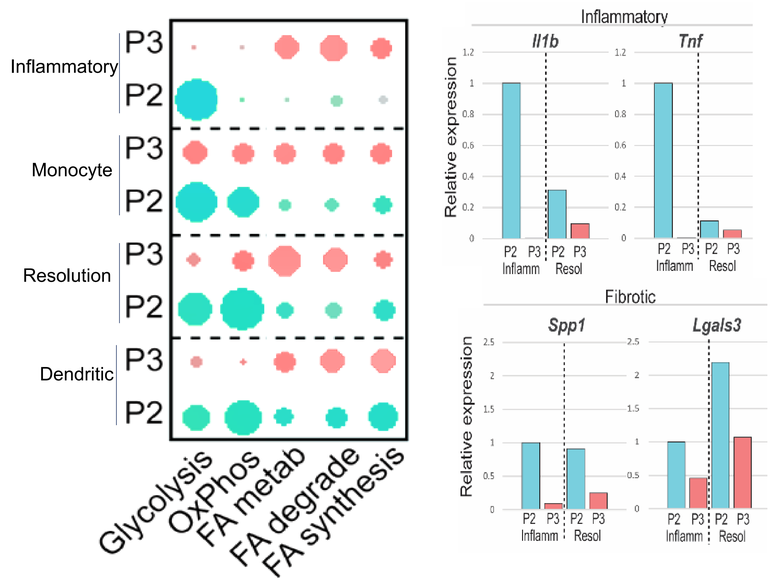
Three sets of mice populations were used for this experiment. In the first, consisting of 2 mice, we euthanized them and amputated their hind limbs to derive their bone marrow. The marrow was used to derive M0 for our samples. The marrow was plated on L929 media which contains macrophage colony stimulating factors that promote the differentiation of the progenitor cells into undifferentiated macrophages.

The two larger populations, each containing 8 mice, had their fingers amputated at the P2 and P3 digits respectively. This tissue was homogenized and placed onto the M0 plates, being left to rest so that differentiation could occur. Next, the samples were filtered, allowing the separation of the cellular structures from the secreted factors which would be analyzed so that the macrophage populations could be determined.

To differentiate between M1 and M2 populations, the Seahorse XF96 was used. This machine analyses the metabolic characteristics of a given sample allowing us to compare our P2 and P3 media. By measuring factors such as extracellular acidification rates and oxygen consumption rates, we are able to determine the predominant macrophage population.

Preliminary Data – P2 and P3 macrophages are metabolically distinct

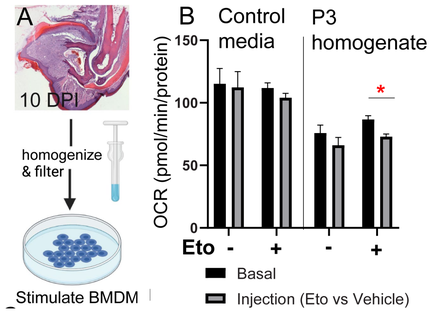




Analyzing a single cell RNAseq dataset from P2 and P3 amputations at 10days post amputation (), we find 4 populations of mononuclear phagocytic cells (inflammatory macrophages, monocytes, resolution macrophages and dendritic cells). In all 4 clusters we find that cells from P2 show an increase in genes involved in glycolysis whereas cells from P3 are more likely to genes related to fatty acid degradation.

We also found that inflammatory and resolution macrophages from P2 (non-regenerative) region of the digit express higher levels of pro-inflammatory cytokines (Il1b, Tnf) and pro-fibrotic genes (Spp1, Lgals3) compared to macrophages from P3.

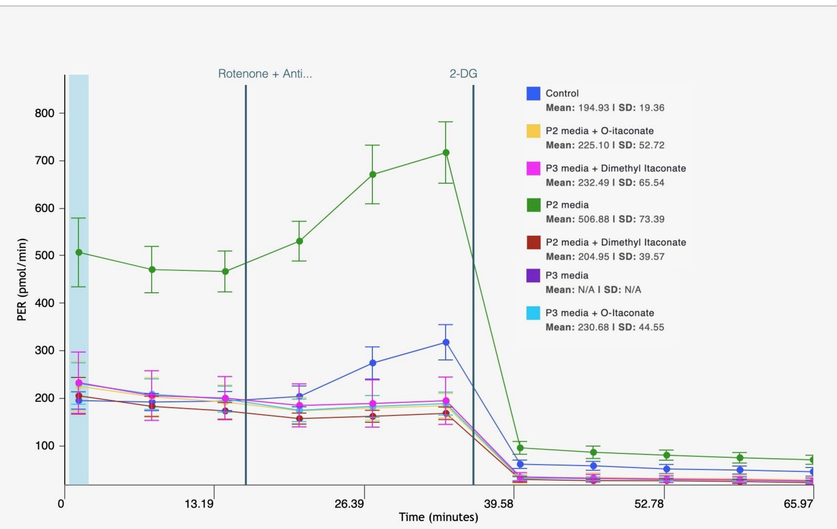
Results



In response to homogenized extract from the P3 wound 10 days after amputation, BMDM increase reliance on fatty acid oxidation (*p<0.05, n=10/group). Eto = etoximir (CPT1 inhibitor). OCR = oxygen consumption rate.

OCR decreases when FAO is blocked in BMDM exposed to P3 homogenate but not when exposed to normal media.

Results



Our experimental results demonstrated that fatty acid oxidation increases in macrophages exposed to P3 tissue. In contrast, high levels of ECAR (signified by the proton efflux rate, PER) due to increased glycolysis was observed in P2 tissue. Cells lost glycolytic capacity in response to itaconate.

Conclusion

These results are supportive of our hypothesis that there exist factors within the P2 tissue that favors the non-regenerative M1 macrophage type while the P3 favors the regenerative M2 type. The deleterious effects of itaconate on the macrophage populations was unexpected and should be further explored to determine if this was an error in application or if this chemical truly does inhibit polarization.

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