Different M1/M2 Macrophage Activation State Markers Capture Different Cell Populations in a Sciatic Nerve Injury and Repair Model in Mice: Redefining this Classification as a Spectrum

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INTRODUCTION: The M1/M2 classification of macrophages was originally developed to provide a framework for understanding their functional polarization; M1 as pro-inflammatory defenders and M2 as anti-inflammatory healers. However, continued investigation has found this binary model to be oversimplified. Macrophage activation state must instead be understood as a continuum or spectrum that is shaped by the complex mix of signals in the tissue microenvironment. Furthermore, macrophage behavior in peripheral nerve after injury is understudied, especially within a full transection and microsuture repair model – a model that is more clinically translatable than crush or chronic constriction models. This study aims to elucidate the fine classification of macrophage subtypes and their spatiotemporal patterns to better assess their role in peripheral nerve regeneration.

METHODS: Five macrophage markers between two transgenic mouse strains and three immunofluorescent antibodies were used to study macrophage state, localization, and migration within the coaptation site of regenerating sciatic nerves. CX3CR-1 GFP knock-in/knock-out mice (B6.129P2(Cg)-Cx3cr1tm1Litt/J) were first used in conjunction with CD206 and iNOS markers to visualize M1 and M2 macrophages, followed by F4/80 for general macrophage visualization. Macrophage Fas-Induced Apoptosis (MaFIA) mice (C57BL/6-Tg(Csf1r-EGFP-NGFR/FKBP1A/TNFRSF6)2Bck/J) were then used in conjunction with CD208, iNOS, and F4/80 to visualize Csf1r expressing general, alongside M1 and M2, macrophages. Both sets of immunofluorescent experiments had neurofilament light chain (NF-L) and DAPI fluorescence which, in combination with H&E histology, offered a tissue reference framework. Four old (age: 21 months) and four young (age: 6 months) male CX3CR-1 GFP as well as twenty-four young male MaFIA mice (age: 6 months) were sacrificed at days 7, 14, 21, and 28 post injury. The macrophages in 12 of the 24 MaFIA mice were selectively ablated (by more than 70%) by administering the dimerizing agent AP20187 retro-orbitally. The time course for the drug treatment started one week before injury and one week after to see the effects of depleted macrophages on nerve healing. Immunohistochemistry was taken of the full nerve with a Leica Stellaris 8 confocal scanning microscope. This study was approved by IACUC and by the institution's ethics committee.

RESULTS: Immunofluorescence showed an increased presence of iNOS positive cells, traditionally M1, during the first week of injury, followed by a decrease on day 14 in the young CX3CR-1 GFP mice. This was accompanied by a concurrent upregulation and distal migration of Cx3Cr1 positive cells, traditionally M2 macrophages, from day 7 to 21, followed by an even distribution on day 28. While quantification, was not yet complete for this abstract due to limited available mice, Figure 1 left panels show this migration from the proximal end (left) to the distal end (right) of the above described sequential waves of cells. This process was delayed by 1 week in the older cohort, but the same milestones were eventually achieved. General macrophages (M1 and M2), defined as Csf1 and/or F4/80 positive cells, showed a smaller cell count differential between the timepoints in the MaFIA mice. Staining for M1 vs M2 macrophages recaptured the trends seen in the Cx3Cr1 mice. Upon closer inspection in figure 1 right panels, CD208 and Cx3Cr1 cells, both typically M2 markers, rarely colocalized, effectively staining different cell populations. Hematoxylin and eosin staining was also performed on the Cx3Cr1 mice to quantify the increase of cell nuclei in the tissue (n=1 due to limited mice, no statistics). Figure 2 graph describes the ratio of nuclei distal to proximal from the coaptation site, as a metric for how much the distal end degenerates and regenerates. Control mice have a ratio of 1. One week after injury, the ratio doubles to two, followed by a decrease back to 1.5 at week 2, and then an increase to 2.5 at week 3, and 3 at week 4.

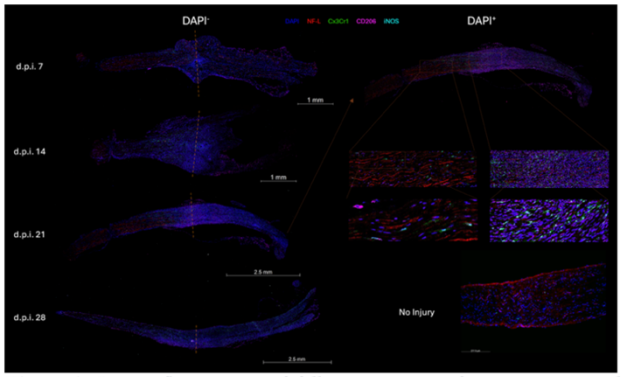

Figure 1: *Immunofluorescence of full sciatic nerve after transection and microsuture repair in Cx3Cr1 GFP mice. Day 7, 14, 21, and 28 post injury with DAPI included (left) zoom of proximal and distal end of Day 21 p.i. without DAPI + control nerve (right)*

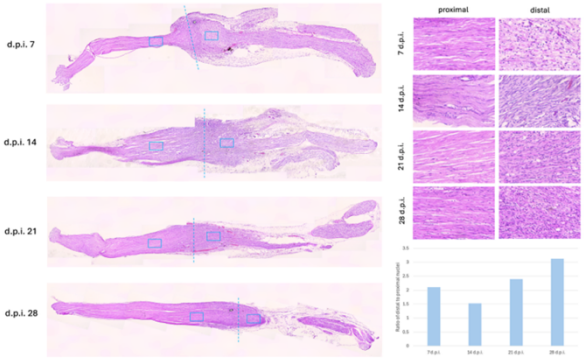

Figure 2: *Hematoxylin and eosin staining of full sciatic nerve Day 7, 14, 21, and 28 after transection and microsuture repair (left) with proximal and distal zooms for each timepoint (right, up) and the ratio of distal to proximal nuclei*

DISCUSSION: The initial wave of iNOS positive M1 macrophages followed by a wave of Cx3Cr1 and CD206 positive M2 macrophages is typical Wallerian degeneration and regeneration in peripheral nerves with gradual return to tissue homeostasis. The trend seen in figure 2 H&E quantification also supports the typical phases of neurodegeneration and regeneration at the same timepoints: more cells at week 1, degraded tissue at week 2, regeneration tissue in the following weeks. This dynamic progression underlines the roles of M1 and M2 macrophages as they are currently classified, where M1 serves to breakdown tissue to facilitate an environment for regeneration led by M2 cells that further create a regenerative niche for Schwann cells and axons. Using M1 and M2 antibodies alongside general macrophage fluorescence, we found that the spatial and temporal dynamics to be complex with fine transition points that would otherwise be unrecognized through general macrophages markers. This brings into question the definition of M1 and M2 if they are defined by mark biological markers, since the populations can vary depending on the chosen markers. The markers used to define macrophages in a given state must be reported and understood correctly. The rare overlap between M2 macrophage markers suggests a more complex transition between macrophage states. Lastly, the depletion of csf1 positive general macrophages results in delayed healing mimicking the delay seen in the aged CX3CR-1 populations, suggesting that an improper macrophage response or transition between states might be a factor in aged nerve repair. The conclusions of this work are twofold, M1 and M2 macrophage populations must be rigorously defined since they may not be the same between studies if different markers are used, and observing the dynamics of M1 versus M2 macrophage populations allows for a finer timing measurement for neurodegeneration and regeneration, which in turn can be used to study pathologies such as aging or macrophage depletion.

CLINICAL RELEVANCE: The transection and repair model used in this abstract allows for a more clinically relevant study pattern as opposed to transection alone or crush. By further understanding the macrophage led response following nerve repair, we are more poised to develop insights to augmenting nerve injury and enhance nerve surgery. Furthermore, the lessons learned about macrophage classification will allow for more specific macrophage targeted therapeutics and support future research on the impact of age on macrophage behavior.

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