Precision-Porous Templated Scaffolds Induce Unique Pro-Healing Cell and Exosome Populations

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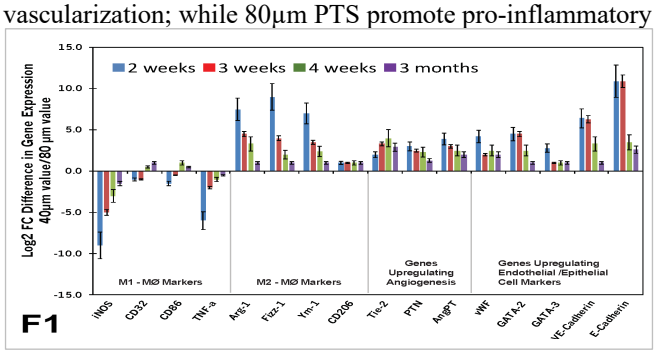
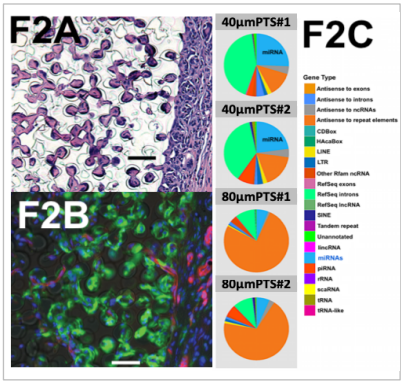
Statement of Purpose: We have previously developed Porous Templated Scaffolds (**PTS**) that are synthetic polymer constructs where pore size is precisely controlled (within a very narrow pore size distribution) uniformly throughout the scaffold and the pore interconnects are also uniform in size. If the pore diameter is ~40µm, PTS show little chronic inflammation or foreign body response while promoting remarkable healing in numerous soft and hard tissue applications. This is accomplished without the need for signaling/stimulating molecules (released or tethered). Using a double transgenic mouse strain (LysM-Cre+/0: mTmG+/0) that uniquely "fingerprints" myeloid cell DNA with an indelible myeloid lineage reporter gene, we provide evidence that only 40µm pore size PTS create an *in vivo* situation that *(a)* promotes an early unique cell community of specific macrophage and T cell phenotypes that *(b)* concomitantly generates extracellular vesicles (**EVs**) with unique pro-angiogenic content capable of directing tissue regeneration.

Methods: We created a new double transgenic mouse strain (LysM-Cre+/0:mT/mG+/0 double transgenic mice) that uniquely "fingerprints" myeloid cell DNA with an indelible myeloid lineage reporter gene, regardless of the cells ultimate phenotypic end point. LysM-Cre+/0:mT/mG+/0 mice express the fluorescent mTomato (mT; **red**) in the membrane of all non-recombined cells, and the fluorescent eGFP (mG; **green**) selectively in the membrane of only myeloid cells. Serendipitously, since the membranes of EVs are composed of the cellular membrane of their parent cells, EVs generated by mT or mG cells retain red or green fluorescence, respectively. Poly(HEMA) PTS of two different sizes (40µm healing and 80µm non-healing) were implanted subcutaneously in both wild-type C57BL/6 and LysM/Cre+/0:mT/mG+/0 mice then explanted at week 2, 3, and 4, and 3 months post-implantation. PTS contents were explanted and processed for *(a)* RNA extraction for qtPCR analysis (macrophage (MØ) cell markers, angiogenesis factors, endothelial and epithelial cell markers to indicate possible transdiffer-entiation), *(b)* fluorescence microscopy (mT non-myeloid cell and mG myeloid cell expression), *(c)* PTS resident cell sub-populations by flow cytometry, and *(d)* tissue histology and immunohistochemistry (endothelial and epithelial cell marker expression). EVs generated *ex vivo* from explanted PTS of different pore size were characterized for *(a)* particle size and membrane markers, *(b)* RNA content by RNAseq, *(c)* their ability to be taken up by various cells, and *(d)* their ability to promote vascular tube networks in an angiogenesis assay.

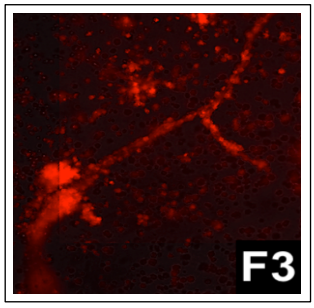
Results: 40µm PTS but not 80µm PTS drive a unique cell community (**F1**). qt-PCR results show that over-expression of genes of all groups dissipate, save for those genes indicative of angiogenesis. In all gene groups, save for M1-MØ markers, cells in 40 µm PTS over-expressed several-fold relative to cells in 80µm PTS, suggesting 40µm PTS scaffolds promote M2-MØ phenotype, angiogenesis and vascularization; while 80µm PTS promote pro-inflammatory M1 phenotype.



Both 40 and 80µm pore size poly(HEMA) PTS explanted from LysM-Cre+/0: mTmG+/0 mice at 4 weeks and histological sections stained with H&E and DAPI stains. Sections were imaged in both visible and UV light; only 40µm PTS displayed tissue in-growth and vascularization (H&E staining) throughout the scaffold (**F2A**); under 450nm excitation, these apparent tissue cells also fluoresced green indicative of their myeloid origin (**F2B**); strongly suggesting the re-programming of the myeloid cells, which may be due to uptake of unique exosomes generated within the 40µm PTS. RNA-seq analysis (**F2C**) of EVs recovered from either 40- or 80-µm PTS explanted after 1-week shows a dramatic abundance of miRNA sequences within the 40-µmPTS myeloid-derived exosomes versus myeloid-derived exosomes generated by cells within 80-µmPTS.



Angiogenesis tube-forming assays were carried out with resting MØ (not endothelial cells) seeded with exosomes derived in *ex vivo* culture of either 40- or 80-µm PTS recovered after 1-week or 4-weeks implantation in *LysM-Cre*+/0:mT/mG+/0 double transgenic mice. Only exosomes extracted from 40µm PTS explanted at Week 1 were able to induce resting MØ to form vascular tube-like structures (**F3**).



Conclusions: *In vivo* 40µm PTS *(a)* promote a cell community of M2-MØ and T cell that *(b)* concomitantly generates extracellular vesicles (**EVs**) with unique pro-angiogenic content capable of directing tissue regeneration.