Effects of Age-Related Changes in ECM Upon Macrophage Polarization

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Statement of Purpose: Extracellular matrix (ECM)-based biomaterials have been used as inductive templates for the reconstruction of damaged or missing tissues in a range of preclinical and clinical applications. These ECM scaffolds have been shown to promote constructive remodeling outcomes downstream of implantation. The ability to promote constructive remodeling has been associated with elicitation of a unique M2 (anti-inflammatory/tissue remodeling)-like macrophage phenotype following ECM implantation. Degradation of ECM scaffolds has been shown to be important to the promotion of this phenotype and to constructive remodeling[1]. Recent studies have demonstrated that source animal age affects a number of properties of decellularized scaffold materials including thickness, stiffness, biochemical content, and degradation rate[2]. These changes are accompanied by a decrease in constructive remodeling with increasing source animal age[3]. This work examines the contributions of degradation products from different source animal age ECM to macrophage polarization.

Materials and Methods: Porcine small intestine submucosa scaffolds were fabricated from young (12 weeks), adult (26 weeks) and aged (>52 weeks) pigs as previously described[3]. The materials were characterized for biochemical and histological differences using collagen, glycosaminoglycan and DNA quantification assays as well as DAPI and hematoxylin and eosin staining. Endotoxin levels in samples were assessed by limulus amebocyte assay. Degradation products were then produced from these materials through a 48-hour pepsin digestion.

Bone marrow macrophages were isolated from young (2 mo.) C57/BL6 mice and cultured for 7 days in L929-supplemented DMEM to differentiate the cells into macrophages. IFN-γ/LPS and IL-4 treatments for 24 hours were used to create canonical M1 and M2 positive controls, respectively. Macrophages were exposed to different source animal age ECM degradation products for 24 and 72 hours to assess ECM-mediated changes in macrophage polarization *in vitro.* Macrophages were also exposed to ECM degradation products for 24 or 72 hours then challenged with IFN-γ/LPS or IL-4 for 24 hours to analyze degradation product effects on the ability of macrophages to polarize to M1 or M2 phenotypes.

Macrophage phenotype was assessed using immunofluorescence, qRT-PCR, and Western blotting, and phagocytosis assays. Cells were labeled with antibodies specific for F4/80, iNOS, Arginase, Fizz1, CCR2, CX_3CR1, and TLR4. Taqman gene expression assays were performed for IL-12β, IL-1β, TNFα, iNOS, Arginase, Fizz1, Mrc1, IL-1ra, IL-10, PPARγ and MHC-II. Western blots were stained with antibodies for iNOS and arginase. Following various treatments, the cells were incubated with Vybrant FITC-loaded E. Coli particles for 2 hours to assess phagocytosis function. Fluorescent staining and phagocytosis assays were quantified using Cell Profiler.

Results & Discussion: H&E and DAPI staining showed a complete reduction in visible nuclei in decellularized tissues compared to native. Gel electrophoresis of DNA extracts from ECM samples showed decell samples were practically devoid of DNA and remnants were highly fragmented. This suggests that the decellularization protocol was effective. Endotoxin levels were not statistically different between scaffold materials indicating observed results were not due to differences in endotoxin contamination.

Positive labeling of cells with F4/80 suggested successful differentiation into macrophages. As expected, IFNγ- stimulated macrophages (M1) showed increased labeling for iNOS, CCR2, Ly6C and TLR4 while IL4-stimulated macrophages (M2) had increased labeling for arginase, Fizz1, and CX_3CR1. ECM exposed macrophages showed a labeling profile which was similar to that observed for M2 controls, but trended toward patterns seen in M1 controls with increasing age.

Gene expression analysis showed increased expression of IL-12, IL1-β, TNFα, and iNOS in M1 macrophages and increased expression of Arginase, Fizz1, CD206 and IL-10 in M2 macrophages. ECM-treated macrophages showed expression trends similar to M2 cells albeit with lower levels, with increasing pro-inflammatory marker expression with increasing source animal age of tissues. Gene expression in samples treated with ECM and then challenged with IFNg or IL-4 showed the baseline trends retained yet amplified. This suggests that aged ECM can affect the macrophage's ability to polarize.

Western blotting demonstrated high levels of iNOS in M1 lysates and arginase in M2 lysates compared to ECM treated macrophages which did not show significant levels of either protein at 24 hours. Western blots of macrophages exposed to ECM prior to polarization showed increased levels of iNOS in aged-ECM treated samples for the M1 polarized group and increased levels of arginase in young ECM-treated samples in the M2 polarized group.

Phagocytosis assay results indicated a higher phagocytic function in M1 cells than M2 cells. ECM-treated cells showed slight increases in phagocytic functionality with increased age of tissue. This trend was accentuated in ECM -> M2 treatments but not ECM -> M1 treatments.

In vitro data suggests that the ability of ECM to polarize macrophages to an anti-inflammatory phenotype is compromised with increased animal source age. This may result from changes in composition or concentrations of certain beneficial extracellular matrix components, alteration of ECM configuration, or changes of degradation product profiles with age. This impaired macrophage response to older source animal age ECM biomaterials has implications for the use of these materials in clinical settings.

Conclusions Extracellular matrix (ECM) biomaterials show compromised ability to polarize macrophages to a distinct anti-inflammatory/tissue remodeling phenotype as the animal source age increases. This agrees with previous findings that the ability for ECM to promote constructive remodeling is lost with increasing source animal age.

References

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