Inflammatory and profibrotic mechanisms of DUOX2 gene mutations and 2'-Fucosyllactose human milk oligosaccharides in Crohn's disease

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Introduction: Crohn's Disease (CD) is a chronic autoinflammatory disorder of the gastrointestinal tract that frequently progresses to intestinal fibrosis. Missense mutations in the *DUOX2* gene, which encodes the NADPH oxidase Dual Oxidase 2, have been associated with impaired epithelial barrier function and enhanced fibrogenesis in CD. Recent evidence suggests that 2'-Fucosyllactose (2'-FL), a human milk oligosaccharide, may mitigate inflammatory signaling and restore epithelial homeostasis. However, the cellular mechanisms linking DUOX2 dysfunction to fibrotic remodeling, and the ability of 2'-FL to counteract these effects, remain unclear. To address this, we developed a novel co-culture system utilizing CRISPR/Cas9-engineered isogenic *DUOX2* variant iPSC-derived macrophages and intestinal organoids. We hypothesized that DUOX2var macrophages would induce fibrotic changes in isogenic human intestinal organoids, and that treatment with 2'-FL would reverse these effects.

Methods: Induced pluripotent stem cells (iPSC) were derived from a pediatric CD patient carrying the *DUOX2* reference genotype (DUOX2ref) and the H678R and R701Q missense mutations associated with strictures were introduced using CRISPR/Cas9 gene editing to generate DUOX2var iPSC. iPSCs were differentiated into macrophages and human intestinal organoids (HIOs), and studied in tissue culture under basal conditions, and following exposure to LPS and/or the candidate antifibrotic agent 2-'FL. Cell surface markers and reactive oxygen species (ROS) were measured by flow cytometry. Inflammatory and fibrotic mediators were assayed using RNA sequencing, real time PCR, immunofluorescence (IF), and a custom Luminex assay. HIO collagen content was quantified using Sirius Red staining with polarized light microscopy.

Results: Genes down-regulated in DUOX2var macrophages and HIOs compared to DUOX2ref were enriched for inflammatory responses (FDR B&H: 1.63E-69) and extracellular matrix (ECM) remodeling (FDR B&H: 2.691E-36), respectively. Consistent with this, flow cytometry demonstrated impaired inflammatory macrophage polarization with *DUOX2* variant carriage, with a reduced frequency of CD14, CD68, and CD163 positive cells compared to DUOX2ref macrophages. Multiplex luminex assays revealed decreased LPS induced secretion of pro-inflammatory cytokines (IL-1β, OSM, TNF-α) and chemokines (CCL2, CXCL5) in DUOX2var macrophages. Under these conditions production of profibrotic PDGFAA, PDGFBB, and OSM was preserved. DUOX2var HIO exhibited increased oxidative stress, with CellTiter-Glo 3D viability assays showing reduced cellular viability with *DUOX2* variant macrophage co-culture. This was associated with an increase in type III collagen content in DUOX2var HIO. Importantly, while 2'-FL attenuated macrophage production of most pro-inflammatory cytokines, it did not reduce HIO collagen content.

Conclusions: *DUOX2* variant macrophages shift from an M1 inflammatory phenotype to an M2 wound healing/profibrotic phenotype and induce greater type III collagen content in isogenic

human intestinal organoids. These findings provide new insight into cellular mechanisms linking *DUOX2* dysfunction to intestinal fibrosis and suggest that 2'-FL holds therapeutic promise for inflammatory responses in CD.

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