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Additions to Oldham Lab manuscript

MCT expression increases in human pulmonary fibrosis and experimental models.

Based on this observation, we characterized MCT1 and MCT4 expression in human IPF lung explants obtained at the time of transplant. Consistent with a pathologic role for MCT 1 and MCT4 in IPF, we found significantly increased expression of these proteins in human IPF lung compared to non-fibrotic control Samples (Figure 1A-B). **Compared to healthy lung, where immunohistochemistry (IHC) revealed that MCT4 expression was sparse, MCT4 localized to myofibroblasts within fibrotic beds, macrophages, and sparse lung epithelial cells in samples from human patients with IPF (Rutter Lab data).**

To determine whether these findings in human IPF were recapitulated in experimental pulmonary fibrosis, we quantified MCT expression in the lungs of mice administered bleomycin (1.25 Ukg i.t.). Similar to human IPF, experimental pulmonary fibrosis was also associated with increased MCT1 and MCT4 expression. **IHC revealed to that MCT4 was increased in myofibroblasts and near areas of fibrotic mass, whereas it was largely absent from non-fibrotic tissue (Rutter Lab data).**

Lactate transport is essential for myofibroblast differentiation in vitro.

(Slide 17 demonstrates that we have produced essentially the same data). The major addition we have:

**Given the robust effect of MCT4 inhibition on myofibroblast differentiation, we compared pharmacologic inhibition of MCT4 to a current IPF standard of care medication, nintedanib. While nintedanib has a broad spectrum of activity (PMID: 25745043) it inhibits TGFβ-mediated fibroblast to myofibroblast transition and decreases Col1a expression both *in vitro* and *in vivo* (PMID 24556663). Both drugs potently inhibited aSMA production, but fibroblast viability was not affected with VB124 treatment, while nintedanib had a moderate cytotoxic effect. These results suggest that VB124 prevents myofibroblast transition and aSMA expression without decreasing cell viability.**

(Data from Slide 19)

**Since MCT4 inhibition reduced myofibroblast differentiation, but not cell viability, we also wanted to determine if MCT inhibition influences other phases of the wound healing/fibrotic process, such as fibroblast migration. To determine whether MCT4 was required for fibroblast migration, we performed a well-established scratch assay with escalating doses of VB124,and found that fibroblast migration was only inhibited at supraphysiologic doses of drug (50 microM), but not at treatment effect doses.**

MCT inhibition reprograms myofibroblast metabolism

This section is well done, but we have little to contribute as there have not been metabolomic experiments done in isolated fibroblasts.

MCT inhibition act downstream of TGF-beta-dependent signaling pathways

Pg 8 (slide 18 contribution)

We next examined whether MCT inhibition affects TGFb-dependent signaling pathways. TGF-beta activates both SMAD and non-SMAD signaling pathways. We found no impact of MCT inhibition on decreasing Smad3 or ERK phosphorylation in **normal** LFs 48h following TGFb stimulation (Figure 5 experiments done in normal lung fibroblasts). **We additionally tested whether MCT inhibition prevented SMAD phosphorylation and nuclear translocation in fibroblasts derived from human IPF patients. Consistent with our results in normal pulmonary fibroblasts (Fig 5A-B), MCT4 inhibition with VB124 did not prevent SMAD3 nuclear localization at any concentration tested, contrary to treatment with SB525334, a known potent inhibitor of SMAD3 nuclear translocation. Taken together, these results suggest that MCT inhibition acts downstream of canonical TGF-b signaling.**

MCT inhibition attenuates myofibroblast transcriptional programs

No data to contribute here: EMT does come up in this section so it may be interesting to add data from slide 23

**RNA-seq data highlighted enrichment of the EMT gene set, which is not surprising given that several of the genes involved in EMT are likely involved in fibroblast activation. However, in the pathogenesis of IPF, injury to epithelial cells can lead to an “epithelial to mesenchymal transition” (EMT). We therefore tested the effects of MCT4 inhibition with VB124 on EMT in lung epithelial cells from IPF patients and healthy donors. Using fibronectin (FN1) expression as a readout of EMT, we found that VB124 attenuated TGF-beta mediated FN1 production from lung epithelial cells in a dose-dependent manner in patients with IPF. A similar, although less pronounced phenotype was observed in pulmonary epithelium from three healthy control patients treated with TGF-beta. Taken together, these results suggest that MCT4 inhibition modestly attenuates EMT in lung epithelial cells.**

MCT inhibition decreases experimental pulmonary fibrosis in mice

Figure 10 – we have identical results on slide 11**.**

**In independently conducted similar experiments, MCT4 inhibition with VB124 had similar, if not superior, protection from bleomycin-induced pulmonary fibrosis as pirfenidone, which is currently used as a standard of care treatment.**

**Given that IPF increases in incidence with age in humans, some studies have suggested that an aged mouse models with the addition of IT bleomycin are the most clinically relevant models of IPF (PMID 28804709, 21743030). In aged mice, disease is more severe and does not spontaneously resolve (PMID 21743030). To enhance the clinical relevance of our findings in young mice, we also treated 60+ week old mice with 1 U/kg of intratracheal bleomycin. On day 7, mice were subsequently treated with VB124, and analysis was conducted on day 21. Like what was observed in younger mice, MCT4 inhibition with VB124 significantly reduced the level of aSMA in the lung, the Ashcroft score, and lung lactate. Taken together, our finding suggest that inhibition of lactate export decreases the pathologic burden of bleomycin induced pulmonary fibrosis and may represent an attractive therapeutic strategy.**

**To further characterize the functional outcomes of a reduced pathologic disease burden, we performed whole body plethysmography, a non-invasive measurement of lung function, Day 20 (20 days after bleomycin administration, and 13 days after treatment with vehicle, VB124, pirfenidone, or nintedanib. Importantly, treatment with VB124 significantly improved broncho-obstruction as measured by plethysmography compared to vehicle, pirfenidone and nintedanib, the current standards of care in IPF.**

Slide 20

**To determine whether the histologic and functional protective effects of VB124 on bleomycin induced pulmonary fibrosis were mediated by both fibrotic production and/or on fibrotic resolution, we performed metabolomic analysis for actin breakdown products on plasma in bleomycin treated animals at day 21. Mice treated with 3 mg/kg V124 had increased plasma levels of 3-methylhistidine, a marker of actin breakdown, compared to those treated with bleomycin alone. Neither pirfenidone nor a sub-therapeutic 0.3 mg/kg dose of VB demonstrated this effect compared to mice treated with bleomycin alone. These results suggest that the reduction in lung aSMA and improved pulmonary function may be due to increased turnover of aSMA and other extracellular matrix components upon MCT4 inhibition.**

**To further elucidate a mechanism for MCT inhibition on whole body metabolism, we performed separate experiments, we performed plasma metabolomics at day 21 of mice treated with bleomycin alone or bleomycin with VB124. Interestingly, we found that VB124 treatment significantly influenced plasma metabolites related to NAD+ biogenesis and degradation. Compared with vehicle treated controls, bleomycin reduced de novo NAD+ intermediates including Tigoneline, Nicotinate Ribonucleoside and Quinolinate, and these effects were further amplified with therapeutic doses of VB124. We also found that there were hallmarks of increased NAD+ consumption, as demonstrated by increased NAD+ degradation intermediates with VB124 treatment, including N-Methyl-2-pyridone-5-carboxamide, 1-Methylnicotinamide and Nicotinamide. Together, these results suggest that MCT4 inhibition increases NAD+ consumption in experimental bleomycin-induced pulmonary fibrosis.**