

PROGRESS REPORT YEAR 1

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Project Title: Upregulation of biliary innate immune responses in PSC

A. LAY SUMMARY OF THE PROGRESS

The pathogenesis of PSC is still unknown; as a consequence, curative treatment is lacking. Association of PSC with Inflammatory Bowel Disease suggests the involvement of innate immune mechanisms that in normal conditions are activated by cells to protect from infections. Indeed, preliminary evidence show that biliary epithelial cells derived from PSC patients, have an enhanced responsiveness to endotoxin bacterial product (i.e LPS) as shown by increased secretion of the inflammatory mediator IL8 and activation of the transcription factor NF-kB, as compared to control non-PSC cholangiocytes.

Therefore, we hypothesize that the biliary epithelium of PSC patients responds with aberrant production of NF-kB-dependent proinflammatory cytokines to factors able to stimulate innate immunity, released during biliary or hepatocellular damage. We have described similar mechanism has been described in Cystic Fibrosis (CF), a known cause of Sclerosing Cholangitis, where negative control of Src/TLR4/NFkB signaling is lost and cholangiocytes have increased pro-inflammatory cytokine production in response to LPS.

To demonstrate the above hypothesis, we aim to study, the effect in PSC of the activation of innate immune pathways in response to bacterial products (PAMPs) and other damage-associated molecule (DAMPs) released by liver cells and known to activate TLR innate immunity. Unfortunately, there is no satisfactory animal or cellular model orthologous to human PSC. Thus, to produce data relevant for the human disease, during the first part of the study, we have generated a cellular model of PSC derived human iPSCs (induced pluripotent stem cells) and 3D liver organoids from PSC patients. We have shown that iPSC can be differentiated in mature and functional biliary cells and we have created a platform for patient-specific disease modeling (Fiorotto et al., Hepatology 2017).

In the second part of this study, we plan to compare the innate immune responses to PAMPs and DAMPs in our human PSC models (primary PSC organoids, iPSCs-derived cholangiocytes and cultures of cholangiocytes immunisolated from liver explants) as respects to their appropriate normal controls. We expect to identify differentially expressed genes that may account for increased TLR-dependent NF-kB activation and are expressed in all the three different cell models as a signature of the disease.

This study will improve the understanding of the pathogenesis of PSC and likely identify novel therapeutic targets

B. STUDIES AND RESULTS FOR YEAR 1 OF THE PROJECT

During the first year of the project:

1. We have completed and published the study describing the protocol to derive functionally mature cholangiocytes from iPSCs and to expand and culture them as polarized monolayer (Fiorotto R et al., Hepatology 2017). Using our system, iPSC-cholangiocytes can be subcultured and expanded for up to ten passages. We have derived iPSC-cholangiocytes from a CF patient with CFTR mutation $\Delta F508$ and from healthy donors. We have used this technology to understand the cellular pathophysiology of biliary epithelial cells and the role of altered epithelial innate immunity in the pathogenesis of CF. Our results show that the absence of CFTR at the apical membrane of cholangiocytes leads to a persistent activation of Src family of tyrosin kinases (SFK) that in turn, phosphorylate TLR4, that triggers an aberrant production of pro-inflammatory cytokines in response to PAMPs, such as LPS. Notably, we show that by targeting SFK we significantly decrease inflammation and we dramatically improve the efficacy of current treatments used to correct the $\Delta F508$ defect in stimulating biliary fluid secretion.

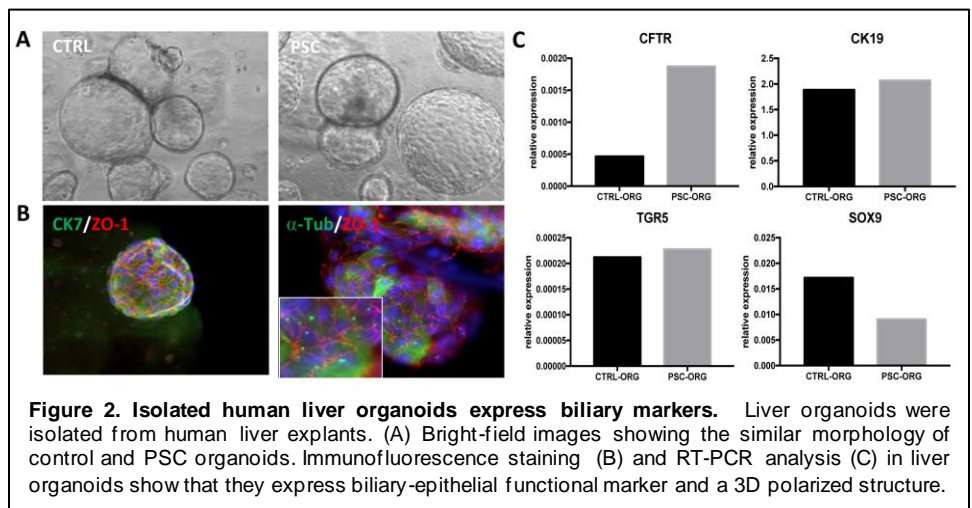
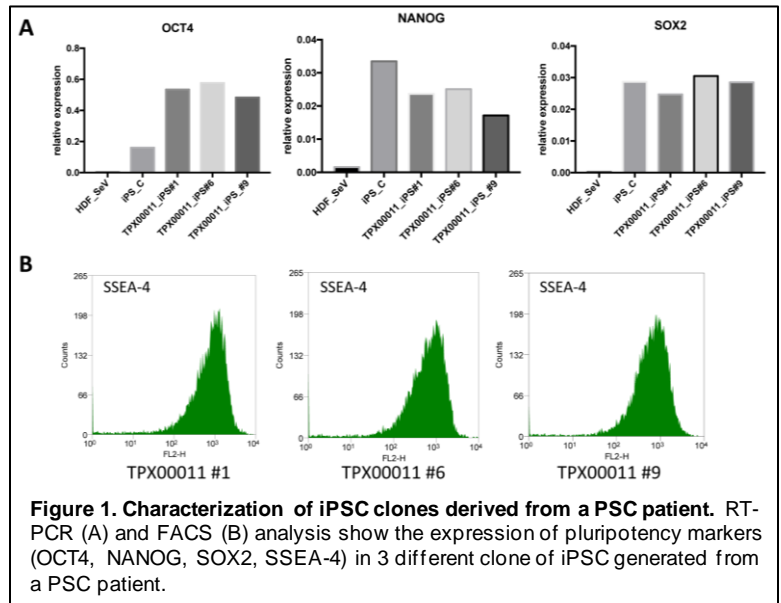
Our study has important implications: **a)** It expands our knowledge about the regulation of innate immunity responses of the biliary epithelium and highlights the pivotal role of the biliary epithelial cell in the pathological process leading to sclerosing cholangitis; **b)** It suggests that an effective treatment of CF-cholangiopathy should target synergistically the basic defect and the inflammation; **c)** It shows for the first time that, using iPSC technology, human diseases of the biliary system can be modeled to obtain results of clinical-translational relevance.

A copy of the manuscript acknowledging the support of Partners Seeking a Cure Foundation is included at the end of this report.

2. We have generated iPSC from a PSC patient and from a healthy control as planned in Aim 1 of the project. Peripheral blood mononuclear cells derived from a blood sample of the patient were used as the source to reprogram iPSC. We have obtained 3 different clones that we have characterized for their pluripotency potential (Fig.1). We are now differentiating them in biliary cells and we are generating new iPSC clones from more patients.

3. We have isolated human liver organoids from liver tissue of a PSC patient undergoing liver transplant and from the normal liver tissue of a patient with HCC also undergoing liver transplant. RT-PCR analysis confirmed that both PSC and control organoids express biliary markers (Fig. 2). We have shown that liver organoids, as well as the other cell preps can be propagated in culture for several passages.

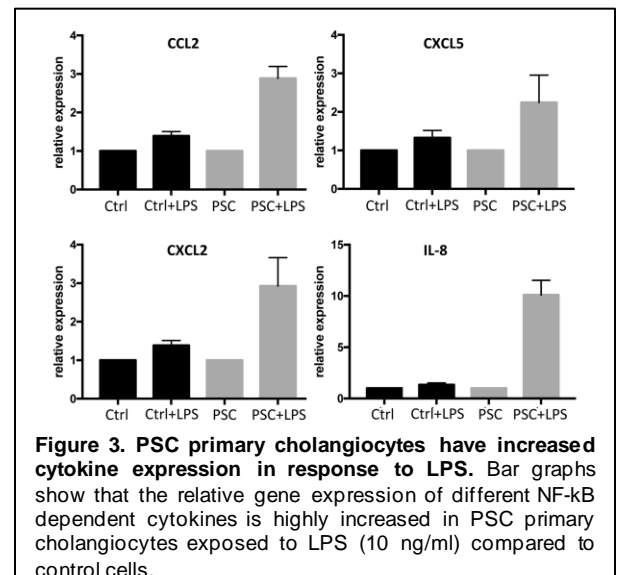
4. Using primary cultures of PSC cholangiocytes (obtained from the group of Dr. Gideon Hirschfield, University of Birmingham), we have performed additional experiments) to confirm that PSC cells have a higher NF- κ B transcriptional activation in response to LPS. In addition we have shown by RT-PCR that PSC cells have a higher expression of several cytokines in response to LPS as compared to control cells (Fig.3)



C. EXPERIMENTAL PLAN FOR THE SECOND YEAR

We have now available three different human cell models: i.e. primary cholangiocytes, biliary organoids and patient derived iPSC. During the second year of the project we will increment the number of patients for the isolation of biliary organoids and iPSC while we will start the experiments as planned in the Aim 2 of the project. Specifically, we will study different components of the innate immune pathway that might be activated in PSC biliary cells by different DAMPs and PAMPs by assessing NF- κ B activation, cytokine secretion and by performing gene expression arrays of genes relevant for TLR signaling.

This part of the study will support our hypothesis that activation of innate immune pathways represents a common



event in the pathogenesis of sclerosing cholangitis and we expect to identify disease-relevant signals that are expressed in all the three different cell models.

Publications:

- Fiorotto R, Amenduni M, Mariotti V, Fabris L, Spirli C, Strazzabosco M. 2017. Src kinase inhibition reduces inflammatory and cytoskeletal changes in $\Delta F508$ human cholangiocytes and improves CFTR correctors efficacy. **Hepatology**. (In Press).
- Strazzabosco M, Fiorotto R, Cadamuro M, Spirli C, Mariotti V, Kaffe E, Scirpo R, Fabris L. 2017. Pathophysiologic implications of innate immunity and autoinflammation in the biliary epithelium. **Biochim Biophys Acta**. (In Press).

Abstract presented at scientific meeting:

- Fiorotto R, Amenduni M, Mariotti V, Spirli C, Strazzabosco M. Establishment and expansion of functional polarized monolayers and 3D tubular structure of cholangiocytes derived by induced pluripotent stem cells (iPSCs) for human biliary disease modeling. **AASLD Liver Meeting 2016, Boston, USA**.
- Fiorotto R, Amenduni M, Cadamuro M, Spirli C, Strazzabosco M. Identification of Src tyrosine kinase as a therapeutic target in cystic fibrosis liver disease using patient specific induced pluripotent stem cells (iPSC)-derived cholangiocytes. **EASL International Liver Congress 2017, Amsterdam, Netherlands**.
- Fiorotto R, Amenduni M, Mariotti V, Fabris L, Spirli C, Strazzabosco M. Inhibition of Src tyrosine kinase restores CFTR function in cystic fibrosis cholangiocytes derived from human induced pluripotent stem cells (iPSC) and improves the response to CFTR potentiators and correctors used in therapy. **Cholangiocytes in Health and Disease: from Basic Science to Novel Treatments. 2017. EASL Monothematic Conference, Oslo, Norway**.