**Università degli Studi di Milano – Bicocca**
**Scuola di Dottorato in Scienze Mediche Sperimentali e Cliniche**
**Indirizzo: TECNOLOGIE BIOMEDICHE, Ciclo XXV**

# EXPRESSION OF αVβ6 INTEGRIN BY PKHD1-DEFECTIVE CHOLANGIOCYTES LINKS ENHANCED DUCTAL SECRETION OF MACROPHAGE CHEMOKINES TO PROGRESSIVE PORTAL FIBROSIS IN CONGENTIAL HEPATIC FIBROSIS

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*To my family,*

*with all my respect, admiration and love...*

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**BACKGROUND AND AIMS:** Congenital Hepatic Fibrosis (CHF) is caused by mutations in *PKHD1,* a gene encoding for fibrocystin, a protein of unknown function, expressed in cholangiocyte cilia and centromers. In CHF, biliary dysgenesis is accompanied by severe progressive portal fibrosis and portal hypertension. The mechanisms responsible for portal fibrosis in CHF are unclear. The αvβ6 integrin mediates local activation of TGFβ1 and is expressed by reactive cholangiocytes during cholestasis. To understand the mechanisms of fibrosis in CHF we studied the expression of αvβ6 integrin and its regulation in *Pkhd1*$^{del4/del4}$ mice.

**METHODS:** In *Pkhd1*$^{del4/del4}$ mice we studied, at different ages (1-12 months): a) portal fibrosis (Sirius Red) and portal hypertension (spleen weight/body weight); b) αvβ6 mRNA and protein expression (RT-PCR, IHC); c) α-SMA and TGFβ1 mRNA expression (RT-PCR); d) portal inflammatory infiltrate (IHC for CD45 and FACS analysis of whole liver infiltrate); f) cytokines secretion from cultured monolayers of primary cholangiocytes (Luminex assay); g) cytokine effects on monocyte/macrophage proliferation (MTS assay) and migration (Boyden chamber); h) TGFβ1 and TNFα effects on β6 integrin mRNA expression by cultured cholangiocytes before and after inhibition of the TGFβ receptor type II (TGFβRII); i) TGFβ1 effects on collagen type I (COLL1) mRNA expression by cultured cholangiocytes.

**RESULTS:** *Pkhd1*$^{del4/del4}$ mice showed a progressive increase in αvβ6 integrin expression on biliary cyst epithelia. Expression of αvβ6 correlated with portal fibrosis ($r=0.94$, $p<0.02$) and with enrichment of a CD45+ve cell infiltrate in the portal space ($r=0.97$, $p<0.01$). Gene expression of TGFβ1 showed a similar age-dependent increase. FACS analysis showed

that 50-75% of the CD45+ve cells were macrophages (CD45/CD11b/F4/80+ve). Cultured polarized *Pkhd1*$^{del4/del4}$ cholangiocytes secreted from the basolateral side significantly increased amounts of CXCL1 and CXCL10 ($p<0.05$). Both cytokines were able to stimulate macrophage migration ($p<0.05$). Basal expression of β6 mRNA by cultured *Pkhd1*$^{del4/del4}$ cholangiocytes (0.015±0.002 2^-dCt) was potently stimulated by the macrophage-derived cytokines TGFβ1 (0.017±0.002 2^-dCt, $p<0.05$) and TNFα (0.018±0.003 2^-dCt, $p<0.05$). Inhibition of TGFβRII completely blunted TGFβ1 (0.014±0.003 2^-dCt, $p<0.05$) but not TNFα effects (0.017±0.001 2^-dCt, p=ns) on β6 mRNA. COLL1 mRNA expression by cultured *Pkhd1*$^{del4/del4}$ cholangiocytes (0.0009±0.0003 2^-dCt) was further and significantly increased after TGFβ1 stimulation (0.002±0.0005 2^-dCt, $p<0.05$).

**CONCLUSIONS:** *Pkhd1*$^{del4/del4}$ cholangiocytes possess increased basolateral secretory functions of chemokines (CXCL1, CXCL10) able to orchestrate macrophage homing to the peribiliary microenvironment. In turn, by releasing TGFβ1 and TNFα, macrophages up-regulate αvβ6 integrin in *Pkhd1*$^{del4/del4}$ cholangiocytes. αvβ6 integrin activates latent TGFβ1, further increasing the fibrogenic properties of cholangiocytes.

# ***INTRODUCTION***

## 1. The liver

*1.1 Liver organization and function*

The liver is the largest gland in the body: in adult humans its weight ranges from 1300 to 1700 g. The liver is located in the abdominal cavity and is anatomically divided into four lobes: right, left, caudate and square. Glisson's capsule, a thin connective tissue layer, covers the entire gland and penetrates within the parenchyma, thereby dividing the liver into the fundamental functional units (hepatic lobules), which are separated by the portal space. The main feature of liver vascularization is a unique pattern in which afferent (supplying) and efferent (draining) vessels connect to a peculiar capillary system, the sinusoids, endowed with enhanced diffusive abilities, running through the lobule.

The liver parenchyma is populated by at least seven distinct cell types, including hepatocytes, cholangiocytes, sinusoidal endothelial cells, macrophages, lymphocytes, dendritic cells and hepatic stellate cells (HSC), which are finely arranged along a matrix framework that facilitates their cooperative interactions.

Liver is known to perform over 500 different functions. They include a huge number of exocrine, endocrine and paracrine activities whereby the different liver cell types are variably involved. The liver is the organ responsible for bile production; the bile produced in the liver is stored in the gallbladder and it is released in the duodenum depending upon the physiological needs to meet. The liver is central in the homeostasis of many metabolic functions: it controls the blood concentration of glucose, supplying it when it is needed and removing it when it is in excess. The

liver removes lipids from the blood and oxidizes them allowing the transfer of energy, it regulates the synthesis and degradation of plasma proteins, and it is the main site of cholesterol synthesis.

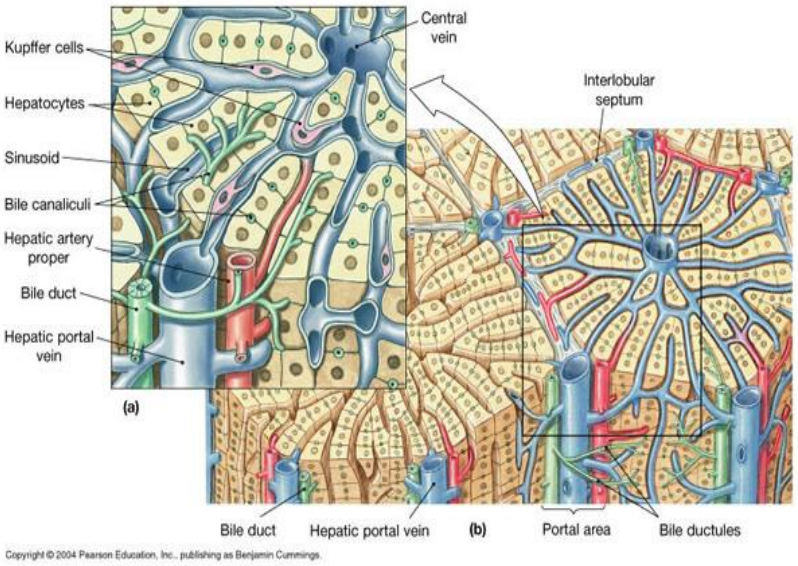



**Fig. 1: Liver structure (anatomy of the liver and biliary tracts)** Copyright© Pearson Education Inc. publishing as Benjamin Cummings.

*1.2 The cholangiocytes and the biliary system*

Cholangiocytes are the epithelial cells that line the biliary tree and they exert distinctive absorptive and secretory functions. Their function is not limited to funneling the bile to the intestinal lumen, as they actively contribute to its formation by regulating its composition, volume and pH. The biliary system consists of two regions: the *intrahepatic ducts* form a complex network of conduits within the liver that begin with the canals of Hering and progressively merge into a system of interlobular, septal and major ducts; the *extrahepatic ducts* are connected to the gallbladder, encompass the hepatic ducts and the common bile duct, which finally delivers the bile to the intestine. They have a different embryologic origin from the intrahepatic counterpart.

Once the primary bile has been produced by the hepatocytes in the bile canaliculi, the luminar structures located between two adjacent hepatocytes, it is funneled to the canals of Hering. These are small channels located at the ductular-hepatocellular junction, lined in part by hepatocytes and in part by cholangiocytes, which represent the physiologic link of the biliary tree with the hepatocyte canalicular system extended within the lobule. Canals of Hering are in direct continuity with the terminal cholangioles, which represent the first tubular structure entirely lined by cholangiocytes: they link the canal of Hering on one side to the interlobular ducts on the other. The terminal cholangioles are the finest ramifications of the biliary tree and traverse the limiting plate between the portal space and the lobule (Strazzabosco & Fabris, 2008). Bile ducts run in parallel with a branch of the portal vein and with one or

two branches of the hepatic artery. This close anatomic association is the basic structure of the portal triad.

Cholangiocytes regulate bile secretion, fluidity and pH by actively pumping $Cl^-$ and $HCO_3^-$ into the ductal lumen. This induces the secretion of water and increases the alkalinity of the bile. In humans, about the 40% of the total bile production is of ductal origin; secretory functions of cholangiocytes are mainly performed by the interlobular, septal and major ducts which express the appropriate ion transport systems and hormone receptors (Boyer JL, 1996). In addition to bile processing and transport, cholangiocytes can interact with the immune system and with different microorganisms, thereby providing a defense barrier against biliary infections (Strazzabosco M., 1997). More recent data have pinpointed a pivotal role of cholangiocytes in the regulation of repair mechanisms in response to many forms of liver injury (Clouston, et al., 2005) (Richardson, et al., 2007) (Fabris, et al., 2007) (Popov & Schuppan, 2009). This function is played by the epithelial cells located to the finest ramifications of the biliary tree, which give rise to a histological lesion called “ductular reaction” (Desmet VJ et al., 1998). Ductular reaction is considered as the main mechanism of progression in chronic liver diseases. In fact, ductular reactive cells actively participate to portal inflammation, by producing cytokines, chemokines, growth factors and other inflammatory mediators, which in turn regulate the activity of other cell elements of the inflammatory microenvironment, leading to the “hepatic reparative complex” (Strazzabosco M, 2012). Reactive cholangiocytes express *de novo* some markers of fetal origin, like the

Neural Cell Adhesion Molecule (N-CAM) and the B-Cell Lymphoma protein (BCL-2) (Fabris et al., 2000), features that are reminiscent of the early biliary morphogenesis.

Among the multiple secretory functions, reactive cholangiocytes are able to secrete VEGF (Fabris, et al., 2008), CTGF (Surveyor & Brigstock, 1999), TNF-α, IL-6 (Jia, 2011), PDGF-BB (Priester, et al., 2010) and SDF-1 (or CXCL12) (Coulomb-L'Hermin, et al., 1999). In liver fibrogenesis, the main cytokine is the TGF-β (Bissell, D. et al., 2001). TGF-β has three isoforms: TGF-β1, TGF-β2 and TGF-β3, being TGF-β1 the most important in the activation of HSC ultimately responsible for collagen synthesis (Sanderson, et al. 1995; Friedman, 2008). Whereas TGF-β2 can be produced by reactive cholangiocytes (Dünker & Krieglstein, 2002), TGF-β1 is mainly secreted by fibroblasts and macrophages. Once released in the extracellular milieu, TGF-β1 can accumulate in the extracellular matrix in a latent form associated with its pro-peptide latency-associated protein (LAP).

## 2. Pathogenetic mechanisms of cholangiopathies

Cholangiopathies are liver diseases in which the biliary epithelial cells (BECs) are the primary target of the pathogenetic sequence. Basically, these diseases feature chronic inflammation centered around the bile duct, variably associated with cholestasis, proliferation/ductopenia, peribiliary fibrosis, eventually leading to the malignant transformation of cholangiocyte (Figure 2).

When bile ducts are damaged, the repair mechanisms follow two distinct processes, regeneration and fibrosis. During regeneration, injured cholangiocytes are replaced by the same cell type leading to the reconstruction of the bile duct if the inflammatory damage is transient. However, if the chronic insult to the bile ducts persists, a wound-healing response characterized by peribiliary fibrosis develops and deposition of connective tissue may obliterate the ductal structure finally resulting in ductopenia.

In primary cholangiopathies, reactive cholangiocytes and mesenchymal cells can establish an intimate contact where a variety of paracrine signals are mutually exchanged. This functional interaction include a number of paracrine and autocrine factors (cytokines, chemokines, inflammatory mediators and growth factors), through which periductular myofibroblasts (MFs) are recruited, proliferate and release components of the extracellular matrix (ECM). The ECM provides an important architectural scaffold along which this interaction may perpetuate. Moreover ECM is an important store of growth factors, matrix metalloproteinase (MMPs), and also signals

to the epithelial cells through specific glycoproteins called integrins. The dynamic interaction between "reactive cholangiocytes" and the mesenchymal cells, the effectors of fibrosis, is considered a fundamental step of biliary fibrosis.

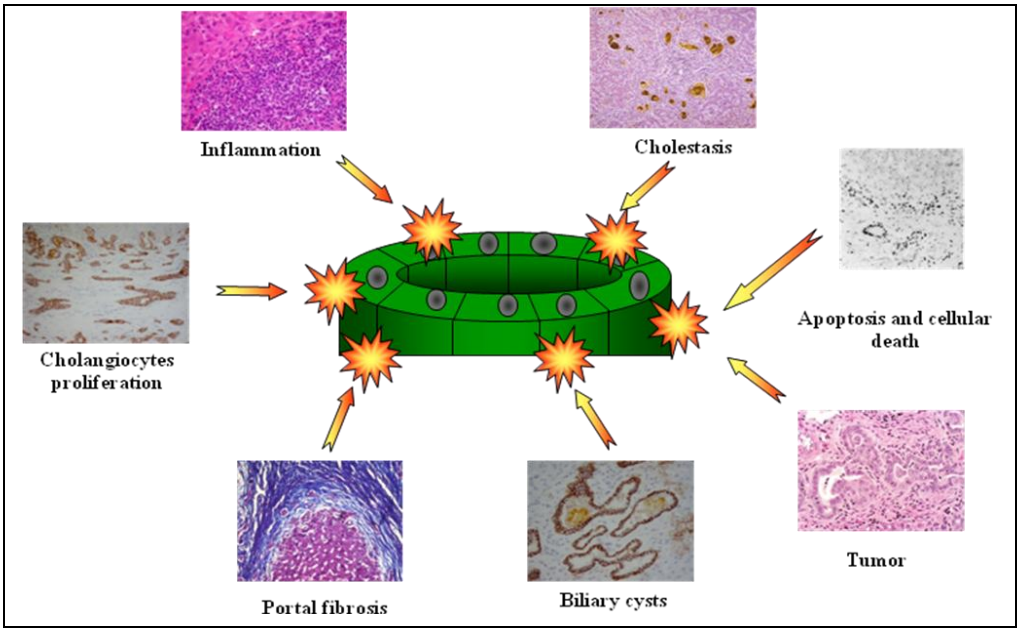



**Fig. 2: Pathogenetic lesions featuring cholangiopaties**

## 3. Hepatic fibrosis

*3.1 Pathogenesis of liver fibrosis*

Development of fibrosis is the common response to chronic liver injury underpinning the progression of liver diseases to cirrhosis and liver failure regardless of the etiology (Pinzani et al., 2011). In the last few years, exciting progress has been made in understanding the cellular and molecular mechanisms of hepatic fibrosis. The general outcome of fibrogenesis is the conversion of normal, low-density basement membrane-like matrix to an high-density interstitial type matrix. When the hepatic injury persists, liver regeneration occurs in conjunction with substitution of damaged epithelial cells with an excess of ECM, including fibrillar collagen. The structural components of the ECM are produced by resident fibrogenic cells, and secreted via excocytosis. Once released in the extracellular milieu, they aggregate with the existing matrix. The ECM is composed of an interlocking mesh of fibrous proteins and glycosaminoglycans (GAGs). The distribution of the fibrous material depends on the origin of the liver injury. In chronic viral hepatitis and chronic cholestatic disorders, the fibrotic tissue is initially located around the portal tracts, while in alcohol-induced liver disease, it locates to the pericentral and perisinusoidal areas. As the liver is getting more fibrotic, significant changes occur both quantitatively and qualitatively. The total content of collagens and non-collagenous components increases three to eight-fold, accompanied by a shift towards the interstitial type of ECM in the sub endothelial space (Disse space).

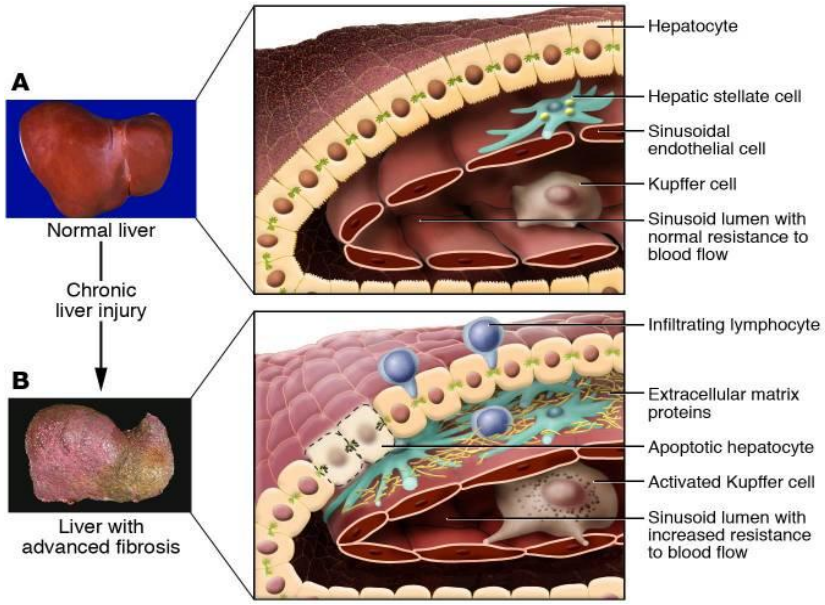


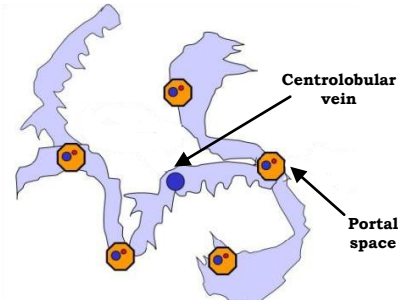
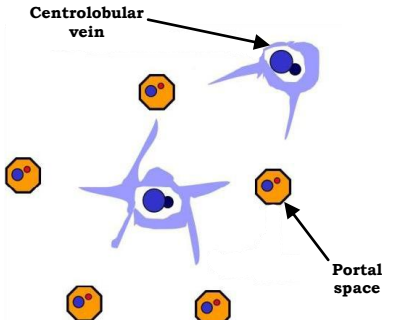
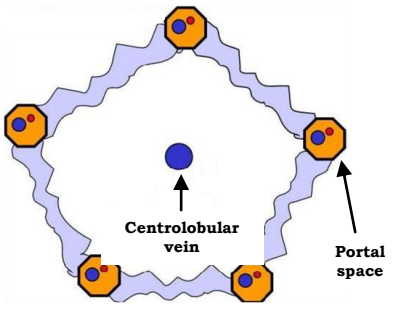
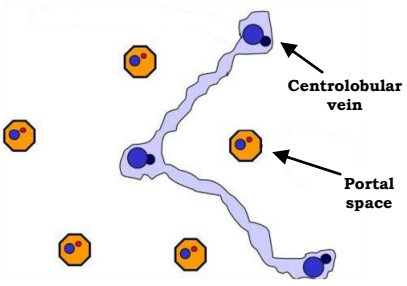

**Fig. 3: Changes in the hepatic architecture** J Clin Invest. 2005 February 1;115(2)

Hepatic fibrosis is the fundamental lesion leading to the development of the life-threatening manifestations of chronic liver diseases related to portal hypertension, and may progress to cirrhosis and hepatocellular carcinoma. In the liver, fibrosis causes important morpho-functional alterations characterized by an altered lobular architecture and by the formation of new vascular branches (Forbes & Parola, 2011). These alterations progressively result on one side, in the loss of hepatocellular function and on the other, in the vascular derangement leading to portal hypertension. In addition, fibrosis sustains an inflammatory microenvironment whereby both hepatocytes and cholangiocytes are prone to neoplastic transformation.

Liver fibrosis can be categorized into four different patterns depending upon the histological structure, the etiology and pathogenesis (Parola & Pinzani, 2009):

- ***Bridging fibrosis:*** typical of chronic viral hepatitis, it is associated with portal-central bridging necrosis, finally resulting in the formation of portal-central and portal-portal fibrotic septa, with marked changes in vascular connections.
- ***Perisinusoidal/pericellular fibrosis:*** typical of non-alcoholic steatohepatitis (NASH) and of alcoholic-induced liver disease (ALD), in this type of fibrosis, deposition of ECM starts to accumulate in in the spaces of Disse, around sinusoids or groups of hepatocytes.
- ***Biliary fibrosis:*** typical of evolving cholangiopathies, it is associated with a pleiomorphic inflammatory portal infiltrate centered around the bile duct, involving a mixed population of lymphocytes, macrophages, and pericytes, and with the development of portal-portal fibrotic septa. Notably, this form of fibrosis may be responsible of early portal hypertension, even before the evolution to cirrhosis.
- ***Centrolobular fibrosis:*** secondary to conditions of altered venous outflow (Veno-Occlusive disease, Budd-Chiari syndrome, chronic hearth failure), it is characterized by fibrotic septa developing from the central vein and can be associated with acute hepatocellular necrosis.

**Tab. 1: Liver fibrosis patterns.**

| ***PATTERNS*** | ***HISTOPATHOLOGY*** | ***STRUCTURE, ETHIOLOGY AND PATHOGENESIS*** |
|---|---|---|
| **BRIDGING FIBROSIS**   | Portal-central bridging necrosis, accompanied by the formation of portal-central fibrotic septa and marked alterations of the vascular architecture | Viral chronic infections (HBV, HCV) and autoimmune hepatitis (AIH)<br><br>Chronic activation of tissue repair and oxidative stress |
| **PERISINUSOIDAL/PERICELLULAR FIBROSIS**   | Deposition of thin collagen fibers in the space of Disse around sinusoids ("chicken wire" pattern) | Non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), and alcoholic hepatitis (ASH)<br><br>oxidative stress |
| **BILIARY FIBROSIS**   | Pronounced ductular reaction, associated with peribiliary fibrosis and portal-portal septa | Chronic cholangiopathies<br><br>Epithelial-Mesenchymal interaction and oxidative stress |
| **CENTROLOBULAR FIBROSIS**   | The fibrotic septa start from the centrolobular vein; acute hepatocellular damage can be present | Veno-occlusive disease (VOD), Budd-Chiari syndrome (BCS), Chronic heart failure<br><br>Altered venous outflow |

*3.2 Cellular elements involved in liver fibrosis*

Liver fibrosis is the result of the abundant deposition of extracellular matrix (ECM) that may occur in different sites. Multiple cell types are variably involved, herein briefly illustrated.

- *Hepatic Stellate Cells (HSC)*. These cells in the quiescent state are localized in the perisinusoidal area and are activated in conditions of chronic hepatocellular damage. They are the main source of the myofibroblast population in the patterns of bridging and perisinusoidal fibrosis (Forbes & Parola, 2011). In addition to these functions, HSC possess a significant contractile activity (Lee & Friedman, 2011), which is able to promotes an increase in the vascular resistance of the sinusoids even in the early stages of fibrosis.

- *Portals Fibroblasts (PFs)*. Portal fibroblasts are present in an inactive form in the connective tissue of the portal tract and can differentiate into MF in response to chronic liver damage (Dranoff & Wells, 2010). Their activation is mainly induced by reactive cholangiocytes as part of the mechanisms of repair/regeneration (Fabris & Strazzabosco, 2011). In this process, production of TGF-β2 by reactive cholangiocytes is a key event (Wells, et al., 2004) (Forbes & Parola, 2011).

- *Myofibroblasts*. MF are an heterogeneous cell population of mesenchymal origin which play a central role in the fibrogenetic mechanisms given their ability to produce a range of factors stimulating the remodeling and deposition of ECM as well as the neoangiogensis. MF are characterized by the expression of different markers associated with the production of several cytokines (Parola & Forbes, 2011) (Novo et al., 2009):

- Positivity for α-Smooth Muscle Actin (α-SMA);
- Production of collagen proteins and tissue inhibitor of matrix metalloproteases (TIMPs);
- Production of autocrine and paracrine signals that promote cell survival as well as angiogenesis, such as Vascular Endothelial Growth Factor (VEGF), Platelet-derived Growth Factor (PDGF), Transforming Growth Factor (TGF)-β1 and Angiopoietins;
- Secretion of pro-inflammatory cytokines such as the Monocyte Chemotactic Protein-1 (MCP1) and ligands for Toll Like Receptor (TLR).

The main growth factors regulating the activity of MFs are TGF-β1, PDGF and interleukin 13 (IL-13). TGF-β1 is the main cytokine which stimulate proliferation and functional activation of MF (Sarrazy, et al., 2011).
PDGF, in particular PDGF-B (Czochra, et al., 2006), is also a potent inducer of proliferation (Pinzani & Rombouts, 2004) and migration (Kinnman, et al., 2000) of MF, whereas IL-13 stimulates the activation of

MF and the production of TGF-β1 by macrophages (Wynn, 2008) (Wynn, 2011).

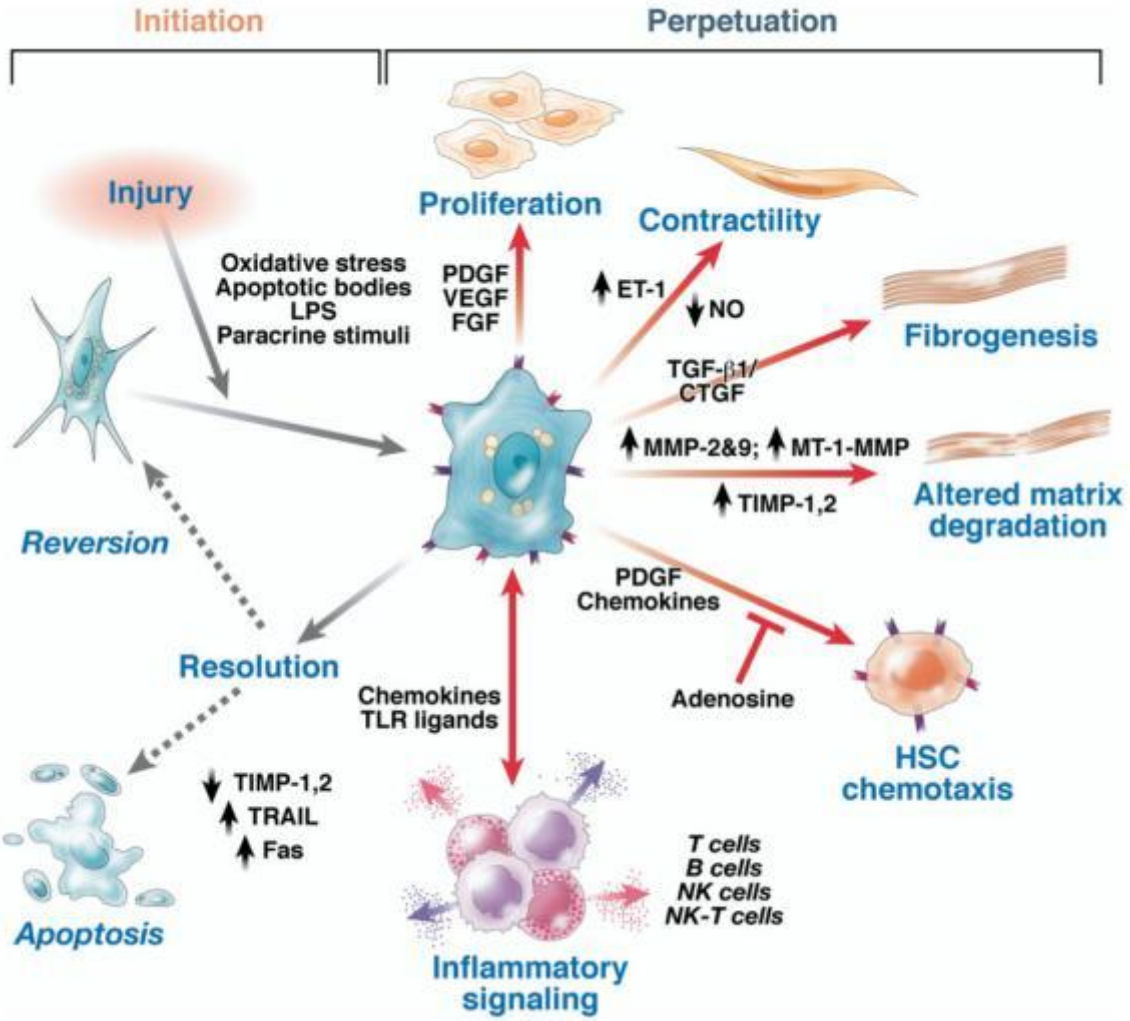



**Fig. 4: Mechanisms of hepatic fibrogenesis.** Gastroenterology. 2008 May;134(6):1655-69. Friedman SL.

- *Fibrocytes*. These are circulating cells of bone marrow origin, characterized by an intermediate phenotype between monocytes (from which they originate) and myofibroblasts (in which they can transdifferentiate) (Bucala, et al., 1994) (Reilkoff, et al., 2011). They may

behave as a reservoir of immature mesenchymal cells involved in the healing processes and in the immunoregulation in different tissues and organs (Reilkoff, et al., 2011). Several cytokines may stimulate the transdifferentiation of monocytes into fibrocytes, including IL-13, IL-4 (Bellini, et al., 2012) and even TGF-β1 (Scholten, et al., 2011). The recruitment of circulating fibrocytes is mainly regulated by CXCR4, the surface receptor of the chemokine Stromal-Derived Factor (SDF-1/CXCL12), which exerts a potent chemotactic effect (Philips, et al., 2004). Although fibrocytes are potentially involved in all patterns of hepatic fibrosis, whether they can actually contribute to the generation of MF is still a matter of deep controversy, but likely only for a small fraction (<10% MF) (Kisseleva, et al. 2006).

- *Monocytes and macrophages.* These two cell populations, consisting of liver resident macrophages (Kuppfer cells) and monocytes derived from circulation, represents an important source of tumor necrosis factor alpha (TNF-α), and also of TGF-β and PDGF-BB, critical factors for the activation of HSC (Marra et al., 2009).

Activation of macrophages is an important feature of reparative fibrogenic processes, and is associated with peculiar secretory functions (Wynn & Barron, 2010; Gordon, 2003; Mantovani et al., 2004), on which macrophages can be classified.

Classically activation (M1 macrophages or CAM) is stimulated by lipopolysaccharide (LPS), interferon-γ (INFγ) and TNF (Mills, et al., 2000). It is characterized by the production of pro-inflammatory cytokines involved in acute inflammation, and of proteolytic enzymes, such as

matrix metalloproteases (MMPs), actively involved in the resolution of fibrosis (Mosser MD, 2003).

Alternative activation (M2 macrophages or AAM) is stimulated by IL-13, IL-4 and IL-10. By releasing TGF-β1, M2 macrophages are relevant in the resolution of inflammation and in the promotion of tissue repair (Gordon & Martinez, 2010).

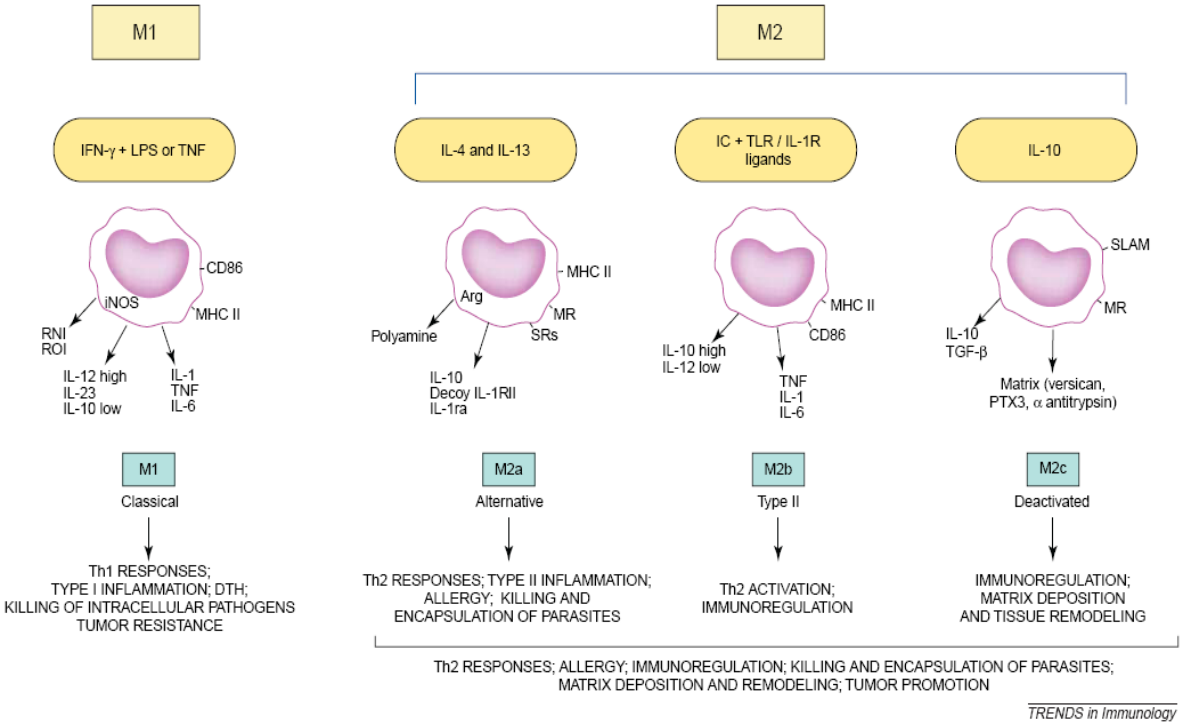



**Fig. 5: The chemokine system in diverse forms of macrophage activation and polarization.** TRENDS in Immunology Vol.25 No.12 December 2004 A. Mantovani

### *3.3 Liver fibrosis and avβ6 integrin*

In the liver, the most prominent profibrogenic molecule is the transforming growth factor β1 (TGFβ1), among several cytokines and growth factors. Following liver injury, TGFβ1 production is strongly up-regulated in multiple cell types, mainly in hepatic stellate cells (HSC) and portal myofibroblasts, but even in macrophages. TGFβ1 is produced as inactive form, and is locally activated after binding with the αvβ6 integrin which cleaves the latency associated peptide (LAP) from the precursor (Annes, J. et al., 2004).

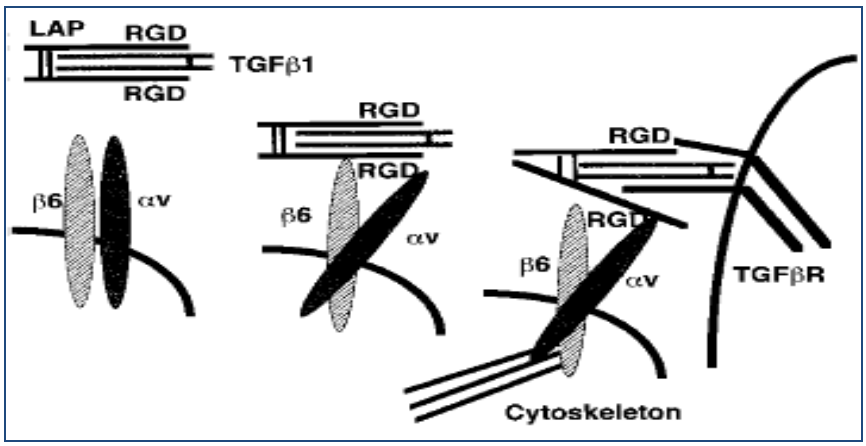



**Fig. 6: Model of TGF*β*1 activation by *αvβ*6 integrin.** The data presented suggest that when latent TGF*β*1 complexes bind to *αvβ*6, sites in the *β*6 cytoplasmic domain become accessible for binding to the actin cytoskeleton (Munger et al.;Cell 1999)

Once activated, TGFβ1 initiates the signaling cascade by bringing together the two TGFβ receptors, type I (TGFβRI) and type II (TGFβRII), on the cell surface. This allows TGFβRII to phosphorylate TGFβRI at the kinase domain, which then propagates the signal by phosphorylating the Smad proteins, critical regulators of different fibrogenic activities.

Integrins are heterodimeric cellular receptors involved in cell-cell and cell-matrix interactions. They are dimers formed by an α and a β subunit, variably assembled in at least 24 different isoforms.

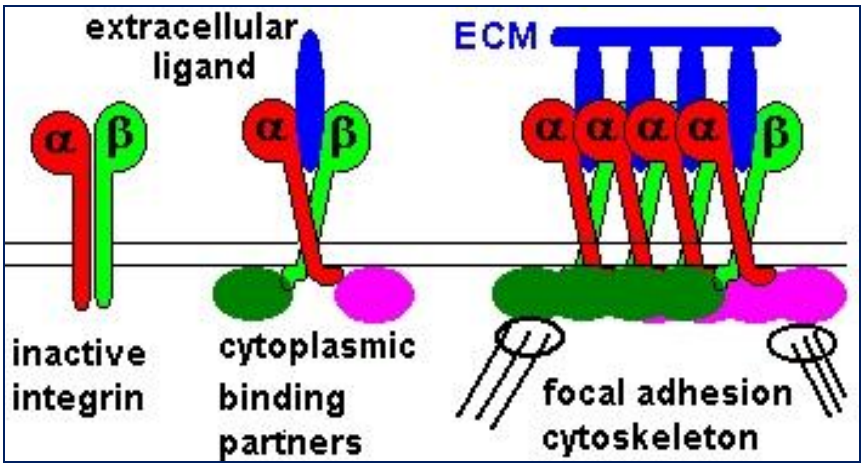



**Fig. 7:** Mechanically and mutually active connections between the cell core and the ECM (extracellular matrix).

Among them, αvβ6 integrin possesses a high affinity for LAP. Once activated, TGFβ1 exerts its biological effects within the limits of the surface of cells displaying αvβ6. αvβ6 integrin expression is restricted to epithelial cells, particularly to ductal epithelia, where it mediates cell adhesion to fibronectin (Fontana L. et al., 2005) (Weinacker, A. et al., 1994) (Busk M et al., J 1992) and to tenascin-C (Prieto et al., 1993), main ECM components of granulation tissues. It is normally expressed during embryonic development, particularly in the lung, skin and kidney (Breuss JM et al., 1995), and decreases thereafter, until disappearing almost entirely in the normal epithelium after birth. In the adult, αvβ6 integrin is promptly up-regulated in response to duct injury and inflammation, and disappears as the inflammatory process resolves (Wang, B. et al., 2007).

In two experimental models of liver fibrosis, the bile duct ligated (BDL) rat and the Mdr2(abcb4)$^{-/-}$ mouse, αvβ6 integrin is expressed by reactive

cholangiocytes, a stereotyped hepatic response to inflammatory damage, and it drives the progression of biliary fibrosis by auto/paracrinally activating TGFβ1 (Patsenker E. et al., 2008) (Popov Y. Et al., 2008). Molecular factors regulating the expression of αvβ6 integrin are largely unknown. Following activation by αvβ6 integrin, TGFβ1 may in turn, increase further the production of this receptor. Whether other pro-inflammatory cytokines can induce up-regulation of αvβ6 integrin is at present uncertain. Studies of the role of this protein in lung injury are consistent with those examining the skin. Intratracheal administration of bleomycin causes fibrosing bronchiolar injury. The response was significantly attenuated in animals lacking αvβ6, with a marked reduction in fibrosis. (Huang X et al., 1998).

*3.4 Experimental models of liver fibrosis*

Several mice models of experimental fibrosis have been established to unravel the molecular and cellular mechanisms of the hepatic fibrosis.

*Pkhd1*$^{del4/del4}$ MUTATIONS IN *PKHD1* are responsible for typical forms of ARPKD. *Pkhd1*$^{del4/del4}$ mice develop intrahepatic bile duct proliferation with progressive cyst formation and associated periportal fibrosis. *Pkhd1*$^{del4/del4}$ mice also develop pancreatic ductal dilation and fibrosis. This model mouse provides evidence that reduced functional levels of fibrocystin are sufficient for cystogenesis and compare with the other model mice (see above), it develops an age-dependent increase of portal fibrosis and hypertension. (Gallagher AR et al., 2008)

*CHEMICALLY INDUCED FIBROSIS USING HEPATOTOXIC AGENTS* Carbon tetrachloride (CCl4) is the most commonly used liver damaging agent to induce liver fibrosis. The trichloromethyl radical, a metabolite produced by cytochrome P-450 in hepatocytes, leads to lipid peroxidation and membrane damage, which results in a reversible acute centrolobular liver necrosis.

*CHOLESTATIC FIBROSIS BY BILE DUCT LIGATION* Bile duct ligation is a convenient and well-studied experimental disease model that induces biliary fibrosis and cirrhosis such as extrahepatic biliary atresia and primary sclerosing cholangitis.

*EXPERIMENTAL LIVER FIBROSIS USING TRANSGENIC* MICE Over the past decade, targeted gene knockouts (loss of function) in mice have become a powerful strategy to address the basis of mono- and polygenic disorders.

**4. Genetic cholangiopathies: Autosomal Recessive Polycystic Kidney Disease (ARPKD), Congenital Hepatic Fibrosis (CHF) and Caroli Disease (CD)**

Genetic cholangiopathies are relatively rare disorders with an estimated prevalence of 1:20,000 live births (Zerres et al., 1998), but with a high mortality rate. Up to 30% of affected people die at birth or within the first year of life or soon develop kidney and liver dysfunctions. Liver fibrosis requires both a well-orchestrated proliferation of cells and the reconstruction of ECM. If the injury persists, the damaged tissues/organs undergo substitution by overabundant ECM and suffer from extensive, pathological fibrosis. Thus, liver fibrosis is of great clinical importance, since normal liver architecture is disrupted and liver function is ultimately impaired. There are several major causes of liver fibrosis and way to study it. Biliary epithelial cells are the primary cell target of several genetic or acquired diseases called cholangiopathies. Cholangiopathies affecting the intrahepatic biliary epithelium can cause high morbidity and mortality. They are the main indication for liver transplant in the pediatric population (Nakanuma, Y. et al., 2010).

Cholangiopathies include a wide spectrum of diseases with different pathogenesis; the biliary epithelium can be damaged by disordered immunity (primary biliary cirrhosis, graft versus host disease and primary sclerosing cholangitis), infectious agent (cytomegalovirus or cryptosporidium), ischemia and toxic compound. In addition, genetic or developmental diseases, that lead to an impaired bile duct biology, are

known to cause progressive cholangiopathies (Alagille Syndrome, Cystic Fibrosis, ADPKD, Caroli Disease, Biliary Atresia etc.). Liver disease is characterized by dysmorphic intrahepatic bile ducts resembling the embryonic ductal plate, which are accompanied by the progressive establishment of portal fibrosis, portal hypertension, hypersplenism, esophageal varices and ascites (Yonem O & Bayraktar Y., 2007). Even though portal fibrosis is the main pathogenic mechanism in CHF and CD, the mechanistic relationships between the defect in fibrocystin and portal fibrosis are not known.

Autosomal Recessive Polycystic Kidney Disease (ARPKD), Congenital Hepatic Fibrosis (CHF) and Caroli's Disease (CD) are genetic disorders of the liver and kidney caused by deficiency in fibrocystin (FPC), the protein encoded the *PKHD1* gene. (Guay-Woodford LM et al., 1995) (Ward CJ et al.,2002).

*4.1 Fibrocystin*

Fibrocystin is a large protein with a single transmembrane domain that is localized in primary cilia and in basal bodies of renal tubular and biliary epithelial cells (Ward CJ et al., 2003) (Zhang MZ et al., 2004). It complexes with polycystin-2 and is involved in cell $Ca^{2+}$ homestasis, but it can also signal via the nuclear translocation of proteolytically clived C-terminus (Wang S. et al., 2007). Its function is not known; likely it is involved in proliferation, secretion, terminal differentiation, tubulogenesis and the maintenance of duct architecture, mediating interactions with the extracellular matrix (ECM). Silencing *Pkhd1* in cultured mouse renal tubular cells, alters cytoskeletal organization and impairs cell-cell and cell-matrix contacts (Mai W et al., 2005).In the liver, FPC deficiency leads to an aberrant development of the bile ducts, which results in biliary microhamartomas and segmental dilations of the bile ducts. In contrast with other cystic cholangiopathies caused by genetic defects in ciliary proteins, such as the polycystic liver associated with the autosomal dominant polycystic kidney disease (ADPKD), in CHF biliary dysgenesis is accompanied by progressive portal fibrosis clinically resulting in portal hypertension. Complications of portal hypertension are a major cause of morbidity and mortality in CHF, especially in young adults. The mechanisms responsible for portal fibrosis in CHF are unknown.

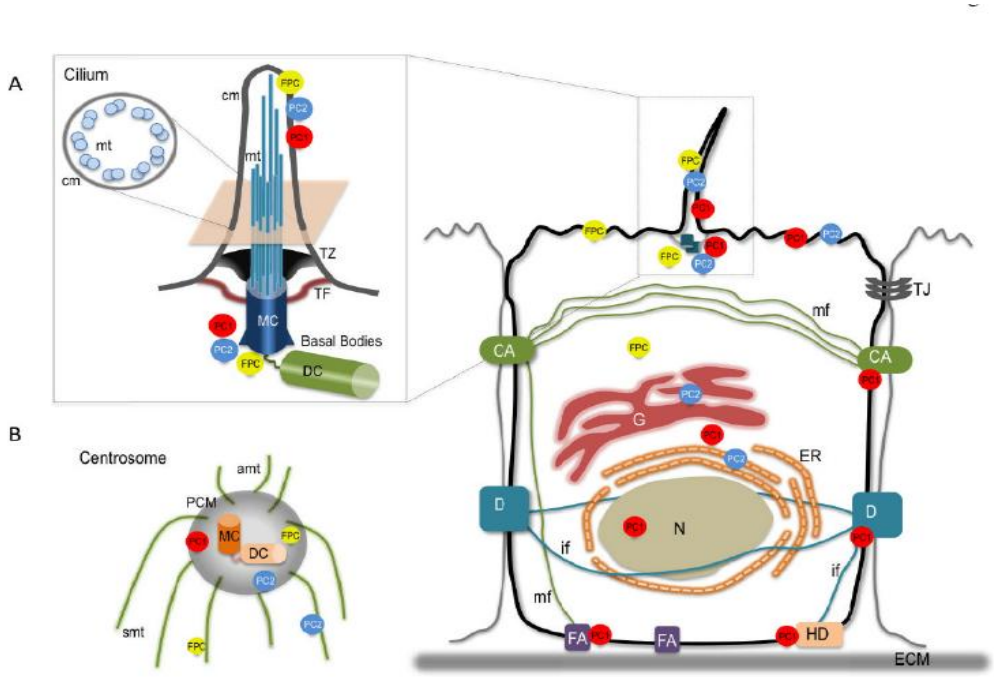



**Fig. 8: Cilium, centrosome and cell cycle regulation in polycystic kidney disease.** Biochim Biophys Acta. 2011 Oct;1812(10):1263-71. 2011 Mar 2. Lee K et al.

The function of FPC remains unknown, however his alterations are actively involved in cystogenesis (Kim, et al., 2008). Several studies have shown how the FPC has a significant role in the regulation of proliferation, in planar polarity (Strazzabosco & Fabris, 2012) and cell apoptosis (Mai, et al., 2005) (Sun, et al., 2010) as well to be implicated in the mechanisms of regulation of $Ca^{2+}$ in synergy with PC2 (Al-Bhalal & Akhtar, 2008).

# ***AIMS***

Congenital hepatic fibrosis (CHF) and Caroli disease (CD) are genetic disorders of the biliary epithelium, with a prevalence in the general population of 1:20000. They are rare diseases causing major morbidity in childhood and young adult population. There are no treatments for these diseases; frequently for their severity is necessary liver transplantation. The peribiliary fibrosis is the main factor determining the clinical phenotype of the disease, but the mechanisms that regulate fibrosis and his development are unknown; an important role in the genesis of fibrosis is played by αvβ6 integrin, whose expression on reactive ducts correlates with the degree of fibrosis. To understand the mechanisms of fibrosis in CHF, we studied the expression of αvβ6 integrin and its regulation in mouse model $Pkhd1^{del4/del4}$ developed by Professor Somlo at Yale University. In particular, the specific aims of my project are:

1. Understand if $Pkhd1^{del4/del4}$ mouse is a good model to study CHF with develop of fibrosis and portal hypertension
2. Assess whether the development of biliary cyst is correlated with the degree of fibrosis and portal hypertension during different ages;
3. Assess whether the αvβ6 integrin is expressed at the level of the biliary epithelium and if its expression is correlated with the degree of portal fibrosis;
4. Assess whether the expression of αvβ6 integrin on the bile duct is due by mechanisms a cross-talk between cholangiocyte and inflammatory cells in the portal tract.

# ***METHODS***

*Experimental mouse model of liver fibrosis*

Pkhd1$^{del4/del4}$, the mouse model of ARPKD used in this study, was a kind gift of Prof. S. Somlo (Yale University, New Haven, CT). The mouse, of a mixed C57BL6/129Sv background, carries a inactivating deletion in the exon 4 of the Pkhd1 gene (Gallagher et al., 2008). These mice mimic the human hepatic disease, developing cysts and progressive portal fibrosis in the liver, already present at 1-3 month (Gallagher et al., 2008). Mice were genotyped by polymerase chain reaction (PCR) analysis of DNA extracts from tail sections. The primers used to screen for the deletions are the forward primer 5'-TTAGGGAAGAATGGCTCTC-3', and the reverse primer 5'-TTCAGAGGGAGGAAAAGCAA-3' for the WT allele, to produce a 580-bp fragment. The reverse primer 5'- GCCAGAGGCCACTTGTGTAG-3', amplifying a 171-bp product was used for the Pkhd1$^{del4}$ allele, as described in Gallagher et al., 2008). WT and mice homozygotes for the deletion were used for this study; heterozygotes were maintained for the breeding.

*Sirius red to evaluate fibrosis extension*

It was used a staining for Sirius Red to analyze the degree of fibrosis in the liver. Sections stained with Sirius Red (n = 5 for each age) were analyzed with a microscope Nikon Eclipse TE2000U polarized light (Nikon, Bloomfield, CT, USA) equipped with a motorized stage (Rockland, MA, USA) that scan, at a magnification of 10X, the whole liver lobes dissected. The images were collected using a digital camera for high quality images (Roper Scientific, Tucson, AZ, USA) and analyzed by Metamorph software (Molecular Devices, Downingtown, PA, USA). We analyzed 10 random

fields containing portal tracts in which the degree of fibrosis was calculated as percentage of the area stained with Sirius Red portal space total.

*Spleen weight quantification*

The spleens of *Pkhd1*$^{del4/del4}$ and WT mice were removed immediately after sacrifice and weighed fresh using precision scale (Kern & sohn, Balingen, Germany). The weight value found was normalized to the total weight of the mouse.

*Immunohistochemistry staining for PanCK, CD45, F4/80, aSMA and avβ6 integrin*

The expression of αvβ6 integrin was evaluated in the same serial sections of those colored with Sirius Red.

After dewaxing, the sections were hydrated with alcohol and subsequently blocked with 30 minutes of incubation in hydrogen peroxide and methanol (10%). After the antigen retrieval, washing with 0.05% Tween 20 in phosphate buffered saline Ultra (PBS, pH 7.4) and blocking with Block V (LabVision, CA), the sections were incubated with primary antibody overnight at 4 ° C. We used the following primary antibodies: αvβ6 integrin (2A1 chimeric Stromedix Cambridge, MA, 1:500), PanCK (DAKO, 1:200), CD45 (rat anti-mouse IgG2b, BD Pharmingen ™, 1:20). After incubation with the antibody selected, the sections were rinsed with 0.05% Tween 20 in PBS for 5 minutes, incubated for 30 minutes at room temperature with horseradish peroxidase and appropriate secondary antibody conjugate and finally developed with 3 - 3'-diaminobenzidine.

In other sections was studied by immunofluorescence staining the co-expression of PanCK and αSMA (Dako, USA), F4/80 (eBioscience inc., San Diego, USA, 1:50), CD45 and F4/80 to characterize the phenotype of CD45-positive inflammatory cells recruited into the portal space. After incubation for one night at 4C° with the primary antibody and subsequent washing, the sections were incubated with the following fluorescent secondary antibodies (Alexa 488 and 594, 1:500, diluted in 1% bovine serum albumin in PBS-Tween, Invitrogen Molecular Probes) for 30 minutes at room temperature in darkness. After washing in PBS and mounting using a kit Vectashield (Vector Laboratories, Inc., Burlingame, CA) with 4,6-Diamidino-2-phenylindole, the slides were examined using the microscope Nikon Eclipse TE2000U fluorescent lamp and the ImageJ software.

*Isolation and characterization of cholangiocytes*

Mouse cholangiocytes were isolated from wild-type and *Pkhd1*$^{del4/del4}$ mice The portal vein was cannulated with a 22-gauge intravenous catheter. The liver was perfused with a buffer solution. Cholangiocytes from WT mice were isolated through micro dissection of intrahepatic bile ducts, whilst, for conditional KO mice, cells were isolated directly from micro dissected cysts (as in Fabris et al., 2006). Cells were cultured on rat tail collagen in a specific medium, NMC (Vroman & LaRusso, 1996) additioned with 10% fetal bovine serum, and eventually formed a monolayer with measurable trans-epithelial resistance. Biliary phenotype was confirmed by cytokeratin-19 staining. Cells were subcultured for up to ten passages.

*Quantitative real – time RT-PCR*

We assessed the amount of mRNA for αvβ6 integrin, Collagen type I and αSMA (Applied Biosytstems, USA) on total liver and on both WT and Pkhd1 mutated polarized cholangiocytes.

To the lysate total hepatic a series of liver biopsies, from the total weight of 100-150 mg, from two lobes of the liver was homogenized with a homogenizer. For RNA extraction from polarized cholangiocytes was used Trizol reagent (Invitrogen, USA).

The total mRNA was extracted using the reagent RNAPure (PeqLab, Erlangen, Germany) according to the manufacturer's recommendations. The corresponding DNA was obtained by reverse transcription of 1 micrograms of total mRNA and the relative transcript levels were quantified by RT-PCR on an instrument ABI 7500 (Applied Biosystem, USA) using the TaqMan probes as previously described (Popov, et al., 2006). The TaqMan probes (dual-labeled with 5'-FAM and 3'-TAMRA) and primers were designed on the basis of the sequences generated by the software Primer Express (Perkin-Elmer, Wellesley, USA) and synthesized by MWG Biotech AG (Ebersberg, Germany). All the probes used in the study are designed to cover the exons terminals in order to exclude genomic DNA of co-amplification.

*Quantification of avβ6 integrin and CD45-positive cells*

To quantify the areas positive for αvβ6 integrin and CD45, 5 random no-overlapping fields for each main liver lobe (10 fields/mouse) were recorded with a digital camera at 200X magnification. In these micrographs the number of bile ducts positive for αvβ6 integrin and the area positive for CD45 ($\mu m^2$) were manually measured using the ImageJ software by 2 investigators blinded to the treatment code (LL, SL). Computed morphometric analysis of CD45 immunolabeling allowed calculating the percentage of pixels above the threshold value with respect to the total pixels per slide area.

*Liver non-parenchymal cell isolation and FACS analysis*

Non-parenchymal cells were isolated from *Pkhd1*$^{del4/del4}$ liver mice in the laboratory of the Liver Center at Yale University.

The livers from mice Pkhd1$^{del4/del4}$ and WT mice of different ages (3, 6 and 9 months in which each group is formed by two elements) were perfused with collagenase / DNase and subsequently removed. It was formed a pellets hepatocytes through centrifugation at low speed spin (2 times to 30 g for 3 minutes), and the fraction non-parenchymal was isolated by centrifugation in density gradient at 4 ° (1500 g for 20 minutes) using a gradient 12-18% Optiprep (Sigma, MO).

FACS analysis was performed to characterize the subpopulation of non-parenchymal cells which constitute the inflammatory infiltrate portal. The cells were resuspended in PBS / 3% FBS and incubated with monoclonal antibodies (1:200, 30 minutes, 4 ° C). The monoclonal antibodies used, were: anti-mouse CD45 PerCP, anti-mouse CD11b APC,

FITC anti-mouse F4/80, APC anti-mouse NK-1.1, FITC anti-mouse CD19, PE anti-mouse Ly-6G/Ly- 6C and PE anti-mouse CD3. It was used a FACSCalibur (BD Biosciences, CA) and analyzed using FlowJo (Tree Star).

*Cytokine secretion (Luminex assay)*

To assess the secretory profile of colangiocytes differentiating the basolateral secretion from the apical cholangiocytes, PKHD1$^{del4/del4}$ and WT mice were isolated at 3 months (Spirli, et al., 2010).

The biliary cells were maintained in culture on a thin layer of collagen derived from mouse tail and cultured as monolayer (Spirlì, et al., 2001). To confirm the biliary phenotype of cultured cells was performed a staining for PanCK and was measured resistance trans epithelial (≥ 1000 Ω × cm2) (Spirli, et al., 2010).

Culture supernatant was used for the analysis of 32 mouse chemokines and cytokines by using the Millipore's MILLIPLEX™ mouse Cytokines/Chemokines kit coupled with BioPlex Luminex platform following manufacturer's instructions.

The Bio-Plex assay contains microspheres conjugated to monoclonal antibodies specific for a target protein or a peptide. Each of the 100 microspheres may contain only a specific antibody capable of capturing a single target protein. The microspheres conjugated to the antibody are allowed to react with the sample. The multiplex assays are created by mixing the microspheres conjugated to different antibodies in order to test simultaneously many analytes in the same sample.

Both the results basolateral and apical were primarily normalized for the amount of total protein of polarized cells, after which the data are expressed in pg/ug.

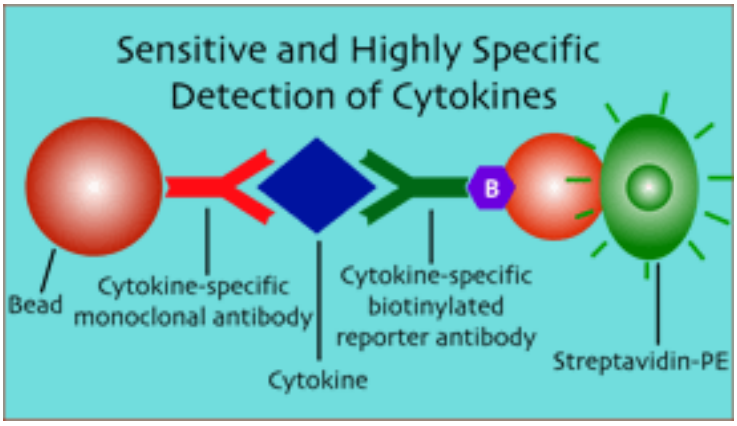



**Fig. 9:** Systems using Luminex technology perform discrete bioassays on the surface of color-coded beads known as microspheres, which are then read in a compact analyzer.

*Functional studies to address the role of cytokines secreted by Pkhd1$^{del4/del4}$ cholangiocytes*

To assess whether the cytokines found to be most abundantly secreted in the basolateral medium of *Pkhd1*$^{del4/del4}$ cholangiocytes CXCL1 (also termed KC) and CXCL10 (also termed IP-10) could be functionally relevant as paracrine signals for the inflammatory cells, we studied the ability of each cytokine to stimulate cell migration in macrophages. We used the macrophage cell line RAW 264.7, derived from Abelson murine leukemia virus-induced tumors in a BALB/c mouse (American Type Culture Collection – ATCC, Manassas, USA).

Assessment of cell migration

Briefly, $5x10^4$ RAW 264.7 macrophages were resuspended in serum-free medium and seeded over a polyvinylpyrrolidone-free polycarbonate membrane 8µm-pore filters (Transwell, Costar, Milan) coated with 50µg/ml matrigel, placed in a Boyden microchamber. Different doses of each single recombinant protein were administered in the lower compartment and compared to controls with no cytokines. Six wells were used for each experimental condition, whereby cells added to the upper compartment of the chamber were incubated for 48 h at 37°C in a 5% CO2/95% air atmosphere. To evaluate the number of fully migrated macrophages, the cells on the upper surface were removed with a cotton swab and the lower surface of the transwell filter was stained with Diff-Quick Staining Set (Medion Diagnostics, Milan) and then micrographs of total membrane were taken on each filter to count the number of clearly discernible nuclei.

*Effects of TGFβ1 and TNFα on β6 mRNA expression in cultured cholangiocytes before and after the inhibition of the TGFβ receptor type II*

After starvation for 24h, polarized cultured cholangiocytes were treated for 24h with recombinant murine TGFβ1 (1ng/ml) and TNFα (1000U/ml) (both from R&D Systems, Minneapolis, USA). β6 mRNA expression was then evaluated in total RNA extracted with Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA) from cultured cholangiocytes, as previously described. To ascertain if TNFα drove separate signaling pathways to directly modulate the β6 expression, independently from a TGFβ1 loop, the same experiments of cytokine stimulation were also run after pre-

incubation for 24h of cultured cholangiocytes with the recombinant soluble TGFβ receptor type II-FC fusion protein (rsTGF-βRII-FC) (10 ng/ml) (Stromedix, Inc., Cambridge, USA). rsTGF-βRII-FC is a specific chimeric molecule inhibiting the TGFβ1 signaling, in which the extracellular portion of TGFβRII was fused to an immunoglobulin heavy chain FC fragment. By blocking specifically the TGFβRII, this antibody prevents the association of TGFβRII with TGFβRI and therefore, its phosphorylation.

*Effects of TGFβ1 on COLL1 mRNA expression on cultured cholangiocytes*

After starvation for 24h, polarized cultured cholangiocytes were treated for 24h with the recombinant murine TGFβ1 (1ng/ml). Before and after TGFβ1 stimulation, COLL1 mRNA expression was determined in total RNA from cultured cells (see above).

*Statistical Analysis*

Results are shown as mean ± SD. Statistical comparisons were made using one-way analysis of variance or the Wilcoxon–Mann–Whitney 2-sample rank sum test, where appropriate. In the latter, the p value was obtained from the exact permutation null distribution. Correlation studies were performed between the following parameters: a) portal tract area positive for Sirius red and spleen weight/body weight, b) cyst expression of αvβ6 integrin and portal tract area positive for Sirius red, and c) cyst expression of αvβ6 integrin and portal tract area positive for CD45 (all continuous normally distributed variable) using the Pearson's coefficient.

The statistical analysis was performed using SAS software (SAS Institute Inc, Cary, NC); p values $<0.05$ were considered significant.

# ***RESULTS***

## 1. Age-dependent increase in peribiliary fibrosis correlates with portal hypertension in *Pkhd1*$^{del4/del4}$ mice

In stark contrast with WT littermates, *Pkhd1*$^{del4/del4}$ mice showed development of Sirius Red stained areas typically restricted to the peribiliary region, which progressively increased with a linear pattern through the different mice maturation (Figure 10A). Progressive development of fibrosis areas was associated with an increase in the spleen weight/body weight (index of splenomegaly) which started to be significant at 6 months, and then increased further (Figure 10B).

There was a strong correlation between Sirius Red staining and splenomegaly ($r=0.88$, $p<0.05$) (Figure 10C). These findings indicate that *Pkhd1*$^{del4/del4}$ mice is a model of slowly progressive peribiliary fibrosis, which is evident since the earliest stages and then becomes clinically relevant with development of portal hypertension, fitting well with the clinical setting of portal fibrogenesis observed in human CHF.

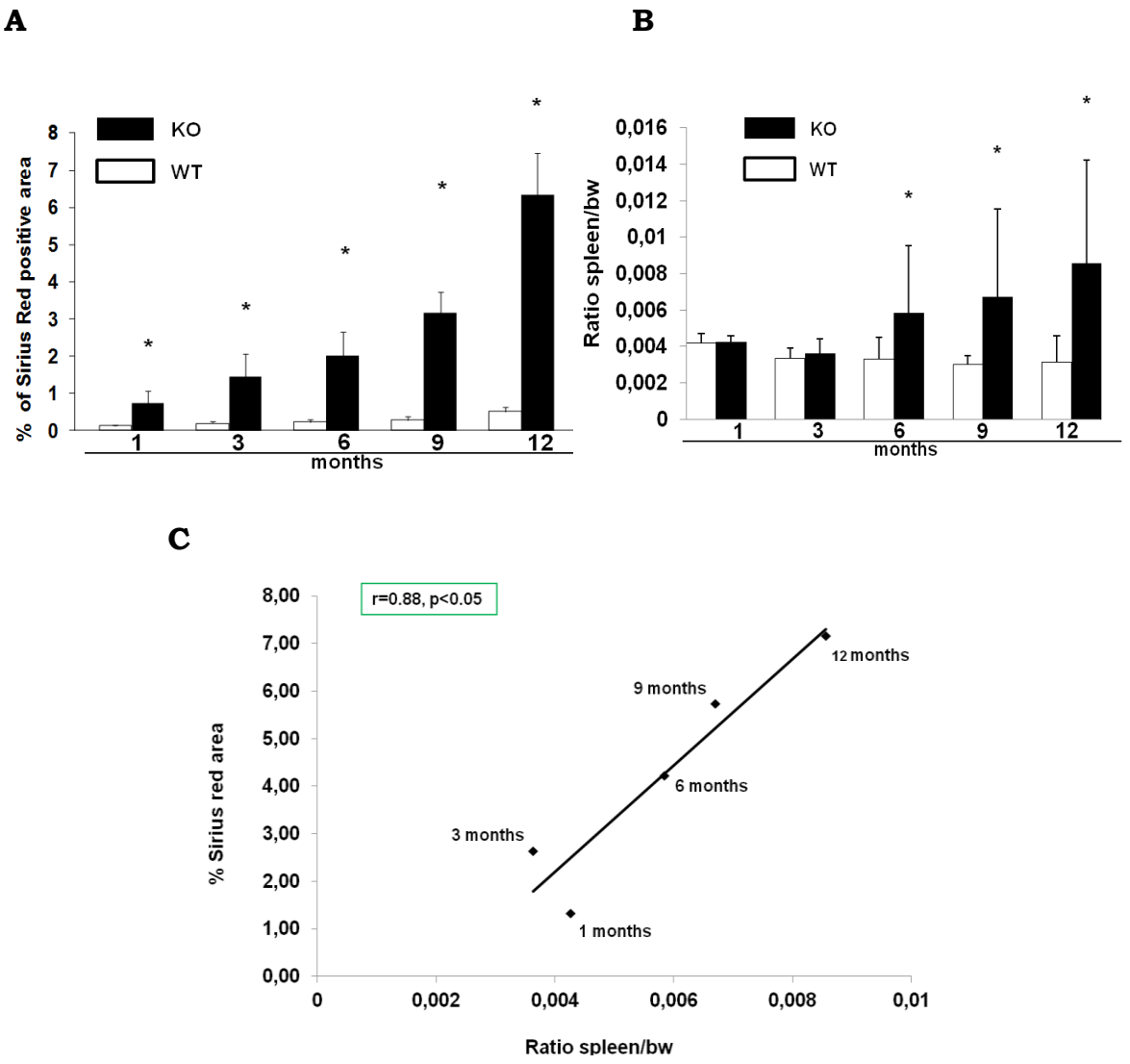



**Fig. 10: Age-dependent increase in peribiliary fibrosis correlates with portal hypertension in *Pkhd1*$^{del4/del4}$ mice** *Pkhd1*$^{del4/del4}$ mice showed an increase of fibrosis which progressively increased through the different maturation ages (A). Progressive increase in the spleen weight/body weight (index of splenomegaly) which started to be significant at 6 months (B). Splenomegaly correlated with portal fibrosis (r=0.94, p<0.02) (C).

## 2. Age-dependent increase in αvβ6 integrin expression on biliary cyst epithelia correlates with portal fibrosis in *Pkhd1*$^{del4/del4}$ mice

Expression of αvβ6 integrin was restricted to biliary cysts, whilst it was constantly negative in the normal bile ducts of WT littermates. In positive biliary cysts, expression of αvβ6 integrin was strong, decorating almost the entire perimeter of the lining epithelium (Figure 11A and 11B), as representative samples of *Pkhd1*$^{del4/del4}$ mice at 3 and 9 months of age, respectively. The age-dependent increase in peribiliary fibrosis was paralleled by the immunohistochemical expression of αvβ6 integrin on biliary cyst epithelia, which was patchy at the earliest stages and then showed a similar steady increase over maturation, from 20% of biliary structures at 1 month to nearly the 70% at 12 months (Figure 11C) confirmed at mRNA level by real time PCR (Figure 11D).

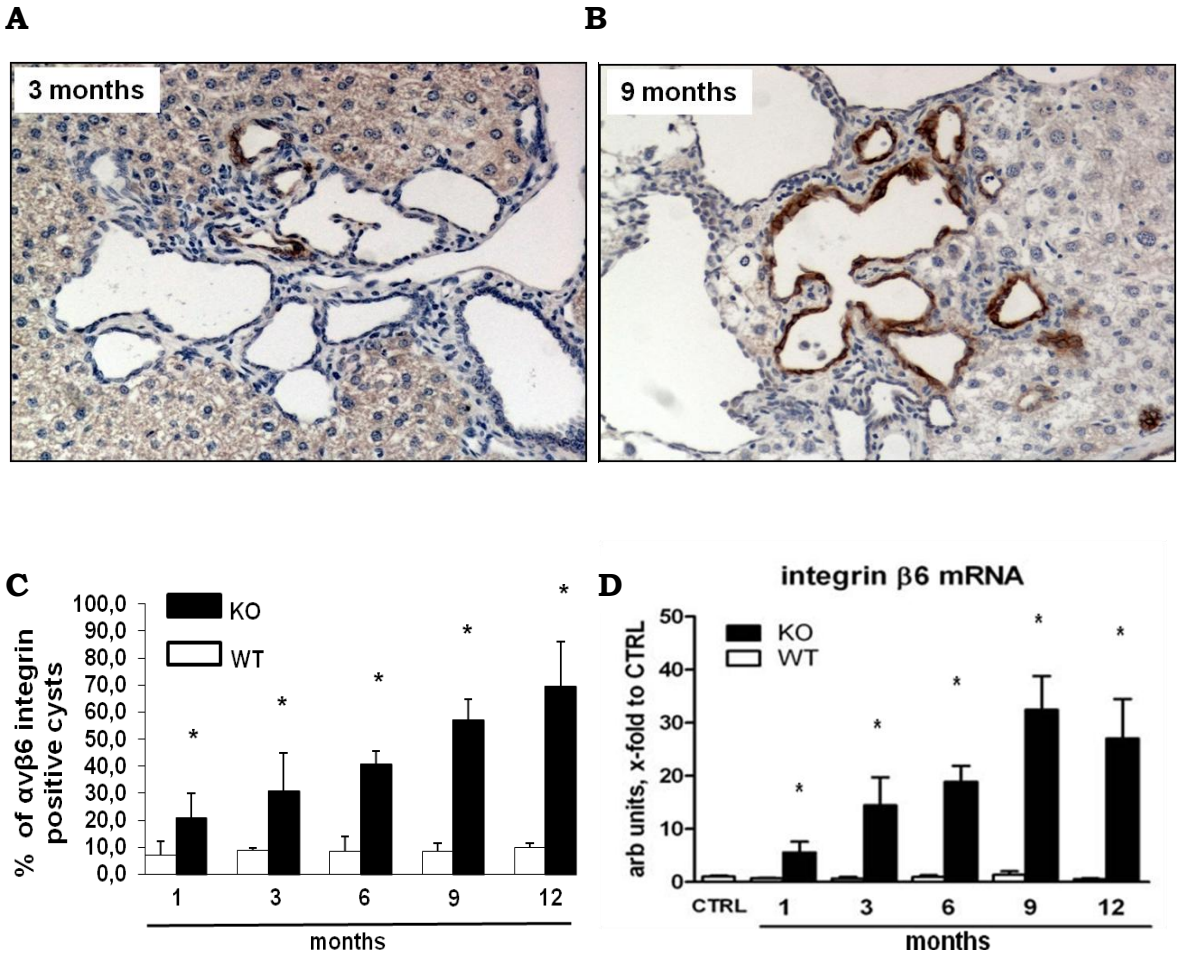



**Fig. 11: Age-dependent increase in αvβ6 integrin expression on biliary cyst epithelia** Pkhd1$^{del4/del4}$ mice showed a progressive increase in αvβ6 integrin expression on biliary cyst epithelia at different ages, representative sample, 3 months of age;(A) and 9 months (B) - immunoperoxidase technique, M=100x (n=5 for each age), which nearly reached the 80% of biliary structures at 12 months (C). *: $p<0.05$ KO vs WT. Expression of αvβ6 integrin was confirmed also by Real Time PCR (D).

Noteworthy, expression of αvβ6 integrin on biliary cysts closely correlated with portal fibrosis (r=0.94, p<0.02) (Figure 12), consistent with an active role played by αvβ6 in peribiliary fibrogenesis in *Pkhd1*$^{del4/del4}$ mice.

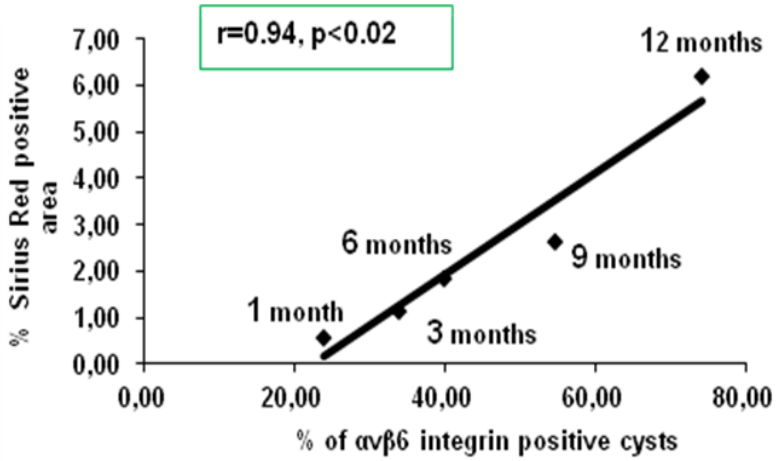



**Fig. 12: Age-dependent increase in αvβ6 integrin expression on biliary cyst epithelia correlates with portal fibrosis in *Pkhd1*$^{del4/del4}$ mice** Expression of αvβ6 strongly correlated with portal fibrosis (r=0.94, p<0.02)

Moreover, the expression of αvβ6 integrin appeared restricted at biliary epithelium and was never expressed by other cell types. Using immunohistochemistry, we also noticed that the bile cysts positive for αvβ6 integrin appeared surrounded by a large inflammatory infiltrate (Fig. 13A), on the contrary, the inflammatory cells around the cysts negative for αvβ6 integrin were much more limited (Fig. 13B).

**A** **B**

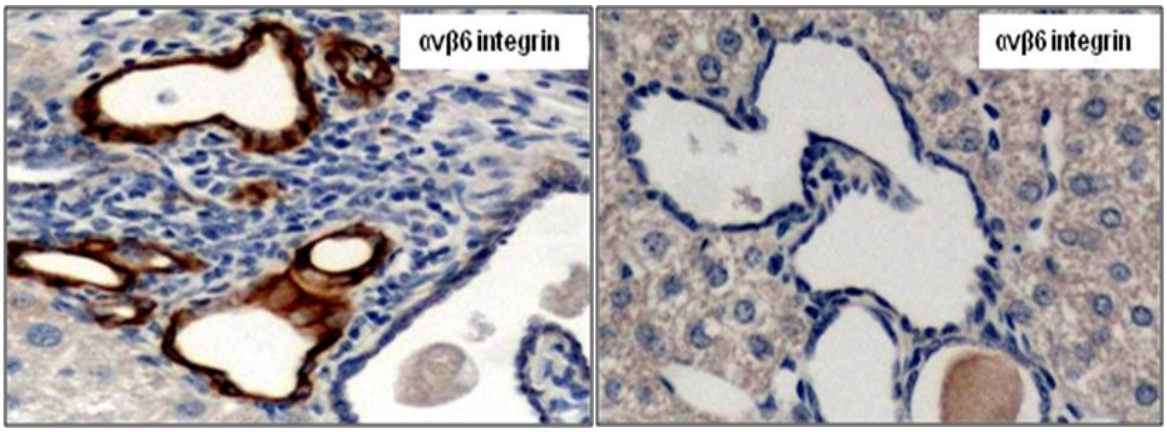



**Fig. 13: Different αvβ6 integrin expression in Pkhd1$^{del4/del4}$ mice** Noteworthy, in the same animal, cysts positive for αvβ6 integrin appeared surrounded by an abundant peribiliary inflammatory infiltrate (A), in contrast, cysts negative for αvβ6 integrin were not surrounded by inflammatory cells (B), magnification: 400x.

### 3. The expression of αvβ6 integrin on biliary cyst is associated with an abundant portal inflammatory infiltrate populated by CD45-positive cells

To characterized the inflammatory cells in the portal tract in mice Pkhd1$^{del4/del4}$ was used a widely marker expressed by leukocytes, the CD45 (Figure 14A and 14B). Inflammatory cells localized in space portal and positive for CD45 increase progressively with age (from 5% of site area measured at 1 month to 25% at 9 months) (Figure 14C).

The CD45-positive inflammatory cells correlates closely with the expression of αvβ6 integrin positive cysts ($r= 0.94$, $p <0.01$) (Figure 14D), suggesting a cause-effect relationship between the recruitment of the inflammatory infiltrate and increased expression of αvβ6 integrin by biliary epithelium. Conversely, any kind of inflammatory infiltrate was observed in WT mice at different ages of growth.

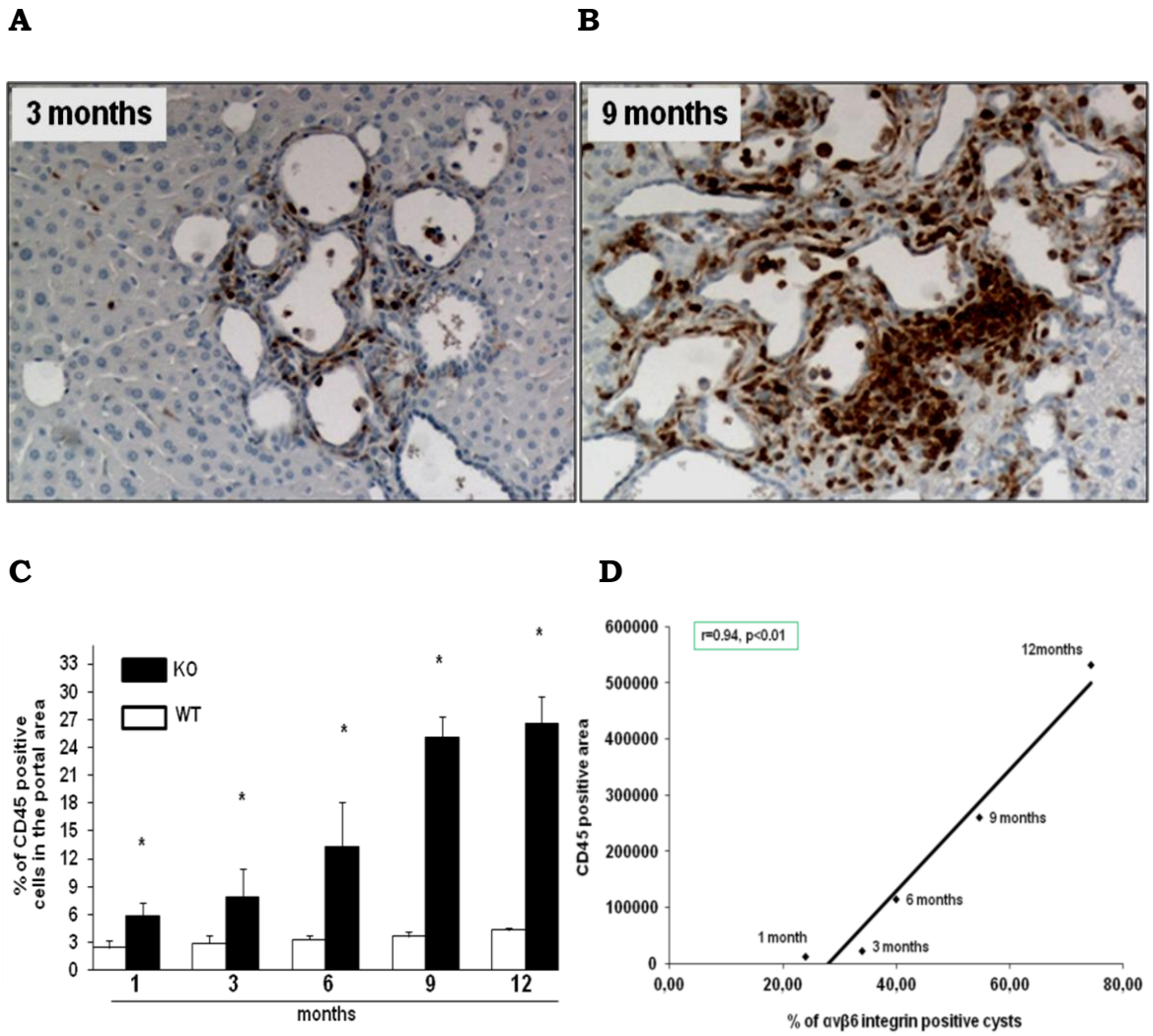



**Fig. 14: Age-dependent increase in peribiliary CD45+ve cell infiltrate correlates with αvβ6 integrin expression on biliary cyst epithelia in Pkhd1$^{del4/del4}$ mice.** Pkhd1$^{del4/del4}$ mice showed a progressive enrichment of CD45+ve cells, strictly adjacent to the biliary epithelium at the different ages, representative sample, 3 months of age; (A) and 9 months (B) – immunoperoxidase, M=200x, (n=5 for each age). The percentage of the portal tract area positive for CD45 started from 5% at 1 month, and increased above 25% at 9 months (C). *: $p<0.05$ KO vs WT. The CD45+ve cell infiltrate in the portal space closely correlated with the expression of αvβ6 ($r=0.94$, $p<0.01$) (D).

It should also be emphasized that compared with a progressive accumulation in the portal space of inflammatory cells positive for CD45, was not however observed a significant representation of myofibroblasts portals α-SMA positive cells classically considered as effector fibrosis.

Just in late stage we have shown an increase of α-SMA positive cells (Figure 15A and 15B). This finding was also confirmed at the level of mRNA, measured by RT-PCR in total liver of mice Pkhd1$^{del4/del4}$ and WT. In contrast to the progressive and linear development of portal fibrosis, present since 3 months in mice Pkhd1$^{del4/del4}$, mRNA expression of α-SMA was significantly increased only in older later (Figure 15D).

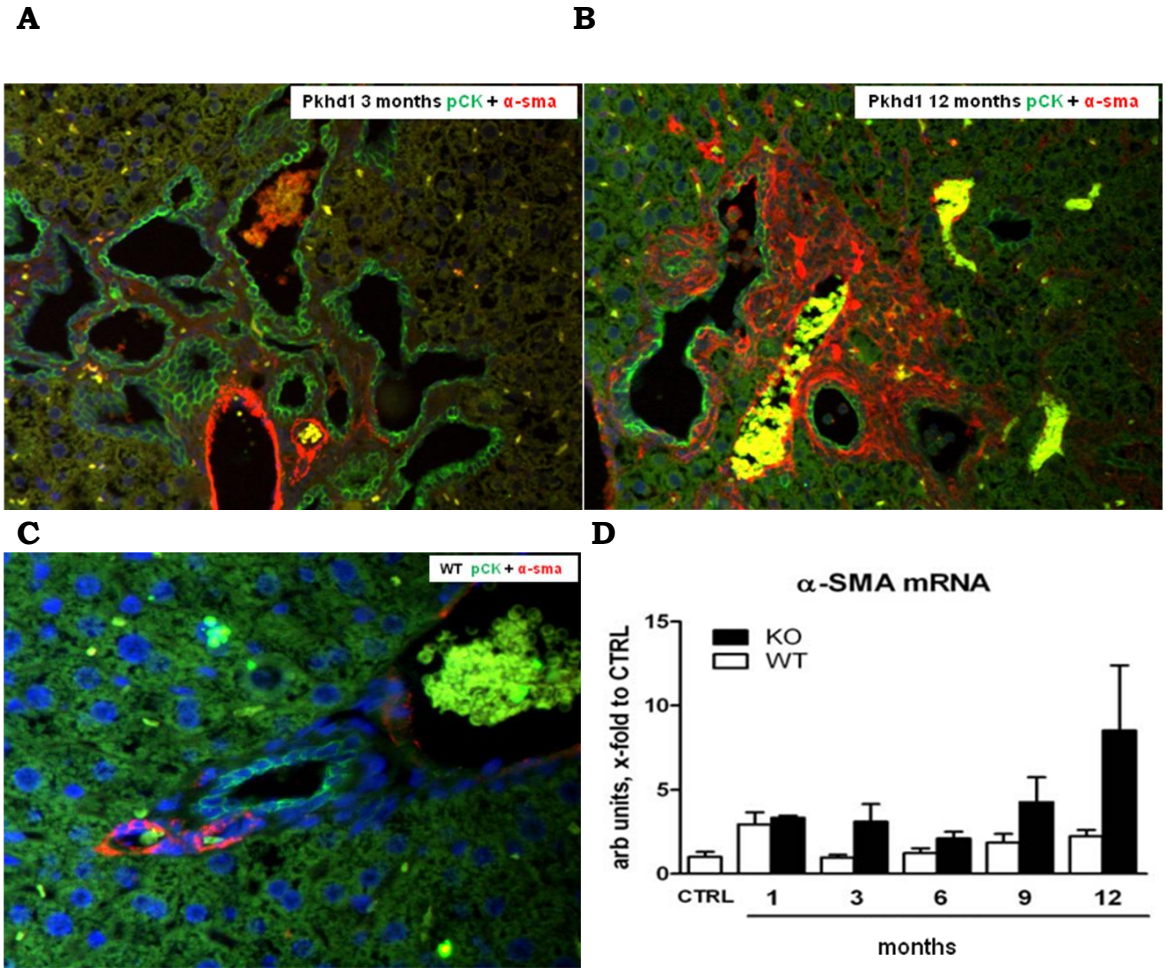



**Fig. 15: Expression of α-SMA in mouse liver *Pkhd1*$^{del4/del4}$.** Immunofluorescence staining in PKHD1$^{del4/del4}$ mice show low expression of α-SMA at 3 months (A) while at 12 months there is an increase of α-SMA expression localized in the portal area (α-SMA in red, PCK in green, magnification 200x (B). The quantification of α-SMA expression by Real Time PCR confirmed a low level of transcripts for α-SMA while at 12 months we found a significant increase (D).

**4. Phenotypic characterization of the portal cell infiltrate: the majority of CD45-positive cells co-express CD11b and F4/80 (macrophage phenotype)**

To characterize the CD45-positive portal cell infiltrate progressively accumulating in *Pkhd1*$^{del4/del4}$ mice, we took a dual approach, based on: a) characterization of non parenchymal cells by FACS analysis of the whole liver infiltrate based on CD45-positive cell selection; b) dual immunofluorescence for CD45 and F4/80 in liver samples from each maturation to confirm the portal tract localization.

*FACS analysis* demonstrated that in *Pkhd1*$^{del4/del4}$ mice the majority of the CD45-positive cells were macrophages, based on their co-expression of both CD11b and F4/80 (Figure 16A shows a representative FACS dot-plots from *Pkhd1*$^{del4/del4}$ mice at 3 months). The percentage of CD45+/CD11b+/F4/80+ cells ranged from 57 to 68% of the total CD45+ cell population over maturation without significant changes between the different ages. Contribution of neutrophils (CD45+/Ly-6G+) and monocytes (CD45+/CD11b+/F4/80-) was much lower than that of macrophages (neutrophils up to 7% at month 3, monocytes up to 13% at month 9), whereas the relative proportions of the different lymphocyte subtypes, including NK (CD45+/CD3-/NK1.1+), NKT (CD45+/CD3+/NK1.1+), T (CD45+/CD3+/NK1.1-) and B cells (CD45+/CD19+), were generally lower than 5%. As shown in Figure 16B illustrating the percentage of the different cell subsets at 3, 6 and 9 months, the relative contribution of each cell fraction to the whole CD45-positive cell population did not change over maturation.

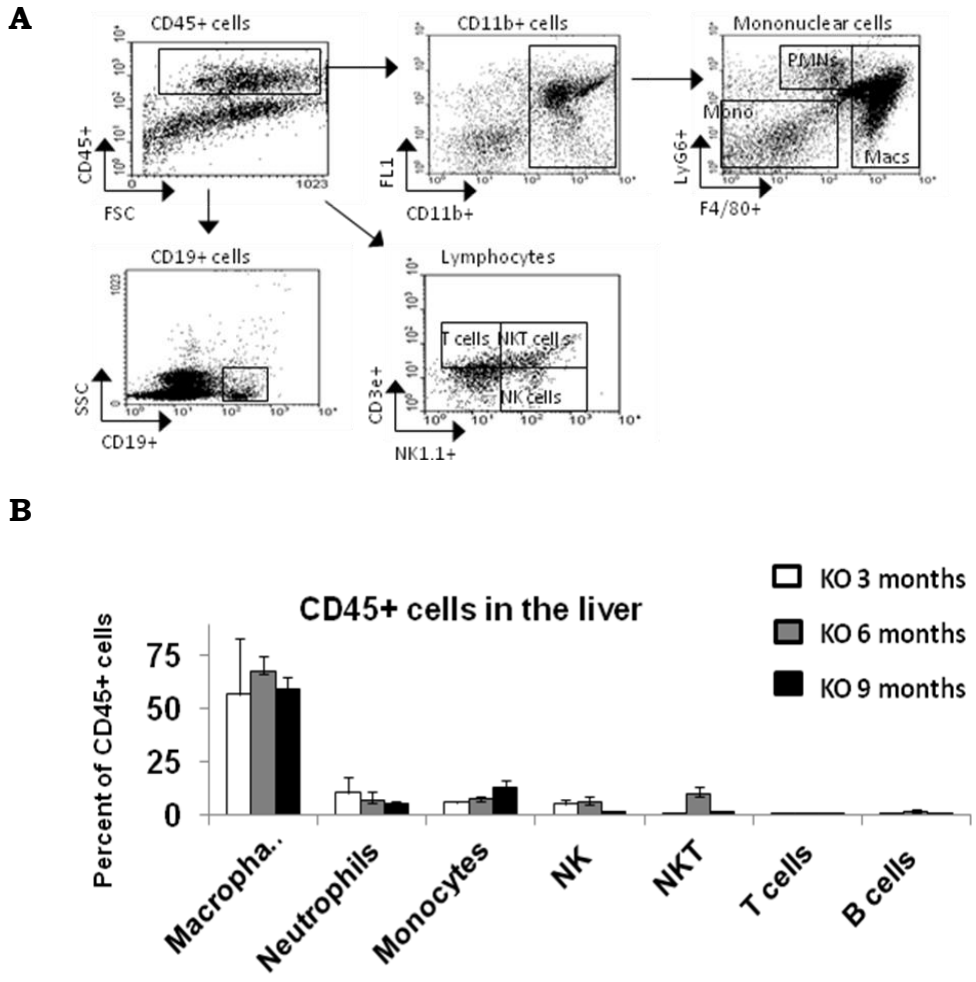



**Fig. 16: FACS analysis of CD45+Liver non-parenchymal cell subpopulations in WT and *Pkhd1*$^{del4/del4}$ mice**. Representative FACS dot-plots shown from PKHD1 null mice (A). CD45+ cell sub-populations in the liver of *Pkhd1*$^{del4/del4}$ mice at 3, 6, and 9 months of age (n=2 and 3, respectively) (B)

*Dual immunofluorescence* for CD45 and F4/80 confirmed that the vast majority of CD45-positive cells co-expressed F4/80 (macrophages) and were localized in the portal space strictly aligning along the cyst profile (dismerged, 17C merged, as representative samples of 3, 6 and 9 months). Moreover the majority of F4/80-positive cells co-expressed CD206

(mannose receptor) a typical marker of alternatively activated macrophages (M2) (Figure 18A, 18B and 18C). In contrast with this observation, the inducible nitric oxide synthase (iNOS), the classical marker for classically activated macrophages, was absent or less expressed (Figure 19A and 19B). Consistent with this observation, microbiological analysis showed that *Pkhd1*$^{del4/del4}$ livers from all maturation ages were sterile. Furthermore, gut decontamination with Polymyxin B and Neomycin had no effect on the progressive accumulation of CD45-positive cells in the portal space (data not shown).

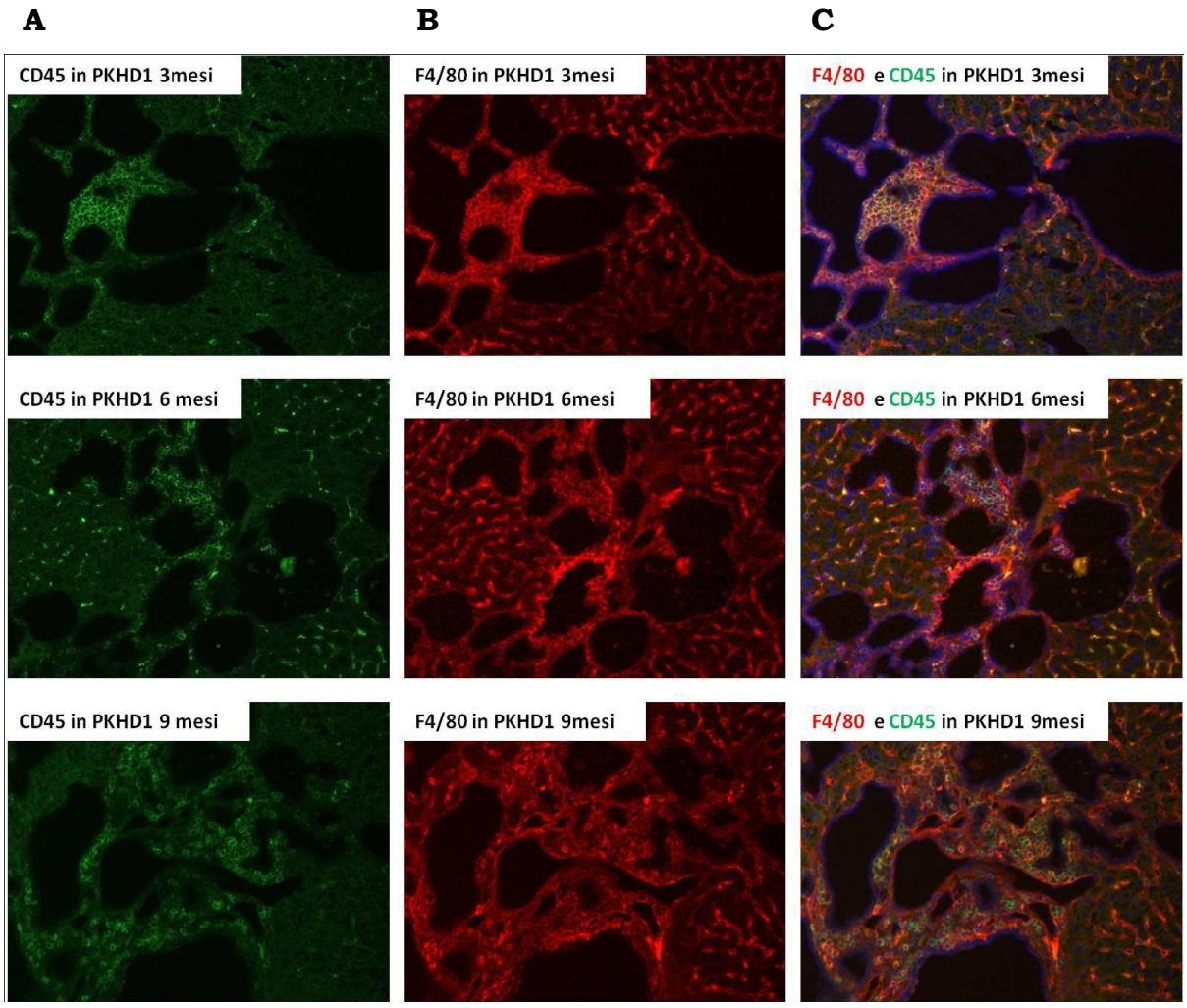



**Fig. 17: The majority of CD45-positive cells co-expressed F4/80 in the portal tract along the cysts profile.** Dual immunofluorescence stainings confirm the peribiliary location of the inflammatory macrophage characterized by double positive for CD45 (green) (A) and F4/80 (red) (B) confirmed merging the two channels (C) (magnification: 200x).

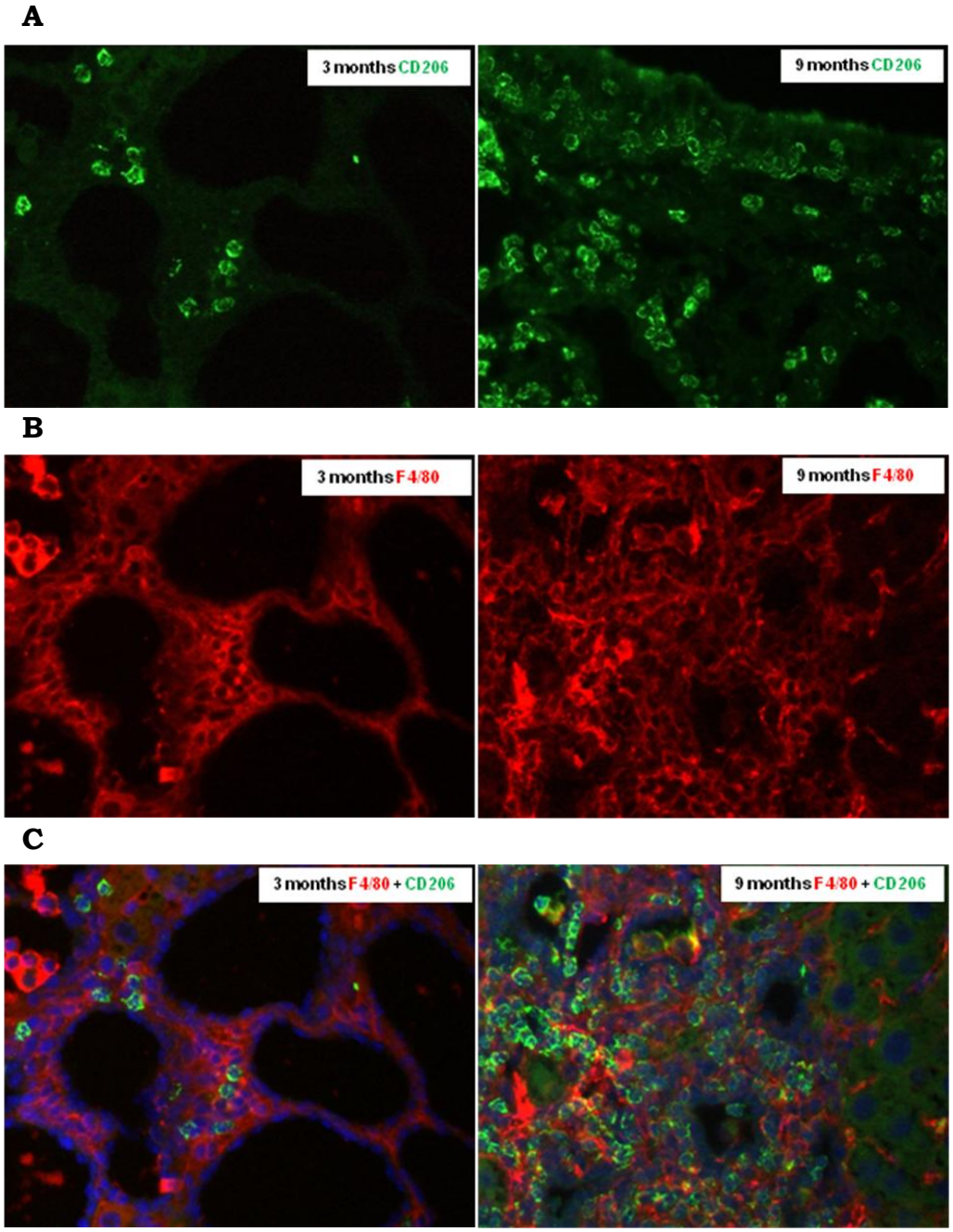



**Fig. 18: The majority of F4/80 positive cells co-expressed CD206 (a typical marker of alternatively activated macrophages M2) in the portal tract along the cysts profile.** Dual immunofluorescence stainings in *Pkhd1*$^{del4/del4}$ mice confirm the peribiliary location of the inflammatory alternatively activated macrophage (M2) characterized by double positive for CD206 (green) (A) and F4/80 (red) (B) at 3 and 9 months of age confirmed merging the two channels (C) (magnification: 200x).

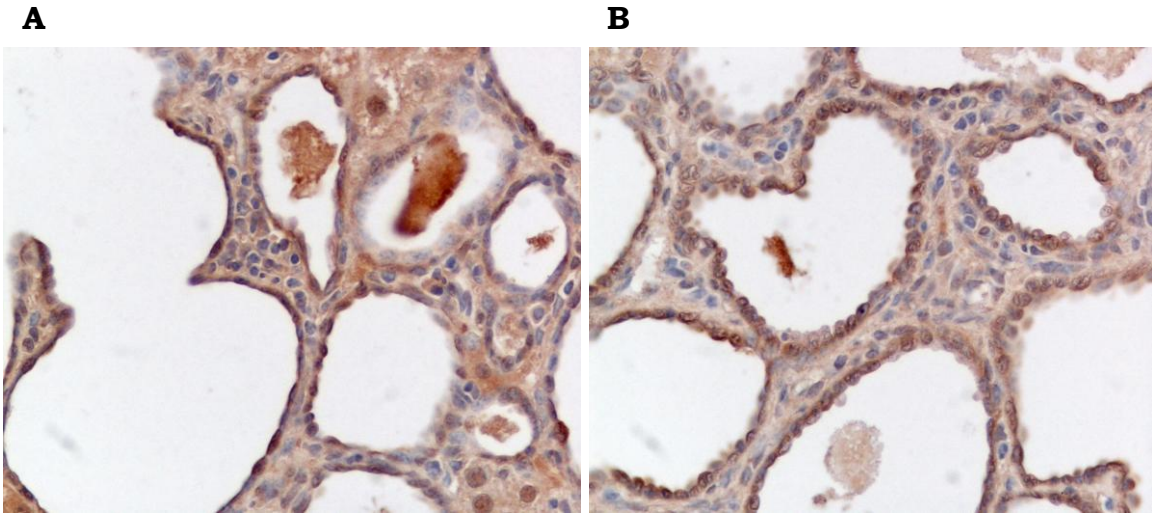



**Fig. 19: Immunohistochemistry staining shows negative or few inflammatory cells positive for iNOS, a classical marker to define classically activated macrophage (M1)** In contrast with the abundant presence of M2 macrophages, immunohistochemistry staning for iNOS (M1) in *Pkhd1*$^{del4/del4}$ mice shows negative or few inflammatory cells positive at 3 months (A) and 9 months of age (B). (magnification: 400x).

**5. *Pkhd1*$^{del4/del4}$ cholangiocytes possess rich secretory functions, featuring several chemokines potentially involved in the recruitment of circulating mononuclear cells (CXCL10 and CXCL1), more pronounced at the basolateral pole**

To investigate whether *Pkhd1*$^{del4/del4}$ cholangiocytes possessed secretory activities able to recruit macrophages, we isolated and cultured primary cholangiocytes from WT and *Pkhd1*$^{del4/del4}$ mice in a polarized manner, as previously described. Thanks to this model, we could measure the differential secretion of multiple cytokines either in the apical or in the basolateral medium using a microsphere-based multiplex immunoassay (Luminex, MILLIPLEX$^{TM}$ MAP)(n=3). The list of all the cytokines tested and their values are shown in Figure 20 A-B, where we reported for each cytokine, the levels in both the apical and basolateral medium of *Pkhd1*$^{del4/del4}$ cholangiocytes, normalized for the quantity of the protein. Interestingly, for some cytokines secretion was found to be much more abundant in the basolateral than in the apical medium (CXCL10 and CXCL1). Among them, the most significant increase in *Pkhd1*$^{del4/del4}$ with respect to WT was found for the IFN-induced protein (CXCL10, $p \leq 0.05$) and the keratinocyte chemoattractant (CXCL1, $p \leq 0.05$). These cytokines have been reported to exert chemoattractant effects on a variety of circulating mononuclear cells, including monocytes and macrophages. Therefore, these data indicate that *Pkhd1*$^{del4/del4}$ cholangiocytes gain a range of secretory functions enabling them to induce extensive peribiliary recruitment of mononuclear cells.

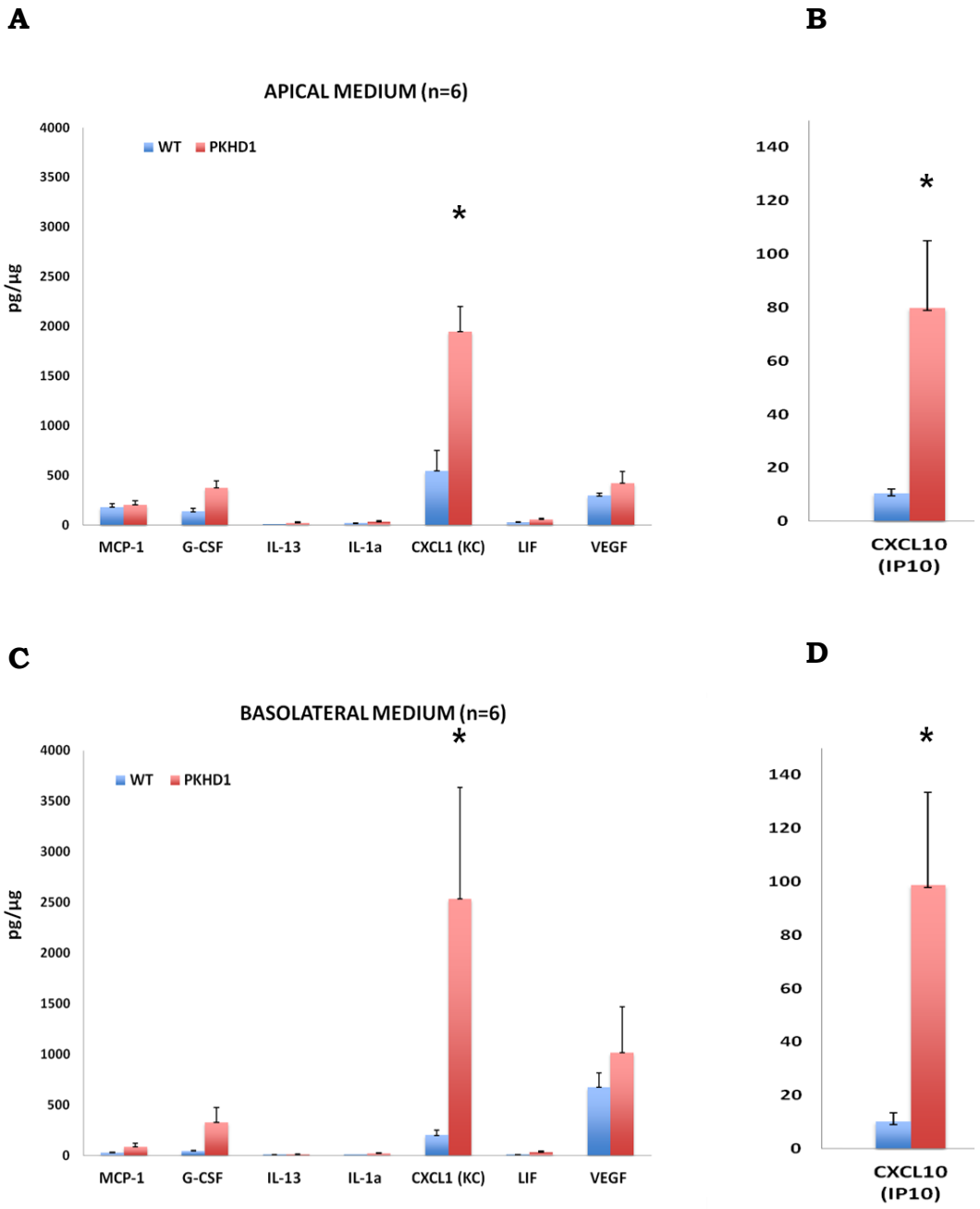



**Fig. 20: Cultured polarized Pkhd1$^{del4/del4}$ cholangiocytes secreted a number of cytokines.** Cytokines concentrations measured in medium collected from the apical (A-B) or basal (C-D) side of cultured Pkhd1 (red) or Wild Type (blue) cholangiocytes. Compared with apical secretion, *Pkhd1*$^{del4/del4}$ mice shown high and significant levels of CXCL1 (C) and CXCL10 (D) by basolateral pole (*: $p<0.05$). Concentrations are in pg/µg normalized on the protein concentration. Graphs B and D have different scales (pg/ng) respect graphs A and C.

### 6. Effects of basolateral/apical conditioned medium from *Pkhd1*$^{del4/del4}$ and Wt cholangiocytes on cell migration in macrophages lines (RAW 264.7)

To see whether the main cytokines secreted from the basolateral pole by *Pkhd1*$^{del4/del4}$ cholangiocytes (CXCL10 and CXCL1). could provide them the ability to recruit macrophages, we studied the ability of collected apical and basolateral medium from Pkhd1 and Wt to stimulate cell migration using murine macrophage (RAW 264.7) (Figure 21A) cell lines. Moreover we further tested each single cytokines (CXCL1 and CXCL10) and the mix (CXCL1 + CXCL10) at 12h (Figure 21B)

Since only RAW 264.7 cells were able to grow in adherence, transwell migration experiments could be performed only with macrophages. As shown in Figure 21A, a significant stimulatory effects were exerted by Pkhd1 basolateral medium ($p<0.05$) compare to Pkhd1 apical medium (n.s). As shown in Figure 21B, CXCL10 induced the strongest motogenic effect in RAW 264.7 (peak at 1 ng/ml, $p<0.05$), but significant stimulatory effects were exerted also by CXCL1 (peak at 1 ng/ml, $p<0.05$) and with the treatment with both chemokines (CXCL10 1 ng/ml + CXCL1 1 ng/ml, $p<0.05$).

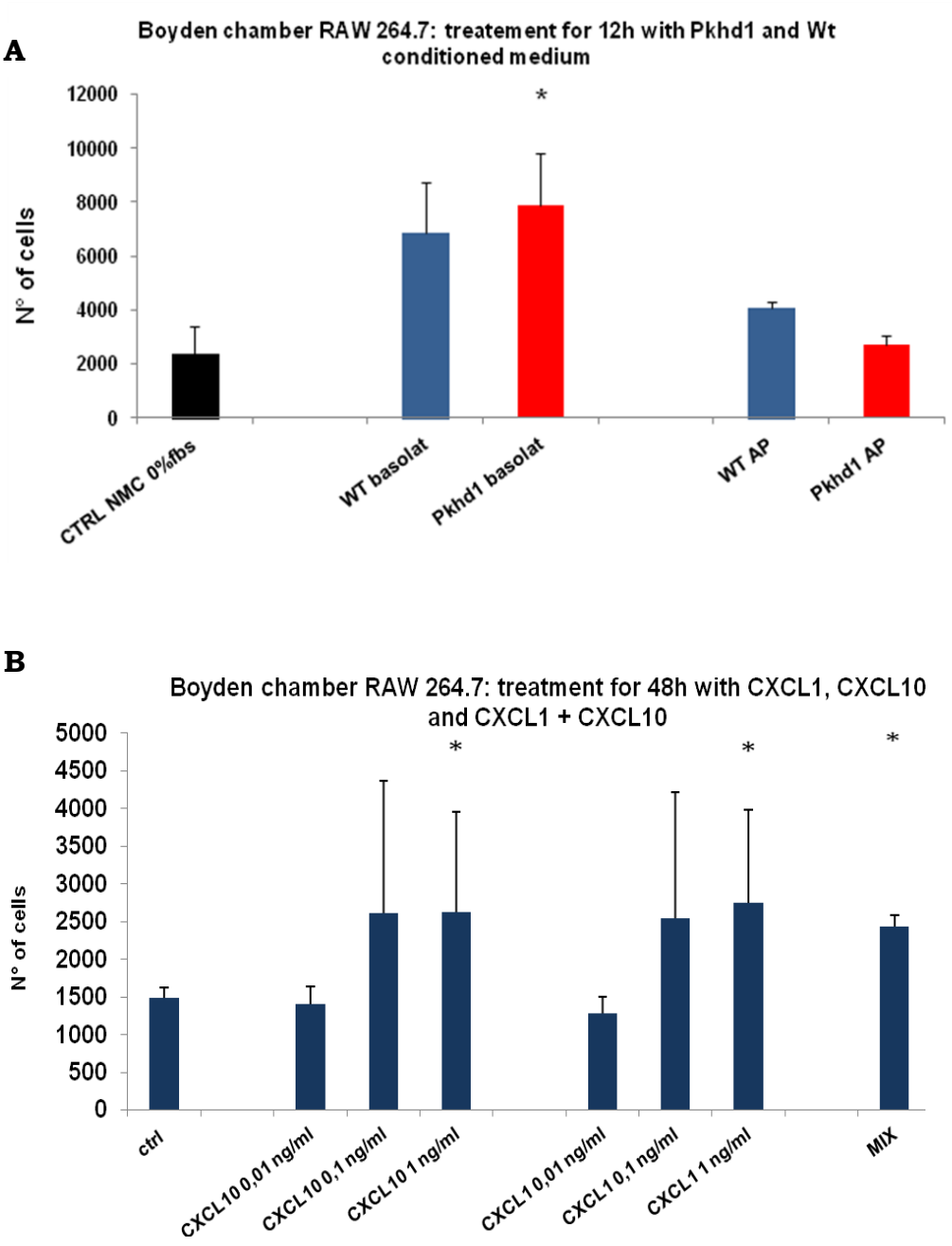



**Fig. 21: Effects of basolateral/apical conditioned medium from *Pkhd1*$^{del4/del4}$ and Wt cholangiocytes and treatment with both CXCL1 and CXCL10 chemokines on cell migration in macrophages lines (RAW 264.7).** *Pkhd1*$^{del4/del4}$ basolateral conditioned medium induced the strongest motogenic effect in RAW 264.7 (*: $p<0.05$) compare to *Pkhd1*$^{del4/del4}$ apical conditioned medium (A) , but significant stimulatory effects were exerted also by CXCL1 (peak at 1 ng/ml, *: $p<0.05$), CXCL10 (peak at 1 ng/ml, *: $p<0.05$) and with the treatment with both chemokines (CXCL10 1 ng/ml + CXCL1 1 ng/ml, *: $p<0.05$) (B).

### 7. The macrophage-derived cytokines TGFβ1 and TNFα stimulate gene expression of β6 integrin in *Pkhd1*$^{del4/del4}$ cholangiocytes; TNFα effect is TGFβ1-independent

Having shown that: a) *Pkhd1*$^{del4/del4}$ cholangiocytes expressed αvβ6 integrin which closely correlated to peribiliary fibrosis and to a portal cell infiltrate positive for CD45, b) most of the CD45-positive cells were phenotyped as macrophages based on their co-expression of CD11b and F4/80, and c) *Pkhd1*$^{del4/del4}$ cholangiocytes possessed rich secretory functions (CXCL1 and CXCL10) enabling them to strongly induce the monocyte and macrophage homing to the peribiliary area, we next studied the ability of two macrophage-derived cytokines, TGFβ1 and TNFα, to regulate the expression of β6 mRNA in cultured *Pkhd1*$^{del4/del4}$ cholangiocytes and compared them to WT littermates, before and after TGFβRII blockade (n=9). As shown in Figure 22, in *Pkhd1*$^{del4/del4}$ cholangiocytes β6 mRNA was significantly increased in basal conditions (0.015±0.002 2^-dCt) with respect to WT (0.010±0.004 2^-dCt, $p<0.01$), and its expression was further and significantly increased after both TGFβ1 (0.017±0.002 2^-dCt, $p<0.05$ vs untreated KO) and TNFα (0.018±0.003 2^-dCt, $p<0.05$ vs untreated KO) stimulation. However, in contrast with TNFα, TGFβ1 significantly increased β6 mRNA expression not only in *Pkhd1*$^{del4/del4}$ but even in WT cholangiocytes (0.018±0.004 2^-dCt, $p<0.01$ vs untreated WT). Noteworthy, TGFβRII blockade abolished the TGFβ1 effects on β6 mRNA expression in *Pkhd1*$^{del4/del4}$ (0.014±0.003 2^-dCt, $p<0.05$ vs TGFβ1-treated KO) as well as in WT (0.014±0.002 2^-dCt, $p<0.05$ vs TGFβ1-treated

WT) cholangiocytes, in contrast with TNFα whose effects on β6 were unaffected by TGFβRII antagonism (0.017±0.001 2^-dCt, p=n.s. vs TNFα-treated KO). These data indicate that pro-inflammatory cytokines, such as TGFβ1 and TNFα, released by macrophages aberrantly recruited into the portal tract stimulate αvβ6 integrin expression in *Pkhd1*$^{del4/del4}$ cholangiocytes, and that the effects of TNFα are specific and independent from TGFβ1 activation.

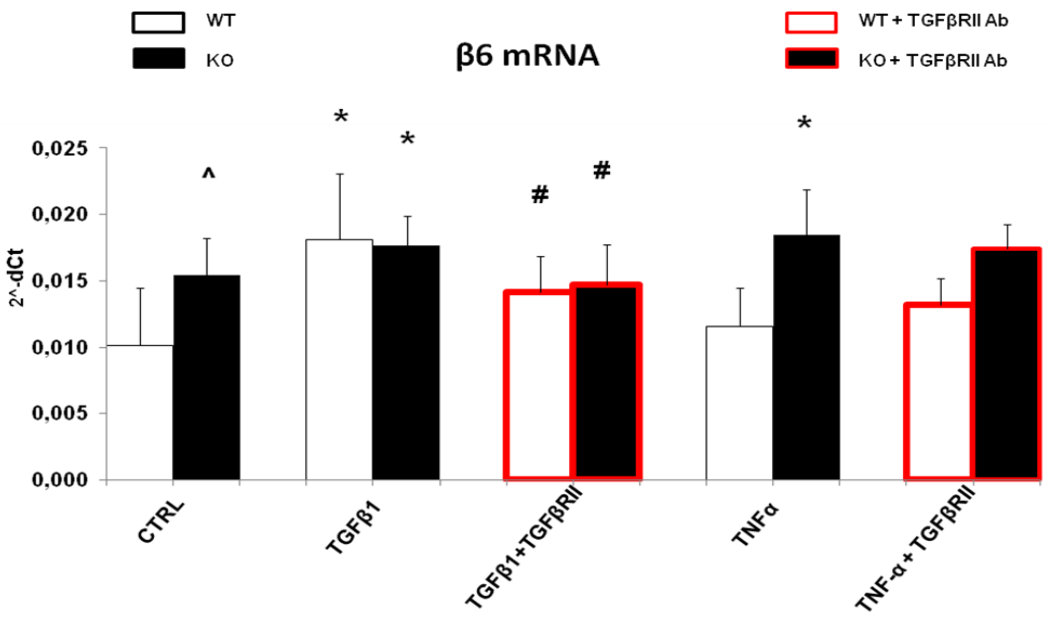



**Fig. 22: Expression of β6 mRNA in cultured Pkhd1$^{del4/del4}$ cholangiocytes was potently stimulated by TNFα (0.018±0.003 2^-dCt) increase as compared to WT (0.015±0.002 2^-dCt) and TGFβ1 (0.017±0.002 2^-dCt) (A, n=3, 3 months of age). *: p<0.05 KO vs WT.** The effects of TNFα and TGFβ1 on the expression of β6 mRNA by Pkhd1$^{del4/del4}$ cholangiocytes are also studied before and after TGFβRII blockade (n=3, 3 months of age). After TGFβRII blockade, the effects of TGFβ1 were abolished (**#**: p< 0.05) in contrast with TNFα whose effects were still detectable.

## 8. *Pkhd1*$^{del4/del4}$ cholangiocytes express increased levels of COLL1, further and significantly stimulated by TGFβ1

Given the evidence that: a) once activated, TGFβ1 exerts its biological effects within the limits of the cells displaying αvβ6 integrin, b) in *Pkhd1*$^{del4/del4}$ mice αvβ6 expression is restricted to cyst cholangiocytes, and c) accumulation of the classic ECM-producing cells, i.e. activated portal myofibroblast, is only a late event in *Pkhd1*$^{del4/del4}$ mice, to understand the role of TGFβ1 in peribiliary fibrosis in FPC-deficiency, we finally studied the effects of TGFβ1 on COLL1 (the main component of ECM in liver fibrosis) mRNA expression in cultured cholangiocytes (n=4). Interestingly, as compared to WT, *Pkhd1*$^{del4/del4}$ cholangiocytes showed increased basal levels of COLL1 mRNA (0.0009±0.0003 2^-dCt), with respect to WT (0.0001±0.00005 2^-dCt, $p<0.01$), which were potently stimulated by TGFβ1 (0.002±0.0005 2^-dCt, $p<0.05$ vs untreated KO) (Figure 23). These data suggest that *Pkhd1*$^{del4/del4}$ cholangiocytes could be actively involved in the generation of ECM components, at least in the early stages of peribiliary fibrogenesis.

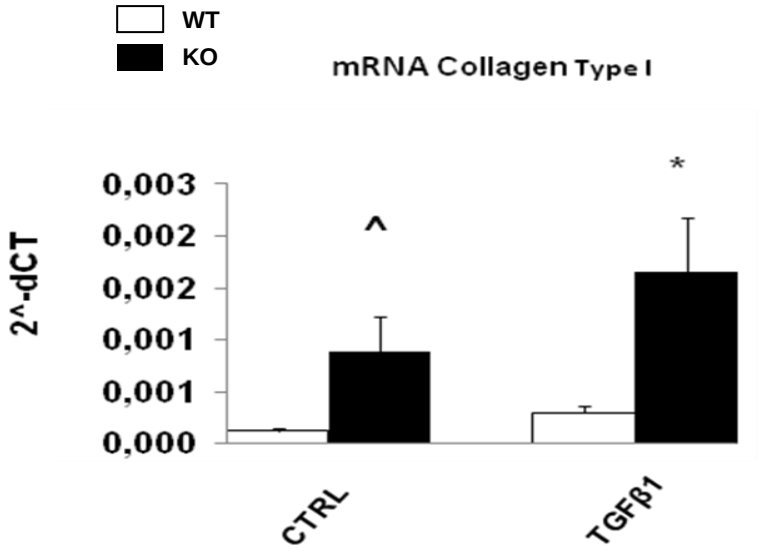



**Fig. 23: Expression of Collagen type I in cultured Pkhd1$^{del4/del4}$ cholangiocytes was potently stimulated by TNFα.** Interestingly, as compared to WT, *Pkhd1*$^{del4/del4}$ cholangiocytes showed increased basal levels of COLL1 mRNA (0.0009±0.0003 2^-dCt), respect to WT (0.0001±0.00005 2^-dCt, **^:** p<0.01), which were potently stimulated by TGFβ1 (0.002±0.0005 2^-dCt, ***:** p<0.05 vs untreated KO).

# ***DISCUSSION***

This study was performed to clarify the expression of αvβ6 integrin and its role in *Pkhd1*$^{del4/del4}$ mice, in particularly we found that: a) αvβ6 integrin is markedly up-regulated in cyst cholangiocytes where it closely correlates with portal fibrosis and the progressive peribiliary accumulation of a CD45-positive inflammatory cells (mainly macrophages); b) *Pkhd1*$^{del4/del4}$ cholangiocytes from their basolateral pole secrete a range of chemokines (CXCL10 and CXCL1) able to recruit peribiliary inflammatory mononuclear cells; c) in turn, inflammatory cytokines (TNFα and TGFβ1) released by macrophages, up-regulate αvβ6 integrin expression in *Pkhd1*$^{del4/del4}$ cholangiocytes; d) once latent TGFβ1 has been activated by αvβ6 integrin, it stimulates Collagen Type I formation in *Pkhd1*$^{del4/del4}$ cholangiocytes.

Compare with other models of biliary fibrosis used to study the role of αvβ6 integrin, such as the rat biliary cirrhosis (BDL) and the mouse primary sclerosing cholangitis-like (Mdr2-KO) (Patsenker E. et al. 2008) (Popov Y. et al., 2008), the mouse used in the present study represents a good model to study spontaneous fibrosis. In *Pkhd1*$^{del4/del4}$ mice, the development of biliary fibrosis is much slower leading to portal hypertension only after 6 months. In contrast with other cholangiopathies, such as biliary atresia, the development of portal hypertension in CHF is generally diagnosed only in the late youth (O'Brien K. et al., 2012). Starting from the defect of fibrocystin in *Pkhd1*$^{del4/del4}$ mice, we studied the levels of hepatic fibrosis-related transcripts to characterize the peculiar fibrogenic behavior. In *Pkhd1*$^{del4/del4}$ mice, levels of procollagen α1(I), the main biomarker of ECM remodeling in liver fibrosis, is augmented respect

to WT littermates, increase more sharply from 6 months onwards. Periportal myofibroblasts are classically considered as the pivotal drivers of biliary fibrosis, and their massive increase is an early feature in Mdr2-KO mice (Popov Y et al., 2005). In contrast, in *Pkhd1*$^{del4/del4}$ mice, increased levels of α-SMA, a surrogate marker of myofibroblast activation, is seen only in the latest maturation stages, once portal fibrosis has already been partly established. Furthermore, immunohistochemistry for α-SMA showed that accumulation of portal myofibroblasts can be observed only at 12 months, thereby ruling out their involvement at least in the early stages of fibrogenesis.

The role of αvβ6 integrin is yet fundamental in portal fibrogenesis of *Pkhd1*$^{del4/del4}$ mice. αvβ6 integrin is expressed exclusively by cyst cholangiocytes, where it is progressively up-regulated over maturation, whilst it is negative in normal bile ducts of WT littermates. Expression of αvβ6 integrin shows a strong correlation with the development of portal fibrosis. αvβ6 integrin is typically up-regulated in activated duct epithelia, where its expression may be regarded as a feature reminiscent of a developmental behavior (Breuss JM et al., 1995). In agreement with this observation, in polycystic liver diseases caused by genetic defects in ciliary proteins, cholangiocytes maintain an immature phenotype related to a lack of differentiating signals from cilium dysfunction (Strazzabosco M. & Fabris L. , 2012). Although the function of FPC is unknown, it is thought that FPC is involved in terminal differentiation of ductal morphogenesis, mediating interactions with the ECM (Israeli S. et al., 2010). Notably, silencing *Pkhd1* in cultured mouse renal tubular cells, alters cytoskeletal

organization and impairs cell-cell and cell-matrix contacts (Mai W. et al., 2005), features consistent with the activation of a developmental program. Therefore, the maintenance of an immature phenotype caused by a defective FPC function may explain the increased basal levels of β6 integrin transcripts that we have found in *Pkhd1*$^{del4/del4}$ cholangiocytes with respect to WT. However, additional factors are likely involved to explain the progressive increase in αvβ6 integrin in *Pkhd1*$^{del4/del4}$ mice through maturation, resulting in an almost 35-fold up-regulation of β6 transcripts at 9 months. By immunohistochemistry, we noticed that expression of αvβ6 integrin on biliary cysts was uneven, and that unlike the negative cysts, the positive cysts were densely surrounded by a mononuclear inflammatory infiltrate stained by CD45. Furthermore, we found a strong correlation between the αvβ6 integrin biliary expression and the mononuclear cells recruited in the peribiliary region. By FACS analysis and immunohistochemistry we have then performed a phenotypic characterization of the CD45-positive cell population, and found that the majority of these cells (around 60-75%) were macrophages and that they were indeed localized in close vicinity to biliary cysts. The finding that in *Pkhd1*$^{del4/del4}$ mice the relative large amounts of macrophages were keeping stable indicating that in FPC deficiency, inflammation likely develops as a chronic, not resolving process (Sica A. et al., 2012). Recruitment of macrophages to cystic areas has been recently shown in the kidney in murine models of different genetic defect (*Pkd1*), where they promote cyst growth and likely contribute to the progression of polycystic kidney disease (Karihaloo A. et al., 2011). The peribiliary accumulation of

macrophages in *Pkhd1*$^{del4/del4}$ mice has led us to hypothesize that *Pkhd1*$^{del4/del4}$ cholangiocytes were able to secrete increased amounts of homing factors. To test this hypothesis, we have isolated and cultured primary cholangiocytes from WT and *Pkhd1*$^{del4/del4}$ mice and measured the apical and basolateral secretion of a panel of cytokines. The secretion of several inflammatory cytokines: KC (also termed CXCL1) and CXCL10 (also termed IP10) was significantly much stronger in *Pkhd1*$^{del4/del4}$ than in WT cholangiocytes, mainly from the basolateral side. The Interferon-ϒ induced protein-10 is a small cytokine of 10kDa, critically regulating the trafficking of effector T cells during several immune-inflammatory processes, including allograft rejection. (Liu et al., 2011) (Qi, X. et al., 2009).

The keratinocyte chemoattractant is a potent chemokine regulating host defense in many infectious diseases by promoting recruitment of blood-derived mononuclear cells, in particular of neutrophils. (De Filippo K. et al., 2008). Since the role of these cytokines in regulating the recruitment of monocytes and macrophages at the sites of tissue injury is not firmly established, we have studied whether CXCL10 and CXCL1, were functionally effective in eliciting migration of a line of macrophages (RAW 267.4). The effects exerted by CXCL10 in particular, but even CXCL1, were motogenic for macrophages. The ability of *Pkhd1*$^{del4/del4}$ cholangiocytes to release a variety of chemotactic factors able to recruit inflammatory monocytes and macrophages into the portal tract, suggests that FPC may normally exert an inhibitory tone on the expression of inflammatory chemokines. Therefore FPC deficiency in cholangiocytes

results in the activation of a persistent inflammatory response leading to the progressive accumulation of macrophages nearby the biliary cysts. If not quickly eliminated, macrophages may be harmful to repair mechanisms, as they are important cell sources of inflammatory cytokines, such as TGFβ1 and TNFα (Duffield JS. et al., 2005) (Murray, P. & Wynn T., 2011). We have therefore hypothesized that in *Pkhd1*$^{del4/del4}$ mice, once recruited into the portal space, infiltrating macrophages lead to scarring by exacerbating the expression of αvβ6 integrin. To test this hypothesis we have evaluated whether the macrophage-derived cytokines, TGFβ1 and TNFα expressed by M1 and M2, were able to modulate the expression of β6 transcripts in cultured cholangiocytes. We found that both cytokines were able to induce a further and significant increase in the β6 mRNA. Notably, whereas TGFβ1 stimulatory effects were present in mutated as well as WT cholangiocytes and were abrogated by blocking the TGFβRII, TNFα stimulated β6 mRNA expression of an extent similar to TGFβ1 but only in mutated cholangiocytes and its effects were unaffected by TGFβRII antagonism. These data indicate a novel TNFα-dependent mechanism for the regulation of αvβ6 integrin expression in *Pkhd1*$^{del4/del4}$ cholangiocytes through a pathway that is independent from TGFβ1.

In the last series of experiments we have addressed the role of TGFβ1 once locally activated by αvβ6 integrin. Following the proteolytic cleavage of the LAP sequence which occurs at the epithelial cell surface, TGFβ1 exerts its effects within the limits of the site of activation. In our model, accumulation of portal myofibroblasts, main target cell type of TGFβ1, is only a late event, developing later than fibrosis. Therefore, we have drawn

our attention on the fibrogenic properties of mutated cholangiocytes. Whereas the ability of reactive cholangiocytes to release important fibrogenic growth factors, such as TGFβ2, platelet derived growth factor and connective tissue growth factor (Fabris L. & Strazzabosco M., 2011) is well recognized, their ability to produce ECM components is still uncertain. We showed that in *Pkhd1*$^{del4/del4}$ cholangiocytes, COLL1 transcripts are increased in basal conditions, and are further and potently stimulated by TGFβ1. Therefore, we provide evidence that cyst cholangiocytes are functionally prominent in portal fibrosis in *Pkhd1*$^{del4/del4}$ mice, since they exclusively express αvβ6 integrin and they are able to produce COLL1 further enhanced by TGFβ1. The direct involvement of cholangiocytes at least in the initial phases of portal fibrogenesis in *Pkhd1*$^{del4/del4}$ mice may explain the two distinctive features of this model as compared to other experimental models of progressive fibrotic liver disease, including the early deposition of matrix components strictly adjacent to the dysgenetic bile ducts, and the slower fibrotic progression. However, whether other cell types different from portal myofibroblasts, such as bone-marrow derived cells (fibrocytes), may additionally contribute to portal fibrosis in *Pkhd1*$^{del4/del4}$ mice is a matter of further investigation.

In conclusion, as illustrated in Figure 24, this study unveils the central role of αvβ6 integrin in CHF. In CHF, portal fibrosis is the result of an intensive cross-talk between epithelial and inflammatory mononuclear cells, originating from the FPC deficient cholangiocytes. These cholangiocytes secrete large amounts of different chemokines (CXCL10

and CXCL1) able to orchestrate the peribiliary recruitment and differentiation of macrophages. In turn, inflammatory cytokines released by macrophages (TGFβ1 and TNFα), up-regulate αvβ6 integrin expression on biliary cysts that enhances fibrosis via activation of latent TGFβ1. Once activated, TGFβ1 enhances production of ECM components (COLL1) in cyst cholangiocytes, ultimately resulting in excessive deposition of matrix in the peribiliary region.

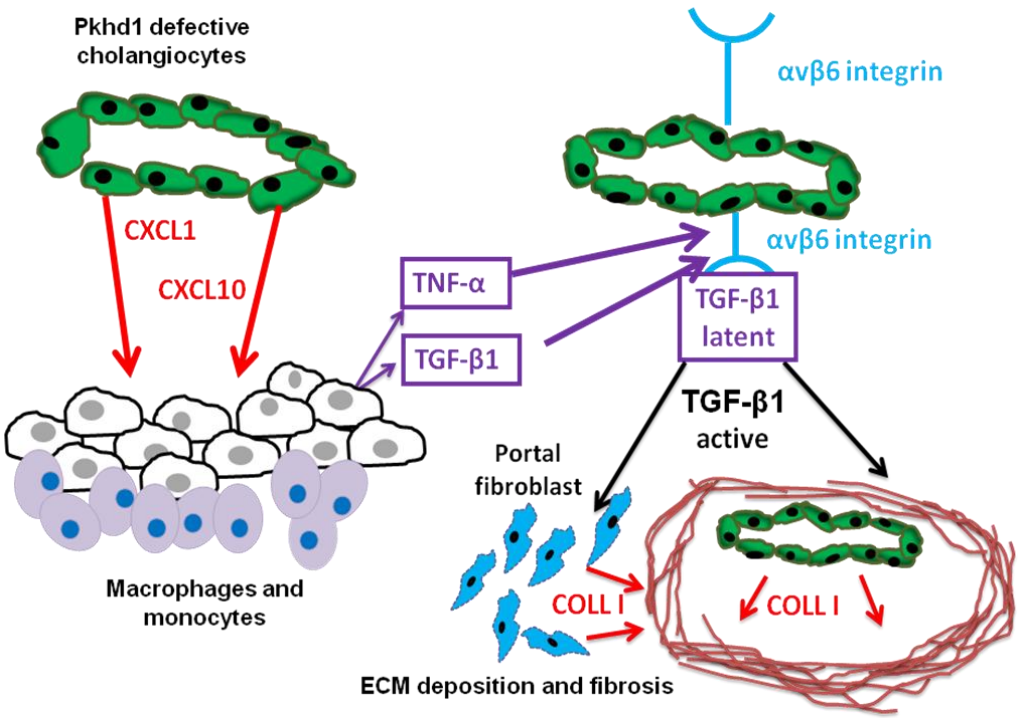



**Fig. 24: Portal fibrosis is the result of an intensive cross-talk between epithelial and inflammatory mononuclear cells, originating from the FPC deficient cholangiocytes.**

# Abbreviations

**ARPKD:** *Autosomal Recessive Polycystic Kidney Disease*

**ASH:** *Alcoholic Steatohepatitis*

**CCA:** *Cholangiocarcinoma*

**CD:** *Caroli Disease*

**CHF:** *Congenital Hepatic fibrosis*

**COLL1:** *Collagen Type 1*

**CTGF:** *Connective Tissue Growth Factor*

**ECM:** *Extracellular Matrix*

**FCP:** *Fibrocystin*

**HCC:** *Hepatocellular Carcinoma*

**HSC:** *Hepatic Stellate Cell*

**IFN-γ:** *Interferon Gamma*

**CXCL10:** *Interferon Gamma-Induced Protein 10*

**CXCL1:** *Keratinocyte-Derived Chemokine*

**MF:** *Myofibroblast*

**MMP:** *Matrix Metalloproteinase*

**NAFLD:** *Non-Alcoholic Fatty Liver Disease*

**NASH:** *Non Alcholic Steatohepatitis*

**PDGF:** *Platelet-Derived Factor*

**PFs:** *Portal Fybroblasts*

**TGF-β:** *Trasforming Growth Factor Beta*

**TNF-α:** *Tumor Necrosis Factor Alpha*

**α-SMA:** *Apha Smooth Muscle Actin*

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