Cell-type specific role of phosphoinositide 3-kinase γ in inflammatory-induced cholestasis

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Summary

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection. Despite advances in treatment strategies, sepsis remains a major health care burden, accounting for 60-80% of lost lives per year in intensive care units (ICU). Current treatments mainly aim to contrast the sepsis-induced development of multiple organ failure (MOF) but they still lack of specificity.

Mounting evidence indicates that the liver plays a key role in both the initiation and the promotion of MOF during sepsis. Among all liver functions, the hepatic formation and secretion of bile is critical to survival and is easily disrupted in septic conditions. Systemic inflammation negatively affects these delicate processes, leading to bile accumulation within hepatocytes (cholestasis), severely affecting liver functions and contributing to poor outcomes.

Phosphoinositide-3 kinase γ (PI3Kγ) is recognized as a key contributor to inflammatory stress responses in both immune and non-immune cells. Previous studies showed that knockout mice for PI3Kγ were protected from cholestasis in experimental mouse models of polymicrobial sepsis. However, the role of this kinase in liver parenchyma remains unknown. Thus, the objective of this study aimed to determine the hepatocyte-specific role of PI3Kγ in the pathogenesis of inflammatory-induced cholestasis.

To elaborate the functional patterns of PI3Kγ, primary hepatocytes derived from PI3Kγ wild-type (WT), kinase-dead (KDγ) and knockout (KOγ) mice were isolated and analyzed *ex vivo*. In parallel, immortalized human (HepG2) and murine (Hepa 1-6) hepatocyte cell lines were used for *in vitro* studies.

Initial investigations disclosed for the first time that liver parenchymal cells express PI3Kγ. Most importantly, both WT primary hepatocytes and cell lines displayed increased PI3Kγ expression upon exposure to a proinflammatory cytokine mix (CM), suggesting the involvement of this enzyme in inflammatory stress responses of liver parenchyma. Furthermore, we find that PI3Kγ regulates the cellular localization of multidrug resistance-associated protein 2 (Mrp2), thus contributing to the pathogenesis of inflammatory-induced cholestasis. Mrp2 is an ABC transporter that is normally expressed at the canalicular membrane of hepatocytes, secreting conjugated endogenous and xenobiotic substances and thus representing an important driving force in bile formation and flow. However, it is well known that Mrp2 undergoes endocytosis processes upon inflammatory stress, strongly contributing to the development of cholestasis. Here we find that the kinase activity of PI3Kγ modulates two distinct signaling pathways

affecting intracellular localization of Mrp2: i) the PKCα/ERM-mediated Mrp2 internalization and ii) the cAMP-dependent insertion of endocytosed Mrp2 particles to the canalicular membrane. Strikingly, both genetic and pharmacological inhibition of PI3Kγ resulted in hepatocyte protection from inflammatory-induced Mrp2 internalization. Furthermore, the lack of functional PI3Kγ in primary hepatocytes increased the expression of heme oxygenase-1 (HO-1), which is known to exert hepatoprotective effects against inflammation-induced oxidative stress.

Overall, our results point out a new cell-type specific role of PI3Kγ in inflammatory-stress responses of liver parenchyma. Based on this study, hepatocyte-specific PI3Kγ inhibition may represent a novel putative therapeutic strategy for preventing sepsis-induced cholestasis, preserving liver organ function of critically ill septic patients and possibly diminishing the mortality in the ICU.

Zusammenfassung

Sepsis ist eine lebensbedrohliche, systemische Fehlreaktion des Körpers auf Infektionen. Trotz Fortschritte in der Behandlung bleibt die Sepsis eine Herausforderung für das Gesundheitswesen und ist für 60-80% der Todesfälle auf Intensivpflegestationen verantwortlich. Aktuelle therapeutische Ansätze sind unspezifisch und zielen hauptsächlich auf die Behandlung des Sepsis-induzierten multiplen Organversagens (MOV).

Zunehmende Hinweise deuten auf eine Schlüsselrolle der Leber in der Entstehung und Entwicklung des MOV. Zu den essentiellen Leberfunktionen gehört die Produktion und Sekretion der Galle. Systemische Entzündungen stören diesen empfindlichen Prozess und führen zu einer Ansammlung der Galle in Hepatozyten (Cholestase). Cholestase während der Sepsis beeinträchtigt die Funktion der Leber nachhaltig und verschlechtert die Prognose.

Das Signalprotein Phosphoinositid-3 Kinase γ (PI3Kγ) gilt als wesentlicher Mediator der Entzündungsreaktion von Immunzellen sowie Parenchym- und Epithelzellen. Frühere Studien zeigen, dass PI3Kγ Knockout Mäuse während einer polymikrobiellen Sepsis vor Cholestase geschützt sind. Die genaue Rolle dieser Lipid-Kinase im Parenchym der Leber ist jedoch noch unbekannt. Daher ist es das Ziel dieser Studie, die Rolle der PI3Kγ in Hepatozyten während der Pathogenese einer entzündungs-induzierten Cholestase zu bestimmen.

Um die Funktionsmuster von PI3Kγ herauszuarbeiten, wurden primäre Hepatozyten von PI3Kγ Wildtyp (WT), *Kinase-dead* (KDγ) und *Knock-out* (KOγ) - Mäusen isoliert und ex vivo analysiert. Parallel dazu wurden immortalisierte menschliche (HepG2) und murine (Hepa 1-6) Hepatozyten-Zelllinien für in vitro Studien verwendet.

Die Untersuchungen zeigten erstmalig eine PI3Kγ Expression in Zellen des Leberparenchyms. In Hepatozyten der Wildtyp-Mäuse und in den Zelllinien wurde eine erhöhte PI3Kγ Expression nach Exposition durch einen pro-inflammatorischen Zytokin-Mix beobachtet. Dies deutet auf eine Beteiligung des Enzyms bei einer Entzündungsreaktion des Leberparenchyms hin. Weitere Ergebnisse zeigen, dass PI3Kγ die zelluläre Lokalisation von Multiresistenz-assoziierten Protein 2 (Mrp2) zu regulieren scheint und dadurch an der Pathogenese einer entzündungs-induzierten Cholestase beteiligt ist.

Mrp2 ist ein ABC-Transporter, welcher normalerweise auf der Kanalikulärmembran der Hepatozyten exprimiert wird und dort konjugierte, endogene und xenobiotische Substanzen sekretiert. Somit ist Mrp2 ein essentieller Bestandteil sowohl bei der Bildung als auch beim Transport der Galle. Es ist ferner bekannt, dass Mrp2 bei inflammatorischem Stress endozytotischen Prozessen ausgesetzt ist. Hier fanden wir heraus, dass die Aktivität der PI3Kγ

zwei unterschiedliche Signalwege reguliert, welche die intrazelluläre Lokalisation von Mrp2 beeinflussen: (1) die Mrp2-Internalisierung vermittelt durch PKCα/ERM, sowie (2) die cAMP-abhängige Insertion von endozytotisch aufgenommenen Mrp2-Partikeln in die Kanalikulärmembran. Erstaunlicherweise führte sowohl eine pharmakologische als auch eine genetische Inhibition von PI3Kγ zur Repression der entzündungs-induzierten Mrp2-Internalisierung in Hepatozyten. Ferner führte der Mangel an PI3Kγ in primären Hepatozyten zu einer Erhöhung der Expression von Hämoxygenase-1 (HO-1), welche einen protektiven Effekt auf Hepatozyten bei entzündungs-induziertem oxidativen Stress hat.

Insgesamt weisen die Ergebnisse dieser Studie auf eine neue zelltypespezifische Rolle von PI3Kγ während einer Entzündungsreaktion des Leberparenchyms hin. Die spezifische Inhibition von PI3Kγ in Hepatozyten könnte eine neue therapeutische Strategie zur Prävention einer Sepsis-induzierten Cholestase darstellen. Mit diesem therapeutischen Konzept könnten die Leberfunktionen lebensgefährlich erkrankter Sepsispatienten erhalten und deren Mortalität auf Intensivpflegestationen reduziert werden.

Abbreviations

ABC	ATP-binding cassette
β2AR	beta 2 adrenergic receptor
˚C	degree Celsius
cAMP	cyclic adenosine monophosphate
CLP	cecal ligation and puncture
CM	cytokine mix
CREB	cAMP response element binding protein
ERM	ezrin-radixin-moesin
fMLP	N-Formyl-Met-Leu-Phe
FRIL	freeze-fracture replica immunogold labelling
g	units of times gravity
GPCR	G protein-coupled receptor
h	hours
HO-1	heme oxygenase-1
ICU	intensive care unit
IFNγ	interferon γ
IL-1β	interleukin-1β
IP	immunoprecipitation
KDγ	kinase-dead γ
KOγ	knockout γ
LPS	lipopolysaccharides
μ	micro
m	milli
M	molar

min	minutes
MOF	multiple organ failure
Mrp2	multidrug resistance-associated protein 2
PCI	peritoneal contamination and infection
PDE3B	phosphodiesterase 3B
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PP-1	protein phosphatase-1
ROS	reactive oxygen species
rpm	rounds per minute
RT	room temperature
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIRS	systemic inflammatory response syndrome
TNFα	tumor necrosis factor α
v/v	volume per volume
w/v	weight per volume
WT	wild-type

Contents

1. Introduction

1.1 Sepsis definition and incidence

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection. Sepsis remains the primary cause of death from infection despite advances in modern medicine, such as vaccines, antibiotics and acute care (Angus and van der Poll 2013). In the developing world, sepsis accounts for 60-80% of lost lives per year, affecting more than 6 million newborns and children annually, with over 100,000 women contracting sepsis in the course of pregnancy and childbirth (Kissoon et al. 2011). Strikingly, in all countries where data on hospitalizations for sepsis are available, the number of cases has increased steadily in the past years (Lagu et al. 2012; La Suarez De Rica et al. 2016; Kaukonen et al. 2014).

According to the classical definition of sepsis, which was used until one year ago, sepsis diagnosis requires the recognition of the systemic inflammatory response syndrome (SIRS), which is frequently caused by infection (Stearns-Kurosawa et al. 2011). SIRS is defined by the presence of two or more criteria, listed in Table 1, and it may follow a variety of clinical insults, including infection, pancreatitis, ischemia, multiple trauma, tissue injury, hemorrhagic shock, or immune-mediated organ injury.

Table 1. Criteria for the systemic inflammatory response syndrome (SIRS).
(Stearns-Kurosawa et al. 2011)

Criterion	Value
Temperature	>38°C or <36°C
Heart rate	>90 beats per minute
Respiratory rate	>20 or $PaCO_2$<32 mm Hg
White blood cell count	>12 K or <4 K mm^{-3}, or >10% bands

For a long time, when SIRS occurred with infection, the diagnosis for the patient was sepsis, and the severity of the disease depended on its association with organ dysfunction, hypoperfusion or hypotension. In particular, patients met the criteria for septic shock when they had persistent hypotension and perfusion abnormalities despite adequate fluid resuscitation (Levy et al. 2003).

In early 2016 the Society of Critical Care Medicine and the European Society of Intensive Care Medicine convened a task force to address definitions and clinical criteria for sepsis (Singer et al. 2016). In effect, since the definitions of sepsis, septic shock, and organ dysfunction remained largely unchanged for more than two decades, the need for re-examination reunited 19 experts in sepsis pathobiology, clinical trials, and epidemiology. These experts pointed out the inadequate specificity and sensitivity of the SIRS criteria, and sustained that the multiple definitions and terminologies used for sepsis, septic shock, and organ dysfunction led to discrepancies in reported incidence and observed mortality. As a conclusion, they agreed that sepsis should instead be defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection. Choosing this definition, they emphasized the importance of organ function preservation, which is critical for patients' outcome. Presently, for clinical operationalization, organ dysfunction is determined by an increase in the Sequential (sepsis-related) Organ Failure Assessment (SOFA) score of 2 points or more (which is associated with an in-hospital mortality greater than 10%). The definition of septic shock was also changed, and is now defined as a subset of sepsis in which particularly profound circulatory, cellular, and metabolic abnormalities are associated with a greater risk of mortality than with sepsis alone (greater than 40%). These updated definitions, based on the considerable advances made into the pathobiology, management, and epidemiology of sepsis in the past years, will help to facilitate earlier recognition and more timely management of patients with sepsis or at risk of developing sepsis.

The reported incidence of sepsis is increasing (Gaieski et al. 2013), likely reflecting aging populations with more comorbidities as well as greater recognition. Although the true incidence is unknown, conservative estimates indicate that sepsis is a leading cause of mortality and critical illness worldwide (Fleischmann et al. 2016; Singer et al. 2016).

The treatment for septic patients usually consists in i) lowering the amount of infectious agents with antibiotics, ii) fluid resuscitation for preventing organ hypoperfusion and iii) administration of anticoagulants to avoid coagulation derangements. All of these treatments aim to contrast the development of multiple organ failure (MOF) and worsened outcome, but they lack specificity (Abraham and Singer 2007). Although novel therapeutic agents have been found to improve sepsis, their results still remain limited, associated with side effects and continued high mortality (Deans et al. 2005). In addition, there is increasing awareness that patients who survive sepsis often have long-term physical, psychological, and cognitive disabilities with significant health care and social implications. Thus, there is an urgent need to

find novel therapeutic strategies, particularly aimed to preserve organ function of critically ill septic patients.

1.2 The liver role in sepsis

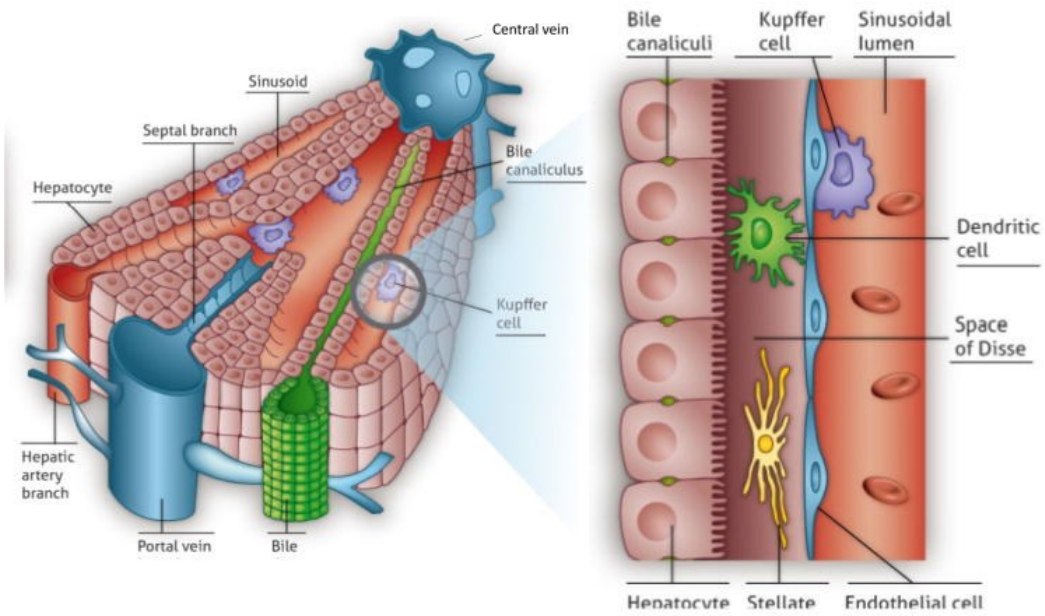
1.2.1 Liver organ structure and functions

The liver is increasingly recognized as a key organ in both the initiation and the promotion of MOF during sepsis (Yan et al. 2014; Hirata et al. 2001). This organ has indeed major roles in the dissemination of infectious pathogens and inactivation of bacterial products, as well as in the production and clearance of inflammatory mediators. However, apart from its important role in the immunological homeostasis, the liver is responsible for over 200 defined functions, such as detoxification, storage, energy production, nutrient conversion, hormonal balance, and coagulation (Yan et al. 2014). These important physiological functions make the liver a critical organ for host survival following severe injury such as sepsis. Notably, evidence has been provided that sepsis-induced liver dysfunction and failure directly contribute to disease progression and death (Canabal and Kramer 2008).

The structure of the liver is characterized by repeated units consisting of a multicellular architecture (liver lobule), where 60-80% of the cell population is represented by hepatocytes (Bhatia et al. 2014). These cells compose liver parenchyma and are arranged in cords that are sandwiched by extracellular matrix (Fig. 1). The space between cords hosts a multitude of supporting cell types, such as sinusoidal endothelial cells, Kupffer cells, biliary ductal cells, and stellate cells. Due to this particular architectural arrangement and cellular heterogeneity, liver cells are continuously exposed to nutrients, hormones, and growth factors, which are delivered via the combined blood supply of the portal vein and hepatic artery.

The structural organization of the liver has profound implications for its immune function (Racanelli and Rehermann 2006). Of particular interest, the liver contains the single largest population of macrophages (Kupffer cells, KCs), which accounts for 80–90% of the total population of fixed tissue macrophages in the body (Sheth and Bankey 2001). KCs have a crucial role both in eliminating bacteria and insoluble waste through phagocytosis and in releasing a wide range of products implicated in liver injury. On the other hand, Zhou et al. recently provided evidence also supporting the role of hepatocytes as key mediators of innate immune responses, since they produce 80–90% of the circulating innate immunity proteins in the body, including acute phase proteins, complements, bactericidal proteins and opsonins (Zhou et al. 2016).

A



B

Cell Type	Functions
Hepatocyte	Protein storage and synthesis, carbohydrate metabolism, secretion of urea and bile, detoxification
Sinusoidal endothelial cell	Mediate nutrient transport, cell extravasation, blood protein scavenging
Hepatic stellate cell	Lipid and vitamin A storage
Kupffer cell	Mononuclear phagocyte system
Biliary epithelial cells	Regulate ductular transport of bile from the liver
Dendritic cell	Antigen presentation, regulate T cell activation and tolerance

Figure 1: The multicellular architecture of the liver. (**A**) The liver possesses a hierarchical structure consisting of repeated functional tissue units (liver lobules), in which hepatocytes are the main parenchymal cells. Within a lobule, oxygenated blood enters through branches of the hepatic artery and portal vein, and flows in specialized sinusoidal vessels towards the central vein. The hepatocytes are polarized epithelial cells that interact closely with a number of non-parenchymal cells types along the sinusoidal tracts of the liver lobule. The bile, which is produced and secreted by hepatocytes, flows in the counter direction relative to blood, towards the intrahepatic bile duct. (**B**) Several cellular types and multiscale tissue structures contribute to the diverse functional roles of the liver. (Bhatia et al. 2014)

However, although the liver has been proposed as an "immunological organ" (Racanelli and Rehermann 2006), the primary functions of the liver are not traditionally considered to be immunological. Among all liver functions, the hepatic formation of bile is critical to survival and is one of the most easily disrupted liver functions (Li and Crawford 2004; Hirata et al. 2001). Importantly, bile assists fat digestion in the intestine and mediates the disposal of poorly water-soluble endogenous compounds and xenobiotics (Stieger and Mahdi 2017). Disturbances in these delicate processes may result in a variety of hepatic, intestinal and systemic disorders, especially in conditions of septic-induce systemic inflammation.

1.2.2 Sepsis-induced liver cholestasis

Maintenance of the enterohepatic bile acid circulation is vital for several liver and gastrointestinal functions including bile flow, solubilization and excretion of cholesterol, clearance of toxic molecules, intestinal absorption of lipophilic nutrients, as well as metabolic and antimicrobial effects (Halilbasic et al. 2013). In addition, the enterohepatic circulation efficiently preserves these precious molecules, since bile acid synthesis from cholesterol involves a series of energy-consuming enzymatic steps (Russell 2009). In particular, bile acids and bile salts reach the liver from the intestine via the portal vein and are taken up by hepatocytes, where they are rapidly and efficiently conjugated to taurine or glycine (Stieger and Mahdi 2017). Since bile acids in hepatocytes are mainly present in their conjugated form, they must be transported via energy-driven transport systems across the membranes of cells involved in the enterohepatic circulation.

Bile acid transporters have different affinities for various bile acid species as well as for other endogenous and exogenous compounds which are insufficiently water soluble to be excreted into urine (Li and Crawford 2004). Hence, both the expression of bile acid transporters and their transport capacity are tightly controlled in order to maintain bile acid homeostasis (Halilbasic et al. 2013). Disturbance of this delicate balance may contribute to cholestasis, which is a common complication in patients with extrahepatic bacterial infections and sepsis (Geier et al. 2006; Li and Crawford 2004; Hirata et al. 2001).

The pathogenesis of cholestasis which develops during sepsis arises from intrahepatic metabolic abnormalities, and is based on disturbance of the intra- and extracellular transportation system of bilirubin in hepatocytes (Hirata et al. 2001). Even though little is known about the mechanisms of sepsis-associated cholestasis, the common pathophysiological denominator is the induction of proinflammatory cytokines and oxidative stress, which are potent inhibitors of hepatocellular bile secretion (Geier et al. 2006; Hirata et al. 2001). Additionally, cholestatic conditions contribute to several complications, such as hypotension, altered glucose and lipid metabolism, increased energy expenditure and renal function impairment, thus aggravating the outcome of septic patients (Horvatits et al. 2013).

About 20% of septic patients develop cholestasis during their stay at the intensive care unit (ICU), and hyperbilirubinaemia in critically ill patients is a strong independent risk factor for mortality in the ICU (Horvatits et al. 2013; Kramer et al. 2007). However, cholestasis in sepsis is generally reversible, since it is usually caused by functional alterations at the hepatocellular and/or bile duct level (Geier et al. 2006). For this reason, the molecular mechanisms implicated

in the pathogenesis of sepsis-induced cholestasis are currently extensively studied in order to identify novel putative therapeutic targets preventing liver dysfunctions.

1.3 Molecular mechanisms of sepsis-induced liver cholestasis

1.3.1 Inflammatory stress-induced mechanisms of cholestasis

During systemic inflammation, microbial products induce signaling pathways within hepatocytes both directly and through activation of pro-inflammatory cytokines, leading to rapid and profound reductions in bile flow (Kosters and Karpen 2010). In particular, the expression and function of key hepatobiliary transporters are suppressed in response to inflammatory stress.

It is now well established that transporters involved in bile formation undergo both transcriptional and post-translational regulation (Anwer 2014; Halilbasic et al. 2013). The transcriptional regulation of hepatocellular transporters is mediated by the nuclear receptor superfamily (Halilbasic et al. 2013) and assures long-term adjustments of transporter functions. Notably, the genes encoding for bile transporter proteins are negatively regulated by a complex interacting network of hepatocyte nuclear factors (HNF1, 3, 4) and nuclear receptors (e.g., FXR, PXR, CAR, RAR, LRH-1, SHP, GR), usually activated by bile acid accumulation within hepatocytes (Geier et al. 2007). On the contrary, post-translational regulations involve short-term rapid changes in plasma membrane localization of transporters, allowing for rapid changes in bile formation (Anwer 2014; Crocenzi et al. 2012). This is a tightly regulated process, and the signaling mediators involved are being actively characterized (Roma et al. 2008).

There is compelling evidence in literature that the physiological recycling of membrane transporters via vesicle-mediated insertion/internalization from and to the endosomal compartment is altered under cholestatic conditions. In particular, under such conditions a shift toward retrieval of bile transporter proteins is induced, decreasing their plasma membrane expression and consequently impairing the transport of their substrates (Crocenzi et al. 2012). This mechanism is schematically shown in Fig. 2.

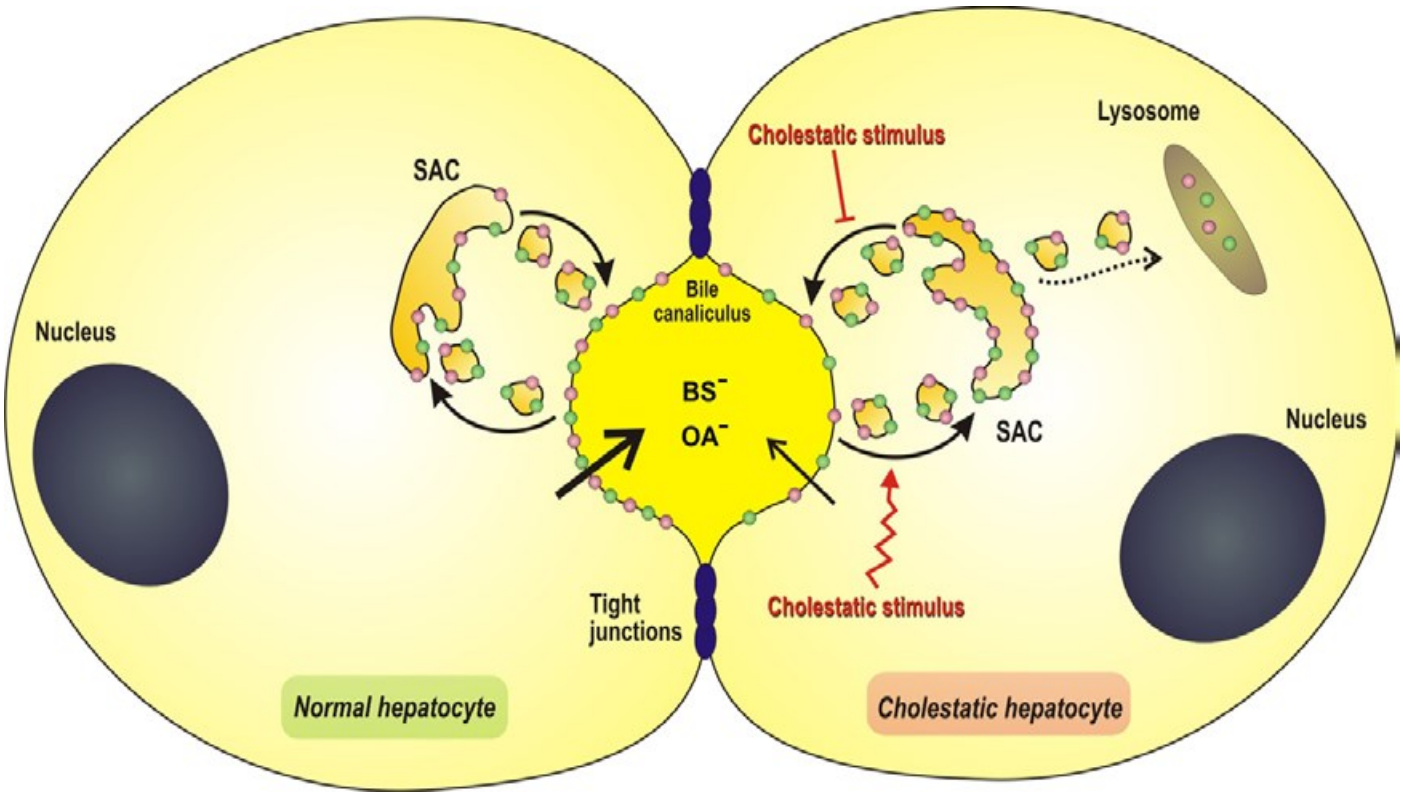


Figure 2: Dynamic localization of canalicular transporters under normal and cholestatic conditions. Under physiological conditions (normal hepatocyte), the density of transporters at the canalicular membrane domain, responsible for the normal biliary excretion of bile-forming solutes such as bile salts (BS^-) and organic anions (OA^-), is regulated by a vesicle-mediated recycling from a subapical compartment (SAC), which serves as a reservoir of transporters available on demand. Cholestatic stimuli disrupt this mechanism, leading to a decrease in the expression of transporters at the canalicular membrane, with a concomitant increase in SAC. This is accompanied by a reduced biliary excretion of their substrates (cholestatic hepatocyte). Transporter internalization may be produced either by stimulating the endocytic internalization of transporters or by inhibiting their normal exocytic insertion from SAC. Sustained cholestatic stimuli could lead to the sorting of endocytosed transporters to lysosomes, followed by lysosomal degradation (Crocenzi et al. 2012).

Changes in transporter localization are considered as the major pathologic mechanism explaining the hepatocyte secretory failure (Crocenzi et al. 2012). A number of signaling pathways have been supposed to be involved in the regulation of these short-term changes (Geier et al. 2006).

1.3.2 The hepatobiliary transport system

Bile formation depends on proper functioning of the parenchymal hepatobiliary transport system (Geier et al. 2006), as illustrated in Fig. 3.

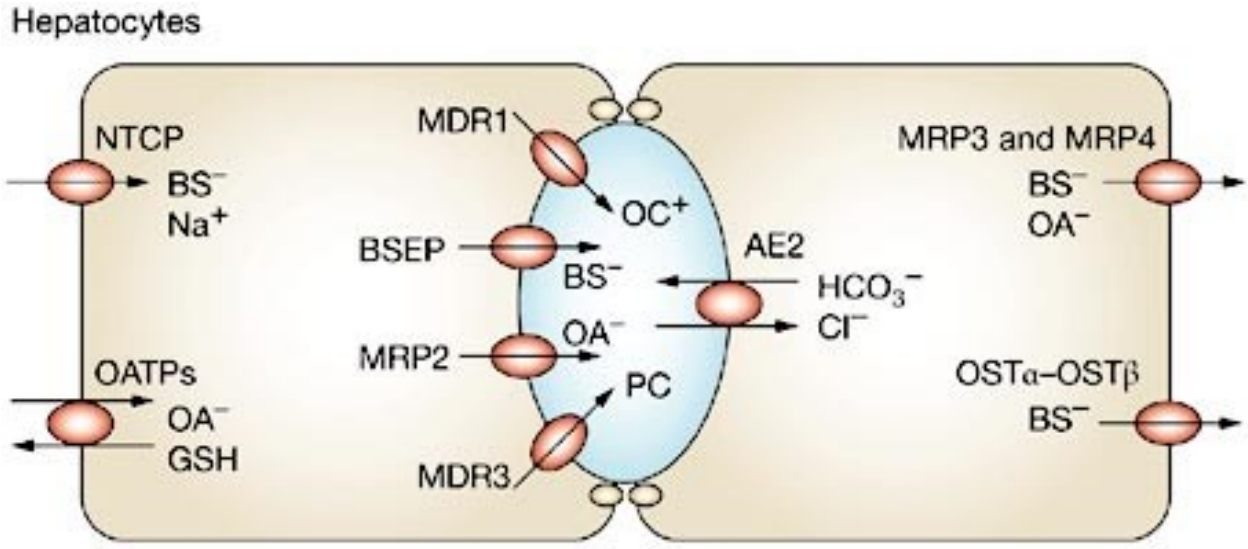


Figure 3: **The hepatobiliary transport system in hepatocytes.** Bile salts are taken up by hepatocytes through the basolateral sodium taurocholate cotransporter and organic anion transporting proteins. Monovalent bile salts are secreted via the canalicular bile-salt export pump, whereas divalent bile salts and anionic conjugates are excreted via the canalicular conjugate export pump. The phospholipid export pump facilitates excretion of phosphatidylcholine, which forms mixed micelles in bile together with bile salts and cholesterol. Cationic drugs are excreted by the multidrug export pump. Basolateral isoforms of the multidrug-resistance-associated proteins MRP3 and MRP4 provide an alternative route for the elimination of bile salts and non-bile-salt anionic conjugates from hepatocytes into the systemic circulation. Abbreviations: AE2, chloride–bicarbonate anion exchanger isoform 2;; BS-, bile salts; BSEP, canalicular bile-salt export pump; Cl-, chloride ions; GSH, glutathione, HCO3-, bicarbonate ions; MDR1, multidrug export pump; MDR3, phospholipid export pump; MRPs, multidrug-resistance-associated proteins; MRP2, canalicular conjugate export pump; Na+, sodium ions; NTCP, basolateral sodium taurocholate cotransporter; OA-, anionic anions or conjugates; OATPs, organic anion transporting proteins; OC+, cationic drugs; OST, organic solute transporter; PC, phosphatidylcholine (Geier et al. 2006).

The hepatobiliary transport system is composed of membrane proteins localized either on the basolateral/sinusoidal membrane, in communication with blood vessels, or on the

apical/canalicular membrane, which faces to bile canaliculi (Geier et al. 2006; Crocenzi et al. 2012).

At the level of basolateral membrane, the hepatic uptake of bile acids from sinusoidal blood is mediated by two main transporter proteins: i) the high-affinity sodium-dependent bile-salt transporter (NTCP) and ii) a family of multispecific organic anion transporters (OATPs) (Geier et al. 2006). On the other hand, the sinusoidal membrane also contains efflux pumps, such as the multidrug-resistance-associated proteins (MRPs) MRP3 and MRP4. These two proteins are normally expressed in hepatocytes at very low levels, but during cholestasis they are upregulated and account for reversed bile-acid transport back into the plasma (Geier et al. 2006). In addition, cholestatic conditions also upregulate the expression of a heterodimeric organic solute transporter (OSTα–OSTβ) for bile acids and sterols.

At the canalicular level, most of the transporter proteins are ATP-binding cassette (ABC) transporters that are either multidrug resistance (MDR) P-glycoproteins (the ABCB subfamily) or MRPs (the ABCC subfamily) (Geier et al. 2006). There are several canalicular transport systems in both rodents and humans. A bile-salt export pump (BSEP/ABCB11) transports monovalent bile acids, whereas a conjugate export pump (MRP2/ABCC2) transports divalent bile acids and various other amphipathic conjugates, including bilirubin diglucuronide and glutathione. There is also a multidrug export pump (MDR1/ABCB1) for transporting amphipathic organic cations (e.g. various drugs), and a phospholipid flippase (MDR3 in humans and Mdr2 in rodents) for phosphatidylcholine translocation. Additionally, the chloride–bicarbonate anion exchanger isoform 2 (AE2) is also present in the canalicular membrane, which is a functionally relevant ATP-independent transport system responsible for bicarbonate excretion.

Among all these components of the hepatobiliary transport system, a crucial role during cholestasis is played by the multidrug resistance-associated protein 2 (MRP2/Mrp2) (Elferink and Paulusma 2015).

1.4 The Mrp2 protein

1.4.1 Mrp2 protein expression and function

The multidrug resistance-associated protein 2 (Mrp2) is an ABC transporter localized exclusively to the apical membrane domain of polarized epithelial cells (Gu and Manautou 2010; Chai et al. 2015). Specifically, this transporter protein is expressed at the canalicular membrane of hepatocytes, the proximal tubule epithelial cells in kidney, and the brush-border membrane in small intestine for excreting conjugated organic anions, including bilirubin, bile acid divalent conjugates and drugs (Gu and Manautou 2010). The localization of Mrp2 to the apical membrane of various polarized cells favors the function of this efflux pump in the terminal phase of detoxification, secreting conjugated endogenous and xenobiotic substances (Nies and Keppler 2007). Substrates for Mrp2 include a wide range of compounds, such as conjugates of lipophilic substances with glutathione, glucuronate, or sulfur containing compounds, as exemplified by leukotriene C4, bilirubin glucuronosides, and some steroid sulfates (Gu and Manautou 2010). Importantly, Mrp2 can also transport uncharged compounds in cotransport with glutathione, thus modulating the pharmacokinetics of many drugs. Hence, hepatic Mrp2 is an important driving force in bile formation and flow.

Deficiency of Mrp2 causes an increased concentration of bilirubin glucuronosides in blood, due to basolateral egress in the absence of proper biliary elimination. Accordingly, the absence of functional Mrp2 from the canalicular membrane causes conjugated hyperbilirubinemia, as observed in the Dubin-Johnson syndrome in humans (Gu and Manautou 2010; Nies and Keppler 2007), in GY/TR^{-} EHBR rats (Paulusma et al. 1996) and in Mrp2$^{-/-}$ mice (Chu et al. 2006).

Previous studies have shown that within cholestatic livers a substantial fraction of Mrp2 is localized in subapical vesicles, thereby largely shutting off canalicular secretion (Elferink and Paulusma 2015; Recknagel et al. 2012; Chai et al. 2015). However, the mechanisms by which endocytosis of hepatocellular membrane transporters occurs in cholestasis remain poorly understood, partly due to their multifactorial nature. At present, this subject is under active investigation.

1.4.2 Mrp2 protein localization in hepatocytes relies on its interaction with intact cytoskeleton

Proinflammatory conditions and oxidative stress caused by infection have a massive influence on the hepatic secretory function, largely due to the retrieval of canalicular membrane trasporters, such as Mrp2 (Crocenzi et al. 2012; Roma et al. 2008; Copple et al. 2010; Geier et al. 2006). Mostly, plasma membrane localization of transporter proteins requires close interactions between the plasma membrane and the underlying cytoskeleton (Crocenzi et al. 2012). Such interactions involve proteins that cross-link membrane proteins with filaments of actin (F-actin). In line with this, canalicular transporter internalization was reported under conditions of disruption of the F-actin cytoskeletal integrity by administration of the poison phalloidin (Rost et al. 1999).

The ezrin-radixin-moesin (ERM) family, which consists of tether proteins that act as cross-linkers between plasma membrane and F-actin, were reported to be involved in anchoring bile transporter proteins in their specific location (Roma et al. 2008; Crocenzi et al. 2012). Indeed, the interaction with one or more ERM proteins seems essential for the proper localization of transporter proteins in general (Crocenzi et al. 2012), and of Mrp2 protein in particular (Elferink and Paulusma 2015), in specific membrane domains.

ERM proteins are not static tether proteins, but possess two states. In the inactive state, when the ERM binding domain interacts with its own PSD-95/Drosophila discs large/ZO-1 domain (PDZ), the interaction between ERM proteins and other PDZ-containing proteins is prevented. Upon phosphorylation, the internal interaction is lost and active ERM proteins can bind to PDZ domains of other target proteins (Jiang et al. 2014).

The expression of individual ERM proteins varies within different tissues (Jiang et al. 2014). Interestingly, the most abundantly expressed ERM proteins in hepatocytes are radixin and ezrin (Elferink and Paulusma 2015). A broad literature supports the importance of both radixin (Kojima et al. 2008; Kikuchi et al. 2002; Sekine et al. 2011) and ezrin (Chai et al. 2015; Elferink and Paulusma 2015) functions in regulating hepatic Mrp2 protein localization, both in humans and in rodents. Intriguingly, the active phosphorylated status of radixin and ezrin has opposite effects in hepatocytes. While phosphorylated radixin (Thr564) associates with Mrp2 at the canalicular plasma membrane (Sekine et al. 2011), phospho-ezrin protein (Thr567) is associated with a redirection of Mrp2 protein to intracellular vesicles (Chai et al. 2015).

Given the important roles of these proteins in the regulation of Mrp2 protein localization, the phosphorylation status of radixin and ezrin proteins has been increasingly studied in experimental models of cholestasis (Anwer 2014; Sekine et al. 2011; Chai et al. 2015).

Collectively, these studies elucidated in detail how both inflammatory stress and the consequent oxidative stress provoke ERM protein modifications in their phosphorylation state, thus leading to Mrp2 internalization. Importantly, these studies showed how the protein kinase C (PKC) plays a key role in mediating Mrp2 withdrawal to endocytic compartments. On one hand, PKC is responsible for the activation of protein phosphatase-1 (PP-1), which in turn de-phosphorylates radixin protein (Anwer 2014; Sekine et al. 2011); on the other, PKC directly phosphorylates ezrin protein, leading to Mrp2 retrieval into cytosolic compartments (Chai et al. 2015) (Fig. 4). However, the role of the different PKC isoforms in regulating rapid changes in Mrp2 localization still remains to be fully elucidated.

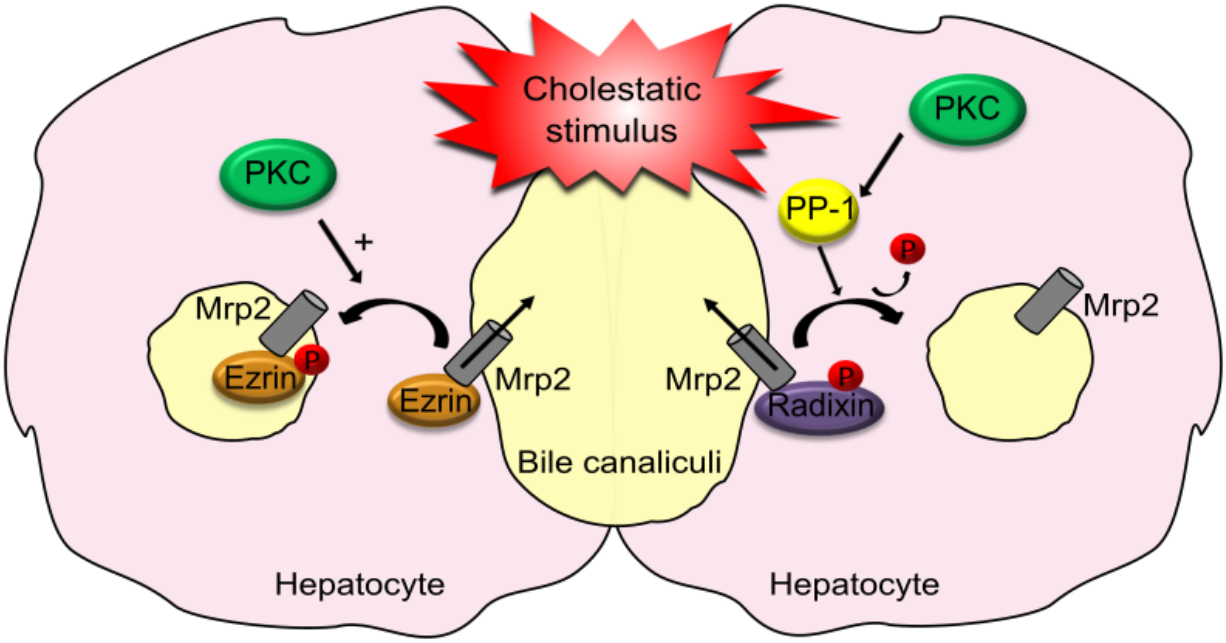


Figure 4: Schematic representation of the PKC-mediated regulation of Mrp2 protein internalization in hepatocytes. Under cholestatic conditions, the protein kinase C (PKC) plays an important role in regulating the phosphorylation status of ezrin and radixin tether proteins, leading to Mrp2 protein retrieval towards endocytic compartments. On one hand, PKC directly phosphorylates ezrin (left side of schematic); on the other, PKC activates the protein phosphatase-1 (PP-1), which in turn is responsible for radixin de-phosphorylation (right side of schematic). In both ways, PKC activation in cholestatic conditions provokes Mrp2 withdrawal from the canalicular membrane of hepatocytes. (Anwer 2014; Sekine et al. 2011; Chai et al. 2015)

1.4.3 cAMP controls Mrp2 protein localization in hepatocytes

The internalization of Mrp2 in cholestatic conditions, which is mainly mediated by ERM proteins, was previously shown to be spontaneously reversed if the cholestatic insult is transient (Crocenzi et al. 2012). Initially, this spontaneous recovery was hypothesized to occur by a microtubule-dependent re-targeting of the endocytosed transporters to the canalicular membrane (Mottino et al. 2005). Based on this, a number of experimental approaches have indicated the key role of cyclic AMP (cAMP) in counteracting cholestasis-induced Mrp2 internalization (Mottino et al. 2002; Park et al. 2014; Schonhoff et al. 2008). For example, it has been proven that the initial drop in both bile flow and transport activity of Mrp2 occurring in the acute phase of drug-induced cholestasis can be partially prevented by administering cAMP as its freely permeable precursor, dibutyryl-cAMP (Mottino et al. 2002; Roelofsen et al. 1998). More significantly, cAMP extensively shortened the spontaneous restoration to normality of bile flow, Mrp2 function, and the localization that spontaneously occurs during the recovery phase (Mottino et al. 2002). A similar anti-cholestatic mechanism was afforded by silibinin, the active component of the hepatoprotector silymarin, most likely due to its capability to inhibit cAMP-degrading phosphodiesterases (Crocenzi et al. 2005).

The major molecular mechanisms by which cAMP protects hepatocytes from cholestatic damage have been partially unraveled by Sánchez Pozzi's research group, which used a model of drug-induced cholestasis in rodents (Zucchetti et al. 2011; Zucchetti et al. 2013). In these studies, the mechanism proposed for cAMP-dependent prevention of cholestatic damage involves two distinct molecular pathways: i) a protein kinase A (PKA)-dependent and microtubule-independent mechanism and ii) the exchange-protein activated by cAMP (Epac)-mediated microtubule-dependent pathway.

However, the signaling pathways mediating cAMP-induced Mrp2 translocation in hepatic cells still remain unclear, and contradictory results exist (Crocenzi et al. 2012). This could be due to the fact that the experimental results obtained rely on the use of chemical inhibitors of the signaling molecules studied, which to some extent may possess cross-reactivity. Nonetheless, cAMP-stimulated translocation of Mrp2 has been suggested to be mediated by phosphatidylinositol 3-kinases (PI3Ks) (Crocenzi et al. 2012; Schonhoff et al. 2008).

1.5 Phosphatidylinositol 3-kinases

1.5.1 The PI3K family

Phosphatidylinositol 3-kinases (PI3Ks) are members of a unique and conserved family of enzymes responsible for the phosphorylation of proteins and lipids. Little is known about the protein kinase function of PI3Ks (Thomas et al. 2013), while the lipid kinase activity of these enzymes has been extensively studied (Kok et al. 2009; Vanhaesebroeck et al. 2010; Cantley 2002). PI3Ks are renowned as catalysts that phosphorylate phosphatidyl-inositol lipids at the D3 position of the inositol ring (Cantley 2002; Engelman et al. 2006). PI3Ks are usually activated in response to cell stimulation by growth factors and hormones, and they contribute to many intracellular processes including cell survival, cell proliferation, metabolism, cell growth, migration and intracellular trafficking (Braccini et al. 2015; Engelman et al. 2006; Hirsch et al. 2007; Beretta et al. 2015). PI3K-produced 3-phosphoinositides regulate intracellular processes through the recruitment of signaling proteins at the plasma membrane, whereby they become activated (Cantley 2002).

According to their lipid substrate preferences and to their structural features, PI3K isoforms have been divided into three classes (class I, class II and class III) (Engelman et al. 2006; Vanhaesebroeck et al. 2010). Although recent studies have pointed to a role of class II and III PI3Ks in vesicular trafficking (Franco et al. 2014) and cell growth (Odorizzi et al. 2000), class I PI3Ks still represent the best characterized subfamily (Fig. 5).

Class I PI3Ks, *in vivo*, primarily convert phosphatidylinositol-4,5-bisphosphate (PIP_2) into phosphatidylinositol-3,4,5-trisphosphate (PIP_3), which interacts with numerous signaling proteins with pleckstrin-homology (PH) domains. Thus, PIP_3 is known to bring the protein serine-threonine kinases Akt (alternatively called protein kinase B, PKB) and phosphoinositide-dependent kinase 1 (PDK1) into proximity, facilitating Akt phosphorylation on Thr308 by PDK1 (Alessi et al. 1996). Subsequently, Akt requires a second mTORC2-dependent phosphorylation on Ser473 for becoming activated (Sarbassov et al. 2005). Fully active Akt, in turn, phosphorylates and regulates compound proteins, such as tuberous sclerosis complex (TSC), glycogen synthase kinase 3 beta (GSKβ), Rac, bcl-2-antagonist of cell death (BAD), p70S6K and forkhead-box-protein O (FOXO), thus triggering multiple intracellular signaling cascades (Cantley 2002; Engelman et al. 2006). All these PI3K-induced signaling pathways are controlled and limited by protein phosphatases, which degrade PIP_3. Of note, Src homology 2 (SH2)-containing-phosphatases (SHIP1 and SHIP2) and phosphatase and tensin homolog

(PTEN) dephosphorylate PIP_3 in positions D5 and D3 respectively, thus terminating PI3K signaling (Cantley 2002; Laurent et al. 2014).

All class I PI3Ks are heterodimers of a p110 catalytic subunit and a regulatory subunit. Depending on the activation mechanism and the differential association with regulatory subunits, class I PI3Ks have been grouped in two subfamilies, class IA and class IB (Vanhaesebroeck et al. 1997; Engelman et al. 2006; Vanhaesebroeck et al. 2010).

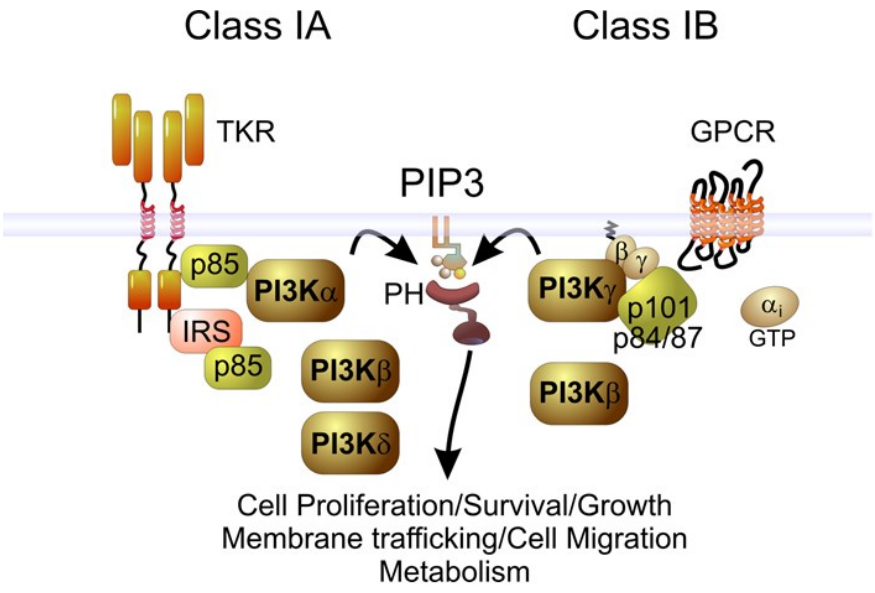


Figure 5: **Class I PI3K family**. Four different class I PI3Ks are expressed in mammalian cells. Depending on their activation mechanisms, they have been divided into two subtypes: class IA PI3Ks (PI3Kα,β,δ) are activated mainly by membrane-bound tyrosine kinase receptor (TKRs), and class IB PI3Kγ is exclusively activated by G protein-coupled receptors (GPCRs), generally of the G_i subtype. Class IA PI3Ks bind to the adaptor subunit p85, which in turn is recruited by its phosphotyrosine binding domain to phosphorylated receptors or adaptor proteins like insulin receptor substrate (IRS). Class IB PI3Kγ binds to adaptor/regulators like p101 or p84/87 and to free $G_{\beta\gamma}$ liberated by active GPCRs. All class I PI3Ks phosphorylate PIP_2 to produce PIP_3, a secondary messenger membrane lipid that functions as a docking site to a large number of proteins containing the pleckstrin homology (PH) domain. These effectors in turn control various cellular responses. (Hirsch et al. 2007)

Class IA catalytic subunits (p110α, p110β and p110δ) bind to the p85 type of regulatory subunits, containing SH2 domains which are able to bind phosphorylated tyrosine on receptor tyrosine kinases (RTKs) (Burke and Williams 2013) and on adapters, such as insulin receptor substrate proteins (IRS-1/2) (Hirsch et al. 2007). On the contrary, class IB catalytic subunit

(p110γ) binds two other regulatory subunits, p101 and p84/87, which facilitate the direct binding with $G_{\beta\gamma}$ subunit of trimeric G proteins. Recently, the p110β isoform of class IA PI3K has been found regulated not only by p85 regulatory subunit, but also by binding directly to $G_{\beta\gamma}$ subunits, thus orchestrating signals from G protein-coupled receptors (GPCRs) as well as from RTKs (Ciraolo et al. 2008; Engelman et al. 2006; Vanhaesebroeck et al. 2010; Guillermet-Guibert et al. 2008).

The differential tissue distribution of PI3K isoforms is a key factor in the distinct biological functions of PI3Ks. For example, p110α and p110β are ubiquitously expressed, and their complete genetic inactivation results in embryonic lethality (Bi et al. 1999; Bi et al. 2002), suggesting a crucial and non-redundant role for the two enzymes in regulating metabolic and house-keeping functions such as cell survival and proliferation. On the contrary, mice deprived of p110δ or p110γ expression are viable and fertile, although they show severely altered phenotypes when their immune system is acutely stressed (Ghigo and Hirsch 2008). p110δ and p110γ have indeed a peculiarly restricted tissue distribution, being abundant in white blood cells (Ghigo and Hirsch 2008; Kok et al. 2009). Given their expression pattern limited to immune cells, p110δ and p110γ have been initially studied preferentially in models of acute and chronic inflammatory diseases, as they have been suggested "partners in crime" in these pathologies (Rommel et al. 2007).

The observation that neutrophil recruitment and dendritic cell movement are attenuated in mice lacking a functional p110γ subunit demonstrated early on the crucial role of this enzyme in promoting inflammation via regulating the in vivo leukocyte migration towards chemokines (Hirsch et al. 2000; Del Prete et al. 2004). Inhibition of PI3Kγ was also found to alleviate symptoms of rheumatoid arthritis, allergic disease and systemic lupus mouse models, mainly due to its involvement in the control of the replenishment of inflammatory cells at sites of inflammation (Wymann and Solinas 2013). Nonetheless, a growing body of evidence indicates a complex pattern of expression of PI3Kγ, that includes the presence of small amounts of this enzyme in tissues other than immune cells, such as heart, smooth muscles, adipose tissue and brain (Patrucco et al. 2004; Becattini et al. 2011; Ghigo et al. 2012; Perino et al. 2011; Perino et al. 2014; Rückle et al. 2006). Of particular interest, in these compartments where the enzyme is less prominently expressed, PI3Kγ was found as a key mediator of cellular responses to stress conditions, mainly induced by inflammation. For this reason, PI3Kγ has gained increasing attention in the last years as a promising drug target for the treatment of inflammatory diseases,

to the extent that PI3Kγ inhibitor could be optimistically considered as an 'aspirin of the 21st century' (Rückle et al. 2006).

1.5.2 PI3Kγ: Kinase-dependent and -independent functions

The catalytic function of PI3Kγ is well known to mediate a wide variety of biological processes, such as inflammation, cardiac remodeling, dendritic cell migration, thrombus formation and allergic responses (Costa et al. 2011; Hirsch et al. 2000; Nienaber et al. 2003; Del Prete et al. 2004; Hirsch et al. 2001; Wymann and Solinas 2013). However, a new layer of complexity in the functions of PI3Kγ has emerged in the past two decades, indicating that this protein functions not only as a kinase, but also as a scaffold protein, activating other signaling molecules by protein-protein interaction (summarized in Hirsch et al. 2009). Remarkably, PI3Kγ enzyme can form a multiprotein complex containing cAMP-degrading phosphodiesterases, thus negatively regulating cAMP pools (Patrucco et al. 2004; Ghigo et al. 2012; Schmidt et al. 2013; Perino et al. 2011). Specifically, it has been well described in cardiomyocytes that PI3Kγ directly interacts with the phosphodiesterase 3B (PDE3B) isoform, thus controlling cAMP-induced heart contractility (Patrucco et al. 2004). Although the specific components of the PI3Kγ-containing complex are yet to be identified, the adaptors p84/87 and p101 might directly interact with PDE3B, thus providing a further layer of action that modulates both p110γ-kinase-dependent and -independent functions (Fig. 6) (Hirsch et al. 2009; Voigt et al. 2006).

However, further studies have recently elucidated the involvement of other interactors in the PI3Kγ-PDE3B macromolecular complex. In cardiomyocytes, for example, p110γ was found to act as an A-kinase anchoring protein (AKAP), tethering PDE3B near its activator protein kinase A (PKA) (Perino et al. 2011), an effect that is important for the negative feedback regulation of cAMP. In addition, different lines of evidence have shown the involvement of Akt for PDE3B activation, especially in adipocytes where this phosphodiesterase is highly expressed (Degerman et al. 2011; Baragli et al. 2011; Ahmad et al. 2007). Since PI3Kγ catalytic function leads to Akt activation, the involvement of PI3Kγ kinase-dependent function for the Akt-mediated PDE3B activation has been investigated. Accordingly, a PI3Kγ/Akt-mediated activation of PDE3B has been described in the brain (Schmidt et al. 2013; Perino et al. 2014; Huang et al. 2015).

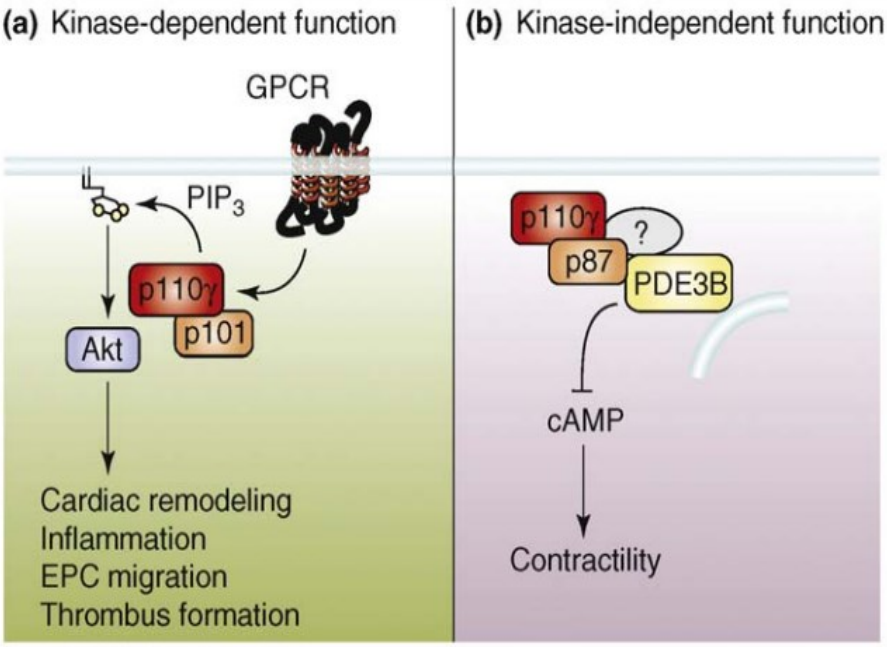


Figure 6: Kinase-dependent and -independent functions of p110γ. (**a**) G protein-coupled receptor (GPCR)-dependent p110γ activation triggers Akt kinase activation through PIP_3 production. The catalytic activity of p110γ leads to different intracellular responses, such as cardiac remodeling, inflammation, endothelial progenitor cell (EPC) migration and thrombus formation. (**b**) The scaffolding role of p110γ in modulating cyclic AMP (cAMP) levels through phosphodiesterase 3B (PDE3B) activation. p110γ and p84/87 bind to PDE3B in a macromolecular complex, in which other members are yet to be identified (as represented by a question mark). PDE3B-mediated cAMP degradation causes a decrease in heart contractility. (Hirsch et al. 2009)

Altogether, these studies underlined that there is a connection between PI3Kγ kinase-dependent and -independent functions, previously described as distinct signaling pathways.

However, the interaction partners identified to date provide only a hint of a potentially larger set of associations and their functional relevance. New studies will be essential to help uncover additional binding partners of PI3Kγ in health and disease.

1.6 Aim of the study

Sepsis-induced organ damage and failure in critically ill patients is a condition involving both infection and inflammation, that nowadays remains associated with high mortality and represents a major clinical challenge (La Suarez De Rica et al. 2016; Lagu et al. 2012). Notably, the liver is a key organ in the initiation and the promotion of multiple organ failure (MOF) during sepsis, playing an active role in the clearance of systemic endotoxins, bacteria and vasoactive byproducts (Hirata et al. 2001).

In the last 20 years, genetic and pharmacologic inhibition of PI3Kγ provided a large body of evidence for the involvement of this enzyme in inflammatory reactions (Wymann and Solinas 2013). Remarkably, the role of PI3Kγ in sepsis has been proposed in previous studies with the lipopolysaccharide endotoxin (LPS), since PI3Kγ-deficient mice developed less LPS-induced lung injury (Yum et al. 2001). Furthermore, genetic and pharmacologic inhibition of PI3Kγ has been demonstrated to prevent MOF and to improve survival in the clinically relevant mouse cecal ligation and puncture (CLP) sepsis model (Martin et al. 2010). Interestingly, in this particular study the authors found that the lack of PI3Kγ catalytic activity is diminishing sepsis-induced liver damage. In 2012, Recknagel et al. found that PI3Kγ-deficient mice were protected from liver damage in another polymicrobial sepsis model, the peritoneal contamination and infection (PCI). Moreover, *in vitro* studies with the human HepG2 hepatocyte cell line revealed that pharmacological inhibition of PI3Kγ suppressed the inflammatory-induced loss of pseudovilli and membrane-bound Mrp2, suggesting a critical role of PI3Kγ in the control of cytokine-induced cholestasis (Recknagel et al. 2012).

On the basis of these findings, we hypothesized that PI3Kγ could mediate hepatocyte-specific inflammatory responses, being involved in the pathogenesis of cholestasis. *ex vivo* and *in vitro* studies were performed to establish the role of PI3Kγ exclusively within the liver parenchyma. In order to inhibit the catalytic activity specifically, the PI3Kγ inhibitor AS605240 was used (Camps et al. 2005). In addition to this pharmacological approach, primary hepatocytes derived from mice lacking or possessing a kinase inactive PI3Kγ protein (Hirsch et al. 2000; Patrucco et al. 2004) were used for investigating and distinguishing PI3Kγ kinase-dependent and -independent functions. This study is aimed to explore the role of PI3Kγ in the inflammatory-induced Mrp2 retrieval from the canalicular membrane of hepatocytes.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

Table 2: Chemicals and reagents

PRODUCT	SOURCE	CAT. NO.
2-Mercaptoethanol	Sigma	M6250
Acetic Acid 100%	Roth	6755
Acrylamide/bisacrylamide mixture Rotiphorese® Gel 30	Roth	3029
Amersham Hybond P 0.45 PVDF membrane	GE Healthcare Life Sciences	10600023
Ampicillin	Roth	K029
AS605240	Alexis Biochemicals	ALX-270462
BLOTTO milk powder	Roth	T145
Bovine serum albumin (BSA) pH 7.0	PAA	K41-001
Bromophenol Blue	Sigma	B0126
$CaCl_2$	AppliChem,	141221
Cell Strainer 100µm, Individual Package	VWR International	10199-658
Collagen Type I solution from rat tail	Sigma	C3867-1VL
D(+)-Glucose anhydrous	Applichem	A3666
Dimethylsulfoxide (DMSO), anhydrous, ≥99.9%	Sigma	276855
Dulbecco's Modified Eagle's Medium - high glucose	Sigma	6429
EDTA	Applichem	A2937
Ethanol	Roth	9065
Fetal bovine serum (FBS)	Sigma	F2442
GammaBind G Sepharose	GE Healthcare Life Science	17088501
Glycine	Roth	3187
H_2DCFDA	Invitrogen	D399
HCl 37%	Roth	4625

Hemin	Sigma	H9039
HEPES	Sigma	H3375
Isoflurane	Abbott	03-A878-R6
KCl	Roth	6781
KH_2PO_4	Sigma	NIST200B
Leupeptin	AppliChem	A2183
Lipopolysaccharides (LPS) from *Escherichia coli* O111:B4	Sigma	L2630
Liver Digest Medium	Gibco	17703-034
Liver Perfusion Medium	Gibco	17701-038
Methanol	Roth	8388
$MgCl_2$	Roth	HN03
N-Formyl-Met-Leu-Phe (fMLP)	Sigma	F3506
N,N,N,N-Tetramethylethylendiamin (TEMED)	Applichem	A1148
Na_2HPO_4	Roth	T877
NaCl	Roth	9265
NaF	Millipore	106449
NP-40 Surfact-Amps™ Detergent Solution	Thermo Scientific™	85124
Page Ruler™- Prestained Protein Ladder	Fermentas	SM0671
Pefablock/AEBSF-Hydrochlorid	AppliChem	A1421
Penicillin/Streptomycin (Pen/Strep)	Sigma	P0781
Pepstatin A	Sigma	P5318
Percoll	Sigma	GE17-0891-02
PMSF	Sigma	329986
Protease inhibitor Complete	Roche Applied Science	11 836 153 001
rm IFNγ	Immunotools	12343536
rm IL-1β	Immunotools	12340013
rm TNFα	Immunotools	12343014
Salbutamol	Sigma	S8260
SDS solution 20 % pure	Applichem	A3942
Sepharose CL4B	Sigma	086K1317
Sodium azide 0.1M solution	Sigma	08591

Sodium deoxycholate	Sigma	302-95-4
Sodium orthovanadate	Sigma	S6508
Tris(hydroxymethyl) aminomethan (Tris)	Applichem	131940
Triton™ X-100	Sigma	T8787
Trypan Blue	Biorad	1450021
Trypsin-EDTA (0.25%), phenol red	Thermo Scientific™	25200056
Tryptone	Sigma	T7293
Tween® 20	SERVA Electrophoresis	37470
William's medium E	Merk Millipore	F 1115
Yeast Extract	Sigma	92144

2.1.2 Buffers and Solutions

For plasmid DNA amplification and purification:

- 5x KCM buffer: 0.5M KCl, 0.15M $CaCl_2$, 0.5M $MgCl_2$.
- LB medium: 10g/l NaCl, 10g/l tryptone, 5g/l yeast extract, pH 7.0.
- LB agar: 10g/l NaCl, 10g/l tryptone, 5g/l yeast extract, 20g/l agar, pH 7.0.
- TE-buffer: 10mM Tris-HCl (pH 7.5), 1mM EDTA.

For preparation of cell lysates:

- Lysis buffer #1 (for organ lysates and for immunoprecipitation experiments): 120mmol/L NaCl, 50mmol/L Tris-HCl pH 8, 0.1% Triton X-100, 4% Protease inhibitor Complete (Roche), 1mM PMSF 50mM NaF, 1mM sodium orthovanadate, and 10mM Na_2HPO_4.
- Lysis buffer #2 (RIPA buffer, for cell lysates): 50mM Tris-HCl pH 8, 150mM NaCl, 1% (v/v) NP-40, 0.5% (v/v) sodium deoxycholate, 0.1% (w/v) SDS, 100µg/ml Pefa-Block, 1µg/ml pepstatin A, 10µM sodium orthovanadate, 1µg/ml leupeptin.

For SDS-PAGE and immunoblotting:

- 5x Laemmli buffer (SDS-PAGE sample buffer): 6% SDS, 25% β-mercaptoethanol, 32% (v/v) Glycerol, 16% 0.5M Tris-HCl pH 6.8, a spatula tip of bromphenol blue.
- Separation gel buffer: 2M Tris-HCl pH 8.8.

- Stacking gel buffer: 0.5M Tris-HCl pH 6.8.
- 10x Running buffer: 2M glycine, 0.25M Tris, 35mM SDS.
- 10x TBS-Tween (TBS-T): 100mM Tris-HCl pH 7.6, 1M NaCl, 1% (v/v) Tween 20.
- Transfer buffer for immunoblotting: 48mM Tris, 39mM Glycin, 0.037% (v/v) SDS, 15% Methanol.
- Blocking solution: 1% BSA in 1x TBS-T.
- Primary antibody solution: 1x TBS-T containing 1% BSA, primary antibody (diluted as listed in table 4), 0.05% NaN_3.
- Secondary antibody solution: 1x TBS-T containing 5% BLOTTO milk powder and secondary antibody diluted 1:10000.
- Stripping solution: 62.5mM Tris-HCl pH 6.7, 2% SDS, 0.7% β-mercaptoethanol.

For cell culture, stimulations and transient transfections:

- 10x Phosphate Buffered Saline (PBS): 1.37M NaCl, 27mM KCl, 100mM Na_2HPO_4, 18mM KH_2PO_4, pH 7.4-7.5 (with HCl).
- 2x HBS solution: 280mM NaCl, 10mM KCl, 1.5mM Na_2HPO_4, 12mM D-Glucose, 50mM HEPES. NaOH was used to bring the solution to pH 7.05.
- Heme stock solution: solved in 0.2 M NaOH, pH 7.4 (with sterile 0.2M HCl).

For ROS detection:

- HEPES-$CaCl_2$ solution: 10mM HEPES pH 7.5, 100mM $CaCl_2$.

For primary hepatocyte isolation:

- Collagen solution: 0.02N Acetic Acid, 1.2% Collagen Type I solution from rat tail.

2.1.3 Kits

Table 3: Kits

PRODUCT	SOURCE	CAT. NO.
cAMP Enzymeimmunoassay Biotrak™ (EIA) System	GE Healthcare	RPN225
cAMP GloAssayKit	Promega	PRV1501
First strand cDNA synthesis kit	Thermo Scientific™	K1612
Maxima SYBR Green/ROX qPCR Master Mix (2X)	Thermo Scientific™	K0221
NucleoBond PC 500	Macherey-Nagel	740574
RNeasy® Mini Kit	Qiagen	74104
Western Lightning® Plus-ECL, ECL Substrate	PerkinElmer	NEL105001EA

2.1.4 Antibodies

Table 4: Primary antibodies

ANTIBODY	CAT. NO.	SOURCE	DILUTION	TECHNIQUE	TYPE (IgG)
Phospho-Akt (Ser473)	9271	Cell Signaling Technology, Danvers, MA	1:1000	WB	Rabbit
Akt (pan)	9272	Cell Signaling Technology, Danvers, MA	1:1000	WB	Rabbit
Phospho-CREB (Ser133)	9198	Cell Signaling Technology, Danvers, MA	1:1000	WB	Rabbit
Anti-β-Actin	A5316	Sigma Aldrich, Steinheim, DE	1:5000	WB	Mouse
Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558)	3141	Cell Signaling Technology, Danvers, MA	1:1000	WB	Rabbit
Ezrin	32759	Santa Cruz Biotechnology, Santa Cruz, CA	1:500	WB	Mouse
Radixin	C4G7	Cell Signaling Technology, Danvers, MA	1:1000	WB	Rabbit
Anti-Heme Oxygenase 1	137749	Abcam, Cambridge, UK	1:1000	WB	Rabbit
PKCα Antibody (C-20)	208	Santa Cruz Biotechnology, Santa Cruz, CA	1:200	WB	Rabbit

PDE3B		Kind gift from E. Hirsch (University of Torino, Italy)	1:100	WB	Rabbit
Anti-his-p110 γ	clone 641	Homemade	1:100	IP (human cells)	Mouse
N-term-p110 γ - GST		Kind gift from E. Hirsch (University of Torino, Italy)	1:100	IP (mouse cells)	Mouse
Anti-p110γ, H1 (Hybridoma supernatant)		Homemade	1:1000	WB	Mouse
Mrp2	20766	Santa Cruz Biotechnology, Santa Cruz, CA	1:50	FRIL	Rabbit
CD11b Antigen FITC	11-0112-41	Thermo Scientific eBioscience™	1:20	FACS	Mouse

Secondary horseradish peroxidase (HRP)-labelled antibodies (anti-rabbit and anti-mouse) were obtained from KPL and used at a concentration of 0.1µg/ml for Western Blot detection.

2.1.5 Primers for qPCR

Table 5: qPCR primer sequences

GENE	PRIMER	SEQUENCE IN 5´ TO 3´-DIRECTION	AMPLICON (bp)
Hmox-1	fwd	CATTGAGCTGTTTGAGGAGC	113
Hmox-1	rev	CAGTATCTTGCACCAGGCTA	113
Actb	fwd	GCTCTTTTCCAGCCTTCCTT	92
Actb	rev	CGGATGTCAACGTCACACTT	92

2.1.6 Cell lines

Table 6: Eukaryotic cell lines

CELL LINE	ORIGIN	CULTIVATION MEDIUM
HEK293	Human Embryonic Kidney 293 cells	DMEM, supplemented with 10% heat-inactivated FBS and 1% Pen/Strep
HepG2	Human hepatoblastoma cells, derived from the liver tissue of a 15-year-old Caucasian American male with a well-differentiated hepatocellular carcinoma	DMEM, supplemented with 10% heat-inactivated FBS and 1% Pen/Strep
Hepa 1-6	Mouse hepatoma cells, derived from BW7756 tumour in a C57L mouse	DMEM, supplemented with 10% heat-inactivated FBS and 1% Pen/Strep
BV2	Mouse microglial cells, expressing a v-raf/v-myc oncogene carrying retrovirus (J2)	DMEM, supplemented with 10% heat-inactivated FBS and 1% Pen/Strep

2.1.7 Bacteria and DNA plasmids

For amplifying DNA plasmids, the bacterial strain Escherichia coli XL-1-Blue was used.

Table 7: DNA plasmids

VECTOR CONSTRUCT	PROTEIN EXPRESSED	SOURCE
pcDNA3.1	Human p110γ-Myc	E. Hirsch (University of Torino, Italy)
pcDNA3.1	Human Ezrin-Myc	H. Morrison (University of Jena, Germany)
pcDNA3.1	Human Radixin-Flag	H. Morrison (University of Jena, Germany)

2.1.8 Animals

PI3Kγ knockout (KO_γ) and PI3Kγ kinase-dead (KD_γ) mice, lacking or possessing a kinase inactive PI3Kγ protein respectively, were obtained (Hirsch et al. 2000; Patrucco et al. 2004). Mice were bred on C57BL/6J background for more than 10 generations and C57BL/6J wild-

type mice were used as controls. Mice were group housed, provided free access to standard chow and water in a controlled facility and maintained with 12h light and dark cycles. Ambient temperature was 29±1°C during the whole experimental period. Experiments were approved by the committee of the Thuringian State Government on Animal Research.

2.2 Methods

2.2.1 Plasmid DNA amplification and purification

For E.coli transformation, the KCM method was performed (Chung et al. 1989). Briefly, 20µl 5x KCM buffer, 80µl ddH_2O and 1µg of plasmid DNA were mixed on ice. Then, 100µl of competent bacteria cells (stored at -80°C) were added and incubated on ice for 40min. Following another incubation step at room temperature (RT) for 10min, 1ml of LB medium was added and incubated at 37°C for 1h shaking (225 rpm). Afterwards, 50µl of this mixture were plated on LB agar containing the antibiotic ampicillin (Amp). LB agar plates were incubated at 37°C overnight, and the next day a single colony was inoculated in 100ml LB medium (+Amp) for the selection of resistant bacteria cells expressing the desired DNA plasmid. Bacteria were grown at 37°C shaking (225 rpm) overnight.

For purification of plasmid DNA, the kit NucleoBond PC 500 was used following manufacturer's instructions. The DNA pellet was washed with 70% ethanol, dried and resolved in an appropriate volume of TE-buffer. DNA concentration and purity were determined by absorbance measurements at 260 nm and 280 nm.

2.2.2 Cell transient transfections

HEK293 cells were plated in 100mm Petri dishes at low density (1 million of cells/dish) and transfected 8h later with the indicated plasmids using the calcium phosphate technique. Briefly, a DNA-containing mix was prepared (for each plate 450µl H_20, 50µl 2.5M $CaCl_2$, 5-10µg DNA) and slowly dropped into a 2x HBS solution (for each plate 500µl) while aerating the mix using a 2ml pipet. Following 20min incubation at room temperature, the final mix was dropped throughout the plate and gently mixed before being placed back to the incubator. 16h after transfection, cells were carefully washed with PBS and lysed as described below.

2.2.3 Primary hepatocyte isolation and culture

The protocol used for primary hepatocyte isolation was a two-step collagenase perfusion procedure. This protocol was a modified version of previously described protocols (Braccini et al. 2015; Gonçalves et al. 2007).

i. Collagen coating

Plates were coated with collagen solution (prepared as described in 2.1.2) the day before primary hepatocyte isolation. Plates were incubated overnight (O.N.) at 37°C and, on the following day, they were washed once in 1x PBS solution and dried completely under sterile laminar flow hood.

ii. Animal preparation *and liver perfusions*

Animals were anesthetized and killed by isoflurane overdose (5% volume). After irreversible cardiovascular dysfunction evidence (heart rate < 30 bpm), the mouse peritoneal cavity was opened. The gastric system was moved to the right and both the portal vein and the inferior vena cava were exposed. Subsequently, the portal vein was cannulated with a 27G needle and the inferior vena cava was cut to allow blood efflux. A Masterflex peristaltic pump was used to perfuse the mouse at a speed of 3 ml/min, firstly with a saline solution (Liver Perfusion Medium, 30ml/mouse) and then with a collagenase-containing medium (Liver Digest Medium, 30ml/mouse). During this perfusion step, a back pressure was created every 30sec by blocking the inferior vena cava with tweezers for few seconds, causing liver inflation. Both perfusion media were pre-warmed at 37°C in order to avoid cold-induced hepatocyte stress.

iii. Primary hepatocyte isolation from perfused livers

At the end of the perfusion procedure, the liver was quickly excised, the gallbladder was removed and the entire organ was placed in a 100mm Petri dish with collagenase containing medium (10ml of Liver Perfusion Medium, pre-warmed at 37°C). Under sterile laminar flow hood, a mechanical disruption of liver capsule lobes was performed, and hepatocytes were released in the medium. Following 10min incubation at 37°C, the digested liver suspension was filtered through a 100μm cell strainer, and collagenase digestion was stopped by adding 30ml of Williams' E complete medium (10% FCS, 1% Pen/Strep). After 1min centrifugation at a speed of 800g RT, the supernatant medium was removed and cell pellets were washed with 18ml Williams' E complete medium. Subsequently, cell viability was assessed by Trypan blue staining (dye exclusion test) and, if a viability percentage >85% was achieved, cell suspension was applied over Percoll solution-containing falcons (for each organ, 15ml Percoll solution was split in 3x 15ml falcons). This step allowed the separation of viable cells from dead ones.

Following vigorous mixing, cells were centrifuged 20min 700g RT. Cell pellets of viable hepatocytes were washed with Williams' Medium E complete medium and centrifuged for 10min 200g.

iv. Cell staining to evaluate the purity of the primary culture

To verify primary hepatocyte isolation purity, cells were stained for the myeloid cell marker CD11b and fluorescence-activated cell scanning (FACS) analysis was performed (Fig. 7). BV2 cells were used as positive controls, while Hepa 1-6 cells were used as negative controls.

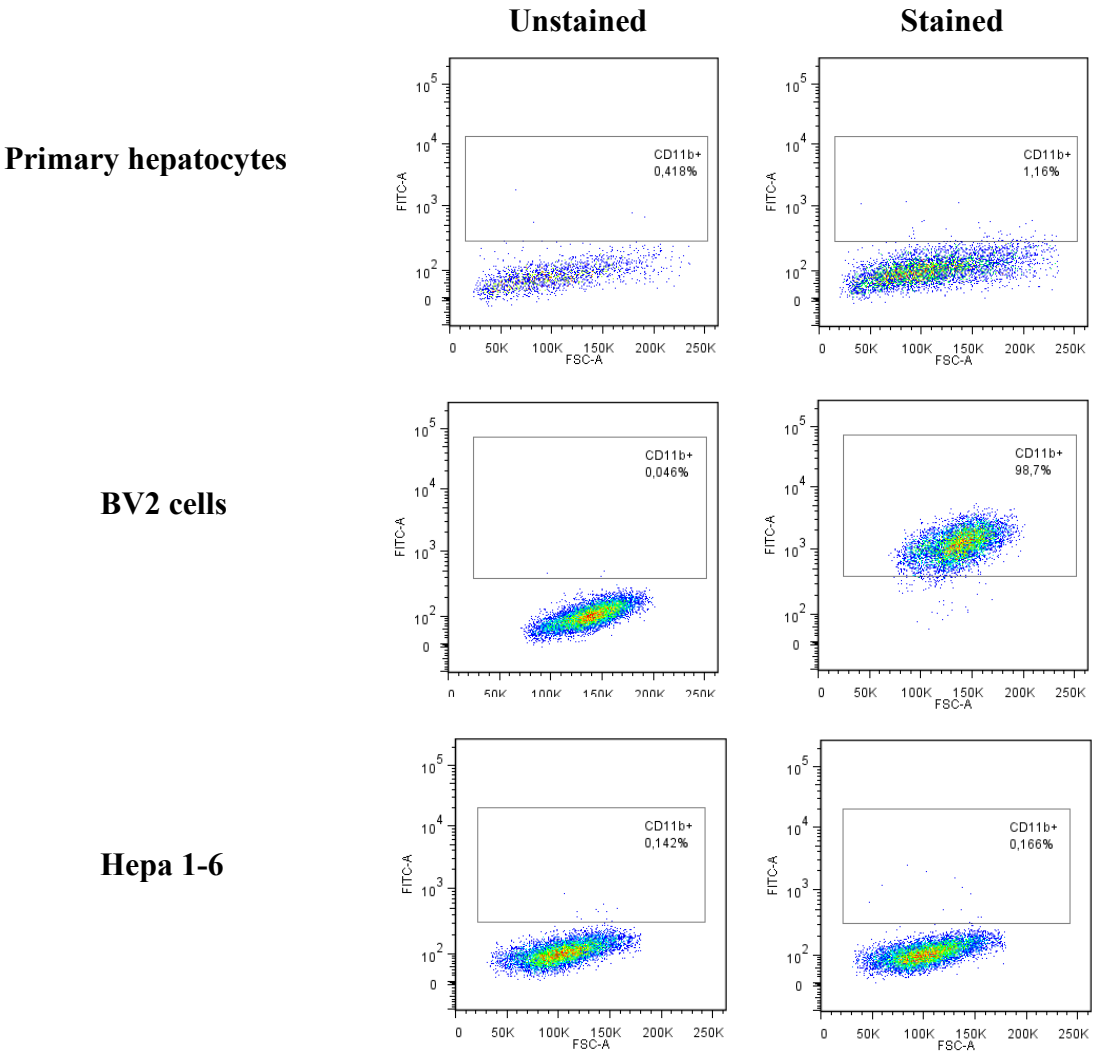


Figure 7: FACS analysis showed the absence of immune cells in primary hepatocyte cultures. Representative flow cytometry analysis of FITC-conjugated CD11b, performed in order to assess the purity of primary hepatocyte isolation. BV2 and Hepa 1-6 cells were used as positive and negative controls, respectively.

Briefly, cells where detached and pelleted by centrifugation. Cell pellets where washed once in 1x PBS solution containing 5% FCS and then re-suspended in the same solution (staining

solution). Cells were incubated with FITC-CD11b antibody for 30min at 4°C in the dark (shaking). Subsequently, cell populations were washed once in 1x PBS solution containing 5% FCS and maintained at 4°C before FACS analysis. The intensity of the fluorochrome-conjugated antibody was measured by flow cytometry using a FACSCalibur and Cell QuestTM software (Becton-Dickinson, Heidelberg, Germany). A minimum of 10000 events was collected.

v. Hepatocytes plating

Freshly isolated hepatocytes were plated onto collagen-coated plates and cultured in Williams' E complete medium. 3h later, cells were washed vigorously with 1x PBS and cultured in Williams' E complete medium overnight. On the following day, the culture medium was changed and hepatocytes were stimulated as indicated.

2.2.4 Cell stimulations

All cells were seeded the day before the stimulation time into 6-well plates (at a density of 300000 cells pro well) or into 12-well plates (at a density of 150000 cells pro well). Cells were maintained in their cultivation medium (see Table 5) at 37°C in an environment with 5% CO2 overnight. On the following day, cells were washed once in 1x PBS solution and cell medium was replaced with a serum-depleted medium 4h prior to the stimulation. For primary hepatocyte stimulations, cells were starved only for 2h in serum-depleted medium.

Cells were stimulated with fMLP, salbutamol or cytokine mix (CM) for the indicated time points and freezed in liquid nitrogen. For heme-TNFα stimulations, primary hepatocytes were first exposed to heme (5μM, 1h) in 1x PBS solution without serum, in order to avoid potential heme scavenging by serum proteins. Subsequently, cells were challenged in complete Williams' E medium with mouse recombinant TNFα (50ng/ml, 8h).

The PI3Kγ specific inhibitor AS605240 (AS) was applied on cells 1h before and together with stimuli, at a final concentration of 500nM. Plates where stored at -80°C and lysed on the same day of SDS-PAGE/immunoblotting assays.

2.2.5 SDS PAGE and immunoblotting

Protein expression and phosphorylation were analyzed by western blotting and immunodetection. Cells or organs were lysed and the proteins extracted were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

i. Protein preparation for Western blot analysis

For total liver lysates preparation, livers were rapidly excised, frozen, pestled in liquid nitrogen and homogenized in ice-cold lysis buffer 1% Triton (lysis buffer #1, described in 2.1.2). Protein extraction from cells was performed in ice-cold RIPA buffer instead (lysis buffer #2, described in 2.1.2). After 30min incubation on ice, lysates were centrifuged at 10,000g for 15min at 4°C, supernatants were transferred into new tubes and mixed with 5x Laemmli's buffer. Samples were denatured by heating at 95°C for 5min and directly used or stored at -20°C until their usage for SDS-PAGE.

ii. Co-immunoprecipitation (coIP) assays

Samples for immunoprecipitation experiments (IP) were lysed in ice-cold lysis buffer 1% Triton (lysis buffer #1, described in 2.1.2). After 30min incubation on ice, lysates were centrifuged at 3000g for 15min at 4°C and supernatants were pre-cleared by rocking for 30min at 4°C with 30µl of 1:1 slurry Sepharose beads. Pre-cleared extracts were then incubated with 15µl of 1:1 slurry of G-Sepharose beads and 1µg of antibody/mg of protein overnight at 4°C with rocking. On the following day, immunocomplexes bound to protein G-Sepharose beads were washed four times in ice-cold lysis buffer 1% Triton, dried using a hamilton syringe and mixed with 1x Laemmli's buffer. Samples were denatured by heating at 95°C for 5min and directly subjected to SDS/PAGE.

iii. Immunoblotting

For immunoblotting, proteins were separated by reducing SDS 7.5% or 10% polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (PVDF) using a Trans-Blot® SD semi-dry machine (Bio-Rad, 10V. 1gel: 60min; 2gels: 90min). After protein transfer, PVDF membranes were blocked in 1x TBST 3% BSA solution for 1h at room temperature, blots were washed and incubated with the respective primary antibodies (O.N., 4°C). Detection was achieved using peroxidase-coupled secondary antibodies (1h, RT). To visualize protein bands, PVDF membranes were subjected to enhanced chemiluminescence reaction (Western Lightning® Plus-ECL, ECL Substrate).

iv. Chemiluminescence detection and densitometric analysis of Western Blots

The chemiluminescence of protein bands was acquired using the LAS 4000 imaging device (Fujifilm) and images were captured with the LAS software. Band signal quantification was performed using the Fujifilm Multi Gauge software. For these quantifications, the background intensities were subtracted from the values of the different bands. The resulting intensities were normalized to loading control (βactin) and indicated as arbitrary units (AU).

2.2.6 ROS measurements

Cellular ROS levels were measured in cells with a technique that detects fluorescence intensity derived from the conversion of the non-fluorescent 2',7' –dichlorofluorescin diacetate molecule (DCFDA) to its oxidized fluorescent form 2', 7' –dichlorofluorescein (DCF). The fluorescence generated is directly proportional to the amount of oxidized DCFDA to DCF. Briefly, primary hepatocytes were seeded on collagen-coated 96-well plates at a density of 30000 cells pro well. On the following day, cells were incubated 30min at 37°C with the DCFDA molecule (5μM in the HEPES-$CaCl_2$ buffer, prepared as described in 2.1.2). After three washes in the HEPES-$CaCl_2$ buffer, 200μl of HEPES-$CaCl_2$ were distributed in each well and fluorescence was measured with the TECAN infinite 200 plate reader (excitation λ= 485nm, emission λ= 535 nm).

2.2.7 RNA isolation, cDNA synthesis and quantitative real time PCR

Briefly, total RNA was extracted from hepatocyte samples using RNeasy® Mini Kit, following the manufacturer's instructions. The concentration of isolated RNA was determined using a UV-VIS spectrophotometer (Thermo Scientific). First strand cDNA was then synthesized from 400ng of sample RNA using the M-MLV reverse transcriptase, according to the kit protocol (Thermo Scientific). Subsequently, quantitative real time PCR was performed with standard procedures, using TaqMan chemistry on an Applied Biosystems ABI 7300 instrument. Primers and probes were designed according to the Universal Probe Library system (Roche) (Table 4). Gene expression levels were analyzed using the 2-ΔΔCt relative quantification method, using βactin as endogenous control for normalization.

2.2.8 cAMP measurements

Livers were excised from mice and directly frozen in liquid nitrogen. The intracellular content of cAMP in liver lysates was detected with cAMP Biotrak competitive enzyme immunoassay system (GE Healthcare) according to the manufacturer's instructions.

For measuring cAMP levels in primary hepatocytes, cells were seeded at a density of 10000 cells per well in 96-well collagen-coated clear-bottom plates, and cAMP was detected with cAMP GloAssayKit (Promega), following the manufacturer's protocol.

2.2.9 Electron Microscopy (FRIL, Scanning Electron Microscopy, Ultrathin Sections)

Freeze-fracture replica immunogold labelling (FRIL) of multidrug resistance-associated protein 2 (Mrp2) was performed as previously described (Recknagel et al. 2012).

To assess if this technique worked with a different Mrp2 antibody, we first tested the protocol with the same cell line used in Recknagel et al. (HepG2 cells).

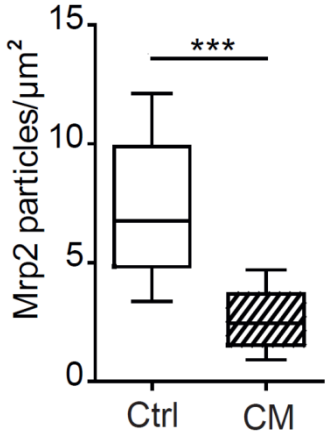


Figure 8: Stimulation of HepG2 cells with pro-inflammatory cytokines triggers internalization of Mrp2 transporter proteins. Cells were stimulated with a cytokine mix (CM, striped box) composed of TNF-α (50ng/ml), IL-1β (10ng/ml), IFN-γ (10ng/ml), and LPS (100ng/ml) for 6h, and analyzed with FRIL technique. The graph shows a quantification of the number of Mrp2 particles (counted on electron microscopy images, data not shown) normalized to the protoplasmic fracture face area. Values of one experiment are presented as boxplots illustrating medians within boxes from first quartile (25th percentile) to the third quartile (75th percentile) and whiskers ranging from the 10th to the 90th percentiles (extreme values are marked outside). n = 24–28 fields of interest per treatment. ***$p < 0.001$, determined by Mann–Whitney U test.

Since we were able to reproduce the results of Recknagel et al. in human cells (Fig. 8), FRIL of Mrp2 was performed in murine cells. Briefly, Hepa 1-6 and mouse primary hepatocytes were seeded in 100mm Petri dish and cultivated as previously described until they reached confluence. Subsequently, cells were treated 6h with a cytokine mix (CM) comprising TNF-α (50ng/ml), IL-1β (10ng/ml), IFN-γ (10ng/ml), and LPS (100ng/ml). Untreated cells and cells stimulated with AS605240 inhibitor served as controls. At the end of the treatment, cells were scraped from the culture dish, centrifuged at 15g for 5min and frozen. FRIL was performed using overnight incubation with a rabbit polyclonal anti-MRP2 primary antibody (1:50), followed by incubation with a gold-conjugated (10nm of gold) goat anti-rabbit IgG secondary antibody (1:50, British Biocell International) for 1h (Westermann et al. 2005). Images were taken as digital pictures on an EM902A transmission electron microscope (Carl Zeiss) using a 1k FastScan CCD camera (TVIPS), and gold particles were counted.

2.2.10 Statistical analysis

Prism (GraphPad) and Sigma Plot software were used for statistical analysis. P values were calculated by using one way analysis of variance (ANOVA), and data were displayed as means ± SD. Data from FRIL experiments are presented as boxplots illustrating medians within boxes from first quartile (25th percentile) to the third quartile (75th percentile) and whiskers ranging from the 10th to the 90th percentiles (extreme values are marked outside).

When Shapiro-Wilk normality test failed, the nonparametric Kruskal–Wallis one-way analysis of variance on ranks was used. Post hoc comparisons were made with the Tukey or the Dunn's methods, as indicated. $p<0.05$ was considered significant (*), $p< 0.01$ was considered very significant (**), and $p< 0.001$ was considered extremely significant (***).

3. Results

3.1 PI3Kγ is induced in liver parenchyma upon inflammatory stress conditions

The PI3Kγ enzyme is abundantly expressed in immune cells, and for this reason its role in acute and chronic inflammatory diseases has been extensively studied in the past 20 years (Rückle et al. 2006; Costa et al. 2011). Nonetheless, recent studies have pointed out that PI3Kγ protein is also expressed in cells other than immune cells (Becattini et al. 2011; Patrucco et al. 2004; Perino et al. 2014). As a matter of fact, PI3Kγ plays important roles in the parenchyma response to inflammatory stress, even if at low expression level (Becattini et al. 2011; Ndongson-Dongmo et al. 2015).

In order to assess whether PI3Kγ protein is expressed within liver parenchyma, livers from wild-type (WT) mice were excised, perfused and analyzed via immunoblotting. Perfused livers derived from two types of PI3Kγ mutant mice were also analyzed: i) mice carrying a targeted mutation in the PI3Kγ gene, causing loss of lipid kinase activity (KDγ), and ii) PI3Kγ-deficient mice (KOγ).

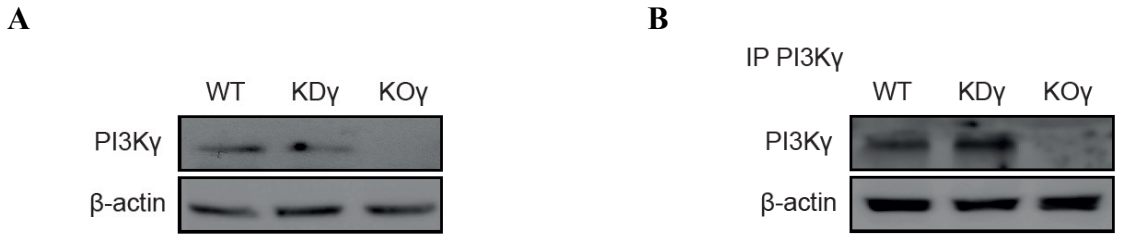


Figure 9: PI3Kγ protein is expressed in liver parenchyma. (**A**) Representative western blot (10% SDS-Page) of PI3Kγ protein expression in PBS-perfused livers derived from wild-type (WT), PI3Kγ kinase-dead (KDγ) and PI3Kγ knockout (KOγ) mice. (**B**) Representative western blot (10% SDS-Page) of immunoprecipitated PI3Kγ in primary hepatocytes derived from WT, KDγ and KOγ mice. IP indicates immunoprecipitation. The western blots shown are normalized on β-actin, and are representative of three independent experiments.

As shown in Fig. 9A, PI3Kγ protein was present in lysates obtained from liver parenchyma of WT and KDγ mice, while it was not detected in KOγ liver lysates. However, liver tissue is not only composed by parenchymal cells (hepatocytes), but also by other cell types of the liver

stroma (Kholodenko and Yarygin 2017). To test whether PI3Kγ is solely expressed in parenchymal cells, primary hepatocytes were isolated from WTγ, KDγ and KOγ mice. Although the amount of protein was too low to be detected in total cell lysates, PI3Kγ protein was found in immunoprecipitated (IP) lysates derived from WT and KDγ primary hepatocyte, but not in KOγ parenchyma (Fig. 9B).

Low PI3Kγ protein expression levels in tissues other than immune cells have already been previously shown in heart parenchyma, adipose tissue and brain (Becattini et al. 2011; Ghigo et al. 2012; Patrucco et al. 2004; Perino et al. 2011). In particular, PI3Kγ in these tissues plays an important role in stress-induced conditions, such as inflammation or metabolic derangements. Consequently, we decided to investigate whether low level of PI3Kγ might be involved in the adaption of liver parenchyma to inflammatory stress.

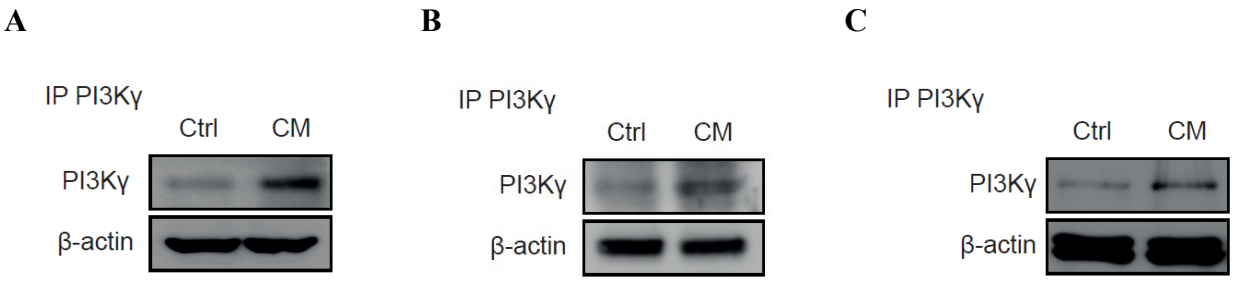


Figure 10: Inflammatory cytokines enhance PI3Kγ protein expression in hepatocytes. (**A**) Representative western blot (10% SDS-Page) of immunoprecipitated PI3Kγ in primary hepatocytes derived from wild-type (WT) mice stimulated with a cytokine mix (CM) composed of TNF-α (50ng/ml), IL-1β (10ng/ml), IFN-γ (10ng/ml), and LPS (100ng/ml) for 6h. (**B** and **C**) Representative western blots (10% SDS-Page) of immunoprecipitated PI3Kγ in HepG2 (**B**) and Hepa 1-6 (**C**) cell lines, stimulated with CM for 6h. IP indicates immunoprecipitation. The western blots shown are normalized on β-actin and are representative of three independent experiments.

Inflammatory stress was induced *in vitro* via stimulating WT-derived primary hepatocytes for 6 hours with a cytokine mix (CM, composed of TNF-α, IL-1β, IFN-γ and LPS). Subsequently, PI3Kγ protein amount was assessed via immunoprecipitation (IP) and immunoblotting. This particular CM treatment was shown in previous studies to recapitulate the *in vivo* physiologic and metabolic hepatic adaptations to inflammatory stress, representing

a reliable *in vitro* model for our study (Recknagel et al. 2012; Ceppi et al. 1996). Indeed, PI3Kγ protein levels increased upon CM treatment of primary hepatocytes, as shown in Fig. 10A. Similar results were obtained using two immortalized hepatocyte cell lines, of human hepatoblastoma (HepG2, Fig. 10B) or murine hepatoma (Hepa 1-6, Fig. 10C) origin. Immunoprecipitated extracts of HepG2 and Hepa1-6 cells also displayed an increased PI3Kγ protein expression following CM treatment (Fig. 10 B and C).

Together, both in primary cells as well as in cell lines the expression of PI3Kγ was enhanced upon cytokine stimulation, suggesting that this enzyme is involved in the inflammatory stress-induced response of hepatocytes.

3.2 Inflammatory stress response of hepatocytes is orchestrated by the PI3Kγ catalytic function

3.2.1 PI3Kγ kinase activity controls Mrp2 bile transporter protein localization upon inflammatory stress

Under inflammatory conditions, hepatocytes adjust the expression and function of basolateral and canalicular hepatobiliary transporters, and this possibly leads to intrahepatic cholestasis (Crocenzi et al. 2012). In particular, cholestatic conditions are usually accompanied by altered bile secretory failure due to endocytic internalization of key transporters involved in bile formation (Roma et al. 2008). The involvement of PI3Kγ in the regulation of bile transporters localization has recently emerged in the study of Recknagel et al.. In this study the freeze-fracture replica immunolabelling technique (FRIL) has been used to localize the resistance-associated protein 2 (Mrp2) upon inflammatory stress. As a result, pharmacological inhibition of PI3Kγ in the human HepG2 cell line upon stimulation with cytokines resulted in a partial block in the retrieval of Mrp2 particles from the canalicular membrane, suggesting that the catalytic activity of PI3Kγ controls Mrp2 localization (Recknagel et al. 2012).
To proof the validity of these data in murine cells, we used the FRIL technique to assay Mrp2 localization in inflamed mouse hepatocytes.

At first, Hepa 1-6 cells were stimulated with cytokine mix (CM) as previously described, and the amount of Mrp2 particles on the hepatocyte plasma membrane was measured upon PI3Kγ-selective pharmacological inhibition with the AS605240 compound. Electron microscopy images in Fig. 11A show that cytokine treatment halved the amount of Mrp2 particles on the plasma membrane, while PI3Kγ inhibition blocked the internalization of these particles. In contrast to HepG2 cells (Recknagel et al. 2012), the quantification of Mrp2 localized on the plasma membrane indicates that a complete rescue of cytokine-induced Mrp2 internalization was obtained in cells treated with the AS605240 inhibitor, as compared to CM-treated cells (Fig. 11B). This observation revealed the important role of the catalytic activity of PI3Kγ in the control of Mrp2 localization in mouse hepatocytes, as compared to human parenchyma (HepG2 cells, Recknagel et al. 2012).

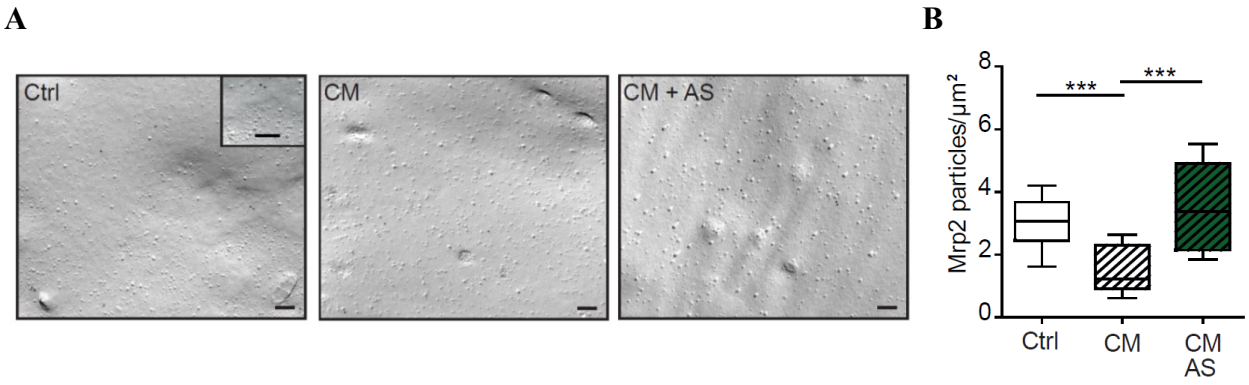


Figure 11: Stimulation of Hepa 1-6 cells with pro-inflammatory cytokines triggers internalization of Mrp2 transporter proteins via PI3Kγ signaling. (**A**) Representative electron micrographs of freeze-fracture-immunolabelled Mrp2 transporters in the plasma membrane of Hepa 1-6 cells. Cells were treated with the specific PI3Kγ inhibitor AS605240 (AS, 500nM, green box) prior to stimulation with a cytokine mix (CM, striped boxes) composed of TNF-α (50ng/ml), IL-1β (10ng/ml), IFN-γ (10ng/ml), and LPS (100ng/ml) for 6h. Untreated and CM stimulated cells were used as controls. (**B**) Quantification of the number of Mrp2 particles (black dots in (**A**)) normalized to the protoplasmic fracture face area. Values of three independent experiments are presented as boxplots illustrating medians within boxes from first quartile (25th percentile) to the third quartile (75th percentile) and whiskers ranging from the 10th to the 90th percentiles (extreme values are marked outside). Scale bars: 100nm. n = 10-13 fields of interest per treatment. ***$p < 0.001$, determined by one-way ANOVA Kruskal-Wallis with Dunn's multiple comparison post hoc test.

In order to assess the relevance of PI3Kγ kinase-dependent function in a more physiological *in vitro* model, we performed FRIL on mouse-derived primary hepatocytes. Using FRIL technique, both pharmacological and genetic PI3Kγ inhibition was used to analyze the role of the signaling protein in Mrp2 localization upon cytokine treatment (CM).

As shown in the electron microscopy images in Fig. 12A, WT-derived hepatocytes treated with CM displayed a reduced amount of Mrp2 particles on the plasma membrane to the same extent as we previously observed in HepG2 and Hepa 1-6 cells. Moreover, PI3Kγ-specific inhibition with the AS605240 compound completely rescued the number of Mrp2 particles on the hepatocytes membrane upon inflammatory stress, consolidating our previous data obtained with the immortalized Hepa 1-6 cell line. Afterwards, we asked if genetic inhibition of PI3Kγ also results in a protection from Mrp2 internalization caused by inflammatory stress, and FRIL technique was performed on hepatocytes isolated from mutant mice for PI3Kγ (KDγ and KOγ).

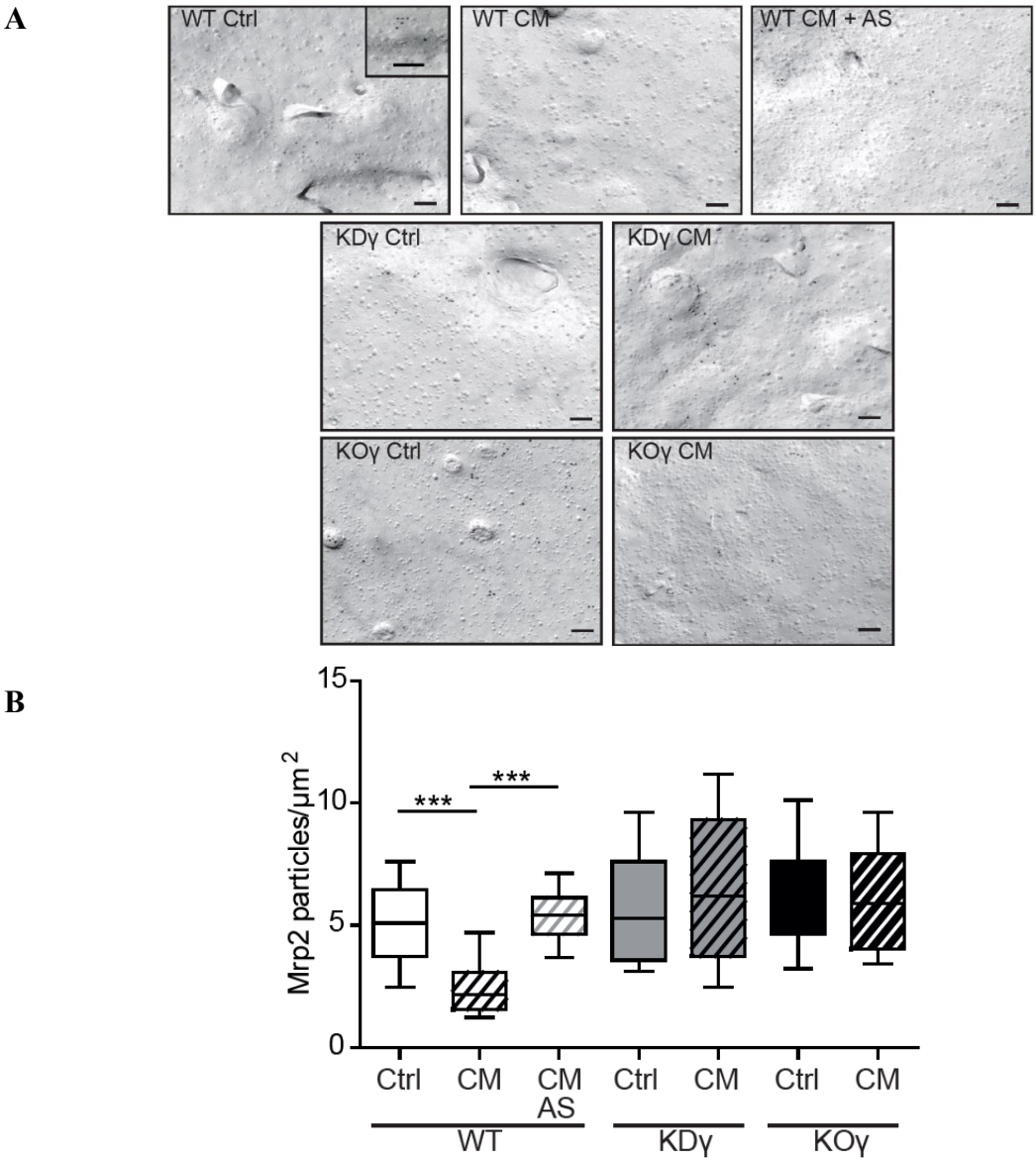


Figure 12: Inflammatory-induced Mrp2 internalization is mediated by PI3Kγ activity in primary murine hepatocytes. (A) Representative electron micrographs of freeze-fracture-immunolabeled Mrp2 transporters in the plasma membrane of primary hepatocytes derived from wild-type (WT), PI3Kγ kinase-dead (KDγ) and PI3Kγ knockout (KOγ) mice. Cells were stimulated with a cytokine mix (CM, striped boxes) composed of TNF-α (50ng/ml), IL-1β (10ng/ml), IFN-γ (10ng/ml), and LPS (100ng/ml) for 6h. WT cells were also subjected to PI3Kγ pharmacological inhibition with the AS605240 compound (AS, 500nM) prior to stimulation. Untreated cells were used as controls. (**B**) Quantification of the number of Mrp2 particles (black dots in (**A**)) normalized to the protoplasmic fracture face area. Values of 3-4 independent experiments are presented as boxplots illustrating medians within boxes from the first quartile (25th percentile) to the third quartile (75th percentile) and whiskers ranging from the 10th to the 90th percentiles (extreme values are marked outside). Scale bars: 100 nm. n = 23-25 fields of interest per treatment. ***$p < 0.001$, determined by one-way ANOVA Kruskal-Wallis followed by Dunn's multiple comparison post hoc test.

In contrast to WT-derived isolated cells, KDγ and KOγ primary hepatocytes were not susceptible to Mrp2 internalization upon CM treatment (Fig. 12A).

The number of Mrp2 particles was counted in electron microscopy pictures of mouse-derived hepatocytes and quantified, as shown in Fig. 12B. Collectively, these results indicate that PI3Kγ kinase activity has a key role in regulating the localization of Mrp2 bile transporter protein within hepatocytes.

3.2.2 The inhibition of PI3Kγ kinase activity affects inflammatory stress responses in hepatocytes

The kinase activity of PI3Ks is responsible for the phosphorylation of phosphatidyl-inositol lipids at the D3 position of the inositol ring, allowing for signaling proteins with pleckstrin-homology (PH) domains to accumulate at sites of PI3K activation by directly binding to $PI(3,4,5)P_3$ (Cantley 2002). Most importantly, the association of the protein serine-threonine kinase Akt with $PI(3,4,5)P_3$ at the plasma membrane brings this protein into proximity with the phosphoinositide-dependent kinase 1 (PDK1), facilitating Akt phosphorylation by PDK1 (Alessi et al. 1996). As a result, the Akt phosphorylation status provides a reliable readout for the catalytic activity of PI3K.

To test whether the kinase activity of the PI3Kγ isoform is involved in the hepatocyte response to inflammatory stress, we subjected primary WT hepatocytes to a short stimulation with cytokines (CM) and evaluated Akt phosphorylation via immunoblotting.

As shown in Fig. 13, the increase in Akt phosphorylation induced by CM treatment was partially abolished when cells were treated with PI3Kγ specific inhibitor (AS605240). Given that the CM we used is composed of cytokines which bind to different receptors and that many intracellular pathways are simultaneously triggered, we suggest that the CM induced Akt phosphorylation might be partially mediated by PI3K isoforms other than γ.

To explore the specific function of PI3Kγ in the control of inflammation-induced Mrp2 internalization we next investigated candidate signaling pathways known to affect Mrp2 localization.

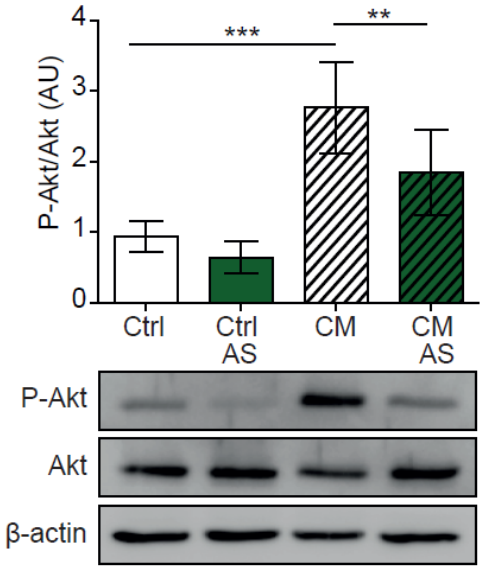


Figure 13: PI3Kγ kinase activity is involved in the response of hepatocytes to pro-inflammatory stress. Representative western blots (10% SDS-Page) and densitometric quantification of Akt phosphorylation (P-Akt, on Ser473) and total Akt protein levels in primary hepatocytes isolated from wild-type (WT) mice. Cells were stimulated with a cytokine mix (CM, striped bars) composed of TNF-α (50ng/ml), IL-1β (10ng/ml), IFN-γ (10ng/ml), and LPS (100ng/ml) for 10min, and subjected to PI3Kγ pharmacological inhibition with the AS605240 compound (AS, 500nM, green bars) prior to stimulation. Untreated and CM-stimulated cells were used as controls. P-Akt/Akt ratios were normalized to β-actin. Values represent mean ± SD of three independent experiments. **p < 0.01, ***p < 0.001, determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test.

3.3 PI3Kγ kinase activity controls both the internalization and the insertion of Mrp2 bile transporter protein

Mrp2 bile transporter protein localization within hepatocytes is tightly regulated (Elferink and Paulusma 2015; Chai et al. 2015). Under cholestatic conditions, such as inflammatory stress, the physiological recycling of these membrane transporters via vesicle-mediated insertion and internalization from/to the endosomal compartment is altered. In particular, inflammatory stress induces a shift towards retrieval of Mrp2 transporter proteins, decreasing their presence on plasma membrane and consequently provoking cholestasis via impairing the transport of their substrates (Crocenzi et al. 2012). PKC and cAMP, as described in paragraphs 1.4.2 and 1.4.3, are key players in the regulation of Mrp2 protein localization under cholestatic conditions (Fig. 14).

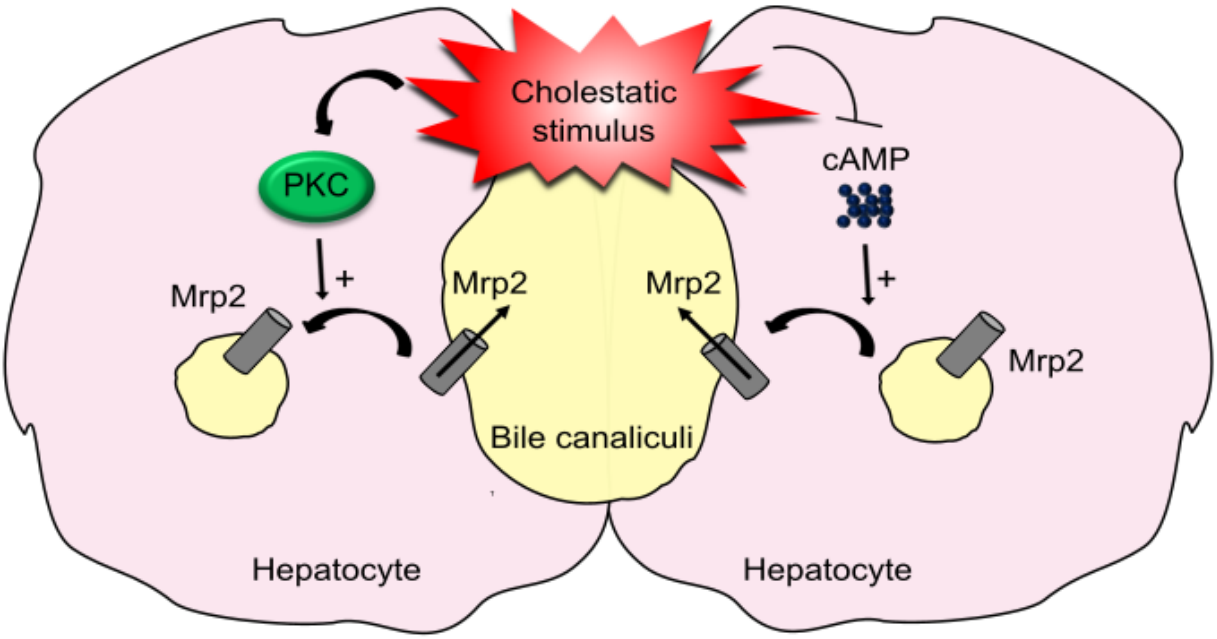


Figure 14: Schematic representation of the molecular pathways regulating multidrug resistance-associated protein 2 (Mrp2) localization. Cholestatic stimuli induce the retrieval of bile transporter proteins on the canalicular membrane of hepatocytes into endosomal compartments. On the left side, the internalization towards endocytic compartments is represented, which mainly involves the activity of protein kinase C (PKC) on Mrp2-connected cytoskeleton. On the right side, the mechanism of regulating the insertion of Mrp2 particles on the canalicular membrane is illustrated, largely controlled by cyclic AMP (cAMP) intracellular levels. (Anwer 2014; Crocenzi et al. 2012)

In the light of these considerations, we hypothesized that PI3Kγ kinase activity can be involved in two Mrp2-related signaling mechanisms: i) its internalization towards endocytic compartments or ii) its insertion in the plasma membrane. Thus, these two distinct pathways were examined.

3.3.1 PI3Kγ kinase activity controls Mrp2 protein internalization via ERM proteins

The localization of bile transporter proteins at the canalicular membrane of hepatocytes is heavily dependent on close interactions between the plasma membrane and the underlying cytoskeleton (Roma et al. 2008; Crocenzi et al. 2012; Sekine et al. 2011). These interactions involve proteins that cross-link membrane proteins, such as Mrp2, with actin filaments (F-actin). Notably, the ezrin/radixin/moesin (ERM) protein family is a well-known mediator of tethering proteins which anchors integral membrane proteins to F-actin (Roma et al. 2008). Previous studies have pointed out the key role of ERM proteins in controlling Mrp2 localization in hepatocytes and its distinct dependence on the ERM C-terminal phosphorylation status (Anwer 2014).

Since the phosphorylation status of ERM proteins (P-ERM) determines the expression of Mrp2 protein at the plasma membrane, we investigated the P-ERM pattern in primary hepatocytes lacking functional PI3Kγ protein compared to WT parenchymal cells.

Intriguingly, primary hepatocytes isolated from KDγ and KOγ mice displayed a reduced amount of P-ERM, thus suggesting the involvement of the PI3Kγ catalytic function in controlling hepatocytic P-ERM status (Fig. 15A). In addition, a similar pattern of ERM phosphorylation was observed when hepatocytes derived from KDγ and KOγ mice were subjected to the pro-inflammatory CM treatment (Fig. 15B). Hence, the PI3Kγ kinase activity in hepatocytes seems to control, either directly or indirectly, the phosphorylation status of the ERM proteins, both in basal conditions and upon inflammatory stress.

Among the proteins participating in the ERM complex, ezrin and radixin are abundantly expressed within hepatocytes (Chai et al. 2015; Sekine et al. 2011). To find a link between PI3Kγ and the phosphorylation of specific ERM proteins, PI3Kγ protein was initially overexpressed in HEK293 cells, either with ezrin or with radixin protein. As a result, PI3Kγ and ezrin could be coimmunoprecipitated in HEK293 transfected cells (Fig. 16, left panel) while no traces of radixin protein could be detected in PI3Kγ immunoprecipitates (Fig. 16, right panel).

These data suggest that PI3Kγ controls Mrp2 protein localization in the plasma membrane by regulating the phosphorylation of ERM complex via direct interaction with ezrin.

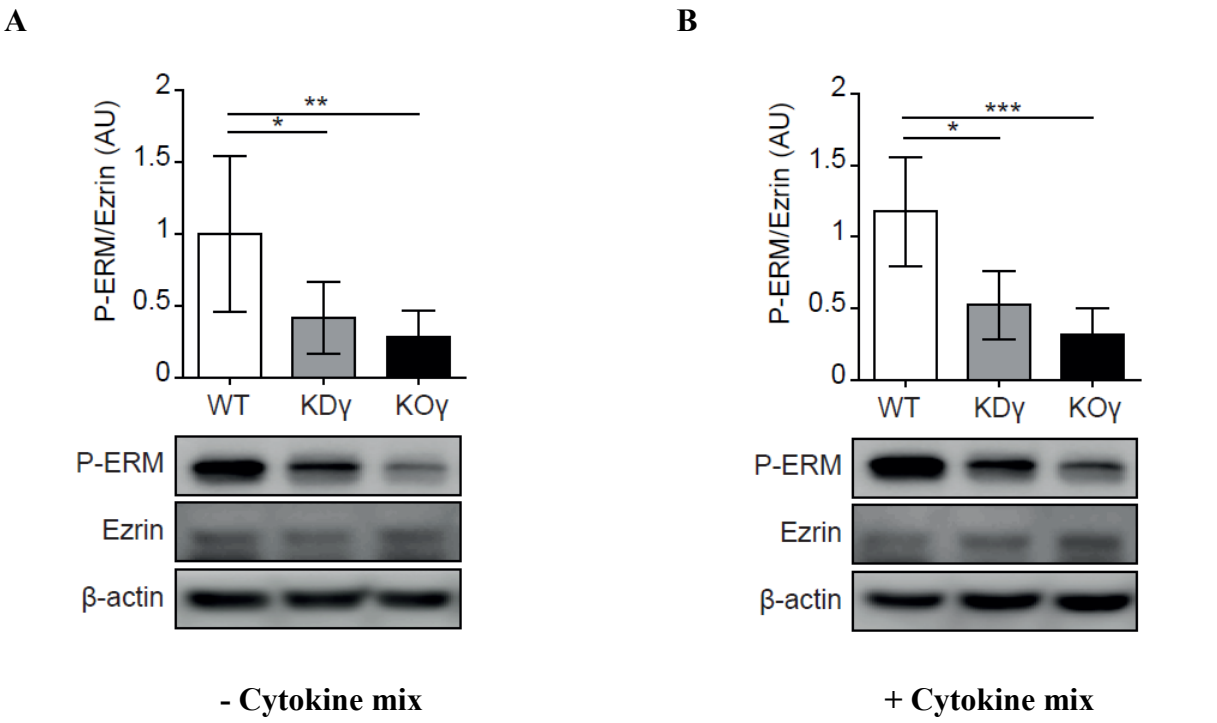


Figure 15: PI3Kγ kinase activity in primary hepatocytes controls the phosphorylation status of ERM proteins. (**A**) Representative western blots (7.5% SDS-Page) and densitometric quantification of ezrin/radixin/moesin phosphorylation (Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558), P-ERM) and total ezrin protein levels in primary hepatocytes isolated from wild-type (WT), PI3Kγ kinase-dead (KDγ) and PI3Kγ knockout (KOγ) mice. Values represent mean ± SD of four independent experiments (n = 4 mice/genotype). *p < 0.05, **p < 0.01, determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test. (**B**) Representative western blots (7.5% SDS-Page) and densitometric quantification of ERM phosphorylation and total ezrin protein levels in primary hepatocytes isolated from WT, KDγ and KOγ mice. Cells were stimulated with a cytokine mix (CM) composed of TNF-α (50ng/ml), IL-1β (10ng/ml), IFN-γ (10ng/ml), and LPS (100ng/ml) for 6h. Values represent mean ± SD of four independent experiments (n = 4 mice/genotype). *p < 0.05, ***p < 0.001, determined by one-way ANOVA Kruskal-Wallis followed by Dunn's multiple comparison post hoc test. P-ERM/Ezrin ratios in (**A**) and (**B**) were normalized to βactin.

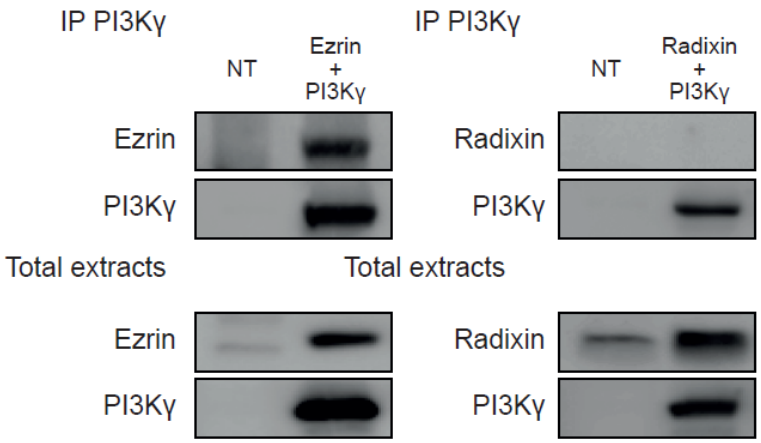


Figure 16: PI3Kγ interacts with ezrin but not with radixin protein. Representative western blots (10% SDS-Page) of PI3Kγ immunoprecipitates (IP) from HEK293 cells transfected with PI3Kγ, radixin and ezrin constructs (all of human origin). Ezrin protein coimmunoprecipitated with PI3Kγ (left panels), while radixin protein did not precipitate together with PI3Kγ protein (right panel). HEK293 non-transfected cells (NT) were used as controls. Western blots of three independent coimmunoprecipitation experiments are shown.

Phosphorylation of ezrin protein negatively correlates with the amount of Mrp2 protein localized at the plasma membrane (Chai et al. 2015; Nakano et al. 2009). In particular, a recent study has described that in human hepatocytes the phosphorylation of Thr567 in ezrin by protein kinase C alpha (PKCα) reduced Mrp2 membrane expression (Chai et al. 2015). Thus, we hypothesized that the effect we observed in mutant KDγ and KOγ hepatocytes on ERM complex phosphorylation might be mediated by PI3Kγ-mediated control of the activity of PKCα.

The association of PI3Kγ and PKCα has already been characterized in activated neutrophils (Lehmann et al. 2009). Notably, in this study the protein kinase activity of PI3Kγ on PKCα was reported as a key regulator of reactive oxygen species (ROS) production upon neutrophil stimulation with the bacterial peptide N-formylmethionyl-leucyl-phenylalanine (fMLP). Based on these reports, we asked whether in hepatocytes PI3Kγ could regulate ERM tether proteins activity through the direct interaction with PKCα, which in turn could phosphorylate ezrin and cause Mrp2 protein internalization.

As expected, PI3Kγ and PKCα could be coimmunoprecipitated in primary hepatocytes (Fig. 17A) as well as in HepG2 and Hepa 1-6 cell lines (Fig. 17B). Furthermore, we observed that the interaction between the two proteins was higher when hepatocytes were stimulated with the

bacterial peptide fMLP (Fig.17 A and B). These data confirm results previously shown in neutrophils (Lehmann et al. 2009).

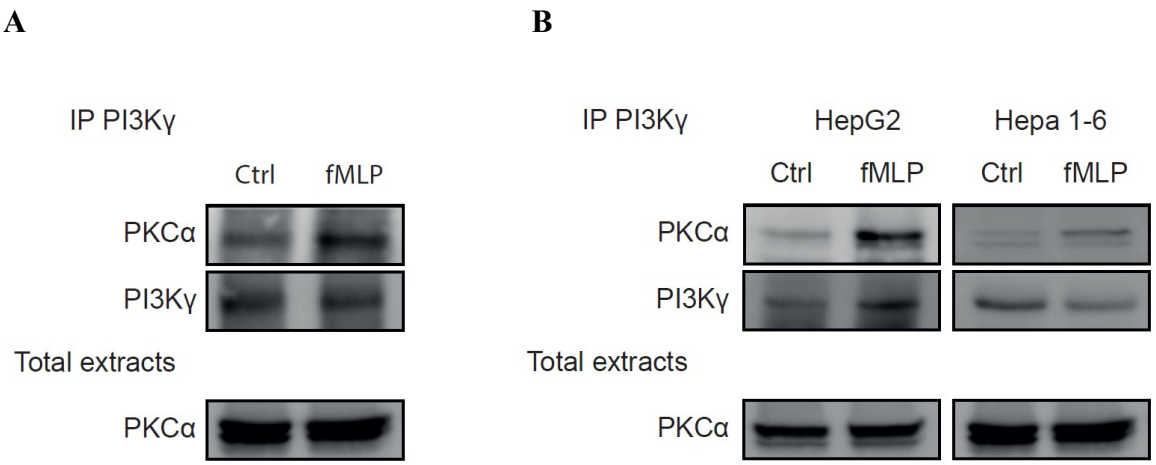


Figure 17: The PI3Kγ-PKCα interaction in hepatocytes is enhanced upon stimulation with bacterial-derived chemotactic factor fMLP. (**A** and **B**) Representative western blots (7.5% SDS-Page) of PI3Kγ immunoprecipitates (IP) from primary hepatocytes isolated from wild-type mice (**A**) and from HepG2 or Hepa 1-6 cells lines (**B**). Cells were stimulated with N-Formylmethionyl-Leucyl-Phenylalanine (fMLP, 10μM) for 10min, and unstimulated cells were used as controls. PKCα protein coimmunoprecipitated with PI3Kγ, and the amount of PI3Kγ-PKCα complexes increased upon fMLP treatment. Western blots of three independent coimmunoprecipitation experiments are shown.

fMLP is a bacterial product best known as a prototype activator of neutrophils and neutrophil chemotactic activity (Hirsch et al. 2000; Sato et al. 2013) but expression of the receptor for fMLP (FPR) has been also found in hepatocytes (McCoy et al. 1995). Since FPR has been characterized as a G protein-coupled receptor that involves PI3Kγ in its signaling responses, we asked whether in hepatocytes fMLP stimulation requires the catalytic activity of PI3Kγ. For this purpose, we subjected primary hepatocytes to a short stimulation with fMLP and evaluated the amount of Akt phosphorylation as readout for the catalytic activity of PI3Kγ.

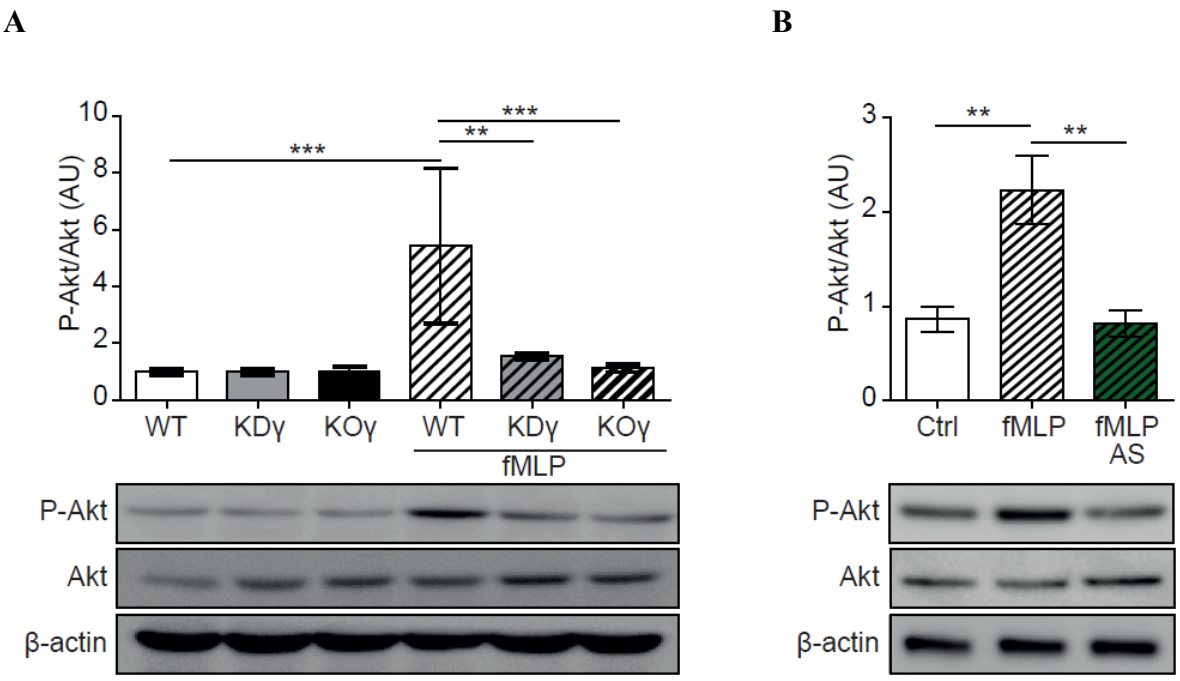


Figure 18: Genetic or pharmacological inhibition of PI3Kγ kinase activity in primary hepatocytes affects fMLP-induced Akt phosphorylation. (**A**) Representative western blots (10% SDS-Page) and densitometric quantification of Akt phosphorylation (P-Akt, on Ser473) and total Akt protein levels in primary hepatocytes isolated from wild-type (WT), PI3Kγ kinase-dead (KDγ) and PI3Kγ knockout (KOγ) mice. Cells were stimulated with N-Formylmethionyl-Leucyl-Phenylalanine (fMLP, 10μM, striped bars) for 10min, and unstimulated cells were used as controls. Values represent mean ± SD of four independent experiments (n = 4 mice/genotype). **p < 0.01, ***p < 0.001, determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test. (**B**) Representative western blots (10% SDS-Page) and densitometric quantification of Akt phosphorylation (P-Akt, on Ser473) and total Akt protein levels in primary hepatocytes isolated from wild-type (WT). Cells were stimulated with fMLP (10μM, striped bars) for 10min, and subjected to PI3Kγ pharmacological inhibition with the AS605240 compound (AS, 500nM, green bar) prior to stimulation. Unstimulated cells were used as controls. Values represent mean ± SD of three independent experiments. **p < 0.01, determined by one-way ANOVA Kruskal Wallis followed by Dunn's multiple comparison post hoc test. P-Akt/Akt ratios were all normalized to β-actin.

Strikingly, fMLP stimulation significantly increased the amount of P-Akt, but this effect was completely abolished by either genetic (Fig. 18A) or pharmacological (Fig. 18B) suppression of PI3Kγ. Analogous effects were also obtained when hepatocyte cell lines were subjected to fMLP treatment in combination with PI3Kγ specific inhibitor (AS605240), as it is shown in Fig. 19A and B.

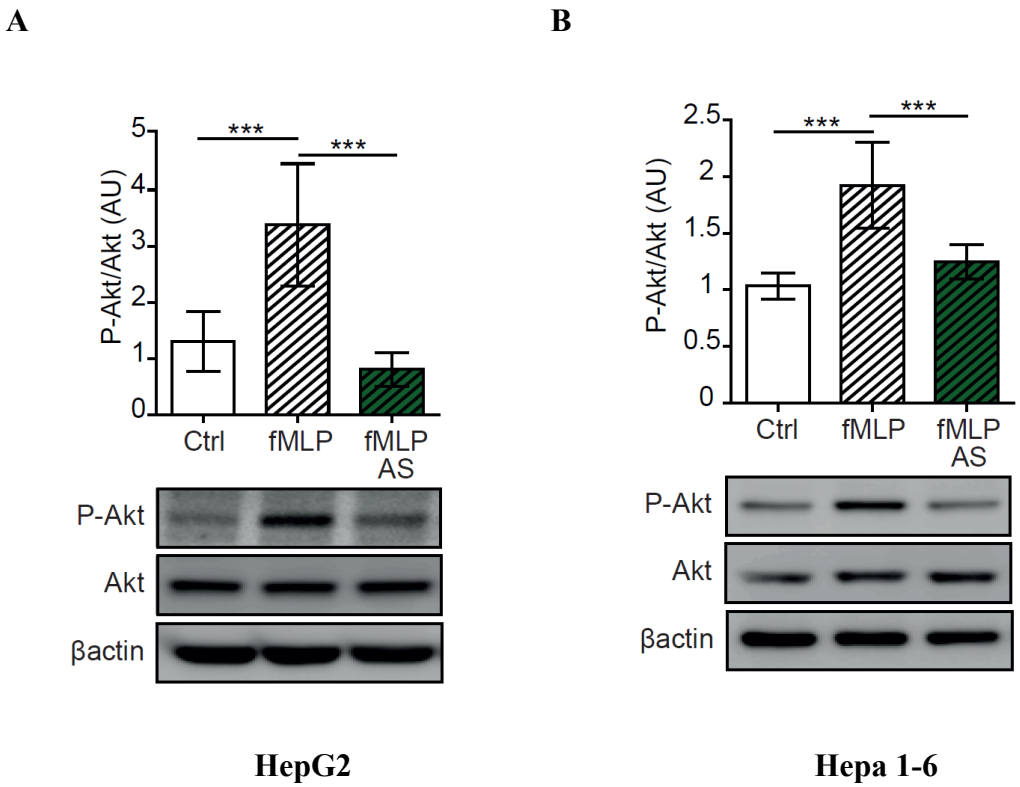


Figure 19: Pharmacological inhibition of PI3Kγ kinase activity in hepatocyte cell lines blocks fMLP-induced Akt phosphorylation. (**A** and **B**) Representative western blots (10% SDS-Page) and densitometric quantification of Akt phosphorylation (P-Akt, on Ser473) and total Akt protein levels in HepG2 (**A**) and Hepa 1-6 (**B**) cells. Cells were stimulated with N-Formylmethionyl-Leucyl-Phenylalanine (fMLP, 10μM, striped bars) for 10min, and subjected to PI3Kγ pharmacological inhibition with the AS605240 compound (AS, 500nM, green bar) prior to stimulation. Unstimulated cells were used as controls. Values represent mean ± SD of three independent experiments. ***$p < 0.001$, determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test. P-Akt/Akt ratios were all normalized to β-actin.

Altogether, our results suggest that the PI3Kγ kinase activity plays an important role within hepatocytes in regulating the inflammatory-induced retrieval of Mrp2 protein from plasma membrane.

3.3.2 cAMP-dependent transport of Mrp2 particles to the canalicular membrane of hepatocytes is regulated by both PI3Kγ kinase-dependent and independent functions

Mrp2 protein retrieval from the canalicular membrane of hepatocytes can be counteracted by increased intracellular levels of cyclic AMP (cAMP) (Zucchetti et al. 2011; Schonhoff et al. 2016). Although little is known about the signaling pathways involved, several studies have previously shown that cAMP favors Mrp2 translocation to the canalicular membrane, leading to a protective biliary excretion (Roma et al. 2008). Given that PI3Kγ has already been found to negatively modulate cAMP pools in different cell types, acting as a scaffold protein and recruiting cAMP-degrading enzymes (Patrucco et al. 2004; Ghigo et al. 2012), we asked if in the liver the amount of intracellular cAMP could be mediated by PI3Kγ as well.

Both cAMP levels as well as the amount of phosphorylated cAMP response element binding protein (P-CREB) were assessed within primary hepatocytes isolated from WT, KDγ and KOγ mice.

Unexpectedly, increased amounts of cAMP and P-CREB were detected not only in KOγ parenchyma, but also in hepatocytes derived from KDγ mice, where the scaffold function of PI3Kγ should be preserved (Fig. 20A and B). Remarkably, KDγ-derived hepatocytes exhibited intermediate cAMP and P-CREB levels as compared to WT and KOγ cells (Fig. 20A and B). Furthermore, even upon cytokine treatment (CM), KDγ-derived hepatocytes maintained cAMP levels midway between WT and KOγ cells (Fig. 20C).

These data suggest that the kinase-dependent function of PI3Kγ may be involved in the control of cAMP production. Despite the fact that the modulation of intracellular cAMP by PI3Kγ is normally attributed to its kinase-independent function, previous studies have demonstrated that the catalytic activity of PI3Kγ can regulate cAMP pools as well (Huang et al. 2015; Perino et al. 2014).

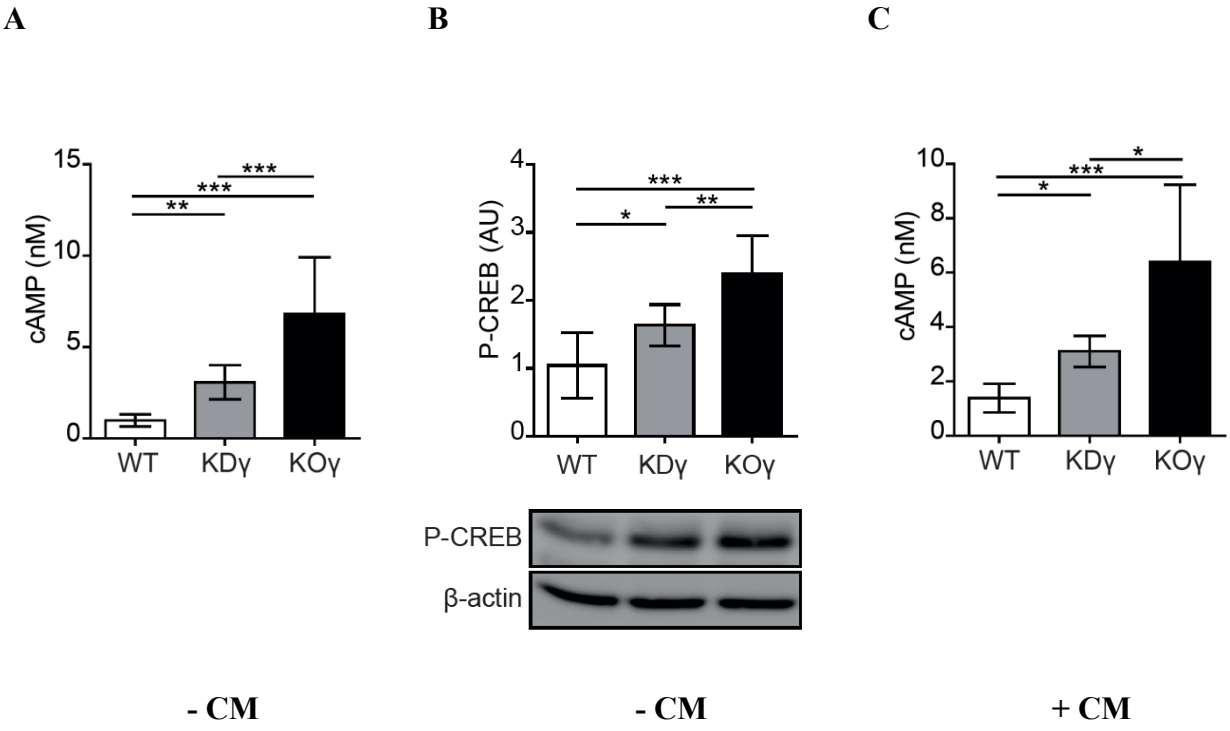


Figure 20: PI3Kγ kinase function regulates cAMP intracellular levels in liver parenchyma. (**A**) Measurement of cAMP levels in primary hepatocytes derived from wild-type (WT), PI3Kγ kinase-dead (KDγ) and PI3Kγ knockout (KOγ) mice (n = 4-5 mice/genotype). cAMP was measured in basal conditions. Values represent mean ± SD of three independent experiments. **p < 0.01, ***p < 0.001, determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test. (**B**) Representative western blot (10% SDS-Page) and densitometric quantification of CREB phosphorylation (P-CREB, on Ser133), normalized on β-actin protein amount, in primary hepatocytes derived from WT, KDγ and KOγ mice (n = 4 mice/genotype). Values represent mean ± SD of four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test. (**C**) Measurement of cAMP levels in primary hepatocytes derived from WT, KDγ and KOγ mice upon stimulation with a cytokine mix (CM) composed of TNF-α (50ng/ml), IL-1β (10ng/ml), IFN-γ (10ng/ml), and LPS (100ng/ml) for 6h. Values represent mean ± SD of three independent experiments. *p < 0.05, ***p < 0.001, determined by one-way ANOVA Kruskal Wallis followed by Dunn's multiple comparison post hoc test.

To assess whether PI3Kγ could act as a cAMP regulator in hepatocytes, the interaction between PI3Kγ and PDE3B was verified through immunoprecipitation experiments. PDE3B could coimmunoprecipitate with PI3Kγ in primary hepatocytes isolated from WT and KDγ mice, while no traces of PDE3B could be detected in extracts derived from KOγ mice (Fig. 21A). In agreement with these data on primary hepatocytes, PDE3B protein could be detected in PI3Kγ immunoprecipitates of HepG2 and Hepa 1-6 cell lines as well (Fig. 21B).

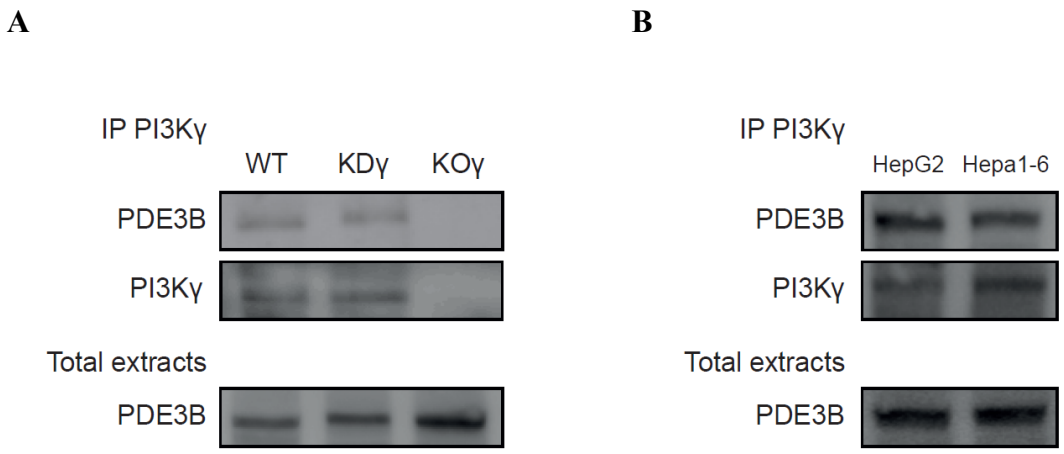


Figure 21: PI3Kγ interacts with PDE3B in hepatocytes. (**A** and **B**) Representative western blots (10% SDS-Page) of PI3Kγ immunoprecipitates (IP) from primary hepatocytes derived from wild-type (WT), PI3Kγ kinase-dead (KDγ) and PI3Kγ knockout (KOγ) mice (**A**) as well as from HepG2 or Hepa 1-6 cells lines (**B**). PDE3B protein coimmunoprecipitates with PI3Kγ in both primary cells and hepatocyte cell lines extracts. Western blots of three independent coimmunoprecipitation experiments are shown.

We have previously demonstrated in neurons that PI3Kγ, by recruiting the phosphodiesterase 3B isoform (PDE3B), expresses ability to phosphorylate and activate PDE3B in an Akt-dependent manner, thus leading to increased cAMP degradation (Perino et al. 2014). Thus, we speculate that the kinase function of PI3Kγ may regulate the activity of PDE3B through Akt activation. Given that hepatocytic cAMP levels are regulated by hormones which signal via the β2 adrenergic receptor (β2AR), such as glucagon and adrenaline (Zucchetti et al. 2011), we analyzed the effects of salbutamol (a β2AR agonist) on the Gi-PI3K-Akt axis. Indeed, compelling evidence indicates that β2AR conducts a duet of signaling including both Gs and Gi proteins, the latter reshaping the spatiotemporal pattern of the Gs signaling via a PI3K/Akt-mediated negative control of cAMP production (Xiao 2001). Consistent with the activation of PI3Kγ downstream GPCRs, salbutamol stimulation greatly increased the amount of Akt phosphorylation in WT primary hepatocytes, and this effect was completely abolished by pharmacological inhibition of PI3Kγ with AS605240 (Fig. 22A). Analogous effects were also obtained when hepatocyte cell lines were subjected to salbutamol treatment in combination with AS605240, as it is shown in Fig. 22B and C.

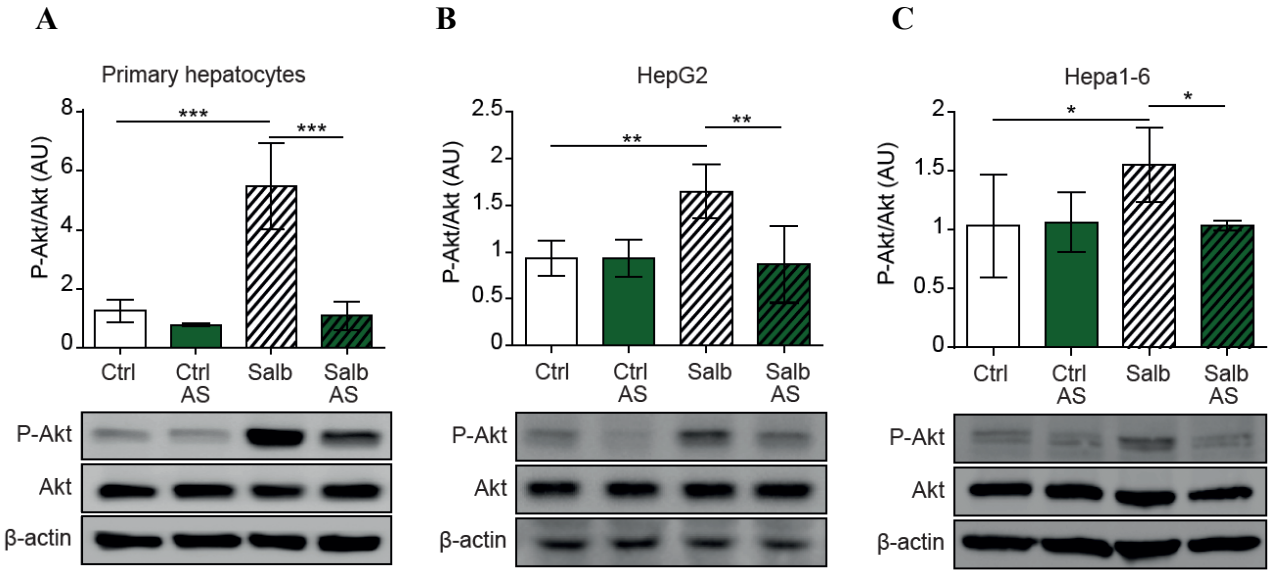


Figure 22: Pharmacological inhibition of PI3Kγ kinase activity in hepatocytes counteracts the Akt phosphorylation induced by the β2AR signaling pathway. (**A** - **C**) Representative western blot (10% SDS-Page) and densitometric quantification of Akt phosphorylation (P-Akt, on Ser473) and total Akt protein levels in primary hepatocytes derived from wild-type (WT) mice (**A**), HepG2 (**B**) and Hepa 1-6 (**C**) cells. Hepatocytes were stimulated with salbutamol (Salb, 50μM, striped bars) for 10min, and subjected to PI3Kγ pharmacological inhibition with the AS605240 compound (AS, 500nM, green bars) prior to stimulation. Unstimulated cells were used as controls. P-Akt/Akt ratios were all normalized to β-actin. Values represent mean ± SD of 3-6 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test.

Collectively, our results suggest an important role of PI3Kγ in modulating cAMP pools in hepatocytes through its catalytic function, regulating the Mrp2 protein insertion at the canalicular membrane.

3.4 PI3Kγ inhibition increases the expression of HO-1 protein in liver parenchyma via enhancing ROS production

Previous reports have shown that increased cAMP levels induce the expression of heme oxygenase-1 (HO-1), which is known to protect against inflammation-induced oxidative stress (Rensing et al. 2004; Larsen et al. 2010). Given the high amount of cAMP observed in hepatocytes derived from KOγ hepatocytes, we next asked whether PI3Kγ might influence the expression of HO-1 protein, possibly affecting ROS-mediated liver damage.

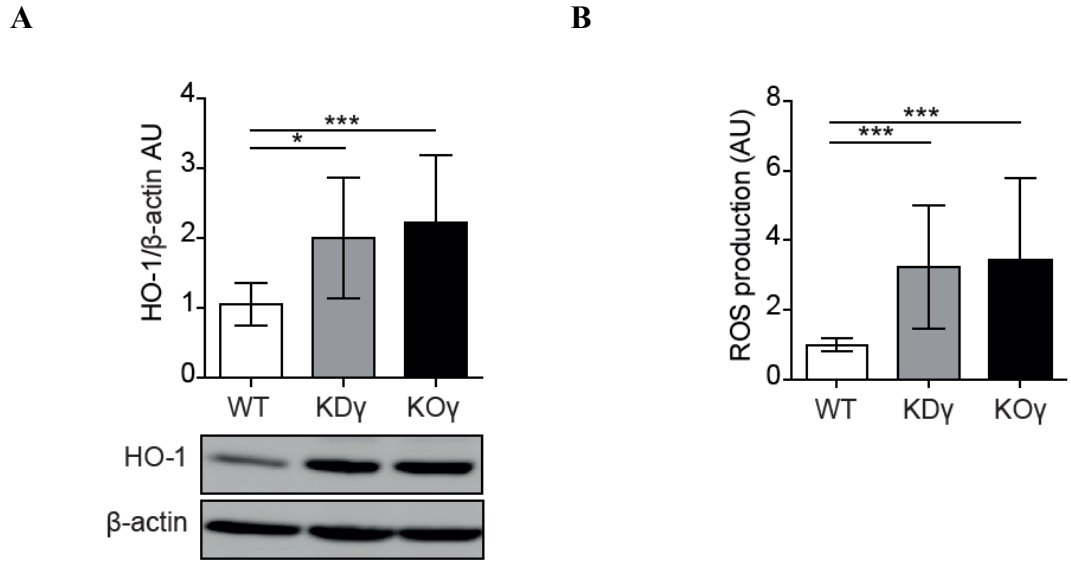


Figure 23: Primary hepatocytes lacking PI3Kγ kinase activity display enhanced HO-1 protein expression and ROS production. (**A**) Representative western blot (10% SDS-Page) and densitometric quantification of heme oxygenase-1 (HO-1) in primary hepatocytes derived from wild-type (WT), PI3Kγ kinase-dead (KDγ) and PI3K γ knockout (KOγ) mice (n = 4 mice/genotype). HO-1 protein amount was normalized on β-actin. (**B**) DCFDA assay for ROS production was performed on primary hepatocytes isolated from WT, KDγ and KOγ mice (n = 4 mice/genotype). Values represent mean ± SD of four independent experiments. *p < 0.05, ***p < 0.001, determined by one-way ANOVA Kruskal Wallis followed by Dunn's multiple comparison post hoc test.

Consistent with the high cAMP levels shown in Fig. 20, HO-1 protein expression was found elevated in KDγ and KOγ primary hepatocytes, as compared to WT controls (Fig. 23A). However, the amount of ROS production was not only lower in KDγ and KOγ parenchyma, but was even higher as compared to WT controls, albeit the HO-1 higher expression (Fig. 23B).

Considering that in this work and in a previous study (Recknagel et al. 2012) the lack of PI3Kγ in hepatocytes results in an overall protection from liver damage, we hypothesize that at the given dose the increased ROS production in KDγ and KOγ parenchyma have hormetic effects (Wetzker and Rubio 2012; Ludovico and Burhans 2014). Beneficial effects of low doses of ROS on vitality parameters has been described in a variety of cellular and organismic systems (Ristow 2014).

Hence, we next asked whether the preconditioning effect of ROS could enhance the resistance against future inflammatory oxidant stress and promote the initiation of tissue repair processes. For this purpose, hepatocytes derived from WT, KDγ and KOγ mice were exposed *in vitro* to heme and TNFα, a treatment which is normally inducing large amount of intracellular free radicals in hepatocytes (Larsen et al. 2010). Afterwards, the amount of HO-1 protein was tested, as an indicator of tissue repair processes.

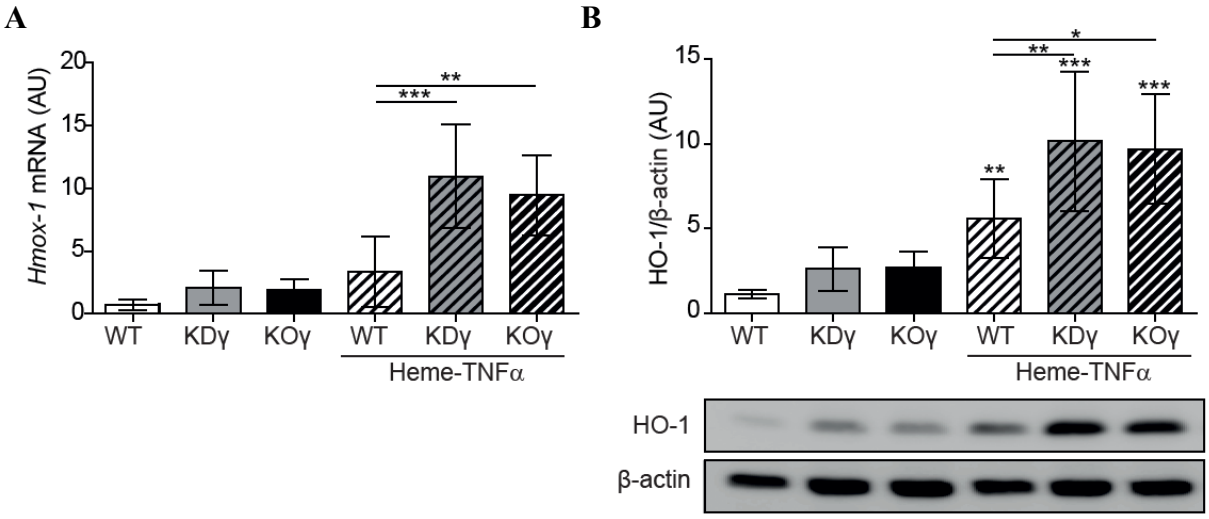


Figure 24: *Hmox-1* gene transcription and HO-1 protein translation are regulated by PI3Kγ kinase activity upon oxidative stress. (**A**) mRNA expression levels of *Hmox-1* in primary hepatocytes derived from wild-type (WT), PI3Kγ kinase-dead (KDγ) and PI3Kγ knockout (KOγ) mice (n = 4 mice/genotype), normalized on the mRNA expression of the housekeeping gene *Actb.* (**B**) Representative western blots (10% SDS-Page) and densitometric quantification of heme oxygenase-1 (HO-1) in primary hepatocytes derived from WT, KDγ and KOγ mice (n = 4 mice/genotype), normalized on the β-actin protein amount. Cells in (**A**) and (**B**) were subjected to oxidative stress using heme (5μM) for 1h following TNFα stimulation (50ng/ml) for 8h (Heme-TNFα, striped bars). Values represent mean ± SD of four independent experiments. Heme-TNFα-stimulated cells were compared to their unstimulated controls. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test.

Remarkably, KDγ and KOγ hepatocytes displayed both increased transcription of HO-1 (*Hmox-1* gene mRNA, Fig. 24A) and translation of HO-1 protein (Fig. 24B) in response to heme-TNFα stimulation, as compared to WT controls. Together, these data suggest that the inhibition of PI3Kγ protects hepatocytes from stress-induced dysfunctions via ROS-mediated hormetic mechanisms.

4. Discussion

PI3Kγ has been extensively studied in leukocytes, where it is abundantly expressed and involved in both innate and adaptive immune responses (Rückle et al. 2006; Barberis and Hirsch 2008; Hirsch et al. 2000). In this study, we established PI3Kγ as an important regulator of the liver parenchymal response to inflammatory stress, opening a new field of investigation for this signaling protein. Here, for the first time, we report that PI3Kγ is expressed in the liver organ and in hepatocytes specifically, albeit in low amounts. This discovery is supported by the growing body of evidence implicating PI3Kγ in regulating stress responses in tissues other than immune cells, such as cardiomyocytes (Patrucco et al. 2004), adipocytes (Becattini et al. 2011), endothelial cells (Huang et al. 2016) and neurons (Perino et al. 2014). Strikingly, in this study we observed that PI3Kγ protein expression increased upon *in vitro* stimulations with cytokines, both in isolated primary hepatocytes and in HepG2 and Hepa 1-6 cell lines. This strongly indicates that PI3Kγ mediates intracellular responses of hepatocytes upon inflammatory stress.

PI3Kγ has been recently found involved in the development of inflammatory-induced liver failure using mouse models of polymicrobial sepsis (Martin et al. 2010; Recknagel et al. 2012). In particular, upon cecal ligation and puncture procedure (CLP), PI3K$\gamma^{-/-}$ mice displayed reduced levels of alanine transaminase (ALT) and aspartate transaminase (AST) in the blood, as compared to WT mice. Although these liver-specific enzymes are released by necrotic hepatocytes and are normally associated with hepatocyte injury, the amounts of ALT and AST within the blood is not considered as a reliable indicator of hepatic failure itself (Bhatia et al. 2014). Bile formation is instead one of the most important functions of the liver that is compromised during hepatic failure. This complex process involves a huge flux of solutes through the hepatocytes and its dysregulation results in cholestasis, leading to very severe liver damage caused by a build-up of pressure and cell lysis due to bile salts accumulation. One of the main pathomechanisms of cholestasis, already established many decades ago, is the withdrawal of bile transporter proteins from the canalicular membrane and the concomitant upregulation of other transporters in the basolateral membrane (Elferink and Paulusma 2015; Paulusma et al. 2000; Scheffer et al. 2002). This prevents excessive pressure formation and damage, allowing for reverse transport of solutes back into the blood. Our research group recently discovered that PI3Kγ is implicated in the pathogenesis of sepsis-induced cholestasis in the experimental mouse model of peritoneal contamination and infection (PCI) (Recknagel et al. 2012). In the present study we unraveled the molecular mechanisms involved.

Our results highlight the importance of PI3Kγ inhibition in protecting hepatocytes from cytokine-induced Mrp2 retrieval form the canalicular membrane, which has been previously identified as a common feature of sepsis-induced cholestasis (Elferink and Paulusma 2015). Cholestatic conditions usually occur early in the onset of sepsis, despite increased cardiac output and hepatic perfusion (Wang et al. 1995). Thus, the hepatocellular dysfunction in sepsis must be associated with the release of proinflammatory cytokines primarily. Therefore, we designed for our studies an *in vitro* model that mimics the physiologic inflammatory environment where we induced hepatocyte cholestasis with a mixture of cytokines.

Mrp2 protein is responsible for mediating the hepatocyte secretion of various amphiphilic anionic compound as well as glutathione conjugates, which are chief determinants of the so-called bile salt-independent fractions of the bile flow (Elferink and Paulusma 2015). Accordingly, mutations in the MRP2 gene in humans result in the autosomal recessive Dubin-Johnson syndrome (DJS), which is a rare disease characterized by a mild conjugated hyperbilirubinemia caused by the impaired hepatobiliary transport system of non-bile salt organic anions across the canalicular membrane (Nies and Keppler 2007; Gu and Manautou 2010). Similarly, the functional absence of Mrp2 in knockout mice is characterized by increased accumulation of bilirubin and its conjugated glucuronides within serum and urine, due to a reduction of Mrp2-dependent biliary secretion (Chai et al. 2015). Upon cholestatic conditions, Mrp2 undergo withdrawal processes, being stored in subapical vesicles underneath the plasma membrane (Paulusma et al. 2000). For evaluating the localization of Mrp2 in hepatocytes, we took advantage of freeze-fracture replica immunogold labeling (FRIL), which is a technique that provides valuable information on the ultrastructural architecture of the plasma membrane. Importantly, immunogold labeling via FRIL technique allows the detection of Mrp2 protein particles exclusively localized within the plasma membrane, and discounts those particles internalized in subapical vesicles. FRIL thus consents a more reliable estimation of the number of Mrp2 within the plasma membrane as compared to other procedures based on immunostainings or plasma membrane fractionation methods, which are limited by the non-specificity of commercially available Mrp2 antibodies.

In agreement with previous results obtained on human HepG2 cells (Recknagel et al. 2012), pharmacological inhibition of PI3Kγ enzymatic activity, using the small molecule AS605240 (AS) in murine Hepa 1-6 cells, confirmed the importance of PI3Kγ kinase activity in controlling the Mrp2 internalization induced by proinflammatory cytokines. Consistently, this observation is supported by the effects of specific PI3Kγ inhibitor, which completely blocked the inflammatory-induced Mrp2 particles retrieval in primary hepatocytes derived from WT mice.

Furthermore, genetic inactivation of PI3Kγ catalytic subunit, as well as genetic deprivation of the entire PI3Kγ protein, mimicked the results obtained with the chemical inhibitor. These results importantly suggest a new potential clinical application for PI3Kγ inhibitors in protecting from cholestatic damage.

To study in details the role of PI3Kγ kinase activity upon inflammatory stress, a cytokine mix (CM) was applied on WT-derived primary hepatocytes in combination with AS for a short time, and Akt phosphorylation was tested as a readout. However, due to the use of a mixture of cytokines that simultaneously activates various receptors and downstream signaling pathways, we could not observe a complete block of Akt phosphorylation by using AS inhibitor. Presumably, upon CM treatment other PI3K isoforms are activated at the same time, and the inhibition of PI3Kγ alone is not sufficient to fully abrogate Akt phosphorylation. For example, other PI3K isoforms like p110α and p110β might be also involved in a variety of intracellular responses to inflammatory cytokines (Tsukamoto et al. 2008; Hawkins and Stephens 2015).

Our data indicate that the kinase activity of PI3Kγ is crucial in restricted signaling responses to a single G protein-coupled receptor, such as the fMLP receptor (FPR). FPR downstream signaling has been characterized in neutrophils, and its activation by the bacterial cell wall-derived chemotactic peptide fMLP triggers multiple intracellular signaling pathways, including a rapid stimulation of PI3Kγ (Hirsch et al. 2000). In contrast to the effects of cytokine mix, fMLP short-time stimulations in hepatocytes induced Akt phosphorylation that was exclusively dependent on PI3Kγ kinase activity, since the fMLP-induced P-Akt signal was completely abrogated by both genetic and pharmacologic inhibition of PI3Kγ catalytic function.

Among the intracellular reactions triggered by fMLP, the PI3Kγ-mediated activation of PKCα seems to play an important role for the neutrophils' defense against pathogen infections (Lehmann et al. 2009; Bertram and Ley 2011). Furthermore, a large body of evidence supports the involvement of PKCα in the pathogenesis of cholestasis within hepatocytes (Wimmer et al. 2008; Pérez et al. 2006; Anwer 2014; Crocenzi et al. 2008). Importantly, here we show that PI3Kγ and PKCα interact in hepatocytes, especially following fMLP stimulation. Thus, we speculate that the kinase activity of PI3Kγ may be important for PKCα activation, although it still remains unclear whether the lipid or the protein kinase function of PI3Kγ is activating PKCα (Lehmann et al. 2009).

Initial studies using broad-spectrum pharmacologic activators and inhibitors of PKCs suggested that stimulation of PKCs causes cholestasis (Anwer 2004). However, PKCs are implicated in both choleretic and cholestatic effects, and it is recently becoming evident that

these opposing effects may be mediated by different PKC isoforms (Anwer 2014). Our observation that PKCα in hepatocytes immunoprecipitates with PI3Kγ suggests the contribution of this specific PKC isoform in the PI3Kγ-mediated cholestatic effects. PKCs have been found involved in mediating the activity of two members of the ezrin/radixin/moesin (ERM) protein family: radixin and ezrin. Radixin is the dominant ERM protein in hepatocytes, and its co-localization with Mrp2 at the canalicular membrane is disrupted in conditions of cholestasis (Sekine et al. 2011; Kojima et al. 2008). Accordingly, knockdown and knockout of radixin in rodents lead to a reduction in membrane Mrp2 (Kikuchi et al. 2002; Wang et al. 2006). However, ezrin protein has also been detected at the canalicular membrane of human hepatocytes (Clapéron et al. 2013; Chai et al. 2015). Importantly, ezrin knockdown mice develop severe intrahepatic cholestasis, characterized by intrahepatic bile acid accumulation without developmental defects of bile duct morphology or infiltration of inflammatory cells (Hatano et al. 2015). Furthermore, studies in Caco2 cells, rat intestine tissue and cholestatic livers indicate that ezrin controls Mrp2 expression and function (Yang et al. 2007; Nakano et al. 2009; Chai et al. 2015).

Interestingly, studies to date suggest that both the dephosphorylation of radixin by PKC-mediated activation of PP-1 (Kojima et al. 2008; Sekine et al. 2011) and the direct PKC-induced phosphorylation of ezrin (Chai et al. 2015; Nakano et al. 2009) lead to Mrp2 retrieval from the plasma membrane (Fig. 4). Given the impact that the ERM phosphorylation status has on Mrp2 localization, we analyzed via western blot WT-derived primary hepatocytes treated with CM using P-ERM antibody. However, P-ERM pattern of CM-treated hepatocytes did not differ from controls, probably due to the failure of the antibody used in distinguishing between P-ezrin and P-radixin, localized at the same molecular weight. Nonetheless, primary hepatocytes derived from PI3Kγ mutant mice showed a considerable decrease in P-ERM, both in basal conditions and upon proinflammatory stimulation. These results point out a major role of PI3Kγ in controlling the phosphorylation of one of the ERM proteins. Strikingly, our immunoprecipitation experiments show that ezrin but not radixin is directly interacting with PI3Kγ, strongly suggesting that a PI3Kγ/PKCα/ezrin signaling pathway may control Mrp2 protein internalization process (Fig. 25).

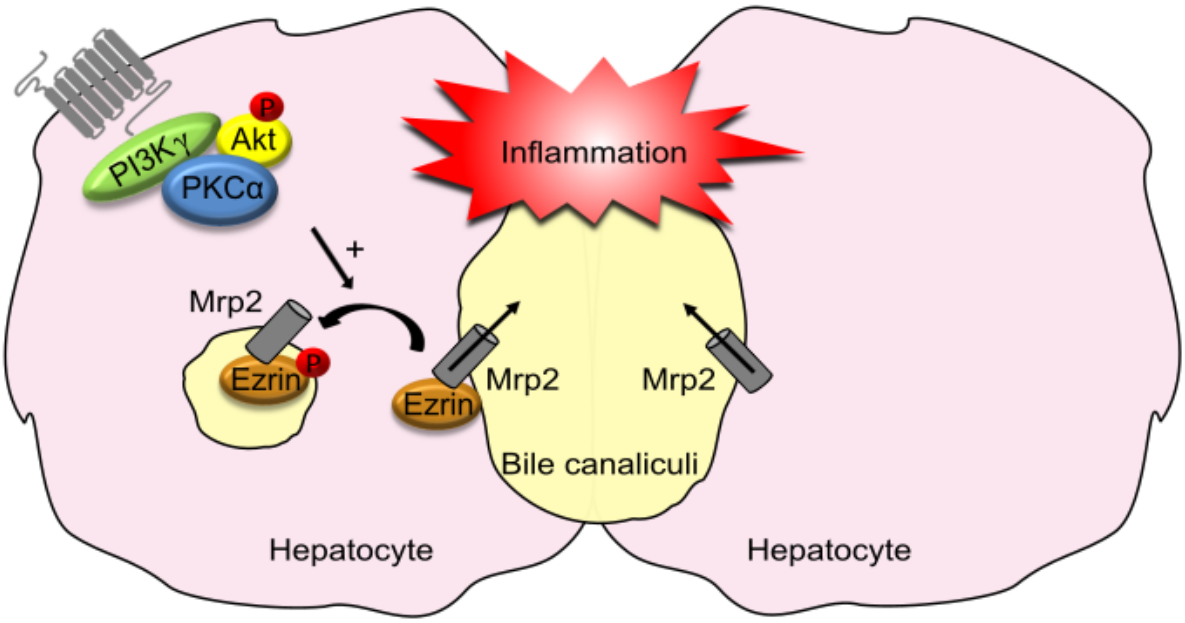


Figure 25: Schematic representation of the PI3Kγ-mediated regulation of Mrp2 protein internalization in hepatocytes. Under inflammatory conditions, ezrin tether protein undergoes a protein kinase C alpha (PKCα)-dependent phosphorylation, and this leads to Mrp2 protein retrieval towards endocytic compartments. This mechanism is controlled by the kinase activity of PI3Kγ, which mediates PKCα activity.

The cholestatic-induced internalization of bile transporter proteins is normally counteracted by a boost of cAMP levels, which redistributes transporters to the canalicular membrane in order to avoid irreversible consequences of sustained internalization, such as accelerated protein degradation (Crocenzi et al. 2012). Our finding that PI3Kγ controls cAMP hepatocytic pools, thus promoting Mrp2 canalicular localization, is in line with previous reports where the scaffold function of this PI3Kγ has been identified as crucial for regulating cAMP levels (Hirsch et al. 2009). Nonetheless, in our study emerged the important role of the sole PI3Kγ catalytic activity in contrast to its kinase-independent function, responsible for the activation of cAMP-degrading phosphodiesterases. We found that cAMP levels in hepatocytes derived from KDγ mice are significantly higher than WT controls both in normal conditions and upon inflammatory stress, strongly suggesting an involvement of PI3Kγ kinase activity in the regulation of cAMP level. Accordingly, hepatocyte treatment with PI3Kγ inhibitor blocked the Akt phosphorylation induced by salbutamol, which is a β2-adrenergic receptor agonist that

negatively modulates cAMP pools through a Gi/PI3K/Akt signaling pathway (Xiao 2001). Furthermore, our immunoprecipitation experiments in both primary hepatocytes and cell lines show that in liver parenchyma PI3Kγ interacts with PDE3B, as it was previously shown in other cell compartments such as heart and brain (Patrucco et al. 2004; Perino et al. 2014). Given that PDE3B activity has been already found controlled by Akt in other cell types (Kitamura et al. 1999; Sahu 2011; Ahmad et al. 2000), we speculate that PI3Kγ catalytic activity in hepatocytes negatively controls cAMP level via an Akt/PDE3B signaling pathway.

Experimental strategies aimed to increase cAMP in hepatocytes through cAMP analogues or PDE inhibitors were successful in protecting from cholestatic damage, especially due to an increased Mrp2 protein insertion at the canalicular membrane of hepatocytes (Roelofsen et al. 1998; Mottino et al. 2002; Crocenzi et al. 2005). Although the molecular mechanisms by which cAMP translocates Mrp2 to the plasma membrane remain incompletely understood, reports to date indicate that this process is mostly controlled by vesicle trafficking along microtubules (Marinelli et al. 2005). For example, Schonhoff et al. have recently elucidated how cAMP mediates microtubule-dependent membrane insertion of Mrp2 via activating the p38α MAPK (Schonhoff et al. 2016). Of note, another recent study revealed Rab11 as a crucial protein involved in the cAMP-mediated reinsertion of Mrp2 at the canalicular membrane of hepatocytes from recycling endosomes (Park et al. 2014). Additionally, an *in vitro* study revealed a novel mechanism involving the interaction of members of the ERM family with the N terminus of Epac, which is a direct effector of cAMP (Gloerich et al. 2010). In particular, ERM protein activation increases binding to Epac1 and results in a clustered localization of Epac1 at the plasma membrane. Thus, an involvement of cAMP/Epac signaling in the inflammatory-induced cytoskeleton reorganization of hepatocytes affecting Mrp2 canalicular localization cannot be excluded.

On the other hand, independently from microtubule trafficking, an alternative route of cAMP-stimulated exocytic insertion is represented by the protein kinase A (PKA). This path has been investigated in rat hepatocyte couples, where the glucagon-induced increase of cAMP has been shown to require PKA activation for preventing drug-induced canalicular secretory dysfunction (Zucchetti et al. 2011). Interestingly, in this study the authors demonstrated that the salbutamol effect is PKA-independent, but instead depends on Epac/MEK and microtubule. These findings highlight the complexity of the mechanisms beneath the potential anti-cholestatic properties of glucagon and adrenaline hormones at the hepatic level.

In the early phase, sepsis is characterized by high concentrations of circulating catecholamines, which boost the initial inflammatory response (Rittirsch et al. 2008). In the

liver, adrenaline predominantly increases cAMP levels by means of the β2 adrenergic receptor (β2AR) (Morgan et al. 1983). β2AR signaling activity and localization at the cell surface are tightly controlled by G protein-coupled serine/threonine kinases (GRKs), which normally phosphorylate agonist-occupied GPCRs and cause their desensitization (Penela et al. 2003). GRK2 is the most extensively characterized member of GRKs, and its kinase activity on β2AR provokes receptor uncoupling from G proteins and clathrin-mediated endocytosis of β2AR via β-arrestin proteins binding. Of note, ezrin has been identified as a novel non-GPCR substrate of GRK2 (Cant and Pitcher 2005), suggesting a possible function for GRK2 linking β2AR activation with hepatocyte reorganization of the actin cytoskeleton following cholestatic insult. Furthermore, the interaction of GRK2 with PI3Kγ has been previously shown to facilitate PI3K recruitment to the membrane upon agonist stimulation, thus contributing to receptor endocytosis and desensitization (Naga Prasad et al. 2002). Hence, given that our results proved that PI3Kγ is involved downstream β2AR in hepatocytes, we do not exclude the participation of GRK2 in the PI3Kγ-dependent regulation of ezrin-mediated Mrp2 localization in liver parenchyma.

In light of the results obtained, we propose that in inflammatory stress conditions kinase-dependent functions of PI3Kγ regulate both Mrp2 retrieval and insertion on the plasma membrane (Fig. 26) via defined effects on ezrin- and cAMP-mediated control of Mrp2 localization.

Apart from this short-term regulation of Mrp2 localization, via PKCα and/or cAMP signaling, in the current study we enlighten the involvement of PI3Kγ in other long-term adaptive responses to infection that hepatocytes exploit to prevent liver failure. Notably, an evolutionary host defense strategy defined as “tolerance to infection”, first discovered in plants and secondarily in animals, has been described to limit disease severity irrespective to pathogen load (Soares et al. 2014). In contrast to the “resistance to infection” defense strategy, mainly consisting in pathogen clearance by innate and adaptive immune systems, disease tolerance does not exert direct effects on pathogens. Instead, the functional outputs of host parenchymal cells are sustained under stress conditions by this “tolerance” strategy, preventing irreversible tissue damage and the consequent compromised host viability (Soares et al. 2017).

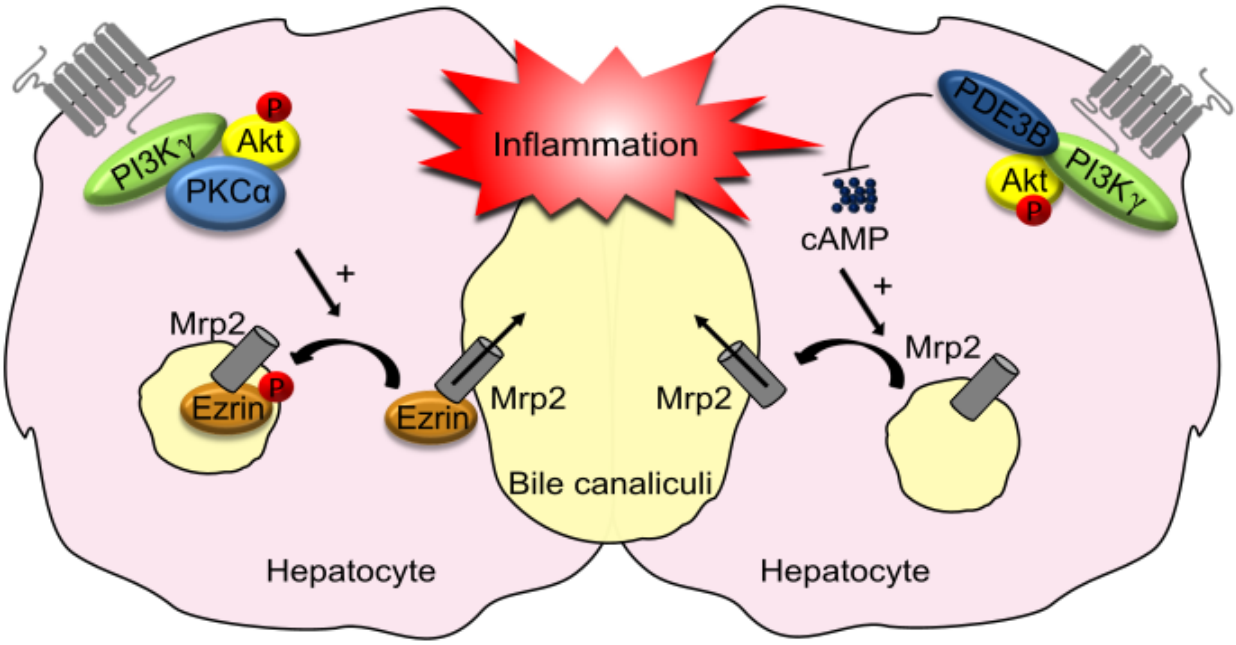


Figure 26: Schematic representation of PI3Kγ-mediated regulation of Mrp2 protein localization. In inflammatory stress conditions, PI3Kγ controls the localization of the Mrp2 bile transporter protein. On the left side of the scheme it is suggested how PI3Kγ kinase activity regulates Mrp2 internalization via PKCα-mediated ezrin phosphorylation. On the right side of the scheme we hypothesize how the insertion of Mrp2 particles on the canalicular membrane is controlled by PI3Kγ-mediated regulation of cAMP intracellular levels.

Among the host genes conferring tolerance to infection, the stress-responsive enzyme heme oxygenase-1 (HO-1) has been recognized as essential for limiting inflammation and preventing cell death, especially in the liver (Gozzelino et al. 2010; Larsen et al. 2010). HO-1 acts as the rate-limiting enzyme in the breakdown of heme (Fe protoportphyrin IX, FePPIX) into equimolar amounts of biliverdin, iron and carbon monoxide (Tenhunen et al. 1968). Importantly, the induction of HO-1 expression in response to microbial infection suppresses the development of severe sepsis in mice (Chung et al. 2008). The safe-guarding function of HO-1 acts irrespective of pathogen load, relying on the cytoprotective effect of HO-1 against excess free heme produced by infection-induced hemolysis. In particular, the cytotoxic effects of free heme mainly depend on a pro-oxidant activity (Gozzelino et al. 2010), driven by the divalent Fe atom contained within the heme protoporphyrin IX ring that can promote the production of free radicals via Fenton chemistry (Wardman and Candeias 1996).

Strikingly, our results show that the lack of PI3Kγ activity increases the amount of HO-1 within hepatocytes, being potentially protective from heme-induced liver damage. Induction of

HO-1 exerts hepatoprotective effects primarily by attenuating Kupffer cell inflammatory response (Devey et al. 2009). However, HO-1 induction using tin protoporphyrin has been also shown to protect hepatocytes from cell death in experimental sepsis *in vivo* and *in vitro* via induction of autophagy (Carchman et al. 2011). Given that autophagy is negatively regulated by the PI3K/Akt-mediated phosphorylation of the mechanistic target of rapamycin complex 1 (mTORC1) (Zhao et al. 2015), we cannot exclude that the increased HO-1 levels found in PI3Kγ mutant mice elicit hepatocellular protection via increasing autophagy. The observation that autophagy activation protects against cholestasis-induced hepatic injury (Gao et al. 2014) additionally supports this hypothesis, although further investigations would still be needed.

Remarkably, it has been recently demonstrated that the HO-1 induction by Hemoglobin-Glutamer 200 (HbG200) attenuates hepatocellular cholestasis in ischemia-reperfused (I/R) rat livers (Donner et al. 2013). Interestingly, in this study it was observed that HbG200-induced HO-1 increase in pericentral hepatocytes largely reverted the I/R-induced downregulation of Mrp2. Given that the lack of PI3Kγ activity results in an increase of HO-1 expression, therapeutic interventions inhibiting the hepatocytic PI3Kγ catalytic activity may improve hepatobiliary function and merits further investigations.

Our data point out the role of PI3Kγ enzymatic activity in the negative regulation of hepatocytic cAMP pools. In light of previous studies where increased intracellular cAMP levels induced HO-1 via a protein kinase A (PKA)-dependent pathway (Immenschuh et al. 1998; Rensing et al. 2004), PI3Kγ kinase function possibly controls HO-1 expression via regulating cAMP hepatocytic amounts. Besides the induction of HO-1 by cAMP, we hypothesize that the increased HO-1 expression we observed in PI3Kγ mutant hepatocytes can be caused by oxidative stress. A large body of evidence indicating oxidative stress as the main cause of HO-1 increase (Ryter and Choi 2005; Gozzelino et al. 2010). These data indeed support our discovery that reactive oxygen species (ROS) production is slightly elevated in KDγ and KOγ-derived parenchymal cells. However, the fact that hepatocytes lacking PI3Kγ display protection from cholestasis excludes that the level of intracellular ROS is noxious. Notably, several studies have provided evidence that oxidative stress occurs in cholestatic conditions, even though the source of ROS appears to be primarily inflammatory cells that accumulate in the liver at early stages of disease (Copple et al. 2010; Roma and Sanchez Pozzi 2008). In particular, reactive oxygen species have been found to induce endocytic internalization of canalicular transporters, especially Mrp2 (Sekine et al. 2006; Sekine et al. 2011). Given that we observed a block of the inflammatory-induced Mrp2 internalization in PI3Kγ mutant hepatocytes, we speculate that the

dose of ROS in KDγ and KOγ liver parenchyma is not sufficiently high to provoke cholestatic damage.

Stress-dependent adaptive responses display pronounced dose dependency. In many cases, the low doses of potentially harmful environmental factor, such as ROS, eventually cause beneficial effects. This dose-dependent phenomenon, characterized on one hand by low dose-induced damage prevention and on the other hand by harmful effects at higher does, has been defined as "hormesis" and it has been well characterized in the past ten years (Calabrese 2008; Wetzker and Rubio 2012). Considering this, ROS, which in high amounts have detrimental effects, at low doses can be rather beneficial by activating signaling molecules that promote health (Ristow 2014). Since the amount of ROS produced in PI3Kγ mutant hepatocytes are not adequately high to cause the detrimental effect of Mrp2 retrieval from the canalicular membrane, we hypothesize that parenchymal liver cells lacking functional PI3Kγ may be protected from inflammatory/oxidative stress-induced damage because of preconditioning by ROS low doses. Consistently, KDγ and KOγ-isolated primary hepatocytes, when exposed to heme and TNFα-induced oxidative stress, showed a pronounced HO-1 response as compared to WT controls, both at transcriptional and at translational level.

In hepatocytes the most important sources of ROS are mitochondria, for two important reasons: i) every liver cell contains hundreds of mitochondria and ii) mitochondrial electron transport can easily be disturbed (Copple et al. 2010). Remarkably, intrinsic cAMP production and PKA activation have been detected in liver mitochondria, where they are proposed to regulate respiration thus adjusting mitochondrial bioenergetics to different metabolic situations (Acin-Perez et al. 2009). In cardiomyocytes, for example, both the ischemic and the glucose deprivation-induced activation of PKA increase the phosphorylation of cytochrome c oxidase subunits, leading to inhibition of mitochondrial respiration and consequent ROS increase (Ferretti et al. 2012). Given that hypoglycemia is frequently observed in septic patients and that hepatocytes possess large amounts of mitochondria, a possible regulation of the mitochondrial cAMP-mediated ROS production by PI3Kγ cannot be excluded. Even though our measurements of ROS using the DCFDA assay did not allow us to define the source of oxidative species, we observed that the increased ROS in PI3Kγ mutant hepatocytes triggered beneficial long-term adaptive responses conferring disease tolerance.

The inflammatory-induced oxidative stress imposed on liver parenchyma, which potentially leads to cholestasis, is mainly counteracted by the adaptive cellular response regulated by the evolutionarily conserved nuclear factor-erythroid 2-related factor 2 (Nrf2) (Hayes and Dinkova-Kostova 2014; Soares et al. 2014). Activation of Nrf2 has been demonstrated to

provide tissue damage control and disease tolerance in mouse models of *Salmonella* (Nairz et al. 2013), *Staphylococcus aureus* (Athale et al. 2012) and polymicrobial infections (Thimmulappa et al. 2006; Kolls 2006), and it has been also found to modulate host resistance mechanisms in many other inflammation-associated diseases (Kim et al. 2010). Nrf2 is a member of the cap'n'collar (CNC) basic-region leucine zipper transcription factor family, and it is considered one of the major protagonists in the host protective mechanisms preventing parenchymal cells from dysfunctions and necrosis (Soares et al. 2014). Of particular interest, within the liver these protective mechanisms enforce tissue damage control and disease tolerance to systemic infections presumably via the expression of Nrf2 regulated effector genes, such as the HO-1-encoding *Hmox-1* gene. Accordingly, KDγ and KOγ hepatocytes exhibited increased *Hmox-1* gene expression both in basal and in oxidative stress conditions. Therefore, the increase of *Hmox-1* gene transcription observed in PI3Kγ mutant hepatocytes, index of augmented Nrf2 activity, can most likely be attributed to ROS.

Nrf2 is renowned as a redox-sensitive transcription factor, which accumulates in the nucleus under stress conditions, enhancing antioxidant and cytoprotective genes (Soares and Ribeiro 2015). It is thus not surprising that the basal activity of Nrf2, as well as the magnitude of its activation in response to stress, are tightly controlled. Under normal homeostatic conditions, Nrf2 is maintained at low level by constant ubiquitinylation and proteasomal degradation. However, the presence of oxidants inhibits Nrf2 constitutive degradation, thereby enabling Nrf2 protein accumulation. The prevailing view is that the E3 ubiquitin ligase substrate adaptor Kelch-like ECH-associated protein (Keap1) is the dominant regulator of Nrf2. Keap1 binds Nrf2 in normal unstressed conditions, promoting its ubiquitination and proteasomal degradation, but ROS exposure causes cysteine modifications which inhibit Keap1 function and promote Nrf2 stability and activity (Suzuki and Yamamoto 2015). Nonetheless, a growing body of evidence is recently supporting the importance of glycogen synthase kinase-3 (GSK-3) as an Nrf2 repressor (Hayes and Dinkova-Kostova 2014; Cuadrado 2015; Salazar et al. 2006). Importantly, PI3K/Akt signaling pathway has been already described to control the activity of GSK-3 (Hayes and Dinkova-Kostova 2014; Sivaramakrishnan and Devaraj 2010), thus suggesting a possible involvement of PI3Kγ in modulating the GSK-3-mediated repression of Nrf2 activity.

5. Conclusions and Outlook

This study reveals an important role of PI3Kγ in the response of liver parenchyma to inflammatory stress. For the first time, here we show that PI3Kγ is expressed in hepatocytes and is involved in the cellular response to inflammatory stress. Our *ex vivo* and *in vitro* data demonstrate that both pharmacological and genetic inhibition of PI3Kγ prevents from inflammatory-induced Mrp2 internalization, thus protecting hepatocytes from cholestatic damage. At the same time, the lack of functional PI3Kγ upregulates HO-1 in liver parenchyma, exerting long-term hepatoprotective effects.

However, further investigations are still required for studying the role that hepatocytic PI3Kγ plays *in vivo* upon inflammatory stress. Although previous studies showed that PI3Kγ inhibition improves the secretory function of hepatocytes in rodent models of peritonitis (Martin et al. 2010; Recknagel et al. 2012), a large body of literature shows that PI3Kγ plays a crucial role within the immune system in protecting the host from infection (Costa et al. 2011; Hirsch et al. 2000; Rückle et al. 2006). Therefore, PI3Kγ inhibition in the whole body in septic conditions on one hand has hepatoprotective effects, but on the other hand causes an immunocompromised state, being detrimental to the host in terms of survival. Thus, strategies aimed to target PI3Kγ specifically in liver parenchyma would prevent sepsis-induced hepatic failure without affecting the immune system.

In light of these considerations, the generation of hepatocyte-specific conditional knockout mice for PI3Kγ would help discriminating the function that this enzyme has in liver parenchyma as opposed to its role within the immune system. These mice could be obtained using the Cre/lox system strategy, by breeding two transgenic mouse lines: i) mice having the PI3Kγ gene flanked by loxP regions and ii) mice having the Cre enzyme expressed under albumin promoter. These two transgenic mouse lines are already available in our animal facility, and will be breed after approval by the local ethics committee. Mice obtained from this breeding would lack PI3Kγ in hepatocytes exclusively, thus representing a valuable model for studying the involvement of this kinase in the pathogenesis of sepsis-induced cholestasis.

Alternatively, a pharmacological strategy aimed to deliver PI3Kγ inhibitors specifically in mouse liver parenchymal cells may also be taken into account. For example, our research group was recently successful in delivering small molecules to hepatocytes using highly specific nanoparticles, based on near infrared fluorescent polymethine dye-derived targeting moieties coupled to biodegradable polymers (Press et al. 2014). Therefore, the efficacy of the

hepatocyte-specific delivery of PI3Kγ inhibitors with these nanoparticles could be assessed *in vivo* as a preclinical study.

Despite the current study is limited by the lack of an *in vivo* proof of anti-cholestatic effects of PI3Kγ inhibition in septic conditions, our results enrich the current knowledge on the molecular pathology in inflammatory-induced cholestasis. Furthermore, given that the liver is a key player in the sepsis-induced MOF syndrome, our findings may help finding novel putative therapeutic strategies for treating the liver secretory failure caused by infection.

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7. Appendix

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Curriculum Vitae

Personal details:

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Education:

October 2013-September 2017: PhD thesis; "Cell-type specific role of phosphoinositide 3-kinase γ in inflammatory-induced cholestasis". Institute of Molecular Cell Biology, Center for Molecular Biomedecine, University Hospital Jena, Germany. Supervisors: Prof. Dr. med. Michael Bauer, Prof. Dr. med. Oliver Kurzai and Prof. Dr. Reinhard Wetzker.

October 2010-September 2013: Master thesis (Medical Biotechnologies); "The role of PI3Kγ and PI3Kβ in the regulation of energy homeostasis". Molecular Biotechnology Center, Department of Molecular Biotechnologies and Sciences for Health, University of Turin, Italy. Supervisor: Prof. Emilio Hirsch.

July 2011: Bachelor degree (Biotechnologies); University of Turin, Italy.

July 2008: High School Diploma; Scientific Lyceum, "Liceo scientifico G. Segrè", Turin, Italy.

Others academic activities:

October 2015-September 2017: Practical assistance of Master's students at the Institute of Molecular Cell Biology (University hospital of Jena, Germany).

October 2009-April 2010: Research activities; "Tissue specific miR-1 and miR-206 as new therapeutic treatment for rhabdomyosarcoma". CeRMS (Center for Experimental Research and Medical Studies), Department of Anatomy, Pharmacology and Forensic Medicine and Center for Experimental Research and Medical Studies, University of Turin, Italy. Supervisor: Prof. Carola Ponzetto.

Publications:

Beretta, Martina; Bauer, Michael; Hirsch, Emilio (2015): PI3K signaling in the pathogenesis of obesity. The cause and the cure. In: *Advances in biological regulation*, 58, pp. 1–15. DOI: 10.1016/j.jbior.2014.11.004.

Perino, Alessia; **Beretta, Martina**; Kilic, Ana; Ghigo, Alessandra; Carnevale, Daniela; Repetto, Ivan Enrico et al. (2014): Combined inhibition of PI3Kbeta and PI3Kgamma reduces fat mass by enhancing alpha-MSH-dependent sympathetic drive. In: *Science signaling* n. 352, 7, ra110. DOI: 10.1126/scisignal.2005485.

Posters and presentations:

June 2016: Gordon Research Seminar on Cyclic Nucleotide Phosphodiesterases (GRS), PGA Catalunya Business and Convention Centre in Girona, Spain. Presented poster entitled: PI3Kgamma controls hepatocytic cAMP production in sepsis-associated liver failure.

March 2015: Telethon convention Riva del Garda, Italy. Presented poster entitled: Combined inhibition of PI3Kβ and PI3Kγ reduces fat mass by enhancing α-MSH-dependent sympathetic drive.

April 2015: Micom conference in Jena, Germany. Presented poster entitled: Cell type-specific role of PI3Kγ in sepsis-associated liver failure.

September 2015: Weimar Sepsis Update in Weimar, Germany. Presented poster entitled: Cell type-specific role of PI3Kγ in sepsis-associated liver failure.

September 2014: JSMC Symposium in Jena, Germany. Presented poster entitled: Cell type-specific role of PI3Kγ in sepsis associated liver failure.

April 2014: Micom conference in Jena, Germany. Presented poster entitled: Cell type-specific role of PI3Kgamma in sepsis-associated liver failure.

Extra-academic activities:

- Volunteer of Croce Verde Turin (Green Cross Turin), an association that operates in Turin (Italy), involved in a variety of activities, e.g. secondary health emergency, transportation by ambulance, urgent transport in collaboration with medical experts. (2010-2017)
- Wedding singer work. (2010-2013)
- Rowing. (2001-2007)

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Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben:

Dr. Eric Seemann: EM Ergebnisse;
Dr. Lars Riecken: Unterstützung bei der zellbiologischer Materials, Diskussion und Interpretation der Ergebnisse;
Prof. Dr. med. Michael Bauer, Prof. Dr. Reinhard Wetzker, Prof. Dr. Emilio Hirsch, Prof. Dr. med. Sebastian Weis: Diskussion und Interpretation der Ergebnisse;
Prof. Dr. Reinhard Wetzker: Redigieren der Dissertation;

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dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

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